

## Research Article

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# Novel insights into the genetics, morphology, distribution and hosts of the global fish parasitic digenean *Proctoeces maculatus* (Looss, 1901) (Digenea: Fellodistomidae)

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**Abstract**

Larval stages of the widely distributed digenean species *Proctoeces maculatus* (Looss, 1901) were reported 40 years ago from South Africa in the common octopus, *Octopus vulgaris* Cuvier (Octopodidae). However, the absence of adult specimens and molecular data from this region has hindered a comprehensive understanding of its distribution. In this study, we collected three species of intertidal and near-shore marine fishes [*Clinus superciliosus* (L.) (Clinidae), *Diplodus capensis* (Smith) (Sparidae) and *Sparodon durbanensis* (Castelnau) (Sparidae)] along the South African coast and discovered adult specimens of *P. maculatus* at five localities. By employing a combination of morphological and molecular techniques, including 28S rDNA, 18S rDNA and COI mtDNA analyses, the first report of adult *P. maculatus* from South Africa is presented. The findings encompass a comprehensive morphological description and molecular data, illuminating the true distribution of this species in the region.

**Introduction**

*Proctoeces maculatus* (Looss, 1901) Odhner, 1911 (Digenea: Fellodistomidae) is a widespread trematode species that parasitizes the gut of a wide range of marine fishes. It was originally described as *Distomum maculatum* Looss, 1901 from the Brown wrasse (*Labrus merula* L.) in Trieste, Italy (Looss, 1901; Odhner, 1911). Over the years, adults of *P. maculatus* have been reported from 65 species of fish; additionally, 26 invertebrate species have been recorded as intermediate hosts for *P. maculatus* (WoRMS, 2023). Additionally, numerous species exhibiting morphological similarities to *P. maculatus* have been described, and a significant proportion of them have subsequently been synonymized with *P. maculatus*. This outcome stems from the conserved morphology observed among isolates, which presents a challenge in discerning clear-cut morphological characteristics to differentiate *P. maculatus* from other species (Freeman and Llewellyn, 1958; Bray and Gibson, 1980).

An interesting trait of *Proctoeces* species is the incorporation of progenetic metacercariae in their life cycles – the larval stages can attain sexual maturity while infecting an intermediate host (Freeman and Llewellyn, 1958; Bray and Gibson, 1980; Oliva and Huaquin, 2000). These trematodes have a near-cosmopolitan distribution and are known to infect a variety of hosts, mainly fishes and molluscs that mostly occur in shallow water (Bray and Gibson, 1980). *Proctoeces maculatus* has been reported only once, 40 years ago, in South Africa, when immature specimens were found in the common octopus *Octopus vulgaris* Cuvier (Bray, 1983).

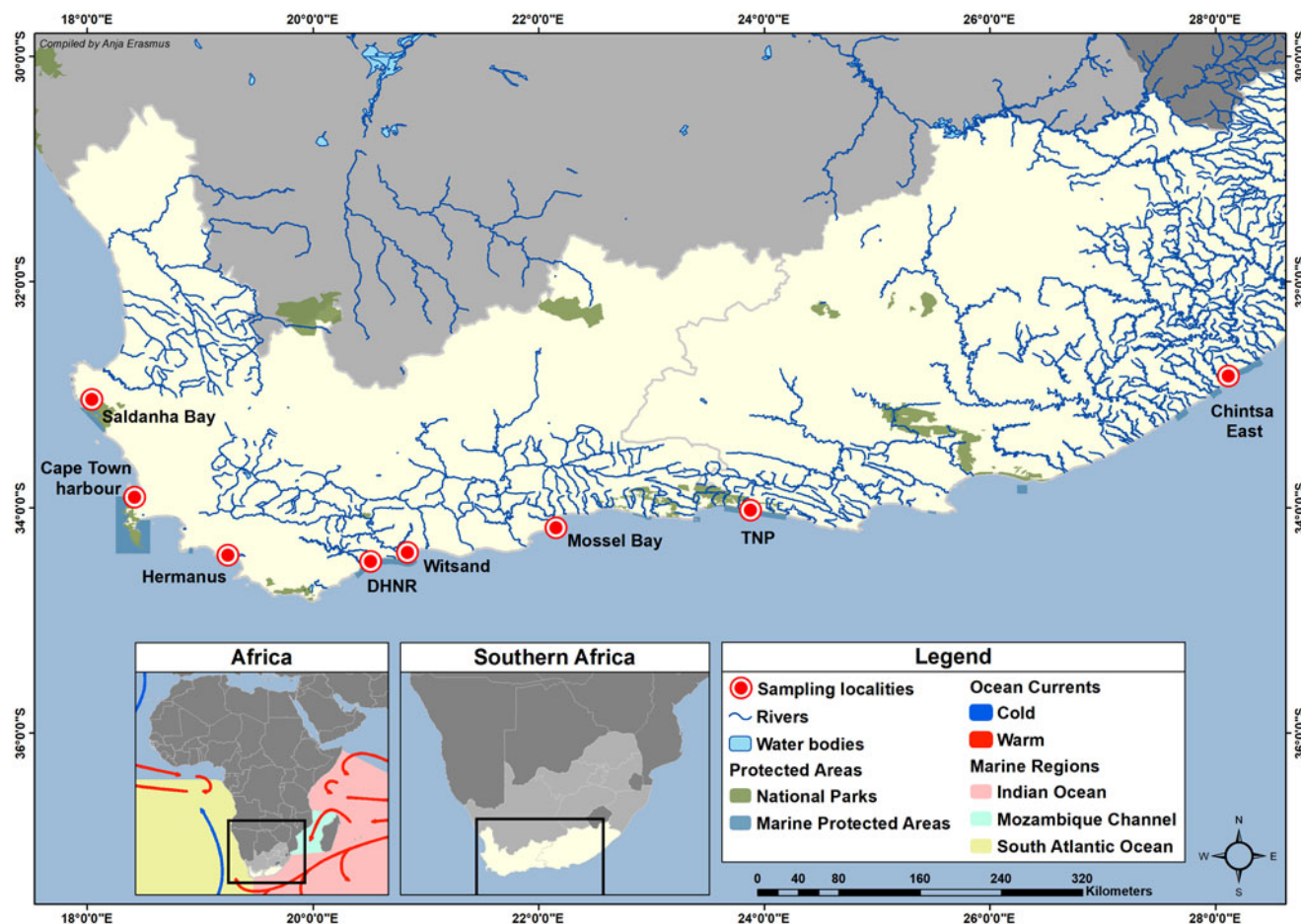
While exploring the trematode biodiversity of fishes along the South African coast, adult specimens of *P. maculatus* were found in three intertidal and near-shore fishes: *Clinus superciliosus* (L.) (Clinidae), *Diplodus capensis* (Smith) (Sparidae) and *Sparodon durbanensis* (Castelnau) (Sparidae). This is the first report of adult *P. maculatus* from marine fishes in South Africa, along with the first molecular characterization of this species from this biodiversity-rich marine environment.

**Materials and methods****Sample collection**

Specimens of *C. superciliosus*, *D. capensis* and *S. durbanensis* were collected from rocky intertidal and near-shore areas along the South African coast (Fig. 1). The collection sites of each species, along with the infection rates, can be seen in Table 1.

Sampling was carried out under the permits MALH-K2016-005a and SMIT-NJ/2020-004 for the Tsitsikamma section of the Garden Route National Park (TNP); RES2018/35 for Hermanus; RES2020/29, RES2021/49 and RES2022/44 for Cape Town harbour, Chintsa East, Langebaan marina in Saldanha Bay (henceforth called Saldanha Bay), Mossel Bay and Witsand; and CN44-87-18289 for De Hoop Nature Reserve. Fishes were collected with baited traps and hand lines and humanely killed using standard methods. Following euthanasia, fishes were subjected to a full helminthological examination by inspecting every organ. Digenean

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**Figure 1.** Map of sampling localities along the South African coast. DHNR - De Hoop Nature Reserve; TNP - Tsitsikamma section of the Garden Route National Park.

trematodes were removed, relaxed in hot saline and fixed in 80% ethanol for further analyses. The prevalence and intensity of each species was calculated according to Bush *et al.* (1997). Fish names and authorities follow FishBase (Froese and Pauly, 2023).

### Morphological analyses

Hologenophores were selected following the concept of Pleijel *et al.* (2008). These, along with additional whole specimens, were rehydrated in distilled water, stained with Mayer's

haematoxylin, destained with 1% hydrochloric acid, neutralized with 1% ammonia, gradually dehydrated in an ethanol series (70, 80, 90, 96, 100%), cleared in methyl salicylate and permanently mounted on slides with dammar gum. These specimens were measured, photographed and used to make detailed drawings for each species. Measurements were obtained using NIS-Elements BR Cameral Analysis software and a Nikon Ni microscope (Nikon Instruments, Tokyo, Japan), and are given as a range followed by a mean in parentheses. All measurements, unless otherwise stated, are given in micrometres ( $\mu\text{m}$ ). Drawings

**Table 1.** Data on fishes collected, localities within South Africa, intensity of infection and prevalence of infection with *P. maculatus*.

Host species	Locality	No. fish	Infection intensity	Prevalence, %
<i>Clinus superciliosus</i>	Cape Town harbour	16	0	0
	Chintsa East	11	3–4	27
	Hermanus	8	1	13
	Saldanha Bay	19	0	0
	Tsitsikamma NP	17	1	30
<i>Diplodus capensis</i>	Chintsa East	16	1–4	25
	De Hoop NR	12	1–22	67
	Mossel Bay	5	0	0
	Tsitsikamma NP	28	1–3	39
	Witsand	3	1–2	67
<i>Sparodon durbanensis</i>	Tsitsikamma NP	12	1–13	42

NP, National Park; NR, Nature Reserve.

were made with the aid of a drawing tube attached to the aforementioned microscope. Digitization of the specimen drawings was done using Adobe Illustrator v. 26.4.1 and Photoshop v. 23.4.2. Voucher material is deposited in the Parasite Collection of the National Museum (NMB), Bloemfontein, South Africa.

### Generation of molecular data

Total genomic DNA was extracted with the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) and the PCR Biosystems Rapid DNA Extraction Kit (PCR Biosystems available from Analytical Solutions, Randburg, South Africa), following the manufacturers' protocols. However, the following adaptations were made to the protocol of the PCR Biosystems Rapid DNA Extraction Kit to obtain quality DNA: only 10  $\mu$ L lysis buffer was used, 5  $\mu$ L proteinase K-containing buffer was used and the final reaction was diluted with 450  $\mu$ L water. The D1–D3 fragment of the 28S nuclear ribosomal RNA gene was amplified using the primers Dig2 (5'-AAG CAT ATC ACT AAG CGG-3') (Tkach *et al.*, 2001) and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Snyder and Tkach, 2001), following the protocol of Tkach *et al.* (2003). Two internal primers were used for sequencing of 28S rDNA: ECD2 (5'-CTT GGT CCG TGT TTC AAG ACG GG-3') (Tkach *et al.*, 2003) and 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') (Littlewood *et al.*, 2000). For the amplification of the 18S rRNA fragment, the universal forward and reverse primers 18SU467F (5'-ATC CAA GGA AGG CAG CAG GC-3') and 18SL1310R (5'-CTC CAC CAA CTA AGA ACG GC-3') (Suzuki *et al.*, 2006) were used; polymerase chain reaction (PCR) conditions were set to 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min and final extension at 72°C for 7 min. The cytochrome *c* oxidase subunit I (COI) genes were amplified using the forward primer Dice1F (5'-ATT AAC CCT CAC TAA ATT WCN TTR GAT CAT AAG-3') (Moszczyńska *et al.*, 2009) and the reverse primer Dice 14R (5'-TAA TAC GAC TCA CTA TAC CHA CMR TAA ACA TAT GATG-3') (Van Steenkiste *et al.*, 2015); PCR conditions were set to 94°C for 4 min, followed by 40 cycles of 94°C for 40 s, 51°C for 40 s, 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were visualized with 1% agarose gel electrophoresis and sent to a commercial sequencing company in Pretoria, South Africa for purification and sequencing (Inqaba Biotechnical Industries [Pty] Ltd.). The resulting sequences were assembled and edited using Geneious v. 11.1.4 bioinformatics software (Biomatters, Auckland, New Zealand). Novel sequence data have been deposited in GenBank (see Table 2).

### Phylogenetic analyses

Sequences included in the phylogenetic analyses were selected based on the results of Wee *et al.* (2017) and Oliva *et al.* (2018). Sequences available for this genus as well as the outgroup sequences were retrieved from GenBank (Table 2).

An alignment was built for each gene, using MUSCLE (Edgar, 2004) as implemented in Geneious v. 11.1.4. The best nucleotide substitution model was predicted using jModelTest 2.1 (Posada, 2008), based on the Akaike information criterion. The general time-reversible model with gamma distribution rate variation among sites (GTR + G) was used to construct both phylogenetic trees. The COI alignment was only used to calculate genetic distance matrices. Both phylogenies are based on Bayesian inference (BI) and maximum likelihood (ML) estimate analyses. BI analyses were performed with MrBayes software and ML analyses were performed with PhyML v. 3.0 (available at <http://www.atgc-montpellier.fr/phyml/>). For the BI analyses of both alignments,

the following parameters were set: Markov chain Monte Carlo chains were run for 3 000 000 generations; the 'burn-in' parameter was set for the first 25% of the sampled trees. A hundred bootstrap pseudo replicates were run to determine the nodal support for ML analyses. Phylogenetic trees were visualized using FigTree v. 1.4.4 (Rambaut 2012) and combined and edited using Adobe Illustrator v. 26.4.1. Pairwise genetic distance matrices were calculated in MEGA v. X using the parameters 'model/method = No. of differences', 'variance estimation method = none', 'substitutions to include = d: transitions + transversions' and 'gaps/missing data treatment = pairwise deletion'.

## Results

### General results

Among all the localities sampled, De Hoop Nature Reserve exhibited the highest prevalence and intensity of infection with *P. maculatus* in *D. capensis* (see Table 1). *Proctoeces maculatus* was most prevalent in *C. superciliosus* from TNP, but had a higher intensity of infection at Chintsa East. However, this species was absent from *C. superciliosus* collected in Cape Town harbour and Saldanha Bay, as well as from *D. capensis* collected in Mossel Bay. Nearly half of the *S. durbanensis* collected from TNP were infected with *P. maculatus*. Having considered the lines of evidence provided by molecular, morphological and ecological (i.e. host) data, we are confident that these collected specimens belong to *P. maculatus*.

### Morphological characterization

Family Fellodistomidae Nicoll, 1909

Subfamily Fellodistominae Nicoll, 1909

Genus *Proctoeces* Odhner, 1911

*Proctoeces maculatus* (Looss, 1902) Odhner, 1911

Type-host: *Labrus merula* L.

Type-locality: Trieste, Italy

New hosts: *Clinus superciliosus* (L.) (Clinidae); *Diplodus capensis* (Smith) (Sparidae); *Sparodon durbanensis* (Castelnau) (Sparidae).

New localities: Chintsa East, De Hoop Nature Reserve, Hermanus, Tsitsikamma section of the Garden Route National Park, and Witsand, South Africa.

Site of infection: Intestine.

Representative DNA sequences: OR724708 (18S); OR724713–OR724718 (28S); OR723765–OR723770 (COI).

Voucher material: A total of 58 voucher specimens deposited in NMB – 22 stained and permanently mounted specimens (accession no. NMB P 999–1020) and 36 specimens in ethanol (accession no. NMB P 991–998).

Description (based on 22 whole mounts; Fig. 2; Table 3). Body subcylindrical, robust, tapering at both ends; widest at level of ventral sucker, occasionally at level of testes; forebody occupying about 26.1% of total body length. Tegument unarmed.

Oral sucker subterminal, spherical to subspherical. Prepharynx absent. Pharynx well developed, globular, muscular. Oral sucker to pharynx length ratio 1:0.7–1.1 (1:0.9). Oesophagus short, often indistinct. Intestine thick-walled. Intestinal bifurcation in mid forebody, often overlaps pharynx dorsally. Caeca end blindly in hindbody between testes and posterior extremity; ends often covered by uterus, thus indistinguishable. Ventral sucker pre-equatorial, transversely oval when viewed ventrally, muscular, larger than oral sucker. Oral sucker to ventral sucker length ratio 1:0.9–1.5 (1:1.2); width ratio 1:1.5–2.0 (1:1.7).

Testes two, intercaecal, obliquely tandem, occasionally tandem, margins entire; anterior testis triangular to elongate, often

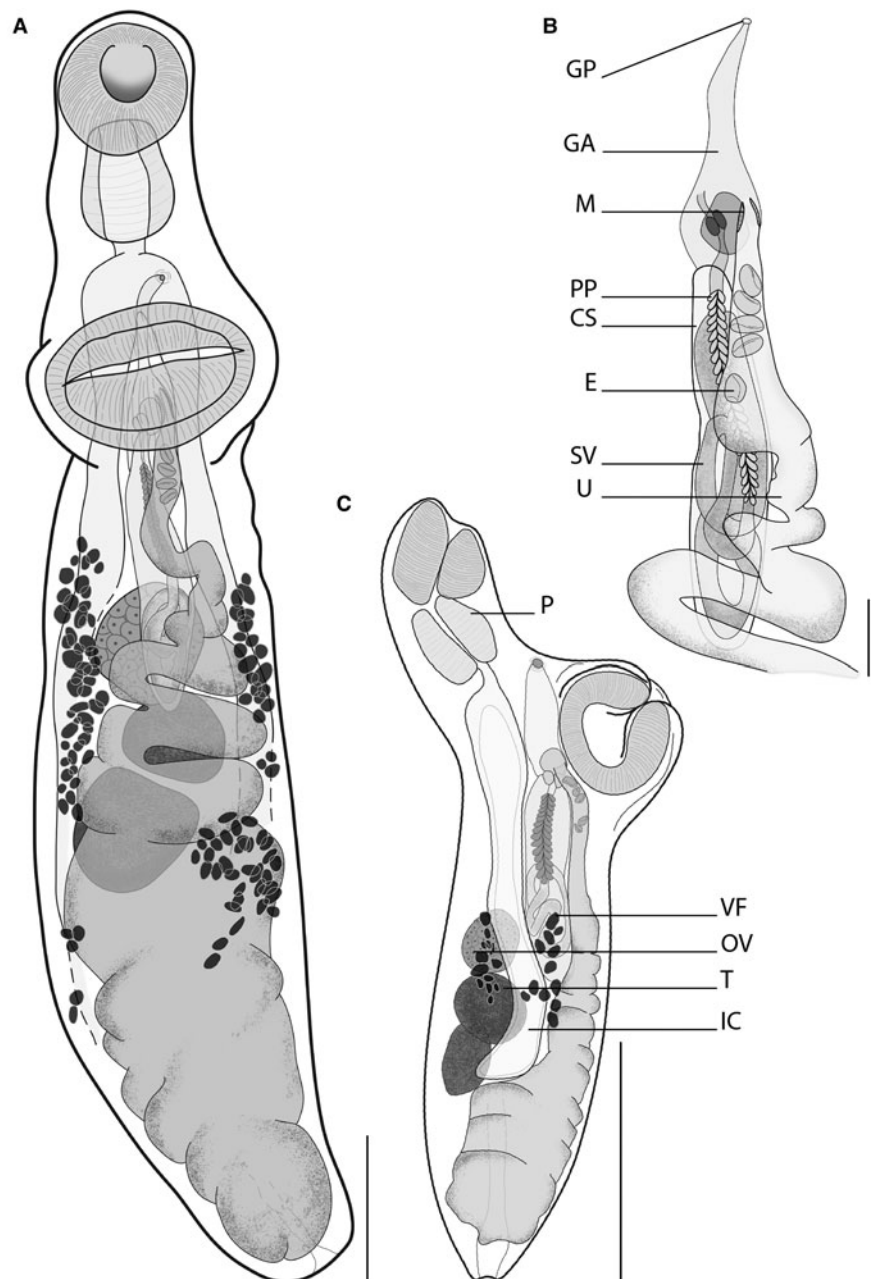
**Table 2.** Sequences used for phylogenetic analyses of the 18S, 28S and COI gene/regions

Species	Host	Locality	GenBank accession numbers			Reference
			18S	28S	COI	
<i>Proctoeces choerodoni</i>	<i>Choerodon cyanodus</i>	Heron Island, AUS	KX671310	KX671299	KY073877	Wee <i>et al.</i> (2017)
<i>Proctoeces humboldti</i>	<i>Semicossyphus darwini</i>	Chile	MF414438	–	–	Ñacari <i>et al.</i> (2018)
	<i>Sicyases sanguineus</i>	Chile	–	KY432601	KY432628	Oliva <i>et al.</i> (2018)
	<i>S. sanguineus</i>	Chile	–	–	KU236023 <sup>a</sup>	Oliva <i>et al.</i> (2018)
<i>Proctoeces insolitus</i>	<i>Acanthopagrus australis</i>	Queensland, AUS	KX671312	KX671300	KY073873	Wee <i>et al.</i> (2017)
<i>Proctoeces cf. lintoni</i>	<i>Fissurella costata</i> <sup>b</sup>	Chile	EU423050 <sup>c</sup>	–	–	Wee <i>et al.</i> (2017)
<i>Proctoeces maculatus</i>	<i>Sparodon durbanensis</i>	TNP, SA	–	–	OR723765	Present study
	<i>S. durbanensis</i>	TNP, SA	–	–	OR723766	Present study
	<i>S. durbanensis</i>	TNP, SA	–	OR724714	–	Present study
	<i>Clinus superciliosus</i>	TNP, SA	–	OR724715	–	Present study
	<i>C. superciliosus</i>	Chintsa East, SA	–	OR724716	–	Present study
	<i>Diplodus capensis</i>	TNP, SA	–	OR724713	–	Present study
	<i>D. capensis</i>	TNP, SA	OR724708	–	OR723768	Present study
	<i>D. capensis</i>	TNP, SA	–	–	OR723769	Present study
	<i>D. capensis</i>	DHNR, SA	–	OR724718	–	Present study
	<i>D. capensis</i>	TNP, SA	–	–	OR723767	Present study
	<i>D. capensis</i>	Chintsa East, SA	–	OR724717	OR723770	Present study
	<i>Archosargus probatocephalus</i>	Mississippi, USA	AY222161	AY222284	–	Olson <i>et al.</i> (2003)
	<i>Sabella pavonina</i> <sup>b</sup>	Tunisia	KX671315	–	–	Wee <i>et al.</i> (2017)
	<i>Sparus aurata</i>	Tunisia	–	KX671302	–	Wee <i>et al.</i> (2017)
	<i>Lithognathus mormyrus</i>	Tunisia	–	KU052937	–	Antar and Gargouri (2016)
	<i>S. pavonina</i> <sup>b</sup>	Tunisia	–	KU052941	–	Antar and Gargouri (2016)
	<i>Thalassoma janseni</i>	Queensland, AUS	KX671325	–	–	Wee <i>et al.</i> (2017)
	<i>Monodactylus argenteus</i>	Queensland, AUS	–	KX671309	–	Wee <i>et al.</i> (2017)
	<i>Chrysophrys auratus</i>	Queensland, AUS	–	–	KY073875	Wee <i>et al.</i> (2017)
	<i>Octopus sinensis</i> <sup>b</sup>	Japan	–	LC618023	–	Izumi <i>et al.</i> (2021)
<i>S. sanguineus</i>	Chile	–	KT865207 <sup>d</sup>	–	Oliva <i>et al.</i> (2018)	
<i>Proctoeces major</i>	<i>S. sanguineus</i>	Chile	KY432595 <sup>d</sup>	KY432618 <sup>d</sup>	–	Oliva <i>et al.</i> (2018)
	<i>S. sanguineus</i>	Chile	JX306110 <sup>d</sup>	–	–	Oliva <i>et al.</i> (2018)
	<i>Perumytilus purpuratus</i> <sup>b</sup>	Chile	JQ782525	–	–	Muñoz <i>et al.</i> (2013)
<i>Proctoeces cf. major</i>	<i>Octopus sinensis</i> <sup>b</sup>	Japan	–	LC618023	–	Izumi <i>et al.</i> (2021)
<i>Proctoeces sicyases</i>	<i>M. argenteus</i>	Hope Island, AUS	AJ224469	–	–	Hall <i>et al.</i> (1999)
	<i>M. argenteus</i>	Moreton Bay, AUS	–	MZ687078	–	Cribb <i>et al.</i> (2021)
	<i>Anarhichas lupus</i>	North Sea, UK	Z12601	AY222282	–	Olson <i>et al.</i> (2003)
	<i>Cerastoderma edule</i> <sup>b</sup>	Wadden Sea, The Netherlands	–	–	KF880498	Feis <i>et al.</i> (2015)
<b>Outgroup</b>						
<i>Coomera brayi</i>	<i>M. argenteus</i>	Hope Island, AUS	AJ224469	–	–	Hall <i>et al.</i> (1999)
	<i>M. argenteus</i>	Moreton Bay, AUS	–	MZ687078	–	Cribb <i>et al.</i> (2021)
<i>Fellodistomum fellis</i>	<i>Anarhichas lupus</i>	North Sea, UK	Z12601	AY222282	–	Olson <i>et al.</i> (2003)
<i>Gymnophallus choledochus</i>	<i>Cerastoderma edule</i> <sup>b</sup>	Unspecified	–	–	KF880498	Feis <i>et al.</i> (2015)

AUS, Australia; TNP, Tsitsikamma section of the Garden Route National Park; SA, South Africa; UK, United Kingdom; USA, United States of America.

<sup>a</sup>Listed on GenBank as *Proctoeces cf. lintoni*.<sup>b</sup>Host is not a fish.<sup>c</sup>Listed on GenBank as *Proctoeces lintoni*.<sup>d</sup>Listed on GenBank as *Proctoeces sp.*





**Figure 2.** *Proctoeces maculatus* whole mount. Ventral view (A), terminal genitalia (B), lateral view (C). **Abbreviations:** E, egg; CS, cirrus sac; GA, genital atrium; GP, genital pore; IC, intestinal caeca; M, metraterm; OV, ovary; P, pharynx; PP, *pars prostatica*; SV, seminal vesicle; T, testis; U, uterus; VF, vitelline follicles. Scale bars: 500  $\mu$ m (A, C); 100  $\mu$ m (B).

contiguous with ovary; posterior testis triangular to elongate, contiguous with anterior testis. Post-testicular field represents 17–42% (26%) of body length. Cirrus sac situated between posterior end of ventral sucker and mid-level of anterior testis, encloses seminal vesicle and *pars prostatica*, ejaculatory duct not observed. Seminal vesicle in posterior part of cirrus sac, tubular, highly convoluted. *Pars prostatica* fills most of anterior cirrus sac, well developed, straight or slightly curved, covered by dense gland cells. Prominent muscular papilla at distal end of cirrus sac. Genital atrium thin-walled, extends from about mid or anterior level of ventral sucker to meet genital pore. Genital pore at about level of intestinal bifurcation, slightly sinistral.

Ovary median to slightly dextral, often contiguous to anterior testis, subspherical to elongate oval but occasionally slightly lobed. Mehlis' gland not observed. Uterus highly convoluted; uterine coils restricted to between mid-level of ventral sucker and posterior extremity, filling most of ventral hindbody, filled with eggs in all specimens. Metraterm at distal end of uterus, enters genital atrium, faint. Eggs oval, operculate, yellow, without filaments.

Vitellarium follicular; follicles vary greatly in size, situated in two lateral fields, extend from slightly anterior to ovary to posterior limit of posterior testis, occasionally overreaching these limits, sometimes difficult to distinguish.

Excretory pore terminal, forming slight concavity at posterior body extremity. Excretory vesicle Y-shaped; site of bifurcation of vesicle not observed due to large number of eggs present in uterus; arms of vesicle terminate in two blind ends near posterior limit of pharynx, often difficult to distinguish.

#### Remarks

The specimens of *P. maculatus* in the present study agree well with the original description of the species by Looss (1901) from brown wrasse, *L. merula* (Labridae) collected off Trieste, Italy, and the redescription by Odhner (1911) based on specimens collected from the butterfly blenny, *Blennioides ocellaris* L., (Blenniidae) collected off Naples, Italy (Table 3), except that the specimens in the present study are smaller, having lower maxima for body length and width, slightly smaller suckers, pharynx and

**Table 3.** Morphometrics of newly collected specimens of *Proctoeces maculatus*, compared to examples of published measurements in literature for adult *P. maculatus*

Host(s)	<i>Diplodus capensis</i> , <i>Clinus superciliosus</i>		<i>Labrus merula</i>		<i>Blennius ocellaris</i>		<i>Crenilabrus</i> sp.	<i>Acanthopagrus schlegelii</i> , <i>Epinephelus akaara</i> , <i>Pagrus auratus</i> , <i>Rhabdosargus sarba</i>	<i>Halichoeres bivittatus</i>	<i>Paraperis colias</i>	<i>Lithognathus mormyrus</i> , <i>Sparus aurata</i> , <i>Trachinotus ovatus</i>	<i>Myoxocephalus stelleri</i> , <i>Platichthys stellatus</i> , <i>Pseudopleuronectes schrenki</i>
Locality	Various, South Africa		Trieste, Italy		Naples, Italy		Black Sea, Russia	Seto Inland Sea, Japan	Bermuda	New Zealand	Bizerte Lagoon, Tunisia	Wakanai, Hokkaido, Japan
Reference	Present study		Looss (1901)		Odhner (1911)		Vlasenko (1931)	Yamaguti (1934)	Bray and Gibson (1980)	Bray (1983)	Antar and Gargouri (2016)	Shimazu (1984)
	Range (n = 19)	Mean	Range (n = unknown)	Max.	Range (n = unknown)	Max.	Range (n = unknown)	Range (n = 9)	Range (n = unknown)	Range (n = 1)	Range (n = 4)	Range (n = 39)
<b>Body length</b>	1151–2870	1811	–	3200	–	2500	~3000	1730–4460	1460	2450	1277–1506	2370–6170
<b>Body width</b>	322–695	484	–	800	300–450	–	700	340–1100	650	950	449–582	700–1420
<b>Forebody length</b>	283–546	428	–	–	–	–	–	–	–	–	420–587	–
<b>Hindbody length</b>	839–2133	1325	–	–	500–700	–	–	–	–	–	612–936	–
<b>Body width:length ratio</b>	1:2.41–5.98	3.73	–	–	–	–	–	–	–	–	–	–
<b>Forebody length as % body length</b>	18.4–35.2	26.1	–	–	–	–	–	–	–	–	–	–
<b>Oral sucker length</b>	167–317	217	–	–	–	–	–	200–560	200	360	146–171	350–700
<b>Oral sucker width</b>	148–270	213	370	–	200–300	–	~250	230–570	280	370	150–191	350–750
<b>Pharynx length</b>	143–278	193	–	–	–	–	–	190–400	230	350	100–137	250–450
<b>Pharynx width</b>	88–244	171	280	–	150–230	–	~200	190–360	170	280	100–142	250–450
<b>Oesophagus length</b>	9–52	28	–	–	–	–	–	–	50	–	20–54	–
<b>Ventral sucker length</b>	223–324	265	–	–	280–420	–	390	230–640	330	430	166–246	470–970
<b>Ventral sucker width</b>	293–465	370	630	–	420–700	–	610	290–840	400	670	237–287	570–1000
<b>Oral sucker length:ventral sucker length</b>	1:0.95–1.48	1:1.23	–	–	–	–	–	–	–	–	1:1.54–1.14	–
<b>Oral sucker width:ventral sucker width</b>	1:1.49–1.98	1:1.74	–	–	–	–	–	–	1:1.4	1:1.80	1:1.59–1.41	1:1.13–1.66
<b>Oral sucker length:pharynx length</b>	1:0.71–1.08	1:0.90	–	–	–	–	–	–	–	–	–	–
<b>Oral sucker width:pharynx width</b>	1:0.57–0.95	1:0.80	–	–	–	–	–	–	–	–	1:0.79–0.67	–
<b>Ovary length</b>	128–244	200	–	–	–	–	–	170–400	–	220	87–129	250–500
<b>Ovary width</b>	112–235	170	–	–	–	–	~220	140–410	–	280	75–87	220–420
<b>Egg length</b>	23–49	41	70	–	72–79	–	74	66–76	–	40–62	–	50–65
<b>Anterior testis length</b>	110–255	186	–	–	–	–	–	–	–	–	83–141	–
<b>Anterior testis width</b>	143–268	204	–	–	–	–	–	–	–	–	71–121	–
<b>Posterior testis length</b>	121–284	197	–	–	–	–	–	–	–	–	87–158	–
<b>Posterior testis width</b>	138–323	220	–	–	–	–	–	–	–	–	79–116	–
<b>Average testis length</b>	116–270	192	–	–	–	–	~220	190–420	160–230	150–160	–	250–750

(Continued)

Table 3. (Continued).

Host(s)	<i>Diplodus capensis</i> , <i>Clinus superciliosus</i>	<i>Labrus merula</i>	<i>Blennius ocellaris</i>	<i>Crenilabrus</i> sp.	<i>Acanthopagrus schlegelii</i> , <i>Epinephelus akaara</i> , <i>Pagrus auratus</i> , <i>Rhabdosargus sarba</i>	<i>Halichoeres bivittatus</i>	<i>Parapercis collas</i>	<i>Lithognathus mormyrus</i> , <i>Sparus aurata</i> , <i>Trachinotus ovatus</i>	<i>Myoxocephalus stelleri</i> , <i>Platichthys stellatus</i> , <i>Pseudopleuronectes schrenki</i>
Average testis width	141–281	121	-	~220	190–570	130–140	250–260	-	270–620
Cirrus sac length	314–633	471	-	-	-	130–340	600	250–374	500–1050
Cirrus sac width	80–148	124	-	-	-	-	160	58–79	120–300
Post-testicular region	223–1217	576	-	-	-	-	-	162–337	-
Post-testicular region as % body length	17–42	26	-	-	-	-	-	-	-

eggs. Those specimens of Odhner (1911) also have a notably shorter hindbody and higher maxima for ventral sucker length and width. A faint metraterm has been noted in some specimens, including our own; Looss (1901) also noted a metraterm, however Bray and Gibson (1980) described the metraterm as being muscular.

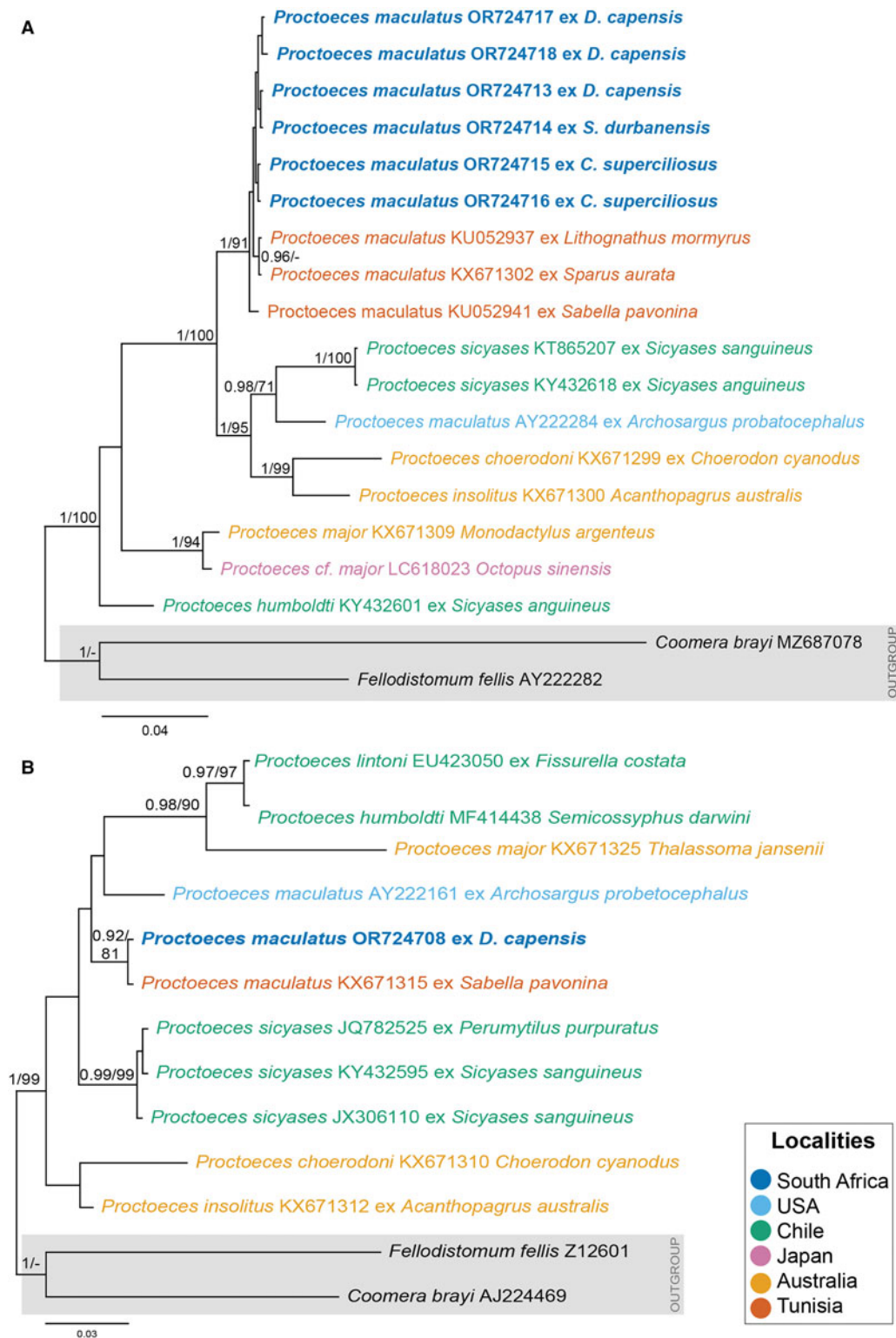
More recent descriptions of *P. maculatus* by Bray and Gibson (1980) and Antar and Gargouri (2016) are also considered. Bray & Gibson (1980) also note the bifurcation site of the  $\gamma$ -shaped excretory vesicle, but this was not observed in any of the specimens of the present study, due to the large number of eggs present in the uterus that fills the hindbody. However, it was observed that the excretory vesicle terminates blindly in the anterior forebody, suggesting that the vesicle might be  $\gamma$ -shaped. Otherwise, the morphometrics of these specimens generally agree well with the specimens in the present study.

The specimens of Antar and Gargouri (2016) from Tunisia are also similar to those of the present study, with the exception of having lower maxima for body length, body width, hindbody length, as well as smaller suckers, ovary, testes and post-testicular field. Specimens collected in the present study are overall slightly smaller than those collected in the Black Sea (Vlasenko, 1931), but contain eggs that are nearly half the length of those observed by Vlasenko (1931). The upper limits of all structures of the specimens collected in Japan are much higher than that noted in the present study, although there is some overlap in the lower limits (Yamaguti, 1934; Shimazu, 1984).

#### Molecular characterization

The alignment of the 28S rDNA dataset generated 729 characters for analyses. Newly sequenced isolates formed a highly supported clade within the 28S analyses (Fig. 3A), together with the *P. maculatus* isolates found from the sparid fish hosts *Lithognathus mormyrus* (L.) (KU052937: juvenile) and *Sparus aurata* L. (KX671302), as well as the polychaete *Sabella pavonina* (KU052941: metacercariae) all collected in the Bizerte Lagoon in Tunisia (Antar and Gargouri, 2016; Wee et al., 2017). An isolate collected from the sheepshead, *Archosargus probatocephalus* (Walbaum) (Sparidae), in the Gulf of Mexico, Mississippi, USA, was identified as *P. maculatus* (AY222284) (Olson et al., 2003), but did not cluster with the abovementioned isolates of *P. maculatus*; our analyses instead recover it in a clade with *Proctoeces sicyases* Oliva, Valdivia, Cárdena, Muñoz, Escribano and George-Nascimento, 2018, *P. choerodoni* Wee, Cribb, Bray and Cutmore, 2017 and *P. insolitus* (Nicoll, 1915). Newly generated sequences differed from each other by 0–0.14% (0–1 nt) and from other isolates of *P. maculatus* (KU052937, KU052941, KX671302) by 0–0.41% (0–3 nt). The isolate identified as *P. maculatus* (AY222284) differed from sequences generated in the present study by 4.40–4.53% (32–33 nt), and from the abovementioned three isolates of *P. maculatus* by 4.53–4.67% (33–34 nt). The overall interspecific variation for the *Proctoeces* isolates in this dataset is 0.14–7.43% (1–54 nt).

The alignment of the 18S rDNA dataset generated 308 characters for analyses. A similar topology was observed for the 18S dataset (Fig. 3B), where the isolate from the present study formed a highly supported clade with *P. maculatus* infecting *S. pavonina* from the Bizerte Lagoon, Tunisia (KX671315) (Wee et al., 2017). These two sequences are identical. Again, the new sequence did not cluster with the isolate identified as *P. maculatus* by Olson et al. (2003) (AY222161); the latter was instead recovered in a clade with *Proctoeces major* Yamaguti, 1934 + [*P. humboldti* George-Nascimento and Quiroga, 1983 + *P. lintoni* Siddiqi and Cable, 1960], sister to the clade formed by sequences of *P. maculatus*. This isolate differed from newly generated sequences and an



**Figure 3.** Bayesian inference (BI) trees based on the 28S rDNA (A) and 18S rDNA (B) datasets of the genus *Proctoeces*. Nodal support given as BI/ML (maximum likelihood). Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site.

isolate of *P. maculatus* (KX671315) by 3.58% (11 nt). The overall interspecific variation for the *Proctoeces* isolates in this dataset is 2.61–10.46% (8–32 nt).

The COI dataset was only used to calculate genetic difference matrices, as there are no COI sequences of *P. maculatus* available in GenBank with which to compare our data. Newly generated sequences of *P. maculatus* differed from each other by 0–0.9% (0–3 nt). The interspecific variation between other species of *Proctoeces* and newly sequenced isolates is 2.4–23.8% (8–79 nt).

This study provides the first COI sequences for this species, which can be used in future phylogenies to study the true diversity of this genus.

### Discussion

Due to a lack of reliable characteristics on which the species of *Proctoeces* can be distinguished and the great morphological variation exhibited within this genus, the species of *Proctoeces* are



notoriously difficult to identify (Freeman and Llewellyn, 1958; Bray and Gibson, 1980). *Proctoeces maculatus* has not been re-collected or genotyped from its type-host at its type-locality (Trieste, Italy). Specimens identified as *P. maculatus* have been recorded and sequenced from Bizerte, Tunisia, which is also in the Mediterranean Basin but somewhat distant from Trieste, being about 950 km straight-distance away in the Tyrrhenian rather than Adriatic Sea and largely separated by the Italian mainland (Antar and Gargouri, 2013, 2016). This geographical distance, and the difference in fish hosts (the type-host being a labrid and those of Antar and Gargouri being from a sparid) further enhance the possibility that the specimens of Antar and Gargouri might not represent *P. maculatus sensu stricto*. This uncertainty can only be resolved with the molecular characterization of specimens collected from the type-host and the type-locality.

In weighing the merits of considering the *P. maculatus* of Looss from the type-locality conspecific with those of Antar and Gargouri from Tunisia, we need to consider two factors: the connectivity of populations and the propensity of *P. maculatus* to both switch fish hosts and use invertebrate (molluscan, annelid and echinoderm) hosts. Looss (1901), in describing *P. maculatus*, reported it from *L. merula* and two other labrid species, *Symphodus cinereus* (Bonnaterre) and *S. tinca* (L.). Linton (1907) subsequently described *Proctoeces subtenuis* (Linton, 1907) (as *Distomum subtenuis*) from Bermuda, recording it from three labrid species and the sparid *Calamus calamus* (Valenciennes). Odhner (1911), while proposing the genus *Proctoeces* and redescribing *P. maculatus*, also described a new species, *Proctoeces erythraeus* Odhner, 1911 from *Acanthopagrus bifasciatus* (Forsskål) (Sparidae) and *Thalassoma lunare* (L.) (Labridae) from the Red Sea. Bray and Gibson (1980) reviewed the case for both species being synonymous with *P. maculatus*; *P. subtenuis* remains as such, although Wee *et al.* (2017) argued that *P. erythraeus* should best be treated as *species inquirenda*. Nevertheless, it had become established relatively early on that species of *Proctoeces* readily infected both labrids and sparids in sympatry. Wee *et al.* (2017) demonstrated that *P. major* Yamaguti, 1934 infected sympatric sparids and labrids (as well as lethrinids, monacanthids, monodactylids and pomacentrids) in Moreton Bay, Australia, but also found that *Proctoeces choerodoni* Wee, Cribb, Bray and Cutmore, 2017, exclusively infected labrids of the genus *Choerodon* Bleeker, showing that the species of *Proctoeces* could (but not always) have wide host ranges incorporating both sparids and labrids. Demarcating the true host-specificity of *P. maculatus* is particularly fraught due to the fact that the majority of records putatively assigned to this species have never been tested with molecular sequence methods, nor accompanied by morphological vouchers or depictions. It is hence highly likely that the host range of *P. maculatus* has, to some extent, been wrongly estimated. That this might be true, however, does not preclude the fact that its host range is wide, nor does a wide host and geographic range mean divergence and speciation cannot occur in certain circumstances.

It is well understood that connectivity in the marine environment is a significant function of population spatial structure, genetic variability and, ultimately, speciation (see Hodge and Bellwood, 2016, for example). Marine taxa in the Mediterranean Basin show varying levels of population connectivity, with even single or two ecologically similar species showing differing levels of genetic variation and connectivity between different Mediterranean regions (Sahyoun *et al.*, 2016; Exadactylos *et al.*, 2019; Falcini *et al.*, 2020; López-Márquez *et al.*, 2021). However, it is clear from many studies that high connectivity and therefore high gene flow is a feature of many Mediterranean marine species at least some of the time (González-Wangüemert *et al.*, 2010;

Exadactylos *et al.*, 2019; López-Márquez *et al.*, 2021), effectively reducing the chances that the *P. maculatus* on the north coast of Africa might have speciated from those on the south coast of Europe. The ability of the species of *Proctoeces* to infect and even reproduce within a wide range of sessile invertebrate hosts as progenetic metacercariae compounds their ability to reduce impediments to connectivity and link populations (Valdivia *et al.*, 2014). Although the lack of molecular sequence data from the type-locality of *P. maculatus* again poses problems, sequence matching of species of *Proctoeces* from sympatric invertebrate and fish hosts has already been achieved (Valdivia *et al.*, 2010; Antar and Gargouri, 2016; Wee *et al.*, 2017). From all this information, we can (with the significant caveat that the status of *P. maculatus* from its type-locality and that of many putative records of this species from around the world is currently unknowable) infer that *P. maculatus* from labrids in the northern Mediterranean being a different species to those from sparids in the south is less likely than them being the same species, and, until the precise molecular nature of *P. maculatus* from its type-locality is known, it is safe and pragmatic to consider those specimens from Tunisia to be the same species.

Since its original description, *P. maculatus* has been reported from 65 fish species (including our three new host records) and 26 invertebrate species globally (WoRMS, 2023). The species is therefore rare among marine trematodes in that it appears to be truly euryxenous, i.e. infecting a wide range of hosts. Only a minority of marine fish-infecting trematodes are euryxenous, with the tendency being firmly towards higher, rather than lower, host-specificity (Miller *et al.*, 2011). The phenomenon is most often observed among species of Hemiuroida, including *Aponurus laguncula* Looss, 1907 (Lecithasteridae), reported from 95 fish species; *Thulinia microrchis* (Yamaguti, 1934) (Lecithasteridae), reported from 34 fish species; and most dramatically in *Derogenes varicus* (Müller, 1784) (Derogenidae), which has been reported from 317 fish species and habitats ranging from tropical coral reefs to abyssobenthic Antarctic waters (WoRMS, 2023). Such vast host ranges intuitively feel over-estimated; in cases of such disparate host and geographical range, they almost certainly are. However, judging their validity is complicated by the dubious reliability of many records, which were often made before the advent of modern molecular sequencing and provided only perfunctory morphological information (Bray *et al.*, 2016). Further complicating the matter is the issue of morphological ‘variation along a theme’, with individuals from disparate localities and hosts showing a degree of variation in size or anatomy, but sufficiently conserved morphology that distinguishing them from one another is difficult or even impossible, and easily confounded by poor specimen condition or preparation practises such as flattening. Renewed scrutiny of such taxa has, in some instances, supported the notion that they actually represent complexes of multiple, often cryptic species (for example Carreras-Aubets *et al.*, 2011), although the converse has also been demonstrated – specimens sampled across a wide host range are shown to be conspecific and thus reinforcing the breadth of the host range [as has happened in the case of *T. microrchis* (Miller *et al.*, 2011)]. It is likely that *P. maculatus* represents both a truly euryxenous and widespread species, and also a complex of multiple species. However, without the ability to access more specimens and generate more molecular sequence data from localities throughout its range, however, no firm conclusions can be drawn.

Using an integrated taxonomic approach (based on a combination of molecular and morphological characteristics), we have identified the specimens in the present study as *P. maculatus*. This study provides the first molecular characterization of *P. maculatus* from South Africa, in combination with morphological

characterization. This is also the first report of adult *P. maculatus* from South Africa, as well as the first report of this species from a fish host in the southern African region. Antar and Gargouri (2016) observed intraspecific variation in their sequences of the partial 28S gene of *P. maculatus* of 0–0.42% (0–5 nt); we consider the 0–0.41% (0–3 nt) difference between the newly generated sequences and the *P. maculatus* sequences available on GenBank as also consistent with intraspecific variation. Newly sequenced isolates are highly similar to isolates collected in the Mediterranean (Antar and Gargouri, 2016; Wee *et al.*, 2017), differing by a maximum of 3 base-pairs. However, the isolate identified as *P. maculatus* collected in the Gulf of Mexico (Olson *et al.*, 2003) did not cluster among other isolates of *P. maculatus*, thus it likely represents another species of *Proctoeces*. This was also noted by Antar and Gargouri (2016). Similar results were seen within the 18S dataset analysed. Bray (1984) reported *P. maculatus* as progenetic metacercariae from the common octopus *O. vulgaris* off Durban, South Africa; it is very likely that the specimens found during the present study represent this species, especially given that these host species share a habitat and similar food sources and are thereby exposed to larval stages of the same parasitic species (Smale and Buchan, 1981; Bennett *et al.*, 1983).

The sequence data generated by Antar and Gargouri (2016) and Wee *et al.* (2017) from sparids and carangids from off Tunisia are the closest available to the type-locality, being also from the Mediterranean Basin. Our *P. maculatus* sequences from sparids and clinids differ from those of Antar and Gargouri (2016) and Wee *et al.* (2017) by a maximum of 3 bp in the partial 28S rDNA region and are identical in the 18S rDNA region, supporting the notion that *P. maculatus* is not only euryxenous, but also has a wide geographical range. This ability to spread across such a wide area is likely facilitated by the versatility of *P. maculatus*, exploiting multiple hosts which are similarly wide-ranging and highly vagile (Feis *et al.*, 2015). South Africa shares several known hosts of *P. maculatus* with the Mediterranean, e.g. the sparids *L. mormyrus* and *Diplodus vulgaris* (Geoffroy Saint-Hilaire), the common octopus, *O. vulgaris* and the Mediterranean mussel, *Mytilus galloprovincialis* Lamarck (Mytilidae), the latter having been introduced to South Africa in the 1970s (Branch and Steffani, 2004). Another known host in the Mediterranean, *Diplodus sargus*, is also found along most of the West African coast and, until recently, was considered conspecific with our new host, *D. capensis*. As discussed above, the ability of *P. maculatus* to incorporate a progenetic stage in its life cycle, thereby thriving even when suitable definitive fish hosts are not present, likely further contributes to the wide distribution and ability of this species to exploit a wide range of hosts.

Interestingly, our results showed that fish sampled from sites within marine protected areas (MPAs) had the highest prevalence of *P. maculatus* (TNP, 30.0% from *C. superciliosus*, 39.0% from *D. capensis* and 42% from *S. durbanensis*; DHNR, 67.0% from *D. capensis*), compared with sites not within MPAs and adjacent to highly urbanized areas (0% in Cape Town harbour, Saldanha Bay and Mossel Bay) (see Table 1). This suggests that these parasites might be sensitive to pollution or other anthropogenic effects and therefore could be good indicators of ecosystem health. Similar results were noted by Erasmus *et al.* (2022), where the parasite diversity of *C. superciliosus* was lower in areas with a higher anthropogenic influence. Such findings are consistent with what we know regarding the deleterious effects that anthropogenic environmental changes have on both the richness and abundance of aquatic parasites (Sures *et al.*, 2023). One possible explanation of this phenomenon could be the absence or reduced presence of suitable intermediate or definitive hosts, which might be more susceptible to the effects of anthropogenic activities in non-MPA areas (Erasmus *et al.*, 2022). Apart from the record of

metacercariae by Bray (1983) from *O. vulgaris*, intermediate hosts of *P. maculatus* are unknown in South Africa. Elsewhere, first intermediate stages of the species have been observed from mytilid bivalves (Stunkard, 1970; Wardle, 1980), while both progenetic and non-progenetic metacercariae have been found in a wide range of invertebrates, including buccinid (Shimazu, 1984), haliotid (Shimazu, 1972), hydrobiid (Belousova, 2022), patellid (Prevot, 1965) and rissoid (Machkevsky and Parukhin, 1981) gastropods; acanthochitonid chitons (Polyplacophora) (Prevot, 1965); pectinid bivalves (Bray, 1983); nereid (Machkevsky, 1985), sabellid (Antar and Gargouri, 2016) and serpulid (Prevot, 1965) polychaetes (Annelida); and stronglylocentrotid echinoids (Echinodermata) (Shimazu, 1979). Most of these host groups, and the definitive fish hosts in which we found *P. maculatus*, are well represented along the South African coast, which means that, in theory, *P. maculatus* is well provisioned with intermediate and definitive hosts. However, the shallow-water marine communities, both in South Africa and elsewhere, are known to be vulnerable to anthropogenic disturbance, such as that caused by excessive harvesting (Crowe *et al.*, 2000; Cole *et al.*, 2011) and urbanization (Celliers and Ntombela, 2015; Momota and Hosokawa, 2021). Further marine environmental parasitological studies, focussing on digeneans and their intermediate and definitive hosts, will be needed to determine the extent to which anthropogenic environmental disturbance could compromise host population/community structure and, by extension, the parasite community.

**Data availability statement.** The data that support the findings of this study are available on request from the corresponding author.

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**Competing interests.** None.

**Ethical standards.** All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Ethical approval for this study was provided by the North-West University's AnimCare Ethics committee (NWU-00565-19-A5 and NWU-00759-22-A5).

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