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*Both authors contributed equally and share the first position.

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Author for correspondence:
Gabriella Gaglio,
Email: ggaglio@unime.it

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

Salvatore Mele[1,*], Giovanni De Benedetto[2,*], Alessia Giannetto[3], Kristian Riolo[3], Sabrina Oliva[3], Olga Reñones[4], Giovanni Garippa[1], Paolo Merella[1] and Gabriella Gaglio[2]

[1]Department of Veterinary Medicine, University of Sassari, 07100 Sassari, Italy; [2]Department of Veterinary Sciences, University of Messina, Messina, Italy; [3]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy and [4]Instituto Español de Oceanografía (IEO, CSIC), Centro Oceanográfico de Baleares, Ecosystem Oceanography Group (GRECO), Palma 07015, Spain

Abstract

The current study provides a morphological and molecular characterization of a new species of *Didymodiclinus* (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 *Didymodiclinus branchialis* (Yamaguti, 1970), *Didymodiclinus epinepheli* (Abdul-Salam, Sreelatha and Farah, 1990) and *Didymodiclinus 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 *Didymodiclinus 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 Didymodiclinae is well separated from Didymozoinae and other gonochoric didymozoids. Following both morphological and genetic results, a key of identification for the genus *Didymodiclinus* 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 *Didymodiclinus 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 *Didymodiclinus* 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 *Didymodiclinus* is proposed.

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

Locality	Year	*n*	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

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: GTCCGATAGCGAACAAGTACCGT
	Rev: AGCATAGTTCACCATCTTTCGGGTCTCAA
ITS-2	For: GTCGTAACAAGGTAGCTGTA
	Rev: TATGCTTAAGTTCAGCGGGT
cox1	For: TTTTTTGGGCATCCTGAGGTTTAT
	Rev: CAACAAATCATGATGCAAAAGG

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 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.

Didymodiclinus 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

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

(*Continued*)

Table 3. (*Continued.*)

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

na, data not available.

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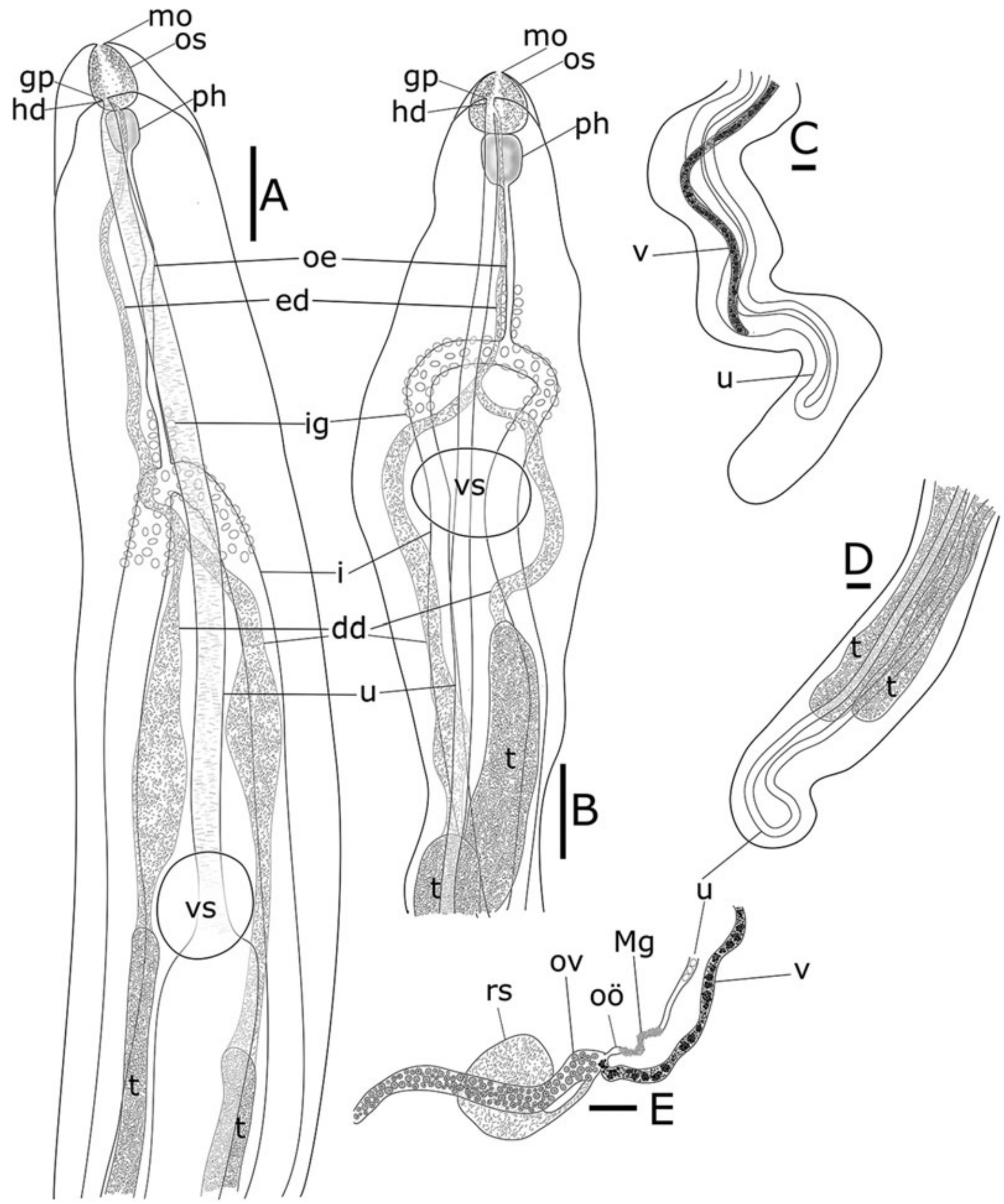

Fig. 1. *Didymodiclinus marginati* n. sp. ex the pseudobranch filaments of *Epinephelus marginatus*. (A) Functional female, holotype, anterior end of the body, ventral view. (B) Functional male, paratype, anterior end of the body, ventral view. (C) Functional female, holotype, posterior end of the body, 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 μm.

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. Gonochoristic, 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 × 57. Pharynx spheroidal, 52 × 38: oesophagus bifurcating into 2 caeca, 437 wide. Ventral sucker 65 × 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 × 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 × 10.

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

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

	BL	BW	OSL	OSW	PL	PW	OL	VSL	VSW	DVS	OW	VW	TL	TW	EL	EW	DGJ
Didymodiclinus branchialis	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			
Didymodiclinus cypseluri	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			
Didymodiclinus epinepheli	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			
Didymodiclinus facialis	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			
Didymodiclinus hainanensis	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			
Didymodiclinus kovaljovae	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			
D. marginati 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			
Didymodiclinus menpachi	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			
Didymodiclinus microovatus	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			
Didymodiclinus pacificus	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			
Didymodiclinus pristipomatis	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			
Didymodiclinus reticulum	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			
Didymodiclinus spilonotopteri	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			
Didymodiclinus tomex	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.

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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 × 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 × 10–11 (Fig. 1E).

Remarks

This species morphologically agrees with the diagnosis of the genus *Didymodiclinus* (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 *Didymodiclinus 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.

Didymodiclinus 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 *Didymodiclinus epinepheli* (Abdul-Salam *et al.*, 1990). *Didymodiclinus marginati* n. sp. has accessory gland cells around the oesophagus, a character not observed in *Didymodiclinus pacificus* (Yamaguti, 1938) and *Didymodiclinus 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 *Didymodiclinus cypseluri* (Yamaguti, 1940) and *Didymodiclinus hainanensis* (Gu and Shen, 1983), but shorter than *D. menpachi* and *Didymodiclinus microovatus* (Reimer, 1980). It also has smaller eggs than *D. cypseluri*, *D. menpachi*, *Didymodiclinus reticulum* (Madhavi and Murugesh, 1994) and *Didymodiclinus spilonotopteri* (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* (Forsskål, 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. *Didymodiclinus marginati* n. sp. (sub-family Didymodiclininae) 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 Didymodiclinidae to include gonochoristic didymozoids, arranging them into 3 subfamilies: Didymodiclininae, 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 Didymodiclininae included only 2 genera separated by the presence (*Didymodiclinus*) or absence (*Paragonapodasmius*) of the ventral sucker. Pozdnyakov and Gibson (2008) rejected the family Didymodiclinidae, including the subfamilies Didymodiclininae within the Didymozoidae. Apart from the general description of *Didymodiclinus* (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

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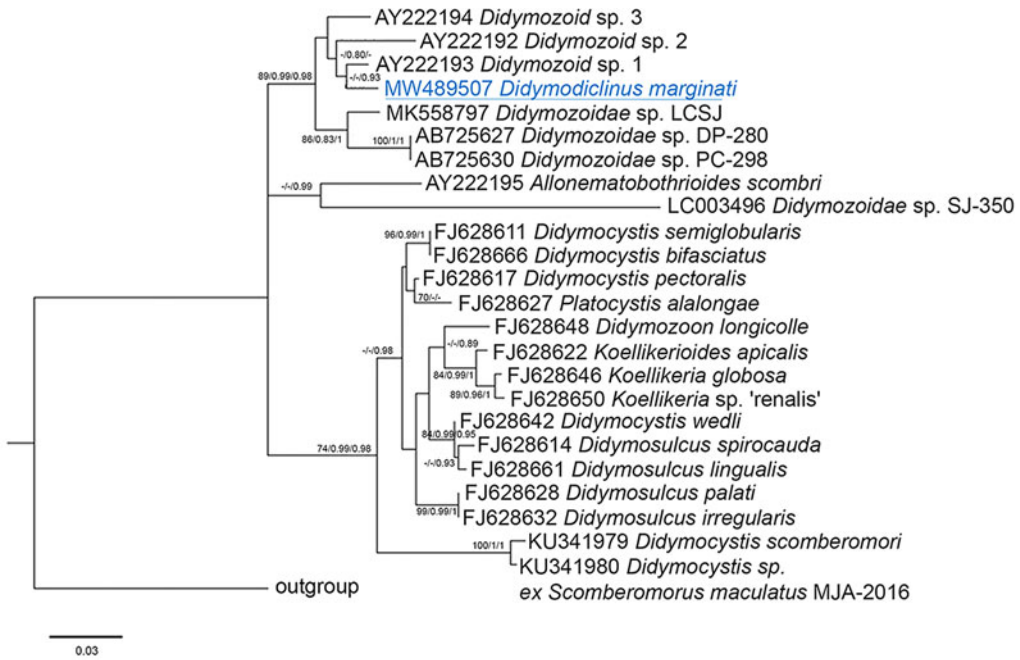

Fig. 2. Phylogenetic relationships between the isolates of the present study and other *Didymozoidae* as inferred from sequences of 28S rDNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for 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*.

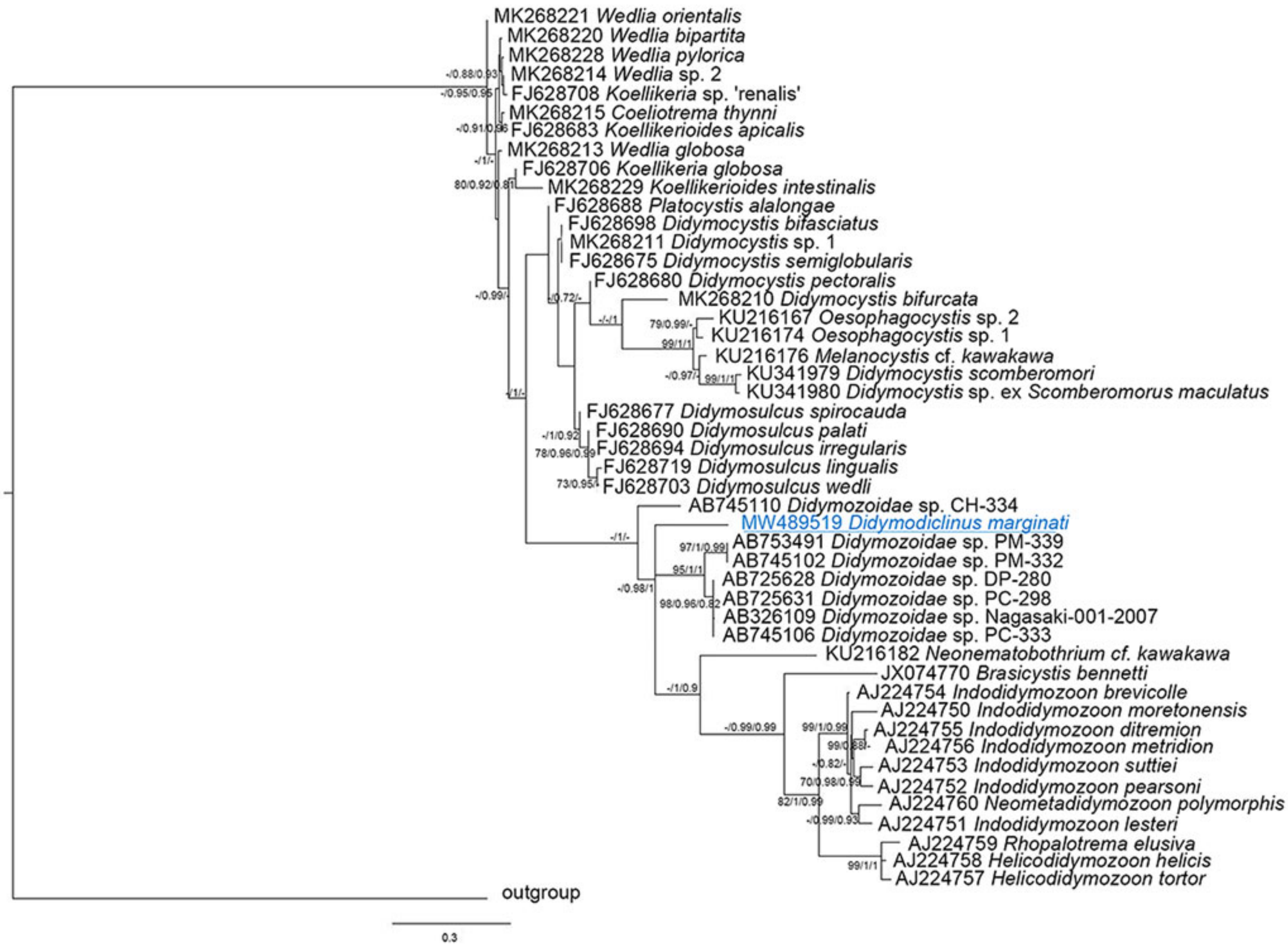

Fig. 3. Phylogenetic relationships between the isolates of the present study and other Didymozoidae as inferred from sequences of ITS-2 rDNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for 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.

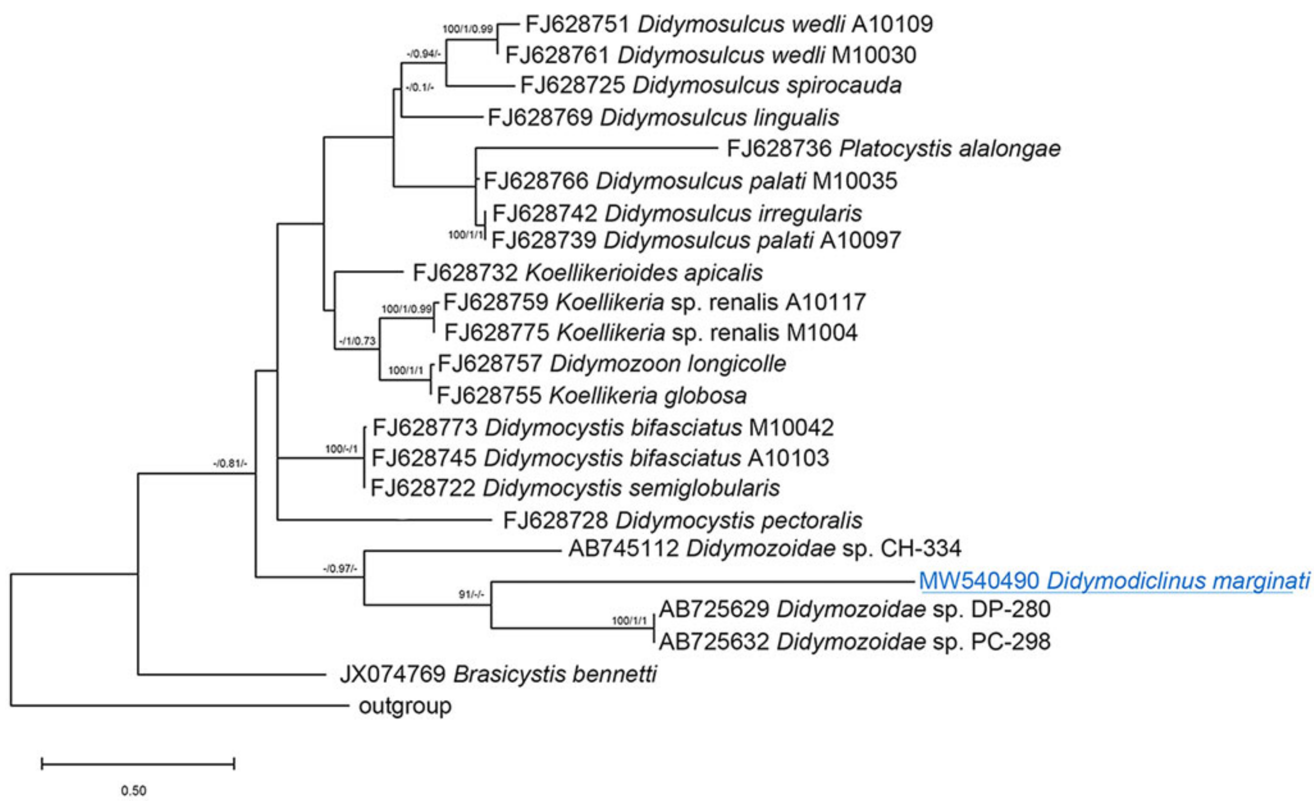

Fig. 4. Phylogenetic relationships between the isolates of the present study and other Didymozoidae as inferred from sequences of cox1 DNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for 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 Didymodiclinus *species*

The following dichotomous keys are provided to facilitate the identification of the trematodes belonging to the genus *Didymodiclinus* 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	*D. spilonotopteri*
3	Eggs small, 15–18 × 9	*D. hainanensis*
	Eggs large, 20–21 × 14–15	*D. cypseluri*
4	Ventral sucker equal or slightly larger than oral sucker	*D. epinepheli*
	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	*D. microovatus*
	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	*D. branchialis*
	Mehlis gland present, ovary width more than 40, testes shorter than 1500	*D. pacificus*
8	Uterus with 3 loops	*D. pristipomatis*
	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	*D. marginati* n. sp.
10	Seminal receptacle absent	*D. menpachi*
	Seminal receptacle present	*D. reticulum*

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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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