

Research Article

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

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Abstract

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

Introduction

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

The present study provides the morphological and molecular description of a didymozoid gill parasite of *E. marginatus* from the Mediterranean Sea (Balearic Islands and Sicily), with the aim of clarifying the taxonomy of this organism. Moreover, a key of identification for the genus *Didymodictinus* is proposed.

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

| Locality | Year | <i>n</i> | Total length (mm) | Total weight (g) |
|----------|------|----------|-------------------|------------------|
| Majorca | 1998 | 30 | 116–802 | 18–9000 |
| | 1999 | 66 | 144–1035 | 58–18 660 |
| | 2001 | 6 | 70–518 | 5–2000 |
| | 2002 | 83 | 68–930 | 84–16 600 |
| | 2003 | 20 | 123–514 | 25–2368 |
| | 2014 | 4 | 317–1070 | 500–22 000 |
| Sicily | 2018 | 16 | 180–700 | 280–3000 |
| | 2019 | 33 | 200–800 | 300–8000 |
| | 2020 | 21 | 270–750 | 600–5000 |

Materials and methods

Fish sampling and parasitological analysis

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

DNA isolation and polymerase chain reaction

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

DNA sequencing and alignment

Extracted samples were sequenced in both the forward and reverse directions on an Applied Biosystems 3730 DNA analyser (Thermo Fisher Scientific, Waltham, Massachusetts, United

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

| Locus | Sequence (5'–3') |
|-------|-------------------------------------|
| 28S | For: GTCGATAGCGAACAAGTACCGT |
| | Rev: AGCATAGTTCACCATCTTTCCGGGTCTCAA |
| ITS-2 | For: GTCGTAACAAGGTAGCTGTA |
| | Rev: TATGCTTAAGTTCAGCGGGT |
| cox1 | For: TTTTITGGGCATCCTGAGGTTTAT |
| | Rev: CAACAAATCATGATGCCAAAAGG |

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

Results

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

Didymodictylus marginati n. sp.

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

Type locality: Majorca, Spain, western Mediterranean Sea.

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

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

Site in host: Gills and pseudobranchs.

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

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

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science

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

| Species | Isolate | Host | Locality | 28S | ITS | COX1 | Author |
|--|-----------|--|---------------------|----------|----------|----------|-------------------------------------|
| <i>Allonematobothrioides scombri</i> | na | <i>Scomber scombrus</i> | UK | AY222195 | | | Olson <i>et al.</i> (2003) |
| <i>Brasicystis bennetti</i> | na | <i>Plagioscion squamosissimus scianid</i> fish | Brazil, Parà, Belem | JX074770 | JX074770 | JX074769 | Melo <i>et al.</i> (2013) |
| <i>Didymocystis bifasciatus</i> | A1 | <i>Thunnus thynnus</i> | Adriatic Sea | | FJ628698 | FJ628745 | Mladineo <i>et al.</i> (2010) |
| <i>Didymocystis bifasciatus</i> | M1 | <i>Thunnus orientalis</i> | California Bay | FJ628666 | na | FJ628773 | Mladineo <i>et al.</i> (2010) |
| <i>Didymocystis lingualis</i> | M1 | <i>Thunnus orientalis</i> | California Bay | FJ628661 | na | FJ628769 | Mladineo <i>et al.</i> (2010) |
| <i>Didymocystis lingualis</i> | M3b/M1a | <i>Thunnus orientalis</i> | California Bay | | FJ628719 | | Mladineo <i>et al.</i> (2010) |
| <i>Didymocystis pectoralis</i> | A1 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628617 | FJ628680 | FJ628728 | Mladineo <i>et al.</i> (2010) |
| <i>Didymocystis scomberomori</i> | na | <i>Scomberomorus maculatus</i> | USA: Gulf of Mexico | KU341979 | KU341979 | | Schrandt <i>et al.</i> (2016) |
| <i>Didymocystis semiglobularis</i> | A2 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628611 | FJ628675 | FJ628722 | Mladineo <i>et al.</i> (2010) |
| <i>Didymocystis</i> sp. ex <i>Scomberomorus maculatus</i> MJA-2016 | MJA-2016 | <i>Scomberomorus maculatus</i> | USA: Gulf of Mexico | KU341980 | KU341980 | | Schrandt <i>et al.</i> (2016) |
| <i>Didymodilinus marginati</i> n. sp. | Em1-2020 | <i>Epinephelus marginatus</i> | | MW489507 | MW489519 | MW540490 | This study |
| <i>Didymosulcus irregularis</i> | A1 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628632 | FJ628694 | FJ628742 | Mladineo <i>et al.</i> (2010) |
| <i>Didymosulcus palati</i> | A10019 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628628 | FJ628690 | FJ628739 | Mladineo <i>et al.</i> (2010) |
| <i>Didymosulcus palati</i> | M10035 | <i>Thunnus orientalis</i> | California Bay | | | FJ628766 | Mladineo <i>et al.</i> (2010) |
| <i>Didymosulcus spirocauda</i> | A10005 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628614 | FJ628677 | FJ628725 | Mladineo <i>et al.</i> (2010) |
| <i>Didymosulcus wedli</i> | A10033 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628642 | FJ628703 | FJ628751 | Mladineo <i>et al.</i> (2010) |
| <i>Didymosulcus wedli</i> | M10030 | <i>Thunnus orientalis</i> | California Bay | | | FJ628761 | Mladineo <i>et al.</i> (2010) |
| Didymozoid sp. 1-PO-2003 | 1-PO-2003 | <i>Epinephelus cyanopodus</i> | Australia | AY222193 | | | Olson <i>et al.</i> (2003) |
| Didymozoid sp. 2-PO-2003 | 2-PO-2003 | <i>Taeniura lymma</i> | Australia | AY222192 | | | Olson <i>et al.</i> (2003) |
| Didymozoid sp. 3-PO-2003 | 3-PO-2003 | <i>Apogon cookii</i> | Australia | AY222194 | | | Olson <i>et al.</i> (2003) |
| Didymozoidae sp. CH-334 | | | | | AB745110 | AB745112 | Abe <i>et al.</i> (2014) |
| Didymozoidae sp. DP-280 | DP-280 | <i>Diagramma pictum</i> | Japan | AB725627 | AB725628 | AB725629 | Abe <i>et al.</i> (2014) |
| Didymozoidae sp. PC-298 | PC-298 | <i>Plectorhinchus cinctus</i> | Japan | AB725630 | AB725631 | AB725632 | Abe <i>et al.</i> (2014) |
| Didymozoidae sp. SJ-350 | SJ-350 | <i>Scomber japonicus</i> | na | LC003496 | | | Abe and Okamoto (2015) |
| Didymozoidae sp. LCSJ-2019 | LCSJ-2019 | <i>Syacium papillosum</i> | Yucatan | MK558797 | | | Vidal-Martínez <i>et al.</i> (2019) |
| <i>Didymocystis bifurcata</i> | na | <i>Thunnus obesus</i> | Solomon Islands | MK268210 | MK268210 | | Moore <i>et al.</i> (2019) |
| <i>Didymocystis</i> sp. 1 | na | <i>Thunnus obesus</i> | Ambon | MK268211 | MK268211 | | Moore <i>et al.</i> (2019) |
| <i>Wedlia globosa</i> | na | <i>Thunnus obesus</i> | Solomon Islands | MK268213 | MK268213 | | Moore <i>et al.</i> (2019) |
| <i>Wedlia</i> sp. 2 | na | <i>Thunnus obesus</i> | Palabuhanratu | MK268214 | MK268214 | | Moore <i>et al.</i> (2019) |
| <i>Coeliotrema thynni</i> | na | <i>Thunnus obesus</i> | Solomon Islands | MK268215 | MK268215 | | Moore <i>et al.</i> (2019) |
| <i>Koellikeria</i> sp. 1 | na | <i>Thunnus obesus</i> | Solomon Islands | MK268216 | MK268216 | | Moore <i>et al.</i> (2019) |
| <i>Wedlia bipartita</i> | na | <i>Thunnus albacares</i> | Bali | MK268220 | MK268220 | | Moore <i>et al.</i> (2019) |

(Continued)

Table 3. (Continued.)

| Species | Isolate | Host | Locality | 28S | ITS | COX1 | Author |
|---|------------|------------------------------------|------------------------|----------|----------|----------|---|
| <i>Wedlia orientalis</i> | na | <i>Thunnus albacares</i> | Solomon Islands | MK268221 | MK268221 | | Moore et al. (2019) |
| <i>Wedlia pylorica</i> | na | <i>Thunnus albacares</i> | Bali | MK268228 | MK268228 | | Moore et al. (2019) |
| <i>Koellikerioides intestinalis</i> | na | <i>Thunnus albacares</i> | Solomon Islands | MK268229 | MK268229 | | Moore et al. (2019) |
| <i>Didymozoon longicolle</i> | A1 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628648 | | FJ628757 | Mladineo et al. (2010) |
| <i>Helicodidymozoon heliciis</i> | na | <i>Platycephalus fuscus</i> | Moreton Bay, Australia | | AJ224758 | | Anderson and Barker (1998) |
| <i>Helicodidymozoon tortor</i> | na | <i>Platycephalus endrachtensis</i> | Moreton Bay, Australia | | AJ224757 | | Anderson and Barker (1998) |
| <i>Indodidymozoon brevicolle</i> | na | <i>Platycephalus fuscus</i> | Moreton Bay, Australia | | AJ224754 | | Anderson and Barker (1998) |
| <i>Indodidymozoon ditremion</i> | na | <i>Inegocia japonica</i> | Moreton Bay, Australia | | AJ224755 | | Anderson and Barker (1998) |
| <i>Indodidymozoon lesteri</i> | na | <i>Platycephalus endrachtensis</i> | Moreton Bay, Australia | | AJ224751 | | Anderson and Barker (1998) |
| <i>Indodidymozoon metridion</i> | na | <i>Suggrundus jugosus</i> | Moreton Bay, Australia | | AJ224756 | | Anderson and Barker (1998) |
| <i>Indodidymozoon moretonensis</i> | na | <i>Platycephalus fuscus</i> | Moreton Bay, Australia | | AJ224750 | | Anderson and Barker (1998) |
| <i>Indodidymozoon pearsoni</i> | na | <i>Platycephalus endrachtensis</i> | Moreton Bay, Australia | | AJ224752 | | Anderson and Barker (1998) |
| <i>Indodidymozoon suttiei</i> | na | <i>Platycephalus fuscus</i> | Moreton Bay, Australia | | AJ224753 | | Anderson and Barker (1998) |
| <i>Koellikeria globosa</i> | A1 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628646 | FJ628706 | FJ628755 | Mladineo et al. (2010) |
| <i>Koellikeria sp. renalis</i> | A1 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628650 | FJ628708 | FJ628759 | Mladineo et al. (2010) |
| <i>Koellikeria sp. renalis</i> | M1 | <i>Thunnus orientalis</i> | California Bay | | | FJ628775 | Mladineo et al. (2010) |
| <i>Koellikerioides apicalis</i> | A1 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628621 | FJ628683 | FJ628732 | Mladineo et al. (2010) |
| <i>Melanocystis cf. kawakawa</i> | MEL6 | <i>Euthynnus alletteratus</i> | Spain, Mediterranean | KU290355 | KU216176 | | Mele et al. (2016) |
| <i>Neometadidymozoon polymorphis</i> | na | <i>Priacanthus macracanthus</i> | Moreton Bay, Australia | | AJ224760 | | Anderson and Barker (1998) |
| <i>Neonematobothrium cf. kawakawa</i> | N18 | <i>Euthynnus alletteratus</i> | Spain, Mediterranean | KU290361 | KU216182 | | Mele et al. (2016) |
| <i>Oesophagocystis sp. 1</i> | DZ14 | <i>Euthynnus alletteratus</i> | Spain, Mediterranean | KU290353 | KU216174 | | Mele et al. (2016) |
| <i>Oesophagocystis sp. 2</i> | DC5 | <i>Euthynnus alletteratus</i> | Spain, Mediterranean | KU290346 | KU216167 | | Mele et al. (2016) |
| <i>Platocystis alalongae</i> | A2 | <i>Thunnus thynnus</i> | Adriatic Sea | FJ628627 | FJ628688 | FJ628736 | Mladineo et al. (2010) |
| <i>Rhopalotrema elusiva</i> | na | <i>Platycephalus fuscus</i> | Moreton Bay, Australia | | AJ224759 | | Anderson and Barker (1998) |
| <i>Genarchopsis goppo</i> (outgroup) | KT254045 | <i>Rhinogobius kurodai</i> | Japan | | | AB703670 | Urabe et al. (2012) |
| <i>Prosogonotrema bilabiatum</i> (outgroup) | 1 DNA-2519 | <i>Rhomboplites aurorubens</i> | USA | KU527431 | | | |
| Hemiuridae sp. (outgroup) | TrM2 | <i>Centropages hamatus</i> | Denmark | | | KM401880 | Skovgaard and Mier-Jedrzejowicz (unpublished) |

na, data not available.

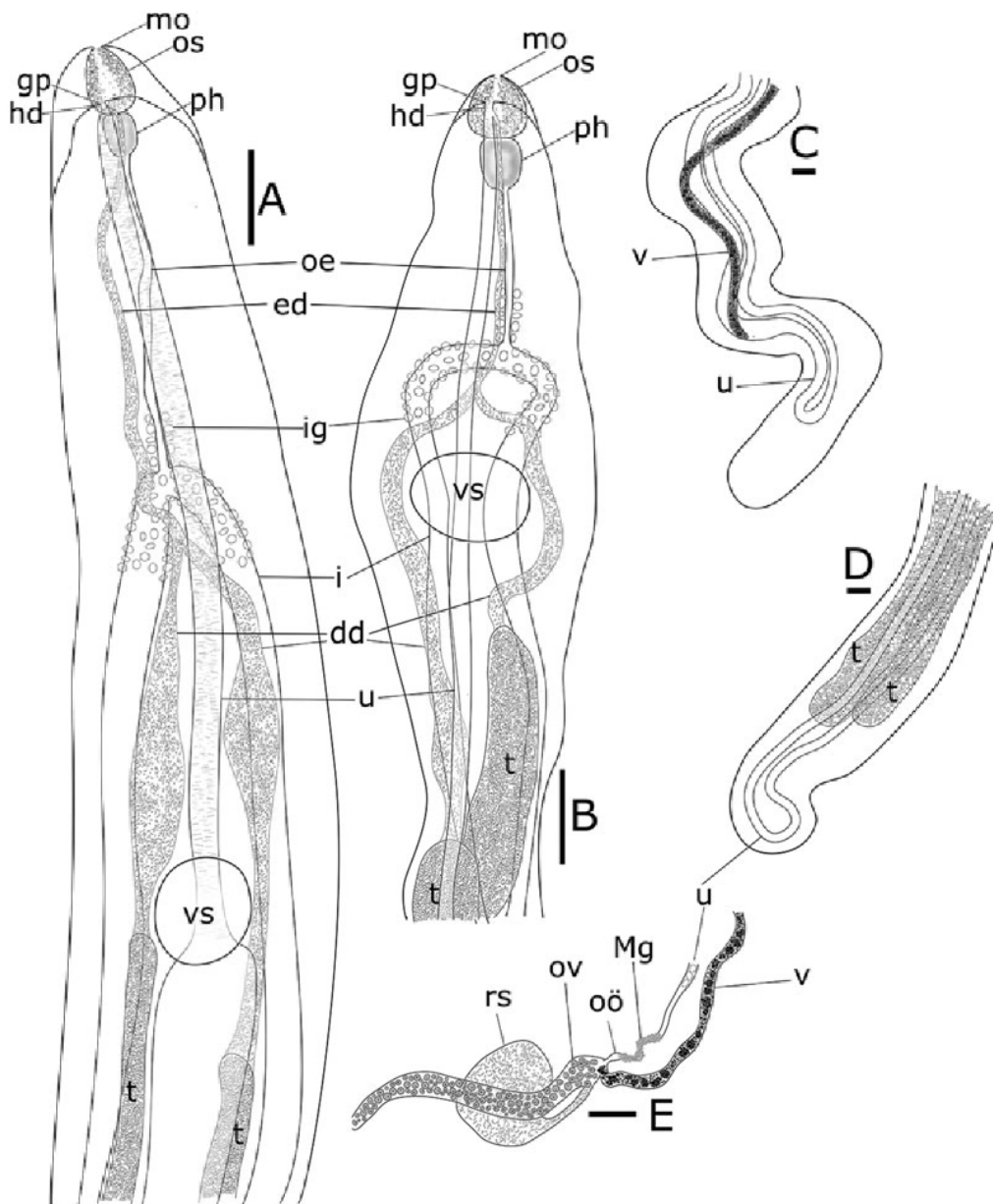


Fig. 1. *Didymodiclinus marginati* n. sp. ex the pseudobranch filaments of *Epinephelus marginatus*. (A) Functional female, holotype, anterior end of the body, ventral view. (B) Functional male, paratype, anterior end of the body, ventral view. (C) Functional female, holotype, posterior end of the body,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

Identifier (LSID) for *D. marginati* n. sp. is urn:lsid:zoobank.org:act:3B39D377-55DC-46BE-BE9B-F5821E39A7C0.

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

Description (Fig. 1)

[Based on 15 adult specimens, 6 functional females and 9 functional males from dusky groupers from Mallorca, western Mediterranean Sea. The values of the holotype NHM UK 2022.4.8.1 (functional female) and paratype NHM UK 2022.4.8.2 (functional male) are expressed in the text, whereas the range of the measurements is reported in Table 4.] Capsule containing a couple of parasites with convoluted posterior ends. Gonochores, with weak sexual dimorphism i.e. underdeveloped male and female organs in both functional female and male specimens, respectively. All measurements are given in micrometres.

Functional male specimen filiform, not divided in longitudinal regions, smaller than functional female, with anterior end dorso-

ventrally flattened and somewhat lanceolate, 53 861 long, 260 wide (Fig. 1A). Oral sucker terminal, spheroidal, 54 \times 57. Pharynx spheroidal, 52 \times 38; oesophagus bifurcating into 2 caeca, 437 wide. Ventral sucker 65 \times 117, at 907 from the anterior end. Pharynx, oesophagus and proximal part of caeca covered with gland cells. Testes 2, parallel, 52 136–52 615 \times 111–128, starting at 50 from the posterior end. Vasa deferentia sinuous and spermatic duct filled with spermatozoa. Vasa deferentia joint at 571 from the anterior end. Rudiments of ovary and vitellarium barely visible. Uterus with 1 loop first descending. Metraterm 60 wide, joined with spermatic duct in a very short hermaphroditic duct, 17 long, reaching genital pore ventrally to the oral sucker. In alive specimens the genital pore is located on an extensible temporary papilla. Descending uterus filled with few (2–100) apparently immature eggs, 15 \times 10.

Functional female filiform, not divided into regions, with anterior end dorso-ventrally flattened, 77 680 long, 226 wide. Oral sucker terminal, spheroidal, 62 \times 47 (Fig. 1B). Pharynx

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

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

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

ellipsoidal, 45×34 . Oesophagus bifurcating into 2 caeca, 156 long, reaching the posterior end of body. Ventral sucker diameter 103, distant 796 from anterior end. Pharynx, oesophagus and proximal part of caeca covered with gland cells. Testes 2, parallel, $6304-7307 \times 44-54$, starting at 55 805 from the posterior end (Fig. 1D). Vasa deferentia straight and spermatic duct filled with spermatozoa. Vasa deferentia joint at 442 from the anterior end. Ovary long, simple, tubular, sinuous, 33 wide, starting 852 from anterior end of body and reaching with a short oviduct the genital junction at 44 960 (3/5 of the body). Seminal receptacle cystic, 191×171 , joining the oviduct. Vitellarium long, simple, tubular, posterior to genital junction, 36 wide, starting at 355 from posterior end and joining the oviduct (Fig. 1C). Mehlis gland present around the first part of the uterus. Uterus with 1 loop, first descending, filled with eggs. Metraterm well developed, 90 wide. Genital pore ventral to the oral sucker. In alive specimens the genital pore is located on an extensible temporary papilla. Mature eggs, $15-17 \times 10-11$ (Fig. 1E).

Remarks

This species morphologically agrees with the diagnosis of the genus *Didymodictinus* (Pozdnyakov, 1993) by the number of characters: the encapsulation in gills and pseudobranchs, a not clearly marked sexual dimorphism, specimens of both sexes with long filiform not regionalized body, a simple short oesophagus, intestinal caeca reaching the posterior extremity, ventral sucker present. Moreover, glandular cells are present around oesophagus and proximal region of the intestinal caeca. Functional males are smaller than functional females, with 2 parallel testes reaching the posterior end, and similarly to *Didymodictinus menpachi* (Yamaguti, 1970) with rudiments of the female reproductive system. Functional females have long and narrow ovaries located in the anterior part of body and vitellarium with similar form, located in the posterior part. The central portion of the female reproductive system is situated approximately in the middle of the body.

Didymodictinus marginati n. sp. can be distinguished from *D. branchialis* because of the larger body length and shorter oesophagus, and also the presence of the seminal receptacle, not observed in the latter species (Table 4). The presence of Mehlis gland, larger pharynx and ventral sucker and shorter oesophagus distinguishes the new species from *Didymodictinus epinepheli* (Abdul-Salam *et al.*, 1990). *Didymodictinus marginati* n. sp. has accessory gland cells around the oesophagus, a character not observed in *Didymodictinus pacificus* (Yamaguti, 1938) and *Didymodictinus pristipomatis* (Yamaguti, 1934). In addition, *D. marginati* n. sp. has smaller oral sucker and eggs and shorter oesophagus than these 2 species. Concerning the differences with the rest of the congeneric species, *D. marginati* n. sp. has larger body than *Didymodictinus cypseluri* (Yamaguti, 1940) and *Didymodictinus hainanensis* (Gu and Shen, 1983), but shorter than *D. menpachi* and *Didymodictinus microovatus* (Reimer, 1980). It also has smaller eggs than *D. cypseluri*, *D. menpachi*, *Didymodictinus reticulum* (Madhavi and Murugesu, 1994) and *Didymodictinus spilonotop-teri* (Yamaguti, 1970), but larger than *D. microovatus*.

All previously described species are also characterized by their different hosts and location within the host tissues, being from other geographical localities. Among the most similar species: *D. branchialis* was recorded in the nostril of *Epinephelus quernus* Seale, 1901 from Hawaii; *D. epinepheli* on the gills of *Epinephelus tauvina* (Forsskal, 1775) from the Arabian Gulf and *Epinephelus coioides* (Hamilton, 1822) from the Philippines; *D. pacificus* on the gills of an epinephelid from the Pacific Ocean; *D. pristipomatis* in the mouth of *Epinephelus akaara* (Temminck and Schlegel, 1842) from the Japan Sea (Yamaguti, 1971; Abdul-Salam *et al.*, 1990; Cruz-Lacierda *et al.*, 2001).

Molecular results

All the isolates showed positive amplification for 28S, ITS-2 and *cox1* genes. Partial sequences of 28S (746 nt, 15 replicates), ITS-2 (640 nt, 15 replicates) and *cox1* (530 nt, 15 replicates) were obtained for *D. marginati* n. sp. The nucleotide sequences of the amplified products of each gene were identical among the isolates from specimens collected in Majorca and Sicily. The representative DNA sequences for 28S, ITS-2 and *cox1* were submitted to GenBank (accession numbers MW489507, MW489519 and MW540490, respectively). The representative sequences of 28S had 97.98 and 97.2% of similarity to that of *Didymozoidae* sp. (AY222193) and *Didymozoidae* sp. (AY222194) from GenBank, with 13 and 18 nt of differences, respectively. The ITS-2 sequences had 90.3% of similarity to that of *Didymozoidae* (KP452505) from GenBank with 74 nt of difference. The obtained sequences of *cox1* had 75.2% of similarity to *Didymosulcus katsuwonicola* (KF379726) from GenBank, with 71 nt of difference.

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

Discussion

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

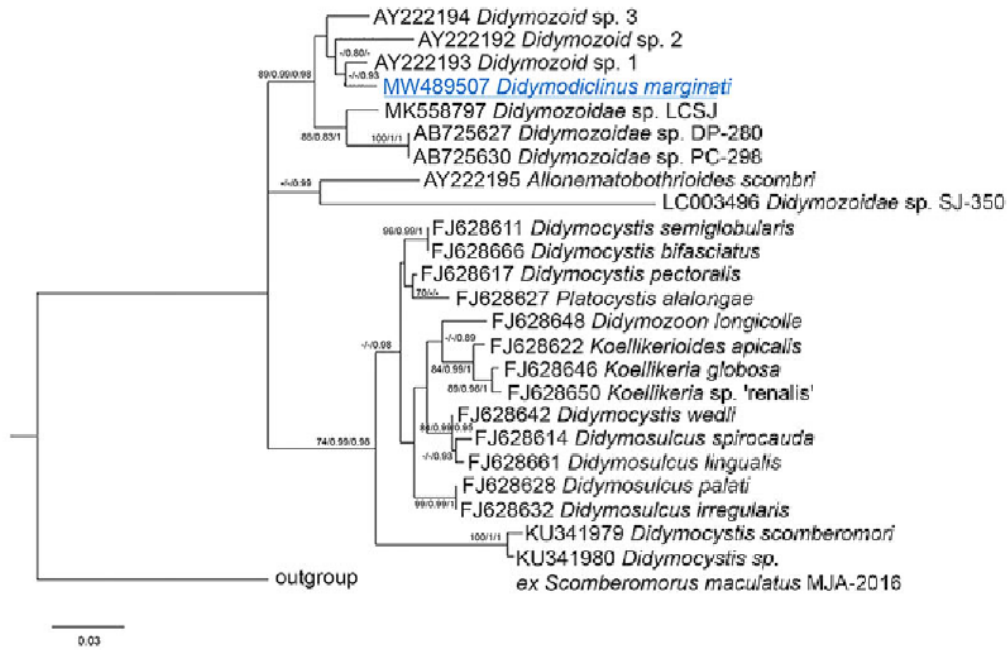


Fig. 2. Phylogenetic relationships between the isolates of the present study and other *Didymozoidae* as inferred from sequences of 28S rDNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for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

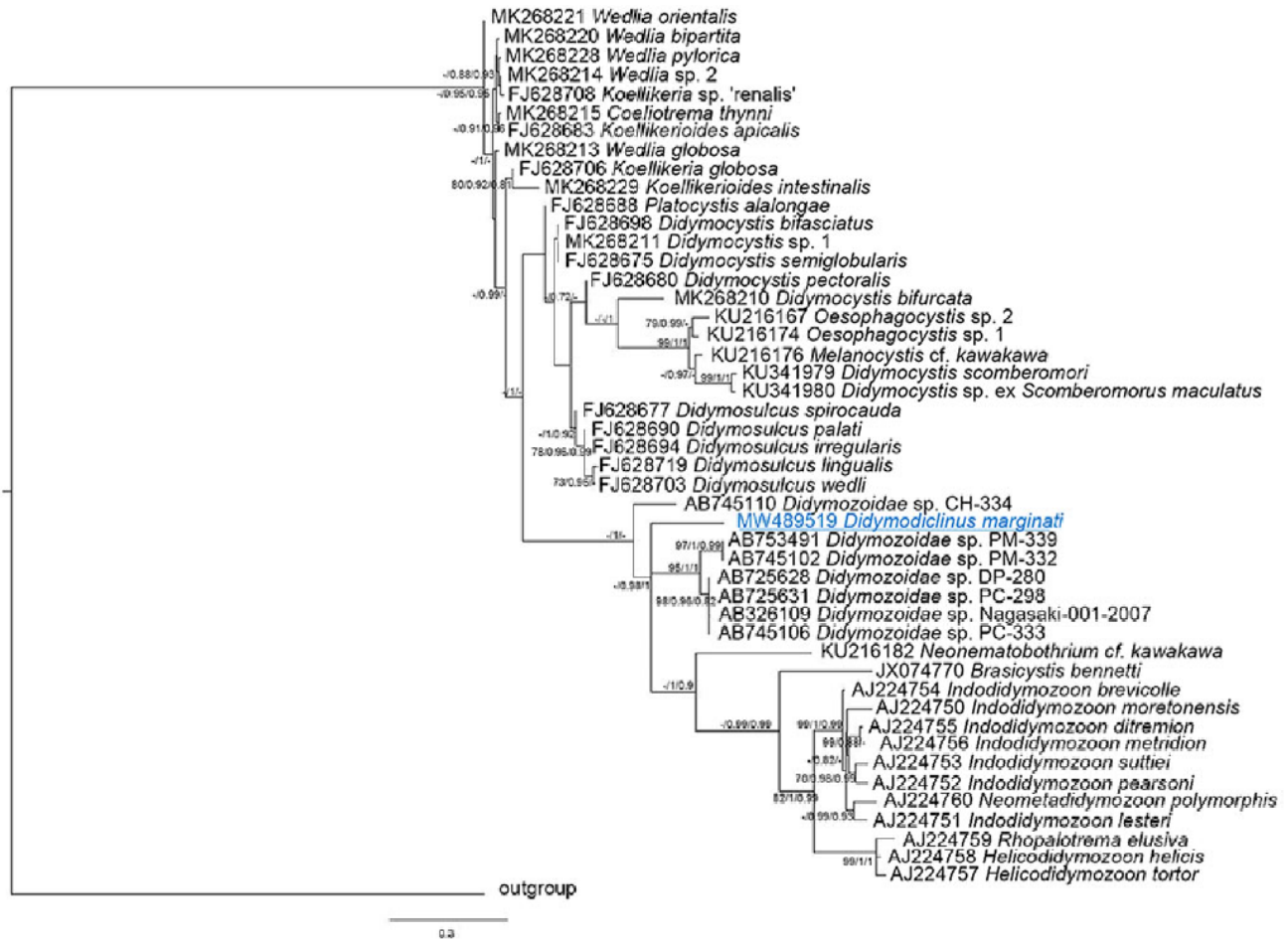


Fig. 3. Phylogenetic relationships between the isolates of the present study and other *Didymozoidae* as inferred from sequences of ITS-2 rDNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for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

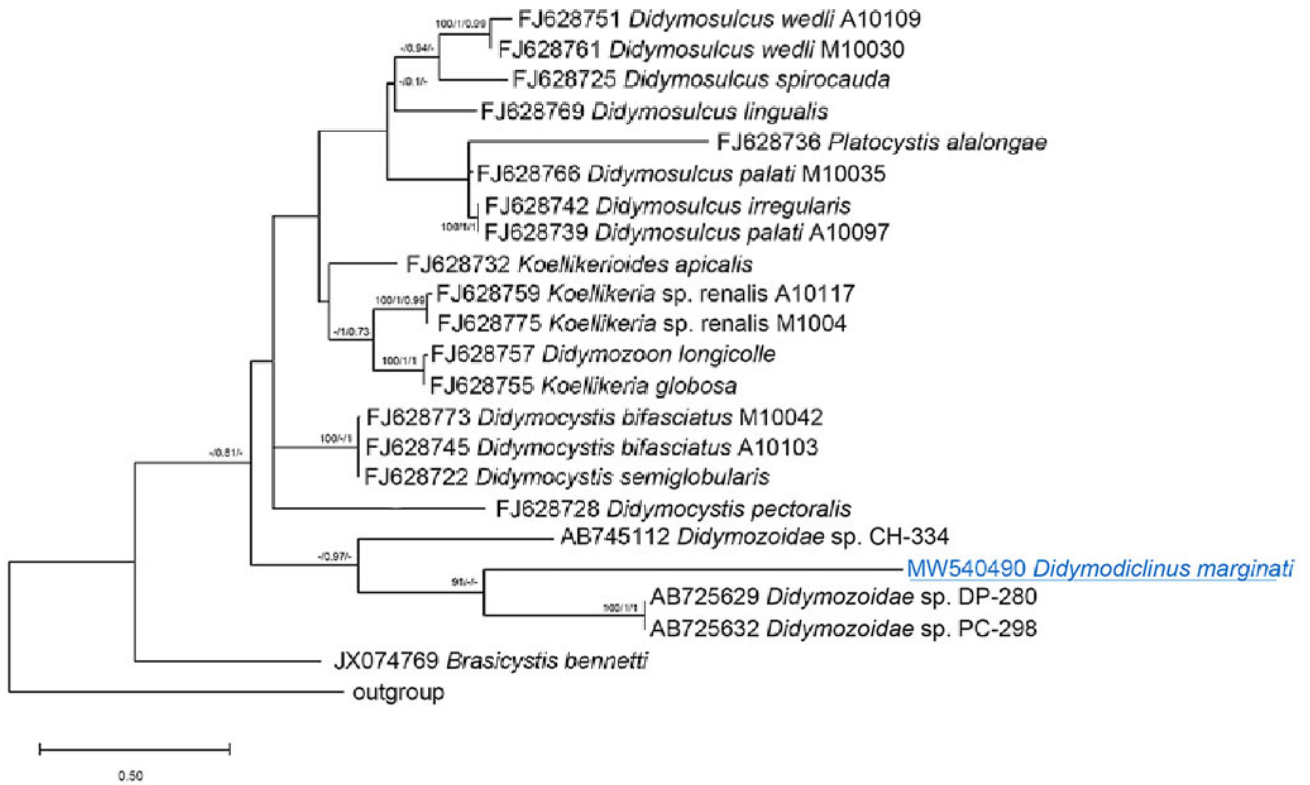


Fig. 4. Phylogenetic relationships between the isolates of the present study and other Didymozoidae as inferred from sequences of *cox1* DNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for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

organ protruded for mating, but it is also evident in stressful situations (e.g. during the dissection of capsules in live specimens; Salvatore Mele personal observation).

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

Key to identification of *Didymodictinus* species

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

| | | |
|---|---|------------------------|
| 1 | Ventral sucker smaller than oral sucker | 2 |
| | Ventral sucker larger or equal than oral sucker | 4 |
| 2 | Seminal receptacle absent | 3 |
| | Seminal receptacle present | <i>D. spilonopteri</i> |
| 3 | Eggs small, 15–18 × 9 | <i>D. hainanensis</i> |
| | Eggs large, 20–21 × 14–15 | <i>D. cypseluri</i> |

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

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

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

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

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