

Postmonorchis sp. inq. (Digenea: Monorchiidae) metacercariae infecting natural beds of wedge clam *Donax trunculus* in Italy

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ABSTRACT: The wedge clam *Donax trunculus* Linnaeus, 1758 is one of the most common bivalve molluscs inhabiting the sandy shores of the Mediterranean Sea and is considered an important commercial resource. In this study, we report the first molecular, morphological and histopathological descriptions of metacercariae from a trematode belonging to the genus *Postmonorchis* (Digenea: Monorchiidae) that infects *D. trunculus* in natural beds of the Italian Tyrrhenian coast (Campania, Lazio and Tuscany). Morphological analysis of the parasite revealed a combination of features that exist in the 3 previously identified species of *Postmonorchis*, viz. *P. donacis*, *P. variabilis* and *P. orthopristsis*, with the addition of new, distinctive morphological characteristics. The pathogen exhibited a predilection for the gill; however, it was also present in the labial palp and mantle in addition to the gut, kidney epithelium and foot. The inflammatory response was characterised by either a focal or diffuse haemocyte infiltration followed by the formation of multiple, large multi-layered capsules associated with tissue destruction. The prevalence of the pathogen ranged from 75 to 100%, while the infection intensity fluctuated among the study areas. Further studies regarding the life cycle of this parasite and the identification of other larval and adult stages and their respective hosts may confirm the identification of a new species of *Postmonorchis* that infects wedge clams in Mediterranean waters. The study of the parasite is completed by molecular analysis of the ITS1 and ITS2 rDNA sequences.

KEY WORDS: Donacidae · Bivalvia · Metacercariae · Monorchiidae · *Postmonorchis* · Trematoda · Parasite · Mediterranean · ITS

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INTRODUCTION

During the last several years, research regarding bivalve diseases has significantly increased, particularly with respect to the bacterial and protozoan agents in cultured species that are listed by the World Organisation for Animal Health (OIE) (Berthe 2008). Studies of Platyhelminthes have focused primarily on taxonomy and rarely on the lesions that are linked to the host–parasite interaction (Doanh et al. 2007, de Montaudouin et al. 2009).

Trematodes have complex life cycles with asexual forms parasitizing the internal organs of molluscs, whereas the sexual stages occur mainly in vertebrates and only occasionally in invertebrates. Depending on the species, the larvae of most trematodes need a second intermediate host, which may be either an invertebrate or a vertebrate (Cribb et al. 2003, Madhavi 2008).

In the Mediterranean Sea, parasitosis in bivalve molluscs is caused mainly by faustulid, fellodistomid, gymnophallid and bucephalid trematodes (Palombi

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1934, Lauckner 1983, Ramòn et al. 1999, Culurgioni et al. 2006). In contrast, reports on the Monorchidae family have mostly described species in the Atlantic or Pacific Ocean, where the cercariae develop within sporocysts of bivalve species such as *Cumingia tellinoides*, *Abra ovata*, *Macoma nasuta* (Martin 1940, Maillard 1975, Bartoli 1984) and *Donax gouldii* (Young 1953, Winter & Hatch 2010). The only reported case in the Mediterranean Sea is *Cerastoderma edule* infected by *Monorchis parvus* (Bartoli et al. 2000).

The wedge clam *Donax trunculus* is an important marine resource obtained by the exploitation of natural beds, distributed from the Atlantic coast of France to Senegal and the shallow bottoms of the Mediterranean Sea (Gaspar et al. 2002, Manca Zeichen et al. 2002, La Valle 2006). Marketable landings of this bivalve are in the range of 535 to 970 t yr⁻¹ in Europe (Food and Agriculture Organisation of the United Nations—Fisheries Global Information System: www.fao.org), and although fishing has slightly declined in recent years, the wedge clam remains a significant commercial species for Italy. Despite the importance of the wedge clam, little is known about the pathogens and parasites that affect the health of *D. trunculus* populations. Recent studies suggest the use of this species as a bioindicator of physiological and cellular alterations caused by pollution (Fishelson et al. 1999, Yawetz et al. 2010). Moreover, some reports refer to the parasitic castration of *D. trunculus* caused by the faustulid *Bacciger bacciger* and damage from the gymnophallid *Meiogymnophallus strigatus* or a *Rickettsia*-like organism (Comps 1979, Bartoli 1983, Ramòn et al. 1999), with no record of Monorchidae infections.

In this report, we present the first study of *Postmonorchis* sp. inq. (Digenea: Monorchidae) metacercariae infecting the wedge clam *Donax trunculus* along the Italian coast of the Mediterranean Sea. The parasite was detected in the years 2009, 2010 and 2012. After a histopathological study on wedge clams, in order to assess the taxonomic position of the parasite, it was characterised morphologically and analysed at the molecular level by sequencing the internal transcribed spacer regions 1 and 2 of the ribosomal DNA.

MATERIALS AND METHODS

Sampling

Donax trunculus specimens were collected during the summer from different geographical areas over a period of several years. The sampling sites in the

Campania region were the mouth of the Volturno River (41° 0' 57.24" N, 13° 56' 49.56" E) during July 2009/2010 (20 clams) and Litorale Domizio (41° 4' 8.39" N, 13° 51' 3.65" E) during July 2012 (20 clams). In September 2012, samples were collected at Formia (41° 14' 20.73" N, 13° 36' 43.42" E) in the Lazio region (20 clams) and at sites in the Tirrenia locality (10° 17' 04" N, 43° 37' 28" E) in Tuscany (100 clams), while samples were also collected at Feraxi Beach (39° 19' 07" N, 9° 13' 19" E) in eastern Sardinia (30 clams; Fig. 1). The clams were collected from the shore using a hand-held semi-circular dredge as used by the local fishermen. The clams were immediately transferred into ~15 l of water collected from the sampling location and maintained in a cool environment in an insulated sealed container for transit to the laboratory with a maximum transit time of 2 h. The samples were opened in the laboratory and examined for external signs under a dissecting microscope (Nikon SMZ-10). To obtain a positive identification of the *Donax* species as the Mediterranean *D. trunculus*, 5 samples were submitted to expert taxonomists at the Zoological Museum of Naples. The observed metacercarial cysts were scraped off the surface of gills and labial palps, and the wet mounts were examined by light microscopy. The cysts were stored in 70 and 100% alcohol for morphological identification and further molecular analysis, respectively. Specimens were also deposited in the *Scientific Catalogue of the Museum Collection* of the Department of Biology, University of Naples Federico II under reference number DBCUnina-Plat00057.

Light microscopy

The bivalves were fixed in Davidson's fixative for 24 to 48 h and then processed for routine histopathology. Two transverse sections, approximately 5 mm thick, including the mantle, gonad, digestive gland, gills, kidney and foot, were excised from each clam and placed into histological cassettes. To obtain a more complete view of the parasitosis, a 5 mm piece of the gill was also removed from each animal and included with the sample in the longitudinal orientation. The tissue samples were embedded in paraffin wax, sliced in 5 µm sections and subsequently stained with haematoxylin and eosin (H&E). Additional stains, such as PAS-BA and Masson's Trichrome (Mazzi 1977), were also used.

Parasite prevalence, infection intensity and immune response efficiency were also evaluated at the sites of infection, including the gill, mantle and labial palps.

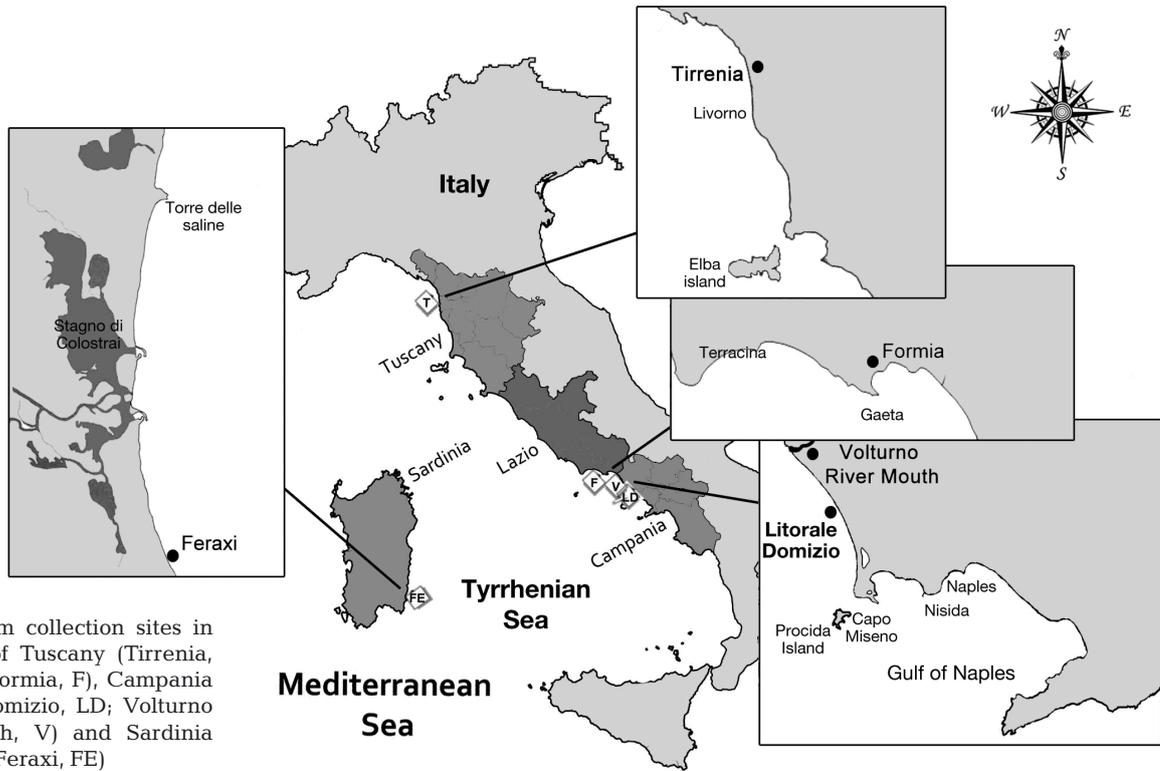


Fig. 1. Clam collection sites in the areas of Tuscany (Tirrenia, T), Lazio (Formia, F), Campania (Litorale Domizio, LD; Volturno River mouth, V) and Sardinia (Feraxi, FE)

For the gill tissues, the data were obtained by histological examination (histological infection density, HID; Bush et al. 1997). In particular, the value of intensity was determined on a fixed area of 8 mm² in 2 consecutive slides under 10× magnification. For the labial palp and mantle the infection intensity (macroscopic infection intensity, MII) was calculated under a dissecting microscope by counting the number of cysts ind.⁻¹. All micrographs were captured using a Nikon DS-Fi1 video camera mounted on a Nikon 50i microscope connected to a computer. The images were shown on the monitor screen, and the number of cysts was obtained using the cell counter plug-in of *ImageJ*, a public domain imaging package (National Institutes of Health, USA: <http://rsb.info.nih.gov/ij/>). For the HID values, we established an arbitrary scale with the following 3 stages: (1) low intensity, values of ≤50 cysts ind.⁻¹; (2) moderate intensity, values of 50 to 70 cysts ind.⁻¹; (3) high intensity, values of ≥70 cysts ind.⁻¹. Moreover, to obtain information regarding the host defence reaction against the parasite, the immune response efficiency (IRE) value was determined. This value was obtained by counting the number of capsules in which the metacercariae were dead from a total of 100 consecutive capsules and is reported as a percentage (%). All other values are reported as the mean ± SD.

For morphological observations, the metacercarial cysts were scraped from the infected tissues and examined as wet mounts. A number of fixed specimens were stained with acetic carmine to highlight the developing organs. Measurements were obtained from 10 well-developed metacercariae using an Olympus BX41 microscope with a calibrated ocular micrometer. Drawings were made with the aid of a camera lucida. All measurements are expressed in micrometres (µm).

Morphological analysis was performed according to the taxonomic keys of the family Monorchiidae (Hopkins 1941, Bray 2008a,b, Madhavi 2008) and the descriptions of species of the genus *Postmonorchis* (Hopkins 1941, Young 1953, Prudhoe & Bray 1973).

DNA amplification, sequencing and analysis

Genomic DNA from the infected gills of 3 animals was extracted by Proteinase K digestion at 56°C for 16 h followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The DNA concentration was measured using a Nanodrop 2000c spectrophotometer (ThermoScientific), and the DNA quality was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide.

The ribosomal DNA (rDNA) region including the internal transcribed sequence 1 (ITS1), 5.8S and ITS2 was PCR amplified using the method of Bowles et al. (1995) and universal primers that anneal within the conserved 3' terminus of the 18S rDNA and the 5' terminus of the 28S rDNA of *Schistosoma* species. The amplification mixture contained 10 ng template DNA, 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM of each deoxynucleotide, 0.4 μM of each primer and 1.25 units of *Taq* DNA polymerase (Invitrogen) in a final volume of 40 μl. The amplification reaction was performed with an initial hot start at 95°C for 4 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension step of 10 min at 72°C. The PCR products were run on 2% agarose gel.

The amplified fragments were gel eluted and cloned with the pGEM-T Easy Vector Cloning Kit (Promega) before being sequenced using the T7 and SP6 plasmid-specific universal primers and nested primers specific for the rDNA of *Schistosoma* (Bowles et al. 1995). The sequencing reactions were run on a 310 Automated Sequencer (Applied Biosystems).

The nucleotide sequences of ITS1 and/or ITS2 from different Trematoda species were downloaded from GenBank (see Table 2) and aligned with the corresponding region of the sequence obtained in this study. Based on the ITS1 and ITS2 alignments, the maximum likelihood trees were separately constructed using the MEGA5 program (Tamura et al. 2011). Bootstrap analysis was conducted on 1000 replicates.

RESULTS

Gross and microscopic observation

The prevalence of infection, macroscopical and histological infection intensity, and immune response efficiency values observed in the examined areas are reported in Table 1.

Macroscopically, the cysts appeared as whitish pearl-like formations in the organs and tissues of infected clams, such as the mantle, labial palps and gills (Fig. 2). In the gills, the cysts were distributed across the upper, middle and lower part of filaments and were usually aligned in a rosary-like pattern (Fig. 2D). Light microscopy also showed a high occurrence of the pathogen in the gill filaments in addition to the labial palps and mantle but not in the siphons. Moreover, in 10% of samples from the Volturno River mouth (2009), parasites were detected in the foot, the stomach and the kidney–pericardium system in cases of severe infection. Within the gills, the encysted metacercariae were located at the haemal sinus and at the connective tissue of the branchial axis. The cysts revealed a hyaline PAS-positive wall. The structure was rounded and consisted of a single layer approximately 2 μm thick (Fig. 3A).

Histopathology revealed different features of the inflammatory response of the host (Fig. 3B,C). Depending on the case, the presence of metacercarial cysts was associated with a moderate/strong immune reaction characterised by an encapsulation-type response (De Vico & Carella 2012a). Specifically, in cases of mild infection, the capsules comprised a monolayer of flattened immune cells around the cyst. In some cases (13%) of severe infection (154 ± 1.2 to 186 ± 2.4), the inflammatory response was characterised by either a focal or diffuse haemocyte infiltration followed by the formation of multiple, large multi-layered capsules associated with tissue destruction. In some cases, parasite encapsulation resulted in rupture of the cyst wall, haemocyte invasion of the cyst and, finally, pathogen destruction. Notably, the early phases of pathogen development were sometimes histologically discernible. The pathogen was smaller (~200 μm) and had a thinner cyst wall with respect to the other metacercariae. In 10% of the cases, the pathogen appeared to be more susceptible to the host inflammatory response (Fig. 3F).

Table 1. Prevalence (P), mean ± SD histological infection density (HID) at the gill and macroscopic infection intensity (MII) at mantle and labial palp, along with immune response efficiency (IRE) of the parasite infecting *Donax trunculus* from all sampled populations. n: number of specimens analysed; nc: not counted

Areas	n	Date of collection	Mean shell length (cm)	P (%)	HID gill	MII mantle	MII labial palp	IRE (%)
Volturno River Mouth	20	July 2009	2.1	100	99±43.1	4.8±1.7	5±1.9	6
Volturno River Mouth	20	July 2010	2.4	100	109±5.2	3.2±2.2	4±0.2	7
Litorale Domitio	20	July 2012	2.7	100	50.8±1.2	4.3±0.1	5.4±0.2	4
Formia	20	July 2012	2.5	85	45±10.2	4±0.3	3±0.1	3
Tirrenia	100	June 2012	2.4	75.2	3.8±2.2	nc	nc	0
Feraxi	30	September 2012	1.9	0	–	–	–	–

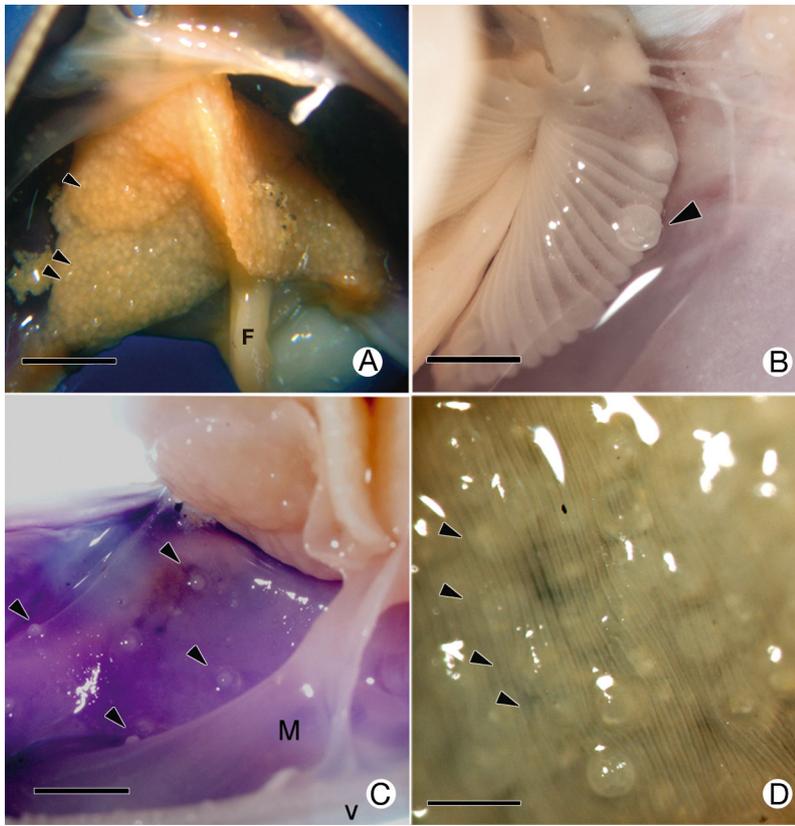


Fig. 2. *Donax trunculus*. Macroscopic views of clams infected with *Postmonorchis* sp. metacercariae. (A) Severe infestation of the gill (arrowheads) in clams from the Volturno River mouth. (B–D) Metacercaria at the labial palp (B), mantle (C) and gill (D) level. F: foot; V: valve; M: mantle. Scale bars = (A) 5 mm, (B,D) 600 μ m, (C) 3 mm

Other histopathological findings included mild hypertrophy or hyperplasia of mucous cells and the connective tissue of the gills as demonstrated by PAS-BA and Trichrome staining, respectively (Fig. 3A,F). The encysted digenean metacercariae were always observed at a high prevalence in the clams (75 to 100%), whereas the infection intensity values fluctuated among the geographical areas. The only exception was the set of samples from Feraxi (Sardinia), which were all negative (Table 1). The HID values were the highest at the Volturno River mouth 2009/2010, and ranged from 35 to 224 cysts ind.⁻¹ in 2009 and from 58 to 206 cysts ind.⁻¹ in 2010. In only a small percentage of the cases did parasite encapsulation resulted in pathogen destruction, with a low immune response efficiency value and a maximum of 8% destroyed cysts in severe infections. In clams from the other geographical areas and years, the histological infection intensity was lower, with minimal values recorded at Tirrenia (3.80 ± 2.21) and no IRE values (Table 1).

Morphological description of the parasite

Gill wet mounts from the infected clams showed the presence of a large number of round cysts of digenean metacercariae (Fig. 2) that were ascribed to *Postmonorchis* sp. inq.

Detailed analysis revealed spherical cysts with a diameter of 270 to 350 μ m (mean 326 μ m) that were transparent and thin-walled so that the eyespots and vitellaria were clearly visible. The early stages of development were also observed. The early cysts were smaller (180–220 μ m) and more thin-walled compared to the older metacercariae, and the worms inside the cysts showed fewer, less distinct characters. Only the spined tegument, compact eyespots, oral and ventral sucker, and a thick-walled excretory bladder were discernible. Therefore, only well-developed metacercariae were used for the morphological description.

A description of the excysted worms, based on 10 specimens, is as follows (Fig. 4): the body is pyriform and elongated and measured 660–710 μ m (mean 655 μ m) long and 232–310 μ m (252 μ m) wide. The tegument is entirely covered with minute spines, and there

are eyespots at the level of the pharynx. The oral sucker is terminal, large and vase-shaped, 130–190 μ m (153 μ m) \times 112–132 μ m (124 μ m). The prepharynx is short. The pharynx is elongated, 72–92 μ m (83 μ m) \times 52–58 μ m (55 μ m). An oesophagus is present. There is intestinal bifurcation anterior to the genital opening. The caeca are long and reach posteriorly to the excretory bladder. The ventral sucker is round to oval, postequatorial, 63–88 μ m (71 μ m) \times 73–102 μ m (81 μ m). The testis is single, close to the posterior extremity, wider than it is long, and oval or reniform, 78–120 μ m (104 μ m) \times 106–198 μ m (159 μ m). The cirrus-sac is dextral and '<'-shaped, it extends posteriorly to the level of the ovarian hind margin, 181–280 μ m (218 μ m) \times 54–88 μ m (69 μ m). The ovary is dextral, compact and adjacent to the testis, 38–72 μ m (47 μ m) \times 30–75 μ m (56 μ m). The metraterm is sinistral and bipartite, 135–155 μ m (146 μ m) \times 58–67 μ m (62 μ m), including a short posterior saccular portion and a terminal portion. The common genital pore is median and is located between the intestinal bifurcation and the

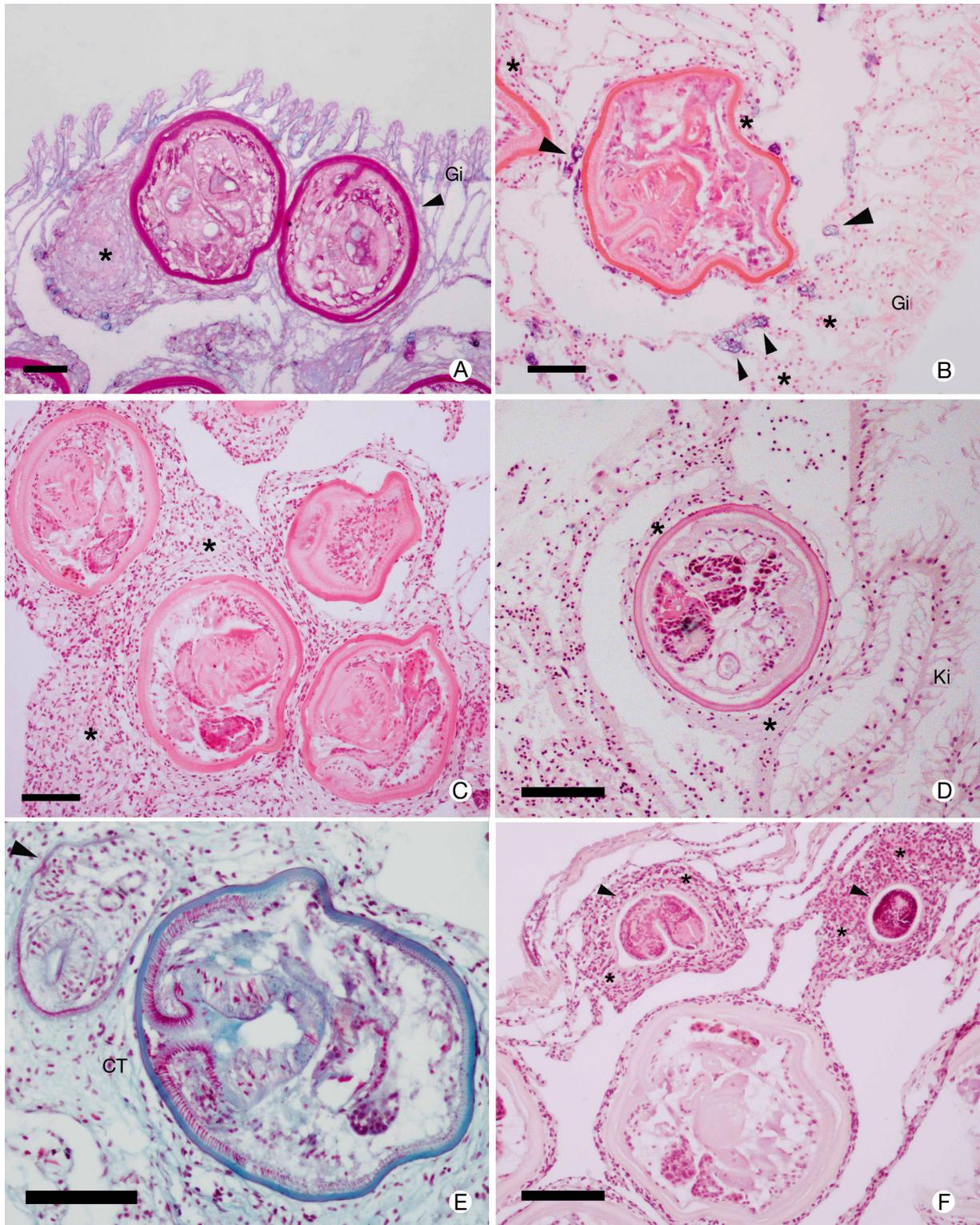


Fig. 3. *Donax trunculus*. Histopathology of wedge clam infected by *Postmonorchis* sp. (A) Parasites infecting the gills (Gi) show PAS-positive cysts; note the efficient capsule; PAS-BA staining. (B) Light inflammatory response accompanied by light mucous cell hypertrophy/hyperplasia (arrowheads); H&E staining. (C) Heavy inflammatory response; H&E staining. (D) Metacercariae located at the kidney (Ki) epithelium; H&E staining. (E) Connective tissue (CT) hypertrophy as demonstrated by Masson-Trichrome staining. (F) Heavy defence response against parasites at the early phase of development (arrowheads). Asterisks: haemocytes. Scale bars = 100 μ m

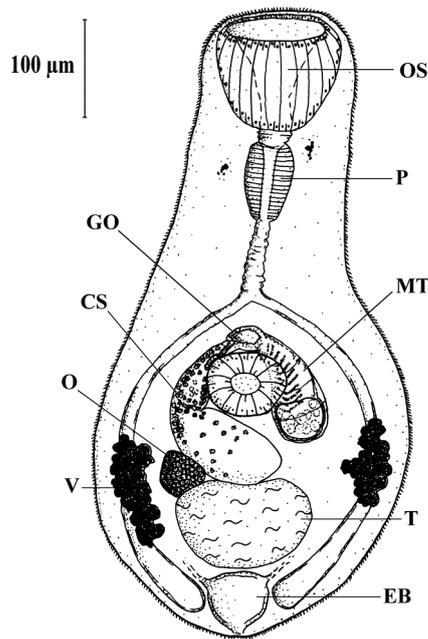


Fig. 4. *Postmonorchis* sp. Morphology of excysted metacercaria infecting the wedge clam *Donax trunculus*. CS: cirrus sac; EB: excretory bladder; GO: genital opening; MT: metaterm; O: ovary; OS: oral sucker; P: pharynx; T: testis; V: vitellaria

ventral sucker. The vitellarium consists of 2 lateral bunches of follicles at the level of the testis and ovary, each bunch measuring 48–91 μm (71 μm) \times 30–58 μm (42 μm). The excretory bladder is small and globular, and the pore is terminal.

Molecular phylogenetic analysis

PCR amplification produced a 1105 bp fragment, and BLAST analysis revealed that the sequenced

fragment (deposited in GenBank under accession KC603478) corresponds to the expected region including the 3'-terminus of the 18S rDNA (44 bp), ITS1 (532 bp), 5.8S rDNA (159 bp), ITS2 (292 bp) and the 5'-terminus of the 28S rDNA (78 bp). The best nucleotide identity score (~70%) for the complete region was found for *Aptorchis megapharynx* (Plagiorchiidae), and the nucleotide alignment contained many indels (>10%). Excluding the sequences corresponding to the 18S, 5.8S and 28S rDNAs, the best nucleotide identity score was obtained for *Monorchis monorchis* (Monorchidae) for ITS1 (83%) and for *Allobacciger centropygis* (Monorchidae) for ITS2 (87%).

The nucleotide sequences of ITS1 and ITS2 obtained in this study were separately aligned with the corresponding region of Trematoda species in GenBank (Table 2). Unfortunately, within the Monorchidae, the ITS1 sequences are known only for species belonging to the genus *Monorchis*, and the ITS2 sequences only for the genera *Allobacciger* and *Hurleytrematoides*. Fig. 5 shows the ITS1 and ITS2 trees obtained from the maximum likelihood analysis of the respective nucleotide alignments using *Homalometron manteri* as the outgroup taxon. In both the ITS1 and ITS2 trees, the Monorchidae form a statistically supported group with a bootstrap value of 100%, including the sequence obtained in the present study.

DISCUSSION

Morphological and molecular data

In this study, morphological observations combined with molecular analysis indicate that the observed

Table 2. Species, taxonomic position and GenBank accession numbers of the sequences used to construct the maximum likelihood trees. *Sequence obtained in the present study

Species	Taxonomy (all Digenea; Plagiorchiida)	Description	Accession number
<i>Aptorchis megapharynx</i>	Plagiorchiata; Plagiorchioidea; Plagiorchiidae	ITS1 and ITS2	EF014727
<i>Macroderoides trilobatus</i>	Plagiorchiata; Plagiorchioidea; Macroderoididae	ITS1 and ITS2	EU850410
<i>Renifer aniarum</i>	Plagiorchiata; Plagiorchioidea; Reniferidae	ITS1 and ITS2	HQ665460
<i>Telorchis bonnerensis</i>	Plagiorchiata; Plagiorchioidea; Telorchidae	ITS1 and ITS2	JF820593
<i>Allobacciger centropygis</i>	Monorchata; Monorchidae	ITS2	JN969511
<i>Hurleytrematoides</i> sp.	Monorchata; Monorchidae	ITS2	JN969577
<i>Monorchis monorchis</i>	Monorchata; Monorchidae	ITS1	Y18939
<i>Monorchis parvus</i>	Monorchata; Monorchidae	ITS1	Y18935
<i>Monorchis</i> sp.	Monorchata; Monorchidae	ITS1	AJ277375
<i>Postmonorchis</i> sp. inq.	Monorchata; Monorchidae	ITS1 and ITS2	KC603478*
<i>Homalometron manteri</i>	Allocreadiata; Allocreadioidea; Apocreadiidae	ITS1 and ITS2	JX400857

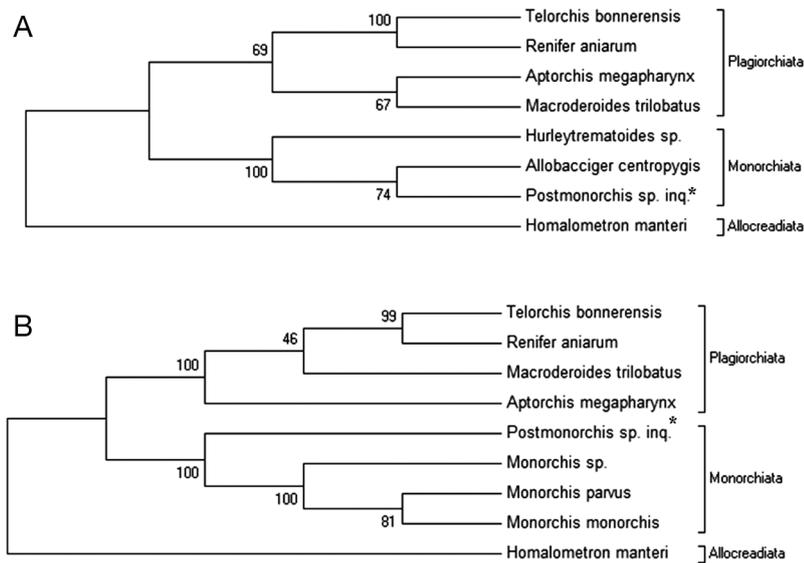


Fig. 5. Maximum likelihood trees of the (A) ITS1 and (B) ITS2 sequences. The numbers indicate the bootstrap percentage values. *Sequence obtained in the present work

parasite that infects the wedge clam *Donax trunculus* belongs to the Monorchhiidae, genus *Postmonorchis*.

In particular, morphological examination of the metacercariae assigned the parasite to the family Monorchhiidae Odhner, 1911 according to Bray (2008a,b) and to the genus *Postmonorchis* Hopkins, 1941 following the classification keys proposed by Hopkins (1941) and Madhavi (2008), except regarding the synonymisation determined by Madhavi (2008) of *Alloproctotrema* Machida, 1973 with *Postmonorchis* (Machida 1973, 2011). The most discriminating morphological feature of the genus *Postmonorchis* is the position of the vitellarium, which is situated at the same level as the testis and ovary, whereas in the related genera *Monorchis* Monticelli, 1893 and *Telolecithus* Lloyd & Guberlet, 1932, the vitellarium is situated at the level of the pharynx and behind the testis, respectively. In *Genolopa* Linton, 1910, the vitellarium is at the same level as the testis, but the latter is located in the middle third of the body, whereas in *Postmonorchis*, the testis is near the posterior end. The genus *Postmonorchis* was established for the type species *P. orthopristsis* Hopkins, 1941. Adults of this species occur in the gut of Haemulidae and Sciaenidae (Teleostea) from the US Atlantic coast (North Carolina), and its life cycle remains unknown. Young (1953) described another species, *P. donacis*, from the Pacific coast of the US. He found the adult worms in Sciaenidae and Embiotocidae (Teleostei) but also found parthenitae, cercariae and metacercariae in the bean clam *Donax gouldii*. Fur-

ther similar observations by Winter & Hatch (2010) support a 2-host life history model for *P. donacis*, which can use *D. gouldii* as both a primary and a secondary host, rather than a 3-host model with a copepod as the primary host (Young 1953). The third species belonging to this genus is *P. variabilis* Prudhoe & Bray, 1973 which is found in Nototheniidae and Harpagiferidae (Teleostei) from sub-Antarctic waters, as reported by Zdzitowiecki (1991) and Rocka (2006). According to Madhavi (2008), the genus *Alloproctotrema* Machida, 1973 should be considered as a synonym of *Postmonorchis*, meaning that the type and only species of that genus, *A. gerres* Machida, 1973 is the fourth member of the genus. However, Machida (2011) redescribed *A. gerres*, stating (p. 1) that '*Alloproctotrema* is valid, distinguishable from *Postmonorchis*

by possessing an unarmed genital atrium and a unipartite terminal organ'.

Here, the metacercariae show a combination of features of the 3 above species but also display some unique characteristics not previously described. The metacercaria more closely resemble *Postmonorchis orthopristsis* because of the following 3 features: first, the testis is reniform and is wider than it is long, while in *P. donacis* the opposite is true and in *P. variabilis* it is globular; second, the body is fully covered with spines, rather than only being covered to the acetabulum, as was reported by Young (1953) for both the larval and adult stages of *P. donacis*; and third, the posterior portion of the metraterm is short, which is very different from the elongated metraterm of *P. variabilis*, as described by Prudhoe & Bray (1973). In contrast, the short, globular, excretory bladder appears identical to that of *P. donacis*, whereas the excretory bladder of *P. orthopristsis* is I-shaped and reaches the anterior margin of the testis, while *P. variabilis* shows both forms. For our specimen, other features, such as the shape of the body, appear to be intermediate.

However, a very distinctive characteristic of our metacercaria, observed in *Postmonorchis* for the first time here, is the vase or funnel shape of the terminal oral sucker, which is similar to other Monorchhiidae, such as *Genolopa beauforti* Hopkins, 1941.

In our case, the molecular analysis confirms that the parasite belongs to the Monorchhiidae family, although it is not *Monorchis*, *Allobacciger* or *Hurley-*

trematoides. This finding strongly supports the hypothesis, based on the above morphological data, that this is the first record for a species of *Postmonorchis* in the Mediterranean Sea.

Host–parasite interaction

In the current study, the inflammatory response was the most relevant lesion. In particular, the encapsulation-type response is a common immune defence reaction towards foreign bodies, such as multicellular parasites, which enter the haemocoel and are too large to be phagocytosed. This reaction comprises a complex multistep process. It consists of a recognition phase, mediated by the coordinated interaction of immunocytes with the parasite surface and its secreted products, and a phase consisting of a response by cooperating activated haemocytes to form multicellular capsules against the foreign body by adhering to and enclosing it. The cytotoxic products are then released by haemocyte degranulation to destroy the invader (De Vico & Carella 2012a,b). In the present case, the latter step was often ineffective, and the parasite was eliminated in only a few cases.

Taken together, the above morphological and molecular data suggest that a new species of the genus *Postmonorchis* affects *Donax trunculus* of the Tyrrhenian coast of the Mediterranean Sea. Further studies accompanied by molecular analysis are necessary to fully elucidate the parasite rDNA sequence and life cycle. The specific differentiation of metacercariae is often difficult because factors such as the lack of distinctive morphological characteristics in larvae make their association with adults very problematic. To fill this gap, attempts at experimental infections and parasitological studies focused on key areas would permit the observation of the early larval stages and adults of *Postmonorchis* sp. inq. in the first intermediate and definitive hosts, respectively. In particular, the adult stages may more likely occur in Sciaenidae, of which some species are preferred definitive hosts for *P. orthopristsis* and *P. donacis* (Hopkins 1941, Young 1953, Winter & Hatch 2010). This is because Looss (1907) established (unfortunately without an adequate description) the new monorchid genus and species *Pristisomum caducum* Looss, 1907 infecting the scienid *Umbrina cirrhosa*, and Yamaguti (1958) synonymised this genus with *Postmonorchis*. Since to date this species has not been observed again, it would be very important to find adult monorchids in *U. cir-*

rhosa from Italian waters, and compare them with our metacercariae both morphologically and molecularly, in order to assess their identity.

With regard to molecular analysis, rDNA sequences clearly distinguish the different trematode species, including combinations for which morphology gives ambiguous results. At present, no sequences from the genus *Postmonorchis* other than those presented here are available in GenBank, with most information provided for the Monorchidae rRNA 18S and 28S regions. Further ongoing research is focused on better characterising the molecular data of other existing *Postmonorchis* species and related genera, collecting additional baseline data regarding infection by this *Postmonorchis* sp. inq. in *Donax trunculus* in the Mediterranean Sea and assessing the impact of these pathogens on the wedge clam stock population.

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