

# Morphological updates, host-specificity, molecular data and phylogenetic analysis of *Acanthobothrium coronatum* (Cestoda: Onchoproteocephalidea), a neglected parasite of the nursehound *Scyliorhinus stellaris*

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## ABSTRACT

*Acanthobothrium* is the most speciose genus of onchoproteocephalidean cestodes, whose adults parasitize the intestine of elasmobranch fishes. *Acanthobothrium coronatum*, the type species of the genus described from Mediterranean elasmobranchs, remains a little known parasite, with the most recent reports dating back to the fifties. We hereby investigate host-specificity and redescribe *A. coronatum* from the same locality of its original description by using light and scanning electron microscopy approaches. Moreover, molecular and phylogenetic data inferred from the analysis of the 28S rDNA gene are reported for the first time. Out of the nine elasmobranch species examined from Gulf of Naples, we only detected *A. coronatum* in the intestine of *Scyliorhinus stellaris*, with infection patterns that supports evident host-specificity for this shark species. The genetic characterization of 28S rDNA showed 99.8–100% similarity with larvae previously found in *Octopus vulgaris* from the same area investigated here. Conspecificity between the present material and the larvae found in the octopus was also confirmed by the tree topology. The host-parasite phylogeny is discussed, even if additional molecular data are needed to clarify potential host-parasite patterns. Notwithstanding this limitation, this is the first molecular study revealing conspecificity between an adult *Acanthobothrium* species from a shark and the larvae found in an intermediate/paratenic host, shedding light on the transmission pathway of *A. coronatum* in *S. stellaris*. Finally, the taxonomic, molecular, and phylogenetic data presented here allow a better characterization of a neglected parasite.

## 1. Introduction

In the last two decades, our understanding of diversity and host-association of cestodes in elasmobranchs has improved substantially worldwide (Caira and Jensen, 2001, 2014). However, this is not the case of the Mediterranean basin, where most of the information is dated and scattered in old opportunistic studies (<https://shark-references.com/species/parasite-hosts-list/A>). Among cestodes, *Acanthobothrium* Blanchard, 1848 is the most speciose genus in the order Onchoproteocephalidea (Caira and Jensen, 2014; Caira and Jensen, 2017), which currently comprise 208 valid species parasitizing the intestine of elasmobranch fishes as adults (Franzese and Ivanov, 2020; Gallagher and Caira, 2020; Zaragoza-Tapia et al., 2020; Van Der Spuy et al., 2020). In addition, while recent studies suggested that members of *Acanthobothrium* are highly host-specific (Jensen and Bullard, 2010; Caira et al., 2014; Caira and Jensen, 2001, 2014), the type species of the genus,

*Acanthobothrium coronatum* (Rudolphi, 1819) Blanchard, 1848, seems to be a generalist parasite with no specific host preference, having been reported from a wide range of elasmobranch hosts. This taxon has been originally described by Rudolphi (1819) mostly based on material declared as collected from *Scyliorhinus stellaris* (Linnaeus, 1758), *Torpedo marmorata* Risso, 1810, and *Torpedo torpedo*, (Linnaeus, 1758) from the Gulf of Naples (Mediterranean Sea). Since then, it has also been reported from a wide range of skates and rays [*Aetobatus narinari* (Euphrasen, 1790), *Amblyraja radiata* (Donovan, 1808), *Bathytoshia centroura* (Mitchill, 1815), *Dasyatis pastinaca* (Linnaeus, 1758), *Hemirhamphysa akajei* (Müller and Henle, 1841), *Dipturus batis* (Linnaeus, 1758), *Dipturus laevis* (Mitchill, 1818), *Dipturus oxyrinchus* (Linnaeus, 1758), *Leucoraja ocellata* (Mitchill, 1815), *Myliobatis aquila* (Linnaeus, 1758), *Narcine timlei* (Bloch and Schneider, 1801), *Neotrygon kuhlii* (Müller and Henle, 1841), *Pastinachus sephen* (Forsskal, 1775), *Raja asterias* Delaroche, 1809, *Raja clavata* Linnaeus, 1758, and *Urogymnus asperimus*

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(Bloch and Schneider, 1801)], and sharks [*Carcharodon carcharias* (Linnaeus, 1758), *Etmopterus spinax* (Linnaeus, 1758), *Mustelus mustelus* (Linnaeus, 1758), *Scyliorhinus canicula* (Linnaeus, 1758), *Somniosus rostratus* (Risso, 1827), *Squalus acanthias* Linnaeus, 1758, and *Squatina squatina* (Linnaeus, 1758)] from several worldwide localities, including Mediterranean Sea, west Japan and Indonesia (Indo-Pacific), U.S.A. (north-western Atlantic), and west coast of Wales (north-eastern Atlantic) (Southwell, 1927; Leon-Borcea, 1934; Baer, 1948; Euzet, 1959; Myers, 1959; Baer and Euzet, 1962; Goldstein, 1967; Williams, 1969; Zaragoza-Tapia et al., 2020; see also <https://shark-references.com/species/parasite-hosts-list/A>). In addition, the most recent data from the Mediterranean Sea dates back to the fifties (Euzet, 1959), thus making this parasite somehow neglected by the recent literature.

During a recent investigation of the parasite fauna of elasmobranch fishes from the Gulf of Naples (Mediterranean Sea), a detailed morphological study revealed that specimens of *Acanthobothrium* found in the intestine of *S. stellaris* belong to *A. coronatum*. Using an integrative approach including patterns of host specificity, morphological (light microscopy and SEM analysis), molecular data and phylogenetic analysis as inferred from 28S ribosomal (r) DNA, we here describe new features of *A. coronatum* from the locality of its original description and first lay the basis for a review of the genus using its type species as a starting point.

## 2. Materials and methods

### 2.1. General data

A total of 345 elasmobranch fishes, representing nine different species, obtained between July 2020 and July 2021 from various depths (~10–600 m) of the Gulf of Naples (Tyrrhenian Sea) were examined for *Acanthobothrium* specimens. Fishes used in the present study mostly constituted the bycatch of scientific and commercial trawling operations (red shrimps' and pink shrimps' fishery activities), held with a commercial fishing vessel equipped with bottom trawl nets (mouth of 3 × 4 m in height and width, respectively; 18–40 mm mesh size) and towed at ~2–2.5 kn on muddy bottoms (see Crocetta et al., 2020; Palomba et al., 2021; Tanduo et al., 2021). However, few additional specimens of *T. torpedo* were also obtained as bycatch of trammel netting activities by local fishermen. Samplings were performed in accordance with the permit n. 0008453 issued by the Italian Ministry of Agricultural, Food and Forestry Policies (May 15, 2020), the guide for the care and use of animals by the Italian Ministry of Health, and the ARRIVE guidelines.

After landing, the fishes were measured (total length) to nearest 0.1 cm. During fish necropsy, the sex was determined by gonadal examination, and stomach and intestine 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 were washed through a 100 µm mesh screen. The remaining material from each organ was examined under a stereomicroscope, and parasites were collected, counted, washed in physiological saline, and preserved in 70% ethanol or frozen at –20 °C (Santoro et al., 2020a, 2020b). Descriptors of infection (i.e., prevalence and intensity of infection) follow Bush et al. (1997).

For morphological identification, 15 *Acanthobothrium* specimens were stained with Mayer's acid carmine, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted in Canada balsam. Cestodes were examined and measured using a compound microscope and a stereomicroscope equipped with the ZEN 3.1 imaging system (Zeiss), and subsequently identified using published identification keys (Euzet, 1959; Rees and Williams, 1965; Goldstein, 1967). Line drawings were prepared with the aid of a camera lucida attached to a compound microscope. Hook measurements follow Euzet (1959) and Ghoshroy and Caira (2001). Measurements are in micrometres unless otherwise stated and reported as mean value ± standard deviation, with ranges in

parentheses followed by the total number of observations.

Designation of proglottid apolysis follows Caira et al. (1999) and Franzese and Ivanov (2018). The categorical method suggested by Ghoshroy and Caira (2001) and Fyler and Caira (2006) was used to facilitate comparisons among species of *Acanthobothrium* recorded in the Mediterranean Sea.

### 2.2. Scanning electron microscopy (SEM) analysis

For SEM, the anterior portion of seven specimens was fixed overnight in 2.5% glutaraldehyde, then transferred to 40% ethanol (10 min), rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO<sub>4</sub> for 2 h, dehydrated in ethanol series, and critical point dried and sputter-coated with platinum. Observations were made using a JEOL JSM 6700F scanning electron microscope operating at 5.0 kV (JEOL, Basiglio, Italy).

### 2.3. Molecular and phylogenetic analysis

Total genomic DNA (gDNA) from the mature proglottids of a subsample of 10 specimens used for the morphological study was extracted using the Quick-gDNA Miniprep Kit (ZYMO RESEARCH), following the standard manufacturer-recommended protocol. A fragment (~1400 bp) of the nuclear large subunit ribosomal RNA gene (LSU; spanning domains D1–D3) was amplified using the primers LSU5 (5'-TAGGTC-GACCCGCTGAAYTTAAGC-3') and 1200R (5'-GCATAGTTCACCATCTTTCGG-3') (Littlewood et al., 2000). PCR reactions were carried out in a 25 µL volume containing 0.6 µL of each primer 10 mM, 2 µL of MgCl<sub>2</sub> 25 mM (Promega), 5 µL of 5× buffer (Promega), 0.6 µL of dNTPs 10 mM (Promega), 0.2 µL of Go-Taq Polymerase (5 U/µL) (Promega) and 2 µL of total DNA. PCR temperature conditions were the following: 94 °C for 3 min (initial denaturation), followed by 38 cycles at 94 °C for 30 s (denaturation), 57 °C for 30 s (annealing), 72 °C for 2 min (extension), followed by post-amplification at 72 °C for 7 min. PCR amplicons were purified using the AMPure XP kit (Beckman Coulter) following the standard manufacturer-recommended protocol, cycle sequenced with primer pair LSU5 and 1200R through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems), by the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Contiguous sequences were assembled and edited using Sequencematrix (Vaidya et al., 2011) and MEGA X v. 11 (Kumar et al., 2018). Sequences were screened using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Morgulis et al., 2008) and then aligned with the available *Acanthobothrium* LSU sequences, using ClustalX v. 2.1 (Larkin et al., 2007). Phylogenetic relationships were inferred using Bayesian inference (BI) with MrBayes, v. 3.2.7 (Huelsenbeck and Ronquist, 2001) and maximum likelihood (ML) with IQ-TREE (Nguyen et al., 2015).

Based on the results of JModelTest v. 2.1.10 (Posada, 2008; Posada and Buckley, 2004), BI setting was set to nst = 6, rates = invgamma (equivalent to the GTR + I + G), ngammat = 4 model of evolution. Four chains (0.2 as the temperature of heated chains) were run for 5,000,000 MCMC generations, with a subsampling frequency of 500 and a burn-in fraction of 0.25. Posterior probabilities were estimated and used to assess support for each node. Values with a 0.90 posterior probability were considered well-supported. ML analysis was performed with 1000 ultrafast bootstrap replicates. Clades were considered to have high nodal support if the ML bootstrap resampling ≥70%. The phylogenetic tree was rooted using *Potamotrygonocestus fitzgeraldae* Marques, Brooks and Araujo, 2003 as outgroup (Caira et al., 2014).

Four representative LSU sequences obtained in the present study and those from GenBank included in the phylogenetic tree are listed in Table 1. Genetic distances (shown as the percentage difference, i.e., no. of base substitutions per sites \*100) were computed using the Kimura 2-Parameters (K2P) model (Kimura, 1980) with 1000 bootstrap resamplings, by MEGA Software, version 7.0.

**Table 1**Species of *Acanthobothrium*, stages (l: larva, a: adult), host, locality, and accession numbers of LSU sequences included in the phylogenetic analyses shown in the Fig. 4.

Species	Stage	Host	Locality	Accession number	References
<i>A. brevissime</i>	a	<i>Dasyatis say</i>	Florida	EU660530	Holland et al., 2009
<i>A. cleofanus</i>	a	<i>Hypanus longus</i>	Nayarit, Mexican Pacific	MZ081426	Unpublished
<i>A. coronatum</i>	a	<i>Scyliorhinus stellaris</i>	Tyrrhenian Sea, Italy	MZ243216-19	Present study
<i>A. filicollis</i>	a	<i>Zearaja nasuta</i>	South Island, New Zealand	MH913266	Bennett et al., 2019
<i>A. hypermekkolpos</i>	a	<i>Rhynchobatus laevis</i>	Australia	HQ917930	Fyler and Caira, 2011
<i>A. katherineae</i>	a	<i>Squaliolus aliae</i>	Gulf of Taiwan	MT395344	Gallagher and Caira, 2020
<i>A. jeanneae</i>	a	<i>Rhynchobatus laevis</i>	Australia	HQ917928	Fyler and Caira, 2011
<i>A. masniae</i>	a	<i>Himantura</i> sp.	Australia	FJ843604	Fyler et al., 2009
<i>A. margieae</i>	a	<i>Orectolobus maculatus</i>	Moreton Bay, Australia	MH729997	Cutmore et al., 2018
<i>A. mattaylori</i>	a	<i>Rhynchobatus laevis</i>	Australia	HQ917927	Fyler and Caira, 2011
<i>A. oceanharvestae</i>	a	<i>Himantura</i> sp.	Australia	FJ843594	Fyler et al., 2009
<i>A. parviuncinatum</i>	a	<i>Urolophus maculatus</i>	Bahia de Los Angeles, Mexico	EF095264	Waeschenbach et al., 2007
<i>A. popi</i>	a	<i>Himantura</i> sp.	Australia	FJ843600	Fyler et al., 2009
<i>A. rodmani</i>	a	<i>Himantura</i> sp.	Australia	FJ843596	Fyler et al., 2009
<i>A. romanowi</i>	a	<i>Himantura</i> sp.	Australia	FJ843598	Fyler et al., 2009
<i>A. santarosaliense</i>	a	<i>Heterodontus mexicanus</i>	Santa Rosalia, Mexico	KF685751	Caira et al., 2014
<i>A. wedli</i>	a	<i>Zearaja nasuta</i>	South Island, New Zealand	MH913270	Bennett et al., 2019
<i>A. zimneri</i>	a	<i>Himantura</i> sp.	Australia	FJ843602	Fyler et al., 2009
<i>A. sp. 1</i>	a	<i>Dasyatis longus</i>	Mexico	AF286953	Olson et al., 2001
<i>A. sp.</i>	l	<i>Paralichthys lethostigma</i>	Northern Gulf of Mexico	GQ470124	Jensen and Bullard, 2010
<i>A. sp.</i>	l	<i>Lagodon rhomboides</i>	Northern Gulf of Mexico	GQ470115	Jensen and Bullard, 2010
<i>A. sp.</i>	l	<i>Diplectrum formosum</i>	Northern Gulf of Mexico	GQ470116	Jensen and Bullard, 2010
<i>A. sp.</i>	l	<i>Cynoscion nebulosus</i>	Northern Gulf of Mexico	GQ470121	Jensen and Bullard, 2010
<i>A. sp.</i>	l	<i>Nectocarcinus antarcticus</i>	Otago, New Zealand	MH924014*	Bennett et al., 2019
<i>A. sp.</i>	l	<i>Trachurus novaezelandiae</i>	Otago, New Zealand	MH924015*	Bennett et al., 2019
<i>A. sp.</i>	a	<i>Himantura</i> sp.	Australia	FJ843592	Fyler et al., 2009
<i>A. sp. 6A</i>	a	<i>Dasyatis say</i>	Northern Gulf of Mexico	GQ470109	Jensen and Bullard, 2010
<i>A. sp. 6B</i>	a	<i>Dasyatis say</i>	Northern Gulf of Mexico	GQ470110	Jensen and Bullard, 2010
<i>A. sp. 6C</i>	a	<i>Dasyatis say</i>	Northern Gulf of Mexico	GQ470113	Jensen and Bullard, 2010
<i>A. sp.</i>	l	<i>Octopus vulgaris</i>	Tyrrhenian Sea, Italy	MN660284-285	Tedesco et al., 2020
<i>Potamotrygonocestus</i> cf. <i>figgeraldae</i>	a	<i>Potamotrygon castexi</i>	Madre de Dios River, Peru	KF685773	Caira et al., 2014

\* Sequence data available for only D2 region of 28S rDNA gene.

### 3. Results

#### 3.1. General data

Number and species of elasmobranchs examined, their biometrical data, depth of sampling, and basic levels of infection for *Acanthobothrium* species are listed in Table 2. Of the nine fish species examined, we only found individuals infecting the intestine of *S. stellaris*. All the specimens found were identified as belonging to *A. coronatum*. Voucher specimens have been deposited at the Collection of the Stazione Zoologica Anton Dohrn in Naples (Italy) with the following accession

numbers: SZN-PLA0001-37. Prevalence and mean intensity of infection in 10 *S. stellaris* were 80% and 12.1, respectively.

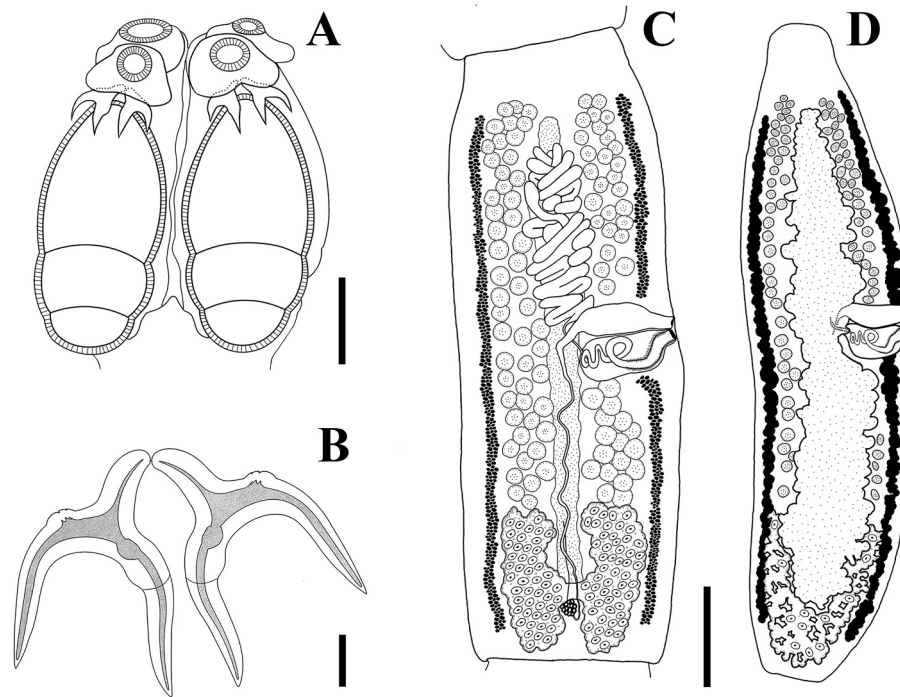
#### 3.2. Morphological analysis

Description (based on 15 mature specimens) (Figs. 1–3): *A. coronatum* is a category 4 species. Strobila acraspedote, apolytic,  $11.8 \pm 1.51$  (9.6–14.8, 15) cm long, composed of  $344.3 \pm 18.21$  (312–373, 15) proglottids. Greatest width at the level of gravid proglottids. Scolex consists of a scolex proper and a cephalic peduncle. Scolex proper  $1038.34 \pm 116.27$  (889.31–1217.35, 15) long by  $954.17 \pm 86.71$

**Table 2**Number, sex (f: female; m: male), total body length (TL: expressed as mean value with range in parenthesis), sampling depth, and basic levels of infection for *Acanthobothrium coronatum* in elasmobranch fishes examined from the Gulf of Naples.

	n	Sex (f/m)	TL (cm)	Depth (m)	Prevalence (%)	Mean intensity (range)
<i>Dalatias licha</i>	3	3 f	99.6 (91–103)	500–600	–	–
<i>Etmopterus spinax</i> *	39	23 f/16 m	28.6 (14–38)	400–600	–	–
<i>Galeus melastomus</i> *	91	48 f/43 m	43.3 (23.5–58)	400–600	–	–
<i>Scyliorhinus canicula</i> *	104	52 f/52 m	40.5 (14–50.2)	400–600	–	–
<i>S. stellaris</i> *	10	9 f/1 m	60.5 (33–81)	100–400	80	12.1 (1–33)
<i>Raja asterias</i> *	11	8 f/3 m	52.3 (48–57.5)	50–250	–	–
<i>R. clavata</i> *	22	16 f/6 m	54.8 (35.5–89.2)	100–250	–	–
<i>Torpedo marmorata</i> *	38	15 f/23 m	23.4 (15–37.5)	100–250	–	–
<i>T. torpedo</i> *	27	21 f/6 m	28.6 (17.5–44)	10–250	–	–

\* Elasmobranch species reported as host of *A. coronatum* in the literature (see Zaragoza-Tapia et al., 2020; <https://shark-references.com/species/parasite-hosts-list/A>).



**Fig. 1.** Line drawing of *Acanthobothrium coronatum* from the intestine of the nursehound *Scyliorhinus stellaris* in the Tyrrhenian Sea. (A) Scolex. (B) Hooks. (C) Mature proglottid. (D) Gravid proglottid. Scale bar: A = 200  $\mu$ m; B = 50  $\mu$ m; C, D = 500  $\mu$ m.

(765.32–1048.38, 15) at maximum width, composed of 4 trilobular bothridia each armed with a pair of bi-pronged hooks surmounted by apical sucker and pad. Each hook is embedded in the scolex immediately in front of each bothridium. Microtriches on the scolex surfaces not observed.

Bothridia free posteriorly,  $869.98 \pm 130.33$  (625.64–966.60, 30) long by  $409.19 \pm 71.38$  (303.31–474.35, 30) wide; maximum width at the level of middle locus. Anterior locus  $488.08 \pm 91.54$  (319.39–551.32, 20) long, middle locus  $205.53 \pm 38.42$  (138.55–259.69, 20) long, posterior locus  $181.64 \pm 24.06$  (149.55–226.24, 20) long. Ratio of locular lengths 1:0.42:0.37 (anterior:middle:posterior). Apical pad  $156.37 \pm 29.69$  (121.82–208.66, 20) long by  $265.20 \pm 25.77$  (232.48–310.46, 20) at maximum width, falciform in shape; suckers  $138.57 \pm 18.48$  (115.58–159.04, 20) in diameter. Hooks hollow, with tubercle on proximal surface of axial prong. Lateral and medial hooks equal in size. Lateral hook measurements ( $n = 10$ ): A:  $105.12 \pm 3.69$  (101.04–109.83), B:  $147.71 \pm 7.77$  (135.43–154.64), C:  $130.75 \pm 8.66$  (118.49–141.86), D:  $238.30 \pm 9.23$  (222.24–244.46). Medial hook measurements ( $n = 10$ ): A:  $105.71 \pm 2.06$  (103.62–108.82), B:  $151.42 \pm 9.37$  (143.35–167.64), C:  $125.76 \pm 6.24$  (105.76–120.50), D:  $239.00 \pm 11.86$  (223.89–244.81).

Cephalic peduncle  $2188.95 \pm 403.74$  (1595.98–2843.66, 15) long by  $599.16 \pm 114.79$  (439.94–701.02, 15) wide, aspinose. Immature proglottids wider than long. Mature proglottids  $2781.04 \pm 315.51$  (2207.92–3443.73, 20) long by  $1110.96 \pm 80.82$  (917.72–1201.34, 20) wide. Gravid proglottids  $7843.92 \pm 738.73$  (7039.51–8992.17, 20) long by  $1383.73 \pm 134.69$  (1231.43–1603.00, 20) wide. Genital pores alternating irregularly, 48.97% (46.41–53.62%, 20) from anterior end of proglottid. Cirrus sac  $505.46 \pm 94.84$  (364.99–673.83, 20) long, containing eversible armed pyriform cirrus  $623.09 \pm 167.70$  (367.81–864.28, 20) long by  $171.86 \pm 12.27$  (158.55–198.85, 20) wide. Testes spherical extending in two lateral fields from anterior margin of ovarian lobes to near anterior end. Testes,  $124.71 \pm 8.64$  (113.68–136.80, 30) in diameter,  $99.3 \pm 6.3$  (88–112, 30) in number per mature proglottid;  $28.81 \pm 6.02$  (19–39) preporally,  $19.31 \pm 1.92$  (17–23) postporally,  $51.18 \pm 3.52$  (44–57) antiporally. Ovary H-shaped

at posterior end of proglottid, symmetrical; lobes  $928.13 \pm 114.36$  (861.03–1160.75, 30) long by  $781.35 \pm 30.79$  (746.81–834.36, 30) wide at isthmus. Mehlis' gland posterior to ovarian isthmus.

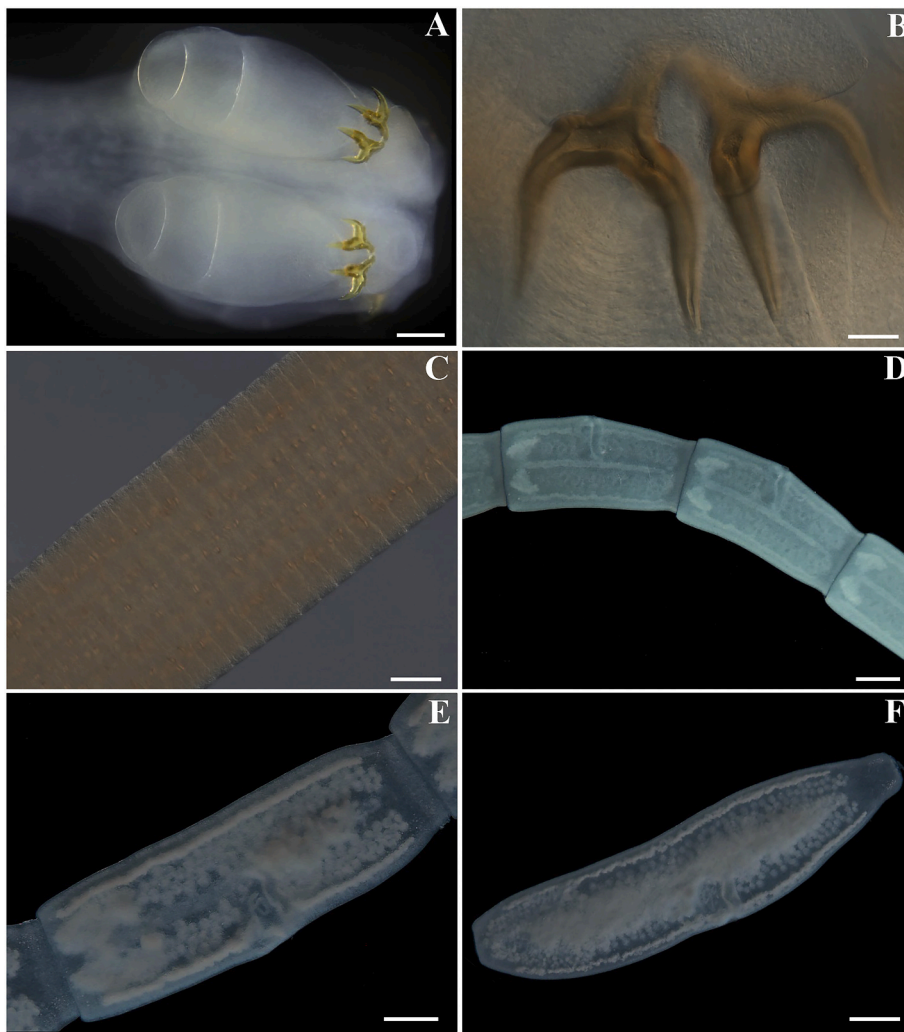
Vagina thick-walled, extending anteriorly from ovarian isthmus along median line to anterior margin of cirrus sac, then laterally along anterior margin of cirrus sac to genital atrium. Minute vitelline follicles in form of lateral bands, each consisting of 1/3 columns of follicles extending from posterior end to near anterior end of the proglottid. Uterus sacciform, extending anteriorly from ovarian isthmus along median line to approximately to anterior margin of primary testicular field. Eggs from the uterus of gravid proglottids, spherical  $79.15 \pm 6.52$  (71.21–93.4, 25) in diameter.

### 3.3. Remarks

With the aim to delimit the number of taxonomic comparisons when identifying *Acanthobothrium* species, Ghoshroy and Caira (2001) grouped the known species into 10 categories (coded from 1 to 10) based on morphologic and morphometric characters. *Acanthobothrium coronatum* is a species of the category 4, which comprises a total of 37 taxa with the following general characters: total length >50 mm, number of proglottids >50, number of testes >80, and symmetrical ovary (Ghoshroy and Caira, 2001; Zaragoza-Tapia et al., 2020).

In the Mediterranean Sea, a total of nine species of *Acanthobothrium* have been found, namely *A. bataillonii* Euzet, 1955, *A. benedenii* Lönnberg, 1889, *A. coronatum* Rudolphi, 1819, *A. filicollis* Zschokke, 1888, *A. magnum* Euzet, 1959, *A. manteri* Hassan, 1983, *A. minus* Tazerouti, Kechemir-Issad and Euzet, 2009, *A. rajaebatis* Rudolphi, 1810, and *A. zschokkei* Baer, 1948. According to Zaragoza-Tapia et al. (2020), only two of those belong to category 4 (namely, *A. coronatum* and *A. magnum*). However, the ovary of *A. magnum* was originally described as that of *A. crassicolle* (i.e., asymmetrical) (Euzet, 1959), and thus this latter anatomical character moves *A. magnum* into the category 3 sensu Ghoshroy and Caira (2001) and Fyler and Caira (2006), although such observation is based on a single specimen. *Acanthobothrium magnum* can be distinguished from *A. coronatum* also by possessing





**Fig. 2.** Microscopic features of *Acanthobothrium coronatum* from the intestine of the nursehound *Scyliorhinus stellaris* in the Tyrrhenian Sea. (A) Scolex showing the trilocular bothridia each armed with a pair of bifid hooks surmounted by the apical sucker and pad. (B) Enlarged view of the bifid hooks surmounted by the pad. (C) Strobila showing immature proglottids just below the cephalic peduncle. (D) Immature proglottids at about the middle of the strobila. (E) Mature proglottids at about the end of the strobila. (F) A free gravid proglottid. Scale bar: A = 200  $\mu$ m; B = 50  $\mu$ m; C = 100  $\mu$ m; D, E, F = 500  $\mu$ m.

a longer strobila length (50 cm), different shape and size of hooks, and the greater number of testes (220 in number) (Euzet, 1959).

Among the Mediterranean species, *A. coronatum* closely resembles *A. rajaebatis*, with which it was commonly confused in the past literature (Euzet, 1959). However, *Acanthobothrium rajaebatis* has only been found in *Dipturus oxyrinchus*, *Rostroraja alba*, and *Dipturus batis* so far (Euzet, 1959). Moreover, this species has been recently listed by Zaragoza-Tapia et al. (2020) in category 5 because the number of testes was considered <80, although Euzet (1959) and Goldstein (1967) reported this number as ranging from 58 to 85. In general, *Acanthobothrium rajaebatis* can be distinguished from *A. coronatum* by the different shape and size of hooks, shorter strobila (5–6 cm), and the smaller number of testes and proglottids (80–120) (Euzet, 1959).

### 3.4. Molecular data and phylogenetic analysis

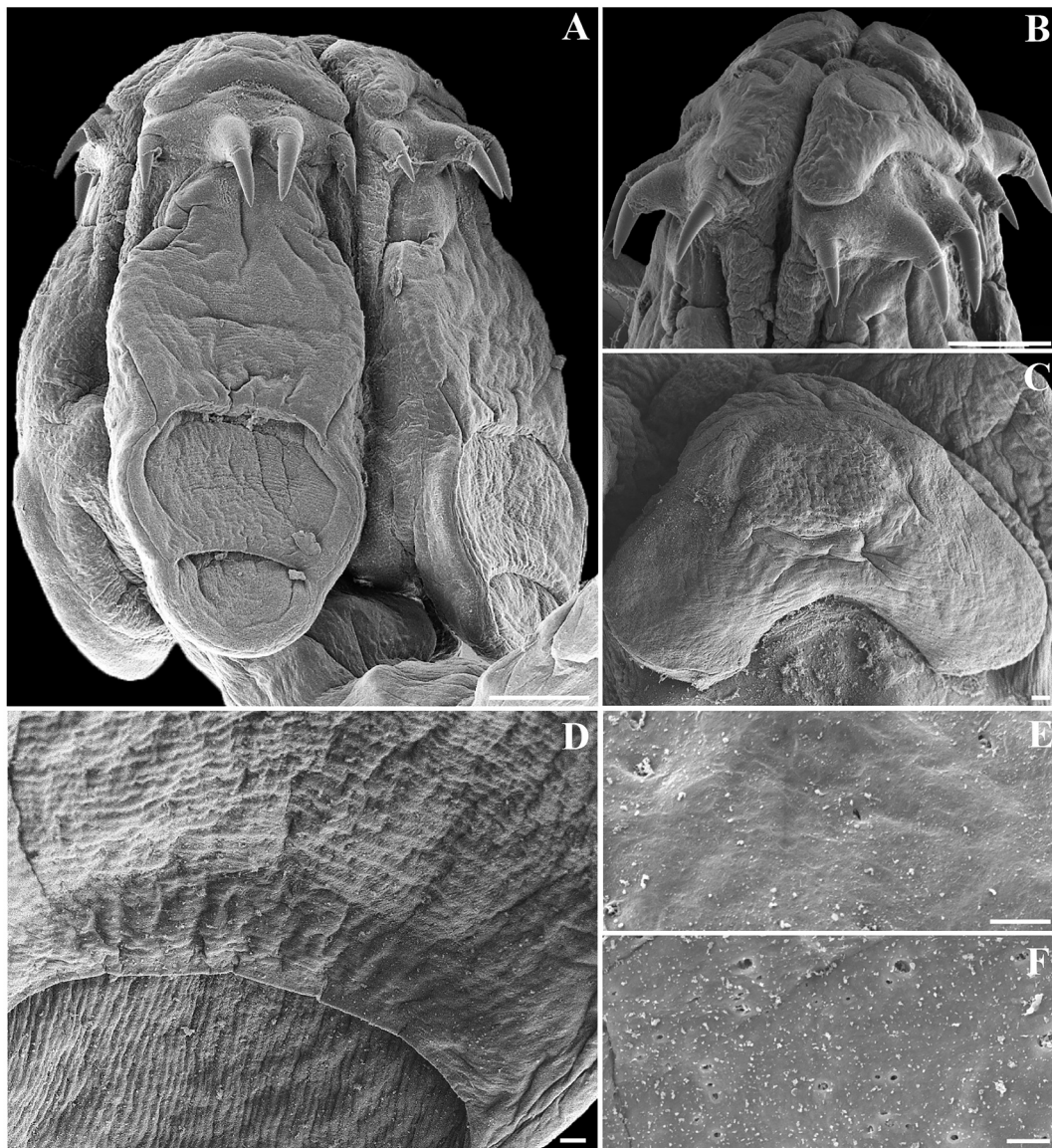
The BLASTn search of the sequences obtained at the LSU gene locus of *A. coronatum* retrieved a percentage of identity of 99.8–100% with the sequences MN660284–285 ascribed to unidentified *Acanthobothrium* larvae found in the common octopus *Octopus vulgaris* Cuvier, 1797 from the Tyrrhenian Sea (Tedesco et al., 2020).

Because ML and BI were 100% congruent, only the ML topology is shown in Fig. 4. The tree topologies placed the sequences of *A. coronatum* within a well-supported clade, which also included the *Acanthobothrium* larvae mentioned above. The sequences included in this clade came out as closely related to *Acanthobothrium wedli* Robinson,

1959 (MH913270), *Acanthobothrium santarosaliense* Caira and Jensen, 2001 (KF685751), and other two unidentified *Acanthobothrium* species (GQ470124 and GQ470115) (Fig. 4). At the intraspecific level, sequences of *A. coronatum* and those of the *Acanthobothrium* larvae (MN660285–286) deposited by Tedesco et al. (2020) showed a distance value of 0.030%, while distance values of 3.1% and 2.6% were recorded between the sequences of *A. coronatum* and respectively the above mentioned sequences of *A. wedli* and *A. santarosaliense*. Sequences of *A. coronatum* obtained in the present study were deposited in GenBank under the accession numbers MZ243216–19.

### 4. Discussion

*Acanthobothrium* species typically exhibit strong (i.e., oioxenous) associations with their hosts (Jensen and Bullard, 2010; Caira et al., 2014; Caira and Jensen, 2001, 2014; Zaragoza-Tapia et al., 2020). All *Acanthobothrium* species found in the Mediterranean Sea were only found in skates and/or rays, except *A. coronatum*, that, in disagreement with previous statements, exhibited non-specific host-associations with at least 26 species of elasmobranchs, including various shark species (Zaragoza-Tapia et al., 2020; <https://shark-references.com/species/parasite-hosts-list/A>). However, the knowledge on Mediterranean *Acanthobothrium* taxa is outdated. In fact, according to Zaragoza-Tapia et al. (2020), the most recent finding of an *Acanthobothrium* species from the Mediterranean Sea dates back Euzet (1959), except for *A. minus*, a taxon recently described from off Algerian coast (Tazerouti et al., 2009a,



**Fig. 3.** Scanning electron micrographs of *Acanthobothrium coronatum* from the intestine of nursehound *Scyliorhinus stellaris* in the Tyrrhenian Sea. Scolex, frontal (A) and lateral (B) views. Enlarged view of sucker and pad (C). Enlarged view of surfaces of middle and posterior loculi (D). Higher magnification of proximal (E) and distal (F) surfaces of scolex showing the lack of microtriches. Scale bar: A, B: = 100 µm; C, D = 10 µm; E, F = 2 µm.

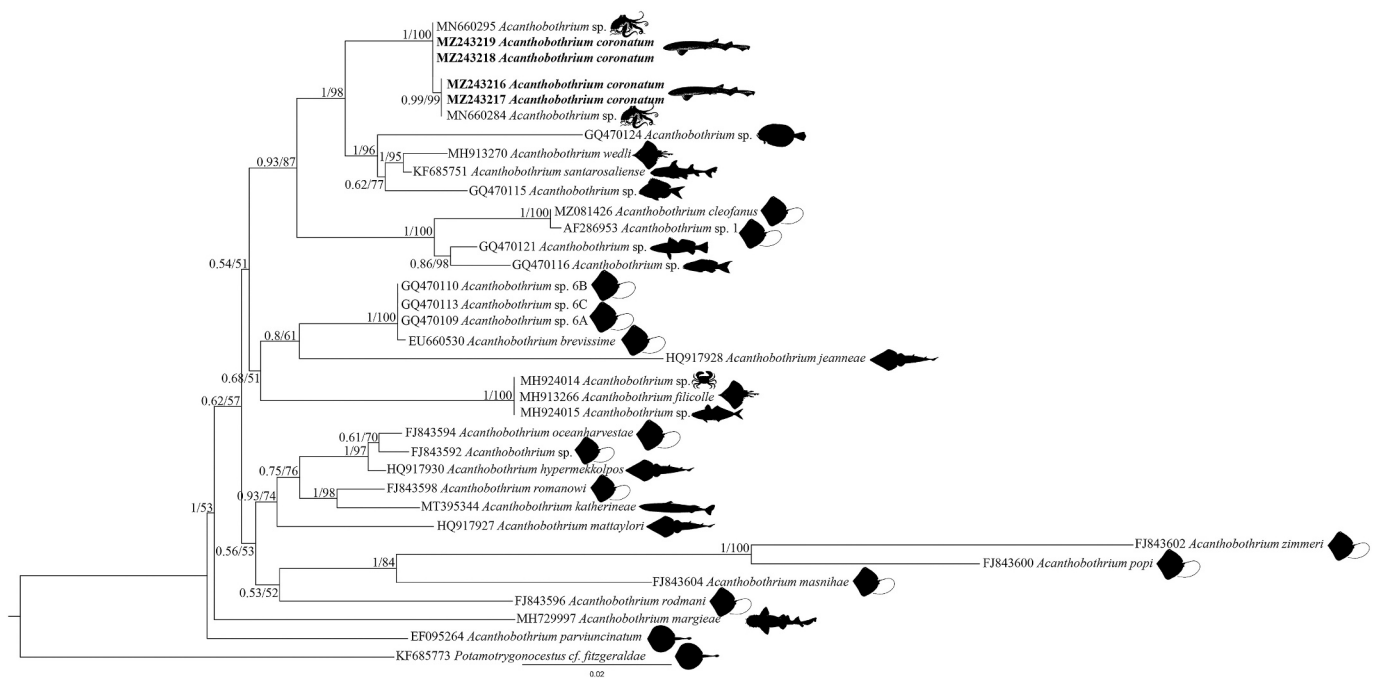
2009b). Present results confirm the necessity to update host-association based on molecular data, as *A. coronatum* was found to be more species-specific than previously reported, at least in the Gulf of Naples. *Scyliorhinus stellaris* was the only final host found among the nine elasmobranch species here studied, including two additional species, namely *T. marmorata* and *T. torpedo*, listed by Rudolphi (1918) as hosts of this cestode from the same locality investigated here (see Table 2). The present results are also in agreement with Euzet (1952, 1959) and Rees and Williams (1965), who already raised doubts about the past identifications of *A. coronatum*. Notwithstanding that, the study of more individuals of species here investigated, as well as additional elasmobranchs, would confirm whether *A. coronatum* is truly a host-specific parasite.

With regards diagnostic characters of *A. coronatum*, present morphometric and morphologic data regarding strobila length, number of proglottids, size and shape of hooks, and genitalia features are congruent with those presented by Euzet (1959) and Rees and Williams (1965), whereas apparent differences were noticed in number of testes per mature proglottid: 106 (range: 80–125) in Euzet (1959), from 81 to

115 in Rees and Williams (1965) and 99.3 (range: 88–112) in the present study. However, a wide range of intraspecific variability in testes number has been already found in many species of *Acanthobothrium* (see Zaragoza-Tapia et al., 2020), and even among the proglottids of a same strobila in *A. coronatum* from *S. stellaris* from Great Britain waters (Rees and Williams, 1965).

With regards life-cycle of *Acanthobothrium* species, a 3–5 host life-cycle has been proposed within onchoproteocephalideans, with copepods and molluscs acting as first and second intermediate hosts and fish as intermediate/paratenic hosts (Caira and Reyda, 2005; Jensen and Bullard, 2010). However, no life-cycle is currently known for any member of the genus *Acanthobothrium*, with one of the primary factors contributing to this dearth of information being that the morphological characters of the larval stages do not resemble their adult counterparts, and thus larvae are difficult to accurately identify using morphological criteria alone (e.g., Joyeux and Baer, 1961). Only in the last decade, the molecular approach has been successfully applied in order to identify cestode larvae, although its usefulness remains limited until the availability of more molecular data.





**Fig. 4.** Phylogenetic tree from maximum likelihood based on 28S rDNA sequences of *Acanthobothrium coronatum* obtained in the present study, with respect to the sequences of the genus *Acanthobothrium*, at the same gene locus available in GenBank. *Potamotrygonocetus fitzgeraldae* was used as outgroup. Nodal supports are indicated for BI (posterior probabilities) and for ML (bootstrap,  $n = 1000$ ). The sequences obtained in this study are in bold. Taxon labels consist of GenBank number followed by species name and icons that indicate if the specimen was collected from a definitive elasmobranch host (i.e., ray, skate or shark) or intermediate/paratenic host (i.e., teleost, crustacean or mollusc).

Most of the previous records of larvae morphologically identified as *Acanthobothrium* come from a wide range of molluscs including cephalopods (Dollfus, 1923, 1929), gastropods, and bivalves (Regan, 1963; Harry, 1969; Cake, 1976). More recent studies from the Gulf of Mexico using the 28S rDNA marker identified larvae of *Acanthobothrium brevisse* Linton, 1909 in the razor clam *Ensis minor* (Chenu, 1843) and the amphioxus *Branchiostoma floridae* Hubbs, 1922 (Holland et al., 2009; Holland and Wilson, 2009), while larvae of an unidentified species of *Acanthobothrium* were found infecting teleost fishes of seven different families (Jensen and Bullard, 2010). By using the same marker, Bennett et al. (2019) revealed that *Acanthobothrium* larvae found in the crab *Nectocarcinus antarcticus* (Hombron and Jacquinot, 1846) and the mackerel *Trachurus novaezelandiae* Richardson, 1843 are conspecific with adults found in the intestine of the rough skate *Zearaja nasuta* (Müller and Henle, 1841) from New Zealand, providing important evidence on the trophic transmission pathways of this tapeworm.

The present molecular characterization confirmed that all specimens analysed here belong to a single species, identified as *A. coronatum* through morphology. The 28S rDNA sequences here obtained showed high levels of similarity with larvae found in the digestive tract of the common octopus from the Gulf of Naples (Tedesco et al., 2020), confirming conspecificity between the samples. Thus, present results suggest that the trophic transmission pathways of *A. coronatum* in *S. stellaris* could occur through the predation on octopuses. Indeed, the presence of an adult tapeworm within the intestine of its definitive host and its larvae within this prey provides convincing evidence for a trophic transmission of that parasite (Randhawa and Brickle, 2011; Bennet et al., 2019). Further supporting our hypothesis is the fact that octopuses are among the most important prey item of *S. stellaris* (Capape, 1975; Ellis et al., 1996). In fact, several cephalopod beaks were found during the present necropsies of *S. stellaris*.

The present phylogenetic analysis included only a relatively small subset of the over 208 valid species of *Acanthobothrium*, because only the 28S sequences of 17 species are available in GenBank, with no sequences known from the Mediterranean Sea. Therefore, it is possible that the

present phylogenetic results may not reflect the true relationships, as a large majority of *Acanthobothrium* species is missing from the present analysis. The tree topologies placed *A. coronatum* within a well-supported clade, which also included the *Acanthobothrium* sequences found in the common octopus (Tedesco et al., 2020). Moreover, the tree topologies placed the species *A. wedli* from the New Zealand rough skate, *A. santarosaliense* from the Mexican hornshark *Heterodontus mexicanus* Taylor and Castro-Aguirre, 1972, and two *Acanthobothrium* species respectively found in two intermediate/paratenic hosts [i.e., the southern flounder *Paralichthys lethostigma* Jordan and Gilbert, 1884 and the pinfish *Lagodon rhomboides* (Linnaeus, 1766)] in a well-supported major clade with sequences of *A. coronatum*. Of these, both *A. coronatum* and *A. santarosaliense* parasitize sharks as adults, while *A. wedli* parasitizes skates. However, the other two species of *Acanthobothrium* recorded in sharks (i.e., *A. katherineae* Gallagher and Caira, 2020 and *A. margiae* Fyler and Caira, 2011) were scattered in the tree. The fact that those *Acanthobothrium* species are distributed within different clades of batoid hosts suggests that the individual host-switching events may underlie the evolutionary history of this host-parasite system, as previously suggested (Caira, 2011; Bennet et al., 2019). A future phylogenetic inference of further taxa of this genus, possibly based on the application of a multigene approach, would clarify the taxonomic status of species belonging to genus *Acanthobothrium*. This could also widen our knowledge on tapeworm colonisation histories and their geographic diversification patterns in elasmobranchs and clarify the patterns of host-parasite co-evolutionary events (Caira and Jensen, 2001; Caira, 2011; Bennet et al., 2019; Gallagher and Caira, 2020).

In conclusion, infection patterns found in the present study support the hypothesis that at least in the investigated area *S. stellaris* is the main definitive host of *A. coronatum*, and that the host-list of this species should be reassessed with modern methodologies. This also represents the first molecular study revealing conspecificity between the adults of an *Acanthobothrium* species hosted by a shark and their larvae found in an intermediate/paratenic host, revealing, at least in part, the trophic

transmission pathways of *A. coronatum* for *S. stellaris*. Finally, our study provides new taxonomic, molecular, and phylogenetic data that allow for a better characterization of this neglected parasite from its type locality.

## Credit author statement

Mario Santoro: Conceptualization, Methodology, Validation, Supervision, Formal analysis, Investigation, Writing – original draft. Fabio Crocetta: Methodology, Writing – review & editing. Marialetizia Palomba: Methodology, Formal analysis.

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## Declaration of Competing Interest

The authors declare no conflict of interest.

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