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Morphological and molecular characterisation of two new species of *Rhipidocotyle* (Digenea: Bucephalidae Poche, 1907) from *Sphyraena putnamae* Jordan & Seale in Mozambique

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Research Paper

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Abstract

Species-level delineation of digenetic trematodes is complex and can be best achieved by integrative taxonomy using both genetic characterisation and morphological analysis. Two new Bucephalidae species of the genus *Rhipidocotyle* Diesing, 1858 are described here based on specimens collected from the intestine of *Sphyraena putnamae* Jordan & Seale following this approach. Adults of *R. siphonyaka* n. sp. and *R. nolwe* n. sp. possess tentacles and a tegument with scales. They are distinguished from their congeners by the arrangement of the digestive structures, the extent of the uterus relative to vitelline fields, and the arrangement of the reproductive structures. *Rhipidocotyle siphonyaka* n. sp. differs from *R. nolwe* n. sp. in having the pharynx and mouth positioned in the pre-uterine field, tandem testes, longer body length, and shorter pre-vitelline and post-testicular distance. *Rhipidocotyle siphonyaka* n. sp. differs from its congeners in having a tube-like intestinal caecum, pharynx and mouth opening positioned in the pre-vitelline field. *Rhipidocotyle nolwe* n. sp. appears to be similar, morphologically and morphometrically, to *Rhipidocotyle khalili* (Nagaty, 1937). Despite their similarities, *R. nolwe* n. sp. has a shorter body length and egg size. Moreover, the molecular analysis of 28S and ITS rDNA fragments indicate that *R. siphonyaka* n. sp. and *R. nolwe* n. sp. are closely related phylogenetically but distinct from one another and other Bucephalidae for which molecular data are available.

Introduction

The Sawtooth barracuda, *Sphyraena putnamae* Jordan & Seale (Family Sphyraenidae), is widely distributed along the Mozambican coast (Fischer *et al.* 1990) and is well known for hosting sexually adult trematodes (Bray and Justine 2011), including Bucephalidae Poche, 1907. Bucephalidae are cosmopolitan Platyhelminthes comprising about 380 parasitic species in marine and freshwater fish, representing 6.1% of the world's fauna (Cribb *et al.* 2014). The family is comprised of nine subfamilies, the most speciose being Bucephalinae Poche, 1907 with eight genera (Montes *et al.* 2023). Within the subfamily, 89% of the 250 species are represented by three genera: *Bucephalus* Baer, 1827 (77 species), *Prosorhynchoides* Dollfus, 1929 (78 species), and *Rhipidocotyle* Diesing, 1858 (67 species) (Corner *et al.* 2020).

The trematode genus *Rhipidocotyle* includes relatively small cosmopolitan parasites which, in their definitive host, inhabit the intestines of a wide range of marine and freshwater piscivorous fishes (Overstreet and Curran 2002; Bott *et al.* 2005; Cribb *et al.* 2014). Currently, the genus contains 14 freshwater and 53 marine species, which have been described or recorded from Europe, Asia, Africa, Australia, and the Americas (Ahyong *et al.* 2024).

Adult members of *Rhipidocotyle* are distinguished from all other typical bucephalids principally by rhynchus morphology that consists of a simple sucker, usually adorned with polygonal cap-like expansion 'hood' protruding in a rim (Gibson 1996; Curran and Overstreet 2009). The rhynchus assumes various formats, sometimes with small papillae that are more or less extensible (Manter 1940). Morphological delimitation of the genus includes a slightly bent *Pars prostatica*, oblique testes, and a pre-testicular ovary (Curran and Overstreet 2009).

The systematics of the subfamily Bucephalinae have been based on broad and general morphological characteristics, leading to several highly speciose genera composed of species that are dissimilar ecologically and phylogenetically (Corner *et al.* 2020). Recently, some systematic studies have employed molecular techniques alongside morphological descriptions to differentiate species, further improving databases available for comparison (Atopkin *et al.* 2022).

Members of the three largest Bucephalinae genera (*Bucephalus*, *Rhipidocotyle*, and *Prosorhynchoides)* have been reported from sphyraenid fishes worldwide (Nagaty 1937; Reimer 1985;

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Bray and Justine 2011). Nine *Rhipidocotyle* spp. are known from African marine waters: *R. eckmanni* Nagaty, 1937; *R. heptatheleta* Stunkard, 1974, and *R. khalili* from the Red Sea; *R. ernsti* Reimer, 1985; *R. lamberti* Reimer, 1985 and *R. tonimahnkei* Reimer, 1985 from Mozambique; *R. paruchini* Gavrilyuk-Tkachuk, 1979 from Cape Agulhas, Indian Ocean; *R. senegalensis* Fischthal & Thomas, 1972 from Senegal; and *R. ghanensis* Fischthal & Thomas, 1968 from Ghana (Nagaty 1937; Fischthal and Thomas 1968, 1972; Stunkard 1974; Gavrilyuk-Tkachuk 1979; Reimer 1985).

Rhipidocotyle bartolli Bray & Justine, 2011 and *R. khalili* are the only *Rhipidocotyle* species currently reported from sawtooth barracuda, *S. putnamae, worldwide (*Bray & Justine, 2011). In our study, adult trematodes from the intestine of *S. putnamae* off the western shore of Inhaca Island in Maputo Bay, Mozambique, were studied using an integrative taxonomic approach involving morphological data and DNA characterisation. Based on morphological and molecular traits, the specimens were found to represent two new taxa and placed in the genus *Rhipidocotyle.*

Material and methods

Fish sampling

Sawtooth barracuda were collected by fishermen at the western Nolwe sand bank offshore of Inhaca Island, Maputo Bay (26°05′43.77″S; 32°50′51.12″E; Figure 1). Thirty-five (n = 35) fish ranging between 25 and 60 cm in length were examined. Fish were dissected, and the intestines opened for collection of trematode parasites.

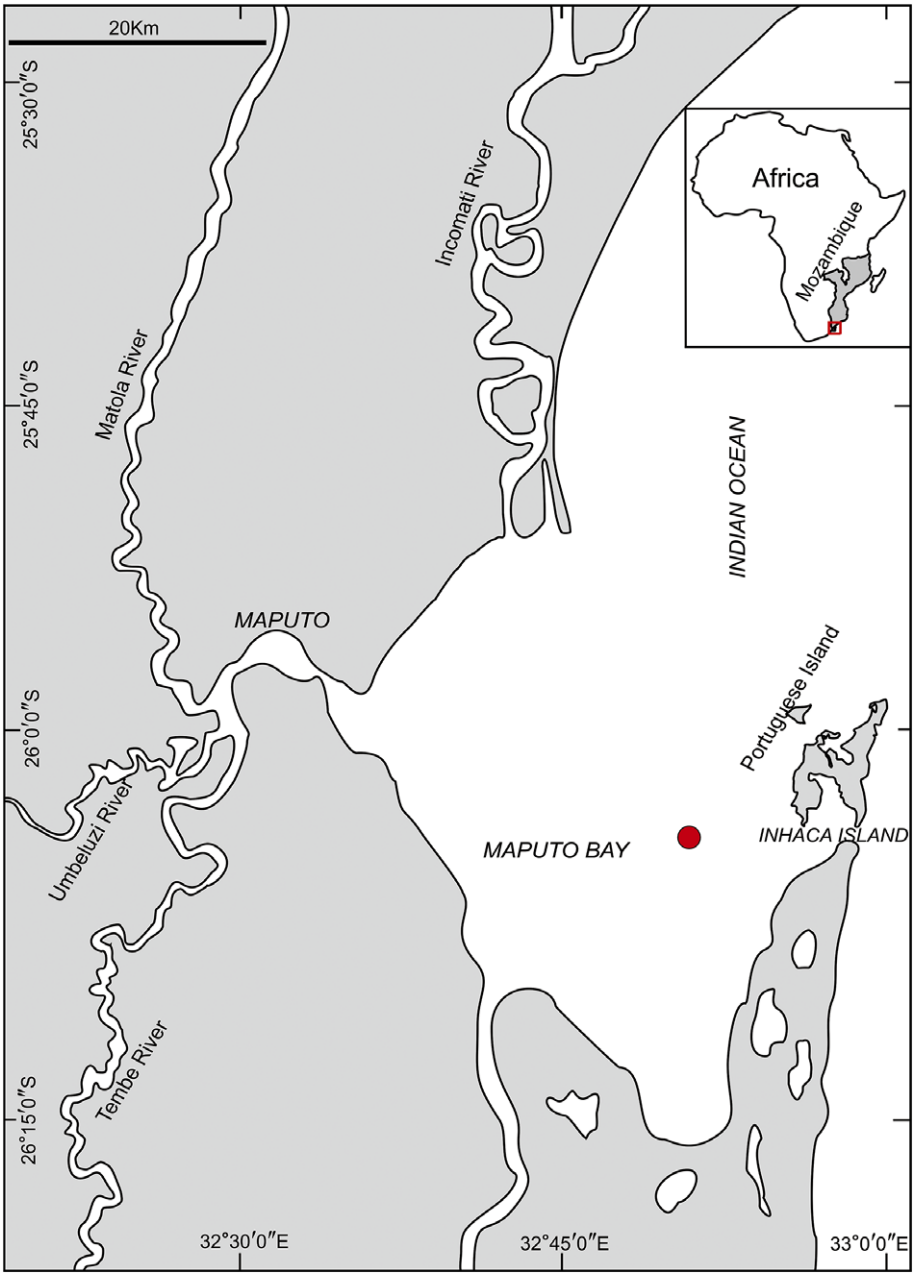

Figure 1. Map of the sample collection site in Maputo Bay, Inhaca Island. Inlay with Mozambique shaded in the African Continent and red square showing the position of the larger map. Red circle/dot shows the specific sampling site for *Sphyraena putnamae* in Maputo Bay off the western shore of Inhaca Island.

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Parasite collection

Detected trematode parasites were isolated from the intestine of naturally infected sawtooth barracuda. Specimens were washed in saline solution (0.85%) and fixed for morphological examination or genetic characterisation. Specimens for morphological examination by light microscopy (LM) were fixed under pressure between a microscope slide and coverslip in formalin–acetic acid–alcohol (FAA) and then preserved with 70% ethanol. For scanning electron microscopy (SEM), unflattened specimens were fixed in 70% ethanol, with those for DNA analyses fixed in 96% ethanol.

Morphological and morphometrical study

For LM, adult trematodes were stained with acetocarmine, followed by a distaining with 0.5% hydrochloric acid in 70% ethanol solution; gradually dehydrated with serial passage through ethanol at 70, 80, 90, 95%, and subsequent repeated exchanges of anhydrous 100% ethanol; cleared in beechwood creosote; and mounted in Canada balsam (Amato *et al.* 1991; Eiras *et al.* 2006) to produce whole-mount preparations. Photomicrographs and morphometric parameters were obtained with an Olympus BX53 compound microscope and Olympus Soft Imaging Solutions (Olympus, Münster, Germany), with photomicrographs used to compile digital illustrations using Corel DRAW® Graphics Suite X6 software (Corel Corporation, Ottawa, Canada). All measurements pertain to whole mounted specimens and are given in micrometres (two significant figures), unless otherwise stated, as the range followed by the mean in parentheses. The standard deviation is also given alongside the mean in parentheses in Table 2. In the present study, we have utilised the visual key to the marine *Rhipidocotyle* spp. first developed by Bray and Palm (2009) for metrical criteria and developed a similar key for comparison. The main criteria included in the visual key are body length and width, egg length, rhynchal length, pre-vitelline distance, pre-uterine distance, pre-mouth distance, post-testicular distance, and cirrus-sac reach, all as a percentage of the body-length.

Scanning electron microscope (SEM)

Unflattened trematodes fixed in 70% ethanol were prepared for SEM analysis by dehydration through a graded ethanol series (70 to 95%) and repeated exchanges of anhydrous 100% ethanol, followed by a graded series of hexamethyldisilazane (40 to 100%) (Merck, Darmstadt, Germany) (Nation 1983; Dos Santos *et al.* 2015). Specimens were dried overnight in a Sanpla dry keeper desiccator cabinet (Sanplatec, Osaka, Japan) and coated with gold using an Emscope SC500 sputter coater (Quorum Technologies, Newhaven, UK). A Vega 3 LMH scanning electron microscope (Tescan, Brno, Czech Republic) was used to study the specimens at 5–6 kV.

Additionally, some specimens fixed in 96% ethanol were bisected, and the anterior was prepared for SEM. This was done by rehydrating the specimens, placing them in DESS (Yoder *et al.* 2006) solution overnight, transferring to distilled water for an hour, transferring to steaming hot (≈ 70 °C) neutral buffered formalin (10% NBF) and allowing to cool to room temperature (10 min), rinsing in 70% ethanol, and then processing as above. The posteriors of these specimens were used for genetic characterisation.

DNA extraction, polymerase chain reaction (PCR) and sequencing

A small piece of tissue was removed from the posterior of 10 specimens fixed in 96% ethanol and genomic DNA extracted using an E.Z.N.A.® Tissue DNA kit (Omega Bio-tek, Inc., GA, USA). The anterior of the specimens was either prepared for LM or SEM study as described above. Genomic DNA was also extracted from three specimens following SEM analyses by removing a piece of dried and gold coated tissue to confirm their identity, again from the posterior. Two ribosomal DNA regions were targeted with partial 28S rDNA amplified using LSU5 (5' - TAG GTC GAC CCG CTG AAY TTA AGC A - 3') (Littlewood 1994) and 1500R (5' - GCT ATC CTG AGG GAA ACT TCG – 3') (Snyder and Tkach 2001), and entire internal transcribed spacer (ITS) rDNA amplified using 81_f (5' – GTA ACA AGG TTT CCG TAG GTG AA – 3') (Gustinelli *et al.* 2010) and ITS2:2 (5' – CCT GGT TAG TTT CTT TTC CTC CGC – 3') (Cribb *et al.* 1998). Internal primer 5.8S-2 (5' – GTC GAT GAA GAG CGC AGC – 3') (Královál-Hromadová *et al.* 2003) was also used to specifically target the second internal transcribed spacer (ITS2) rDNA if the entire ITS rDNA amplification failed. PCR reactions (30µl) were run using the following conditions: 5 min at 95°C followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 52°C, 2 minutes at 72°C, and a final extension of 5 minutes at 72°C. Amplification was verified on a 1% agarose gel impregnated with SafeViewTM Classic (Applied Biological Materials Inc., Richmond, Canada) and visualised using a SmartDocTM 2.0 gel visualisation and smartphone imaging system (Accuris instruments, Edison, NJ, USA). Amplicons were sequenced in both directions following Avenant-Oldewage *et al.* (2014).

Obtained sequence data were aligned and edited, if necessary, and reads were merged and compared using Geneious Prime version 2023.2.1 (http://www.genious.com). Generated data were aligned to closely related taxa following Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) analyses (see Table 1), with *Dollfustrema hefeiense* Liu in Zhang *et al.* 1999 included as outgroup following previous studies (Nolan *et al.* 2015; Hammond *et al.* 2018, 2020; Corner *et al.* 2020; Curran *et al.* 2022). Only data covering at least 80% of the alignment were retained, and identical sequences for conspecific data were removed. Data with doubtful identification were also excluded. The ITS rDNA alignment was trimmed to only ITS2 (at internal primer 5.8S-2) to better accommodate available data from other studies. Data were aligned using MAFFT (Katoh *et al.* 2002; Katoh and Standley 2013) via the EMBL-EBI portal and refined manually in MEGA7 (Kumar *et al.* 2016). Genetic distances (uncorrected p-distance) and base pair differences were calculated using MEGA7. Evolutionary histories were reconstructed using both maximum likelihood (ML) and Bayesian inference (BI) approaches. The Tamura-Nei nucleotide substitution model (Tamura and Ne, 1993) was selected for both ML and BI analyses using the model selection tool in MEGA7, with discrete Gamma distribution (five categories) included. Topologies were supported by 1,000 bootstrap replicates (Felsenstein, 1985) for ML and 10 million Markov chain Monte Carlo (MCMC) generations were for BI analyses. A single topology per marker analysed is presented based only on BI analyses due to the similarity between ML and BI approaches and the superior BI nodal support. Nodes annotated with support in format BI/ML, with nodes of inconclusive support (less than 50% bootstrap support or 0.5 posterior probability) not annotated or marked with "-". All obtained sequence data were deposited to GenBank.

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Table 1. List of 28S and ITS rDNA sequence data included in genetic analyses

Species	Host	Locality	28S rDNA	ITS rDNA	Isolate	Reference
***Rhipidocotyle nolwe* n. sp.**	***Sphyraena putnamae***	**Maputo Bay, Inhaca Island, Mozambique**	**PQ453500**	**PQ453487**	**TJD1 (NHM 2024.9.23.12)**	**Present study**
			PQ453501	**PQ453488**	**TJD24**	**Present study**
			PQ453502	**PQ453489**	**TJD25**	**Present study**
			PQ453503	**PQ453490**	**TJD27**	**Present study**
***Rhipidocotyle siphonyaka* n. sp.**	***Sphyraena putnamae***	**Maputo Bay, Inhaca Island, Mozambique**	**PQ453504**	**PQ453491**	**TJD2 (NHM 2024.9.23.5)**	**Present study**
			PQ453505	**PQ453492**	**TJD3**	**Present study**
			PQ453506	**PQ453493**	**TJD5**	**Present study**
			PQ453507	**PQ453494**	**TJD6**	**Present study**
			-	**PQ453495**	**TJD7**	**Present study**
			PQ453508	**PQ453496**	**TJD18**	**Present study**
			PQ453509	**PQ453497**	**TJD22**	**Present study**
			PQ453510	**PQ453498**	**TJD23**	**Present study**
			-	**PQ453499**	**TJD33**	**Present study**
Prosorhynchoides sp. TW-2019	*Plecoglossus altivelis*	Tanabe Bay, Wakayama Prefecture, Japan	LC498576	LC498575	TW-2019 Ayu_MC_204	Shirakashi *et al.* (2020)
Prosorhynchoides caecorum	*Bairdiella chrysoura*	Davis Bayou, Mississippi, USA	KT273393	KT273393	SSC-175	Nolan *et al.* (2015)
Prosorhynchoides galaktionovi	*Tylosurus crocodilus*	Lizard Island, Australia	MN310395	MN310393	LI (LI1 & LI2)	Hammond *et al.* (2020)
			MN310396	–	–	Hammond *et al.* (2020)
Prosorhynchoides kohnae	*Tylosurus crocodilus*	Lizard Island, Australia	MN310397	MN310394	LI	Hammond *et al.* (2020)
Prosorhynchoides megacirrus	*Sciaenops ocellatus*	Off Deer Island, Mississippi, USA	KT273391	KT273391	SSC-129	Nolan *et al.* (2015)
Prosorhynchoides moretonensis	*Tylosurus gavialoides*	Moreton Bay, Australia	MG953230	MG953233	–	Hammond *et al.* (2018)
Prosorhynchoides waeschenbachae	*Tylosurus gavialoides*	Moreton Bay, Australia	MG953231	MG953234	–	Hammond *et al.* (2018)
Aenigmatrema sp. A	*Sphyraena obtusata*	Off Green Island, Moreton Bay, Australia	MT809142	MT809218	THC17657 (A&B)	Corner *et al.* (2020)
Aenigmatrema grandiovum	*Sphyraena obtusata*	Off Amity Point, Moreton Bay, Australia	MT809145	MT809227	THC16655	Corner *et al.* (2020)
Aenigmatrema inopinatum	*Sphyraena obtusata*	Off St Helena Island, Moreton Bay, Australia	MT809144	MT809220	THC17295A	Corner *et al.* (2020)
Aenigmatrema undecimtentaculatum	*Sphyraena obtusata*	Off Dunwich, Moreton Bay, Australia.	MT809141	MT809217	THC17652A	Corner *et al.* (2020)
			MT809143	MT809208	THC17888F	Corner *et al.* (2020)
Bucephalus gorgon	*Seriola dumerili*	Gulf of Mexico, Louisiana, USA	KT273400	KT273400	SSC-105	Nolan *et al.* (2015)
Bucephalus polymorphus	*Dreissena polymorpha*	Lepelskoe Lake, Belarus	AY289238	AY289238	3B	Stunžėnas *et al.* (2004)
Paurorhynchus hiodontis	*Hiodon alosoides*	Red River, Minnesota, USA	KT273401	KT273401	SSC-183	Nolan *et al.* (2015)
Rhipidocotyle angusticolle	*Euthynnus alletteratus*	Louisiana, USA	KT273383	KT273383	SSC-107	Nolan *et al.* (2015)
Rhipidocotyle cf. *angusticolle*	*Auxis thazard*	Cabo Frio, Rio de Janeiro, Brazil	OP458334	OP458341	CP7	Pantoja *et al.* (2022)

(*Continued*)

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Table 1. (*Continued*)

Species	Host	Locality	28S rDNA	ITS rDNA	Isolate	Reference
Rhipidocotyle campanula	*Anodonta anatina*	Kaunas Water Reservoir, Lithuania	KF184355	KF184360	L583	Petkevičiūtė *et al.* (2014)
	Unio crassus	River Kiauna, Lithuania	KF184357	–	L618	Petkevičiūtė *et al.* (2014)
Rhipidocotyle fennica	*Anodonta anatina*	Lake Saravesi, Finland	JQ346716	JQ346723	F6_4n-28	Petkevičiūtė *et al.* (2014)
		Lake Vilkokšnis, Lithuania	–	KF184365	L649	Petkevičiūtė *et al.* (2014)
Rhipidocotyle galeata	*Eutrigla gurnardus*	North Sea, United Kingdom	AY222225	–	–	Olson *et al.* (2003)
Rhipidocotyle lepisostei	*Lepisosteus oculatus*	Pascagoula River, Mississippi, USA	KT273390	KT273390	SSC-311	Nolan *et al.* (2015)
Rhipidocotyle santanaensis	*Acestrorhynchus pantaneiro*	Colonia Carlos Pellegrini, Ibera Lagoon, Argentina	OQ244080	–	1	Montes *et al.* (2023)
Rhipidocotyle transversale	*Strongylura marina*	Off Deer Island, Mississippi, USA	KT273394	KT273394	SSC-301	Nolan *et al.* (2015)
Dollfustrema hefeiense	*Rhinogobius giurinus*	Fish market, Guandong, China	KT273386	KT273386	SSC-231	Nolan *et al.* (2015)

Results

Class Trematoda Rudolphi, 1808
Subclass Digenea Carus, 1863
Superfamily Bucephaloidea Poche, 1907
Family Bucephalidae Poche, 1907
Subfamily Bucephalinae Poche, 1907
Genus *Rhipidocotyle* Diesing, 1858

Rhipidocotyle siphonyaka *n. sp.*

Description (Figures 2, 3, 6 and Table 2)

Based on 14 whole-mount and 10 SEM preparations of mature specimens. Body 2000–3082 (2580) µm long, 172–311 (253) µm wide, tapering anteriorly, rounded posteriorly, widest at level of ovary (Figure 2). Tegument with scales (as mulitpointed spines, 29 points), retractable, with retracted scales appearing pit-like (Figures 3B; 6). Rhynchus oval 75.3–243 (120) long, 61.7–189 (98.6) wide, with six small tentacles (Figure 3A). Mouth sinistral opening ventral at pre-uterine region, on anterior half of body, always preceded by protuberance (Figures 2A; 3A, C). Mouth contains inner layers with expanded structures (Figure 3C). Pharynx elliptical, 47.1–160 (78.9) long, 29.9–118 (65.9) wide, positioned in anterior half of body. Oesophagus very short. Caecum tube-like, 235–1310 (601) long, 40–261 (122) wide, extending anteriorly from pharynx, in pre-uterine region, then recurving posteriorly to level of ovary; anterior extremity tapered, posterior extremity rounded. Gonads in post-equatorial region (Figure 2A).

Testes two, sub-spherical in ventral view, smooth, tandem, post-equatorial, partially overlapped, both larger than ovary; anterior testis 98.3–277 (135) long, 85.5–180 (115) wide; posterior testis 92.5–287 (140) long, 82.9–293 (133) wide. Cirrus-sac 401–1200 (649) long, 48.8–167 (92.6) wide, tube-like, sinistral, parallel-sided reaching posterior testis. It encloses oval seminal vesicle in its proximal part. *Pars prostatica* uniform, straight, 255–1030 (427) long, 44.3–78.3 (60.7) wide; ejaculatory duct large in diameter opens on genital lobe; genital lobe bi-lobed inside genital atrium, 200–376 (272) in diameter; genital atrium large (Figure 2C). Genital pore sub-terminal opens ventrally at very short distance from posterior extremity of body (Figures 2A, C; 3A).

Ovary oval round, smooth, equatorial, pre-testicular, medial in between two vitelline fields, 98–287 (154) long, 79.4–269 (139) wide. Oviduct descends from posterior part of ovary. Mehlis' gland well developed, large, immediately posterior to ovary surrounding oviduct. Vitelline duct connects to vitelline follicles in sinistral field (Figure 2B). Vitellarium composed by two lateral fields of vitelline follicles on each side, commences at level of posterior margin of posterior testis, proceeding longitudinally up to level anteriorly to ovary; one vitelline follicle field slightly longer than the other; vitelline follicles vary from oval to irregular in shape, dextral vitelline follicles numbering 10–13, sinistral ones 11–15; each vitelline follicle measuring 35.3–81 (57.9) long, 23.7–60.7 (38.2) wide. Laurer's canal not observed, likely obscured by uterus. Genital pore elliptical, sub terminal at short distance from posterior extremity (Figure 3A); tegument around genital pore corrugated with transversal ridges. Uterus extends from genital atrium, fills most available space between genital pore and posterior testis, passing medially between vitelline fields reaching anterior 40% of body, distinctly anterior to vitelline fields, but posterior to pharynx (midway between the anterior vitelline field and the pharynx) (Figure 2A). Eggs oval, very numerous, operculate, appear golden yellow in colour, 17.3–21 (19.3) long, 13–17 (14.5) wide (Figure 3D). Metraterm not observed, likely obscured by uterus.

Excretory pore terminal (Figure 3A). Excretory vesicle saccular.

Taxonomic summary

Type-host: sawtooth barracuda, *Sphyraena putnamae* Jordan & Seale (Carangaria: Sphyraenidae)
Type locality: Maputo Bay, western shore of Inhaca Island
Site of infection: Intestines
Infection parameters: Prevalence 42.86% (8 of 35 fish infected); Intensity 1–2.

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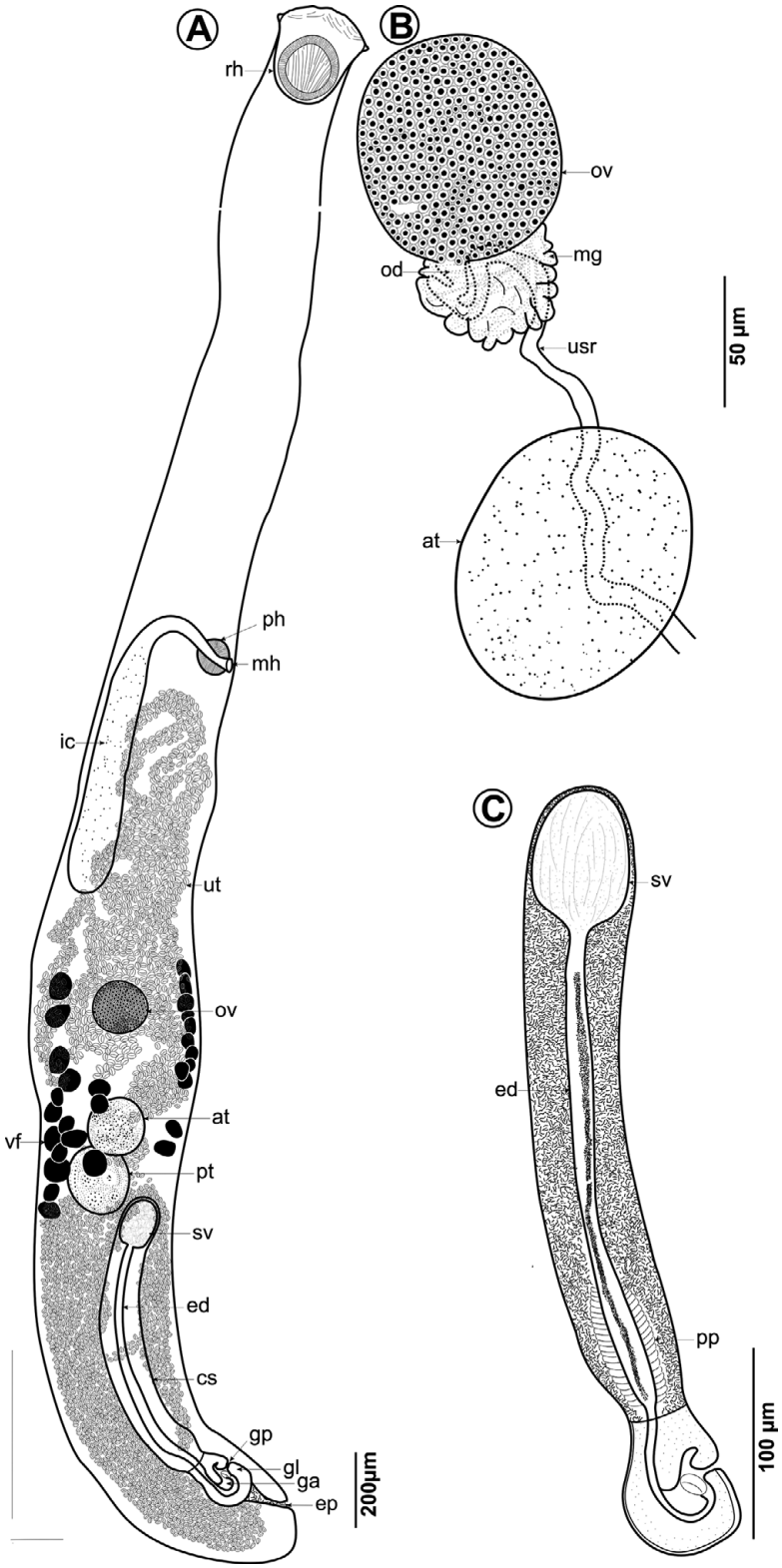

Figure 2. Line drawing of *Rhipidocotyle siphonyaka* n. sp. from *Sphyraena putnamae* collected in Maputo Bay. **A**. Entire body showing anatomy of internal organs. **B**. Position of the gonads. **C.** Anatomy of the cirrus-sac of *R. siphonyaka* n. sp. *Abbreviations:* at – anterior testis; cs – cirrus-sac; ed – ejaculatory duct; ep – excretory pore; ga – genital atrium; gl – genital lobe; gp – genital pore; ic – intestinal caecum; mg – Mehlis gland; mh – mouth; ov – ovary; od – oviduct; ph – pharynx; pp – *Pars prostatica*; pt – posterior testis; rh – rhynchus; sv – seminal vesicle; usr – uterine seminal receptacle; ut – uterus; vd – vitelline duct; vf – vitelline follicle.

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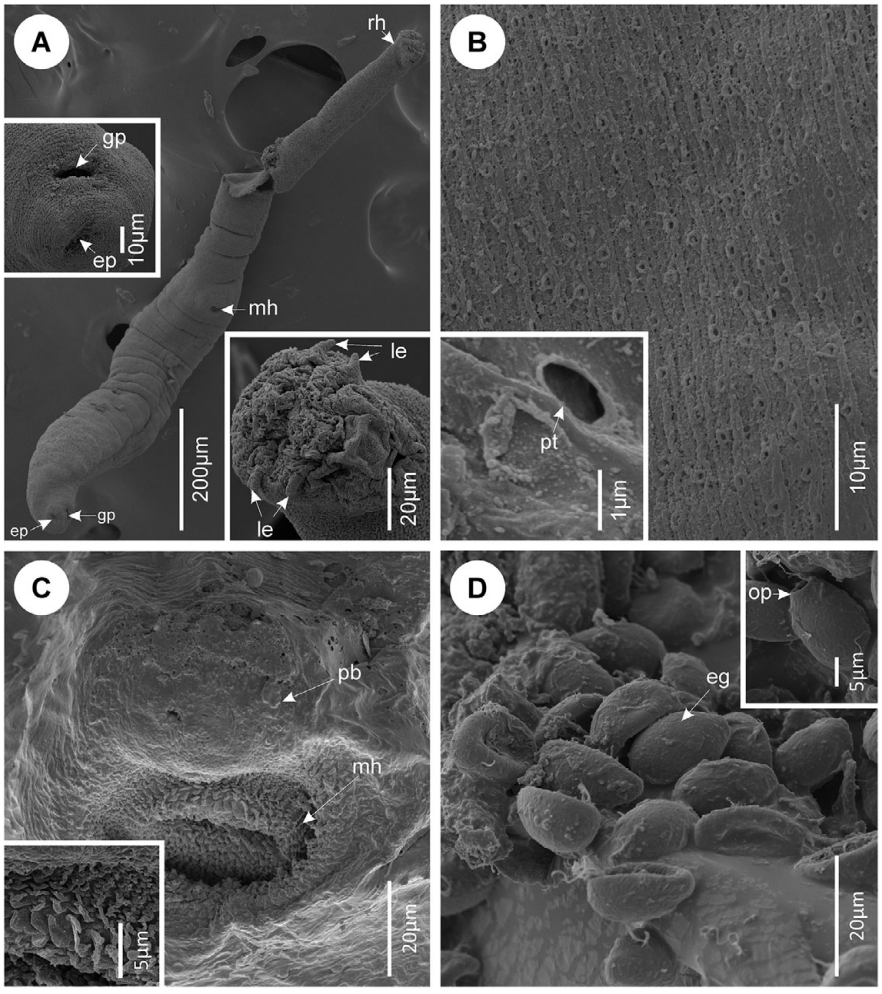

Figure 3. Scanning electron micrographs of *Rhipidocotyle siphonyaka* n. sp. collected from *Sphyraena putnamae* in Maputo Bay. **A**. Ventral view of whole specimen; upper inlay picture shows the position of the excretory pore and genital pore; lower inlay picture show six small tentacles around the rhynchus. **B**. Topography of the tegument with pits; inlay shows enlarged single pit. **C**. Elliptical mouth with inner layers with expanded structures (inlay). **D**. Many oval eggs, operculate (inlay). *Abbreviations:* eg – egg; ep – excretory pore; gp – genital pore; mh – mouth; op – operculum; pb – protuberance; pt – pits; rh – rhynchus.

Specimens deposited: Holotype – ovigerous adult specimen deposited at the Iziko South African Museum, Cape Town, South Africa (SAMC-A096876); paratypes: four specimens deposited at the Iziko South African Museum, Cape Town, South Africa (SAMC-A096877 - 80); four paratype (NHM 2024.9.23.1 - 4) and one hologenophore (NHM 2024.9.23.5) specimens deposited at the Natural History Museum, London, UK.
Representative DNA sequences: 28S rDNA – PQ453504-PQ453510; ITS rDNA – PQ453491-PQ453499.
Zoobank: urn:lsid:zoobank.org:act:31149D12-1DD6-4CC9-9656-488E1C4CC47F
Etymology: The species '*siphonyaka*' refers to the name of Inhaca's King (Carlos Sipho Nhaca), whose family nominated 'KaNyaka' to an Island with western and southern coasts facing Maputo Bay, where the fish host was collected. It is to honour his commitment to environmental conservation.

Remarks

The current material was first compared with marine *Rhipidocotyle* spp. described from *Sphyraena* spp. and thereafter to those morphometrically similar according to the updated visual key for the metrical criteria (See Supplementary Table S1).

Among representatives of *Rhipidocotyle*, six species are acknowledged from *Sphyraena* spp. – namely, *R. khalili*; *Rhipidocotyle longleyi* Manter, 1934; *Rhipidocotyle longicirrus* (Nagaty, 1937); *Rhipidocotyle barracudae* Manter, 1940; *Rhipidocotyle sphyraenae* Yamaguti, 1959; and *R. bartolli*, which are opposed to the present new species by having a caecum and a mouth opening in the post-vitelline field or almost midway through the uterus versus a tube-like caecum extending to pre-uterine region and the mouth opening at the pre-uterine field (Nagaty 1937; Manter 1940; Yamaguti 1959; Bray and Justine 2011).

Specifically, *R. longleyi* and *R. khalili* have a longer body length, shorter pre-uterine distance, and larger egg size versus shorter body length, longer pre-uterine distance, and smaller egg size in *R. siphonyaka* n. sp. (Manter 1934; Nagaty 1937). However, *R. barracudae, R. longicirrus, R. bartolli*, and *R. sphyraenae* have a shorter body length, larger width, and shorter pre-vitelline distance, whereas *R. siphonyaka* n. sp. has a longer body length, shorter width, and longer pre-vitelline distance (Manter 1940; Yamaguti 1959; Bray and Justine 2011).

The new species, *R. siphonyaka* n. sp., resembles *Rhipidocotyle fluminensis* Vicente & dos Santos, 1973 from Rio de Janeiro State, Brazil and *Rhipidocotyle pseudorhombi* Nahhas, Sey & Nakahara, 2006 from the Arabian Gulf morphometrically.

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Table 2. Comparison of the measurements (μm) of adult *Rhipidocotyle siphonyaka* n. sp. and *R. nolwe* n. sp. in the intestine of *Sphyraenae putnamae* in Maputo Bay, Mozambique along with the three other species.

Species	*R. siphonyaka* n. sp.	*R. nolwe* n. sp.	*R. khalili*[a,c]	*R. bartolii*[a]	*R. fluminensis* [b]	*R. pseudorhombi* [b]
Locality	Maputo Bay, Mozambique	Maputo Bay, Mozambique	Red Sea	New Caledonia	Rio de Janeiro	Arabian Gulf
Reference	Present study	Present study	Nagaty (1937)	Bray and Justine (2011)	Vicente and dos Santos (1973)	Nahhas *et al.* (2006)
Body length	2000–3080 (2580 ± 333)	1550–2740 (2030 ± 397)	2558–3300 (2863)	1369–1877 (1625)	2460	2050–2500 (2250)
Body width	172–311 (253 ± 46)	237–386 (281 ± 57.1)	314–363 (343)	239–276 (259)	560	200–260 (234)
Rhynchus length	75.3–243 (120 ± 57.5)	67.7–212 (116 ± 45.3)	122–152 (132)	77–127 (107)	160	80–130 (108)
Rhynchus width	61.7–189 (98.6 ± 47.5)	73.7–262 (111 ± 67.1)	122–139 (128)	89–97 (94)	180	75–125 (93)
Rhynchus to uterine loop	755–1360 (1050 ± 175)	333–1140 (728–310)	–	575–956 (779)	–	–
Rhynchus to caecum	705–1180 (966 ± 153)	561–1230 (865 ± 263)	–	572–951 (765)	–	–
Rhynchus to vitellarium	1050–1640 (1360 ± 210)	774–1660 (1080 ± 369)	–	378–577 (493)	–	–
Pharynx length	47.1–160 (78.9 ± 35.8)	47.4–122 (79.7 ± 30.5)	57–76 (65)	59–65 (62)	–	33–53 (44)
Pharynx width	29.9–118 (65.9 ± 24)	54.4–146 (83.6 ± 35.4)	–	69–78 (73)	–	38–68 (47)
Caecum length	235–1310 (601 ± 335)	313–725 (486 ± 190)	–	103–187 (134)	310	410–550 (483)
Caecum width	40–261 (122 ± 74.5)	59.5–74.6 (67.9 ± 6.85)	–	67–87 (76)	160	50–90 (63)
Pre-vitelline distance	1130–1730 (1410 ± 189)	845–1740 (1190 ± 315)	–	486–701 (598)	–	–
Post-vitelline distance	617–1720 (901 ± 332)	392–803 (660 ± 153)	–	624 835 (746)	–	–
Pre-uterine distance	830–1430 (1130 ± 179)	444–1110 (750 ± 307)	–	686–959 (826)	–	–
Post-uterine distance	36.8–134 (65.6 ± 44.1)	34.7–131 (67.7 ± 36.6)	–	–	–	–
Pre-caecal distance	834–1270 (1060 ± 144)	730–1330 (965 ± 248)	–	678–953 (810)	–	–
Post-caecal distance	903–1400 (1230 ± 141)	936–1500 (1110 ± 263)	–	–	–	–
Pre-mouth distance	806–1420 (1140 ± 185)	721–1260 (958 ± 245)	–	–	–	–
Vitelline field length dextral	263–1060 (500 ± 250)	275–631 (463 ± 146)	–	243–344 (282)	–	–
Vitelline field length sinistral	244–693 (514 ± 150)	325–796 (543 ± 187)	–	217–295 (264)	–	–
Follicle number dextral	10–13	12–13	14	–	–	14
Follicle number sinistral	11–15	13–16	21	–	–	21
Vitelline follicle length	35.3–81 (57.9 ± 16.1)	41.5–66.8 (55 ± 10.3)	38–57 (51)	–	–	–
Vitelline follicle width	23.7–60.7 (38.2 ± 12)	27–63.2 (40.4 ± 14)	19–68 (39)	–	–	–
Ovary length	98–287 (154 ± 65.2)	91.6–252 (141 ± 54.1)	122–167 (146)	100–120 (111)	210	100–150 (128)
Ovary width	79.4–269 (139 ± 55.3)	86.1–198 (113 ± 38.9)	91–152 (124)	74–91 (83)	170	80–138 (113)
Ovary to anterior testis distance	93.6–375 (172 ± 81.6)	92.2–184 (141 ± 38.3)	–	–	–	
Pre-ovarian distance	1190–1790 (1520 ± 189)	745–1720 (1280 ± 334)	–	737–1037 (883)	–	
Post-ovarian distance	744–1820 (1050 ± 336)	619–1350 (1030 ± 235)	–	522–737 (637)	–	
Anterior testis length	98.3–277 (135 ± 52.9)	99.8–316 (172 ± 75.4)	152–190 (166)	102–164 (144)	–	75–180 (130)
Anterior testis width	85.5–180 (115 ± 33.4)	82.2–249 (153 ± 61.9)	152–190 (166)	78–131 (108)	–	60–160 (113)
Posterior testis length	92.5–287 (140 ± 55.3)	109–221 (144 ± 40.9)	114–152 (138)	136–152 (143)	270	113–180 (148)
Posterior testis width	82.9–293 (133 ± 61.1)	85.2–214 (136 ± 43.6)	114–152 (138)	98–109 (102)	–	130–160 (145)
Pre-testicular distance	1380–2130 (1750 ± 200)	1160–2040 (1490 ± 345)	–	812–1111 (949)	–	–
Post-testicular distance	435–796 (590 ± 126)	384–159 (718 ± 403)	–	319–485 (387)	–	–
Inter–testicular distance	0	0–29 (4.15 ± 10.9)	–	–	–	–
Posterior testis to cirrus–sac	0–197 (51.9 ± 69.2)	0–133 (73.1 ± 54)	–	0	–	–

(*Continued*)

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Table 2. (*Continued*)

Species	*R. siphonyaka* n. sp.	*R. nolwe* n. sp.	*R. khalili*[a,c]	*R. bartolii*[a]	*R. fluminensis*[b]	*R. pseudorhombi*[b]
Locality	Maputo Bay, Mozambique	Maputo Bay, Mozambique	Red Sea	New Caledonia	Rio de Janeiro	Arabian Gulf
Reference	Present study	Present study	Nagaty (1937)	Bray and Justine (2011)	Vicente and dos Santos (1973)	Nahhas *et al.* (2006)
Cirrus-sac length	401–1200 (649 ± 246)	423–687 (538 ± 94.8)	594–825 (652)	440–482 (463)	700	410–763 (556)
Cirrus-sac width	48.8–167 (92.6 ± 35.6)	68.9–192 (96.2 ± 43.2)	103–133 (121)	70–97 (84)	190	73–100 (85)
Seminal vesicle length	69.6–180 (94 ± 32.7)	69.3–258 (116 ± 67.1)	–	0–121 (48)	–	88–113 (102)
Seminal vesicle width	32.7–122 (58.8 ± 25.1)	45.6–117 (69.8 ± 31.5)	–	0–75 (32)	–	50–93 (69)
Pars prostatica length	255–1030 (427 ± 222)	295–517 (388 ± 84.2)	–	0–377 (184)	–	350–650 (471)
Pars prostatica width	44.3–78.3 (60.7 ± 10.2)	54.2–70.5 (64.1 ± 6.06)	–	0–54 (35)	–	55 (55)
Genital lobe	200–376 (272 ± 62.3)	171–342 (286 ± 63.6)	–	–	–	–
Genital pore to the posterior end	47.9–340 (155 ± 83.6)	44.5–250 (136 ± 67)	–	–	–	–
Egg length	17.3–21 (19.3 ± 1.1)	18.6–21.9 (20.1 ± 1.11)	19–23 (20)	26–26 (26)	21	18–20 (19)
Egg width	13–17 (14.5 ± 1.41)	11.8–16.8 (14.1 ± 1.7)	13–15 (14)	12–17 (14)	14	10–13 (12)
Width (%)[d]	7.12–13	8.63–20	–	12.7–18.2 (16.2)	–	–
Pharynx length (%)[d]	1.92–5.8	1.88–5.17	–	–	–	–
Pre-mouth distance (%)[d]	37.7–65.4	35.9–65.3	–	56.9–60.1 (58.4)	–	–
Rhynchus length (%)[d]	3.04–9.22	3.63–9.02	–	4.92–7.70 (6.60)	–	–
Pre-vitelline distance (%)[d]	45.8–64.4	51.5–90.2	–	35.5–37.7 (36.7)	–	–
Pre-uterine distance (%)[d]	30.4–55.5	22.1–57.5	–	49–52.8 (50.8)	–	15–20
Pre-caecal distance (%)[d]	34.5–48.3	37.1–69.3	–	47.6–51.1 (49.8)	–	–
Caecum length (%)[d]	9.88–49.5	11.9–42.1	–	7.27–9.97 8.14	–	–
Inter-testicular distance (%)[d]	0–5.15	0–1.63	–	–	–	–
Post-testicular distance (%)[d]	18.3–27.4	23–67.6	–	22.6–25.8 (23.7)	–	–
Pre-testicular distance (%)[d]	61.4–78.9	57.5–86.3	–	57–59.3 (58.4)	–	–
Post-vitelline distance (%)[d]	26.6–62.4	25.4–41.7	–	44.5–46.9 (46)	–	–
Vitelline field length dextral (%)[d]	10.9–38.4	15.4–40.1	–	16–18.3 (17.3)	–	–
Vitelline field length sinistral (%)[d]	12.2–24.5	16.2–44.6	–	–	–	–
Ovary length (%)[d]	3.99–10.4	4.11–10.7	–	5.91–7.68 (6.89)	–	–
Seminal vesicle length as % of cirrus-sac length	7.63–18.2	13.5–41.4	–	0–25.3 (10.3)	–	–
Cirrus-sac reach (%)[d]	16.9–43.6	18.1–41.4	–	33.2–44.2 (38.8)	–	20–31 (25)

[a]described from the same host species.
[b]most similar to *R. siphonyaka* n. sp.
[c]most similar to *R. nolwe* n. sp.
[d]percentage relative to total body length of specimen.

However, *R. fluminensis* and *R. pseudorhombi* are distinct from *R. siphonyaka* n. sp. in having a shorter pre-vitelline distance, and the mouth and pharynx positioned at the level of vitelline field (Vicente and dos Santos 1973; Nahhas *et al.* 2006). Morphologically, *R. pseudorhombi* is more similar to *R. siphonyaka* n. sp. with the uterus reaching the pre-vitelline field and a caecum which is tubular. However, the mouth opens at the pre-uterine field in the new species, whereas it opens almost midway through the uterus and vitellaria in *R. pseudorhombi* (Nahhas *et al.* 2006).

American *Rhipidocotyle* species also differ from *R. siphonyaka* n. sp. by having a shorter body length and a sac-like intestinal caecum (Chandler 1935, 1941; McFarlane 1935; Linton 1940; Hopkins 1954). The majority of Asian species differ from *R. siphonyaka* n. sp. by having a shorter body length, uterus and digestive organs positioned at post-vitelline field, and a

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shorter pre-vitelline distance, except for *R. pseudorhombi* discussed upward (Chauhan 1943; Yamaguti 1959; Wang 1985; Nahhas *et al.* 2006; Bray and Palm 2009; Madhavi and Bray 2018).

The intestinal caecum, pharynx, mouth, and ovary of the European *Rhipidocotyle* are arranged in the post-vitelline field (Dimitrov *et al.* 1996; Bartoli *et al.* 2006; Bray and Justine 2011), therefore differing from *R. siphonyaka* n. sp., which are placed in the pre-vitelline field. The only Australian species, *R. labroidei*, resembles Asian species in having a uterus and caecum in the post-vitelline field. However, *R. laroidei* differs from *R. siphonyaka* n. sp. by a shorter body length and pre-vitelline distance, and a longer cirrus-sac reach and rhynchal length (Jones *et al.* 2003).

African *Rhipidocotyle* – namely, *R. eckmanni*, *R. ernsti*, *heptatheleta*, *R. lamberti*, *R. paruchini*, *R. senegalensis*, *R. tonimahnkei*, and *R. ghanensis* – are also distinct from *R. siphonyaka* n. sp. by possessing a short saccular caecum, and the internal organs are arranged in the post-vitelline field versus a tube-like caecum and organs arranged in the pre-vitelline field (Nagaty 1937; Fischthal and Thomas 1968; Stunkard 1974; Reimer 1985).

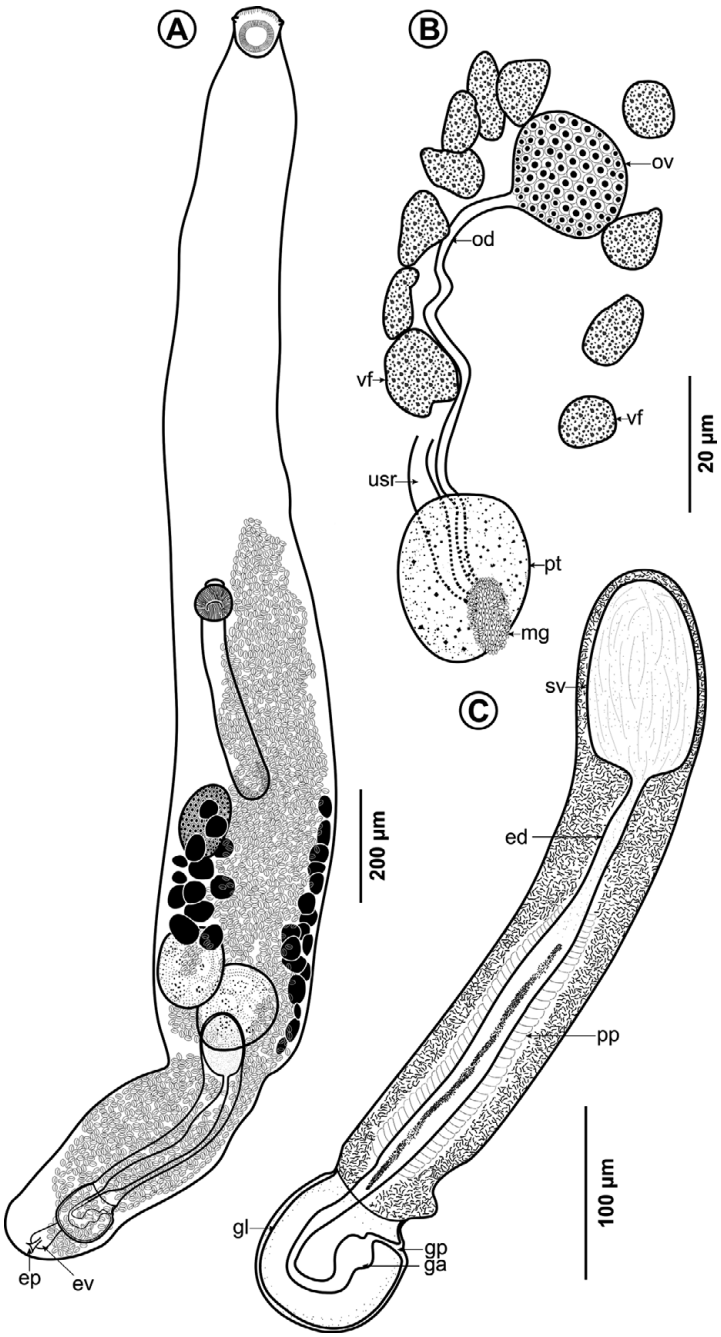

Figure 4. Line drawing of *Rhipidocotyle nolwe* n. sp. from *Sphyraena putnamae* collected in Maputo Bay. **A**. Entire body showing organs. **B**. Arrangement of the female gonad. **C**. Anatomy of the cirrus-sac of *R. nolwe* n. sp. *Abbreviations:* ed – ejaculatory duct; ep – excretory pore; ev – excretory vesicle; ga – genital atrium; gl – genital lobe; gp – genital pore; mg – Mehlis gland; od – oviduct; ov – ovary; vd – vitelline duct; vf – vitelline follicle; pp – *Pars prostatica*; pt – posterior testis; sv – seminal vesicle; usr – uterine seminal receptacle.

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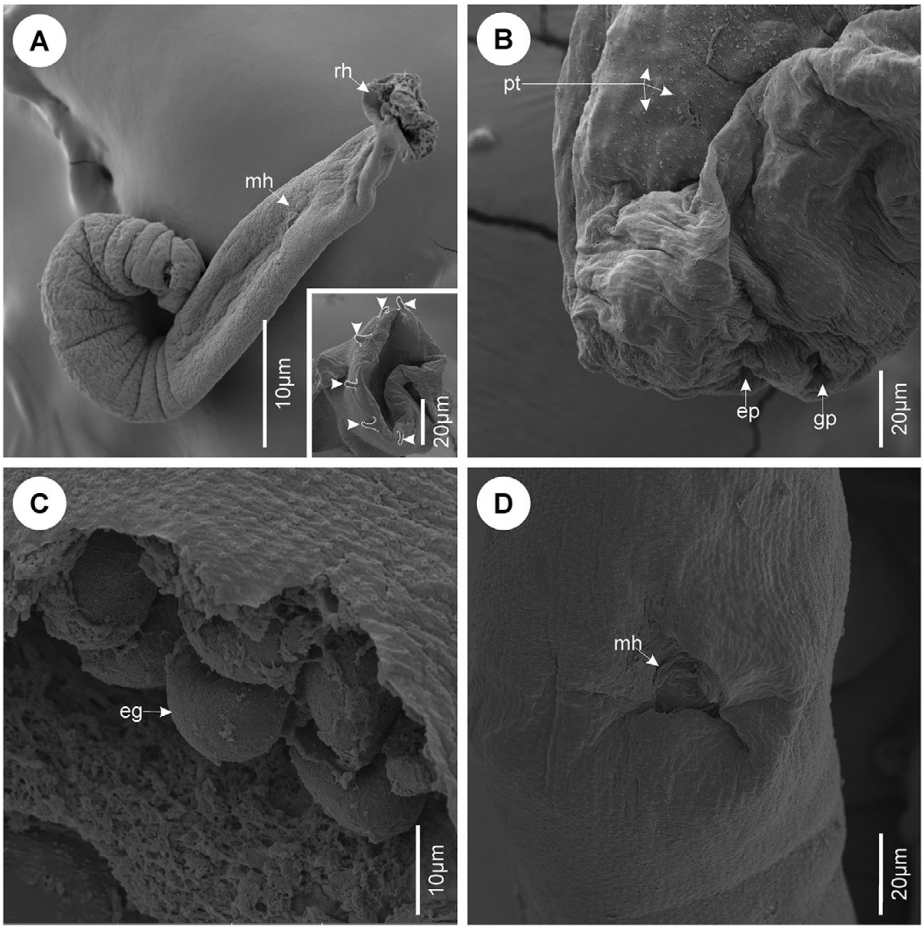

Figure 5. Scanning electron micrographs of *Rhipidocotyle nolwe* n. sp. from *Sphyraena putnamae* in Maputo Bay. **A**. Ventral view of whole specimen; inlay picture shows the presence of small tentacles on anterior margin of the rhynchus (represented with broken line). **B**. Tegument with pits (openings) on entire surface. **C**. Part of uterus with eggs. **D**. Mouth. *Abbreviations:* eg – egg; ep – excretory pore; gp – genital pore; mh – mouth; pt – pit; rh – rhynchus.

Rhipidocotyle nolwe *n. sp.*

Description (Figures 4, 5, 6 and Table 2)

Based on 26 whole-mount and 8 SEM preparations of mature specimens. Body elongate 1550–2740 (2030) µm long, 237–386 (281) µm wide, tapering towards anterior, rounded posteriorly, widest at level of ovary (Figure 4A). Tegument with scales (as multipointed spines, 29 points), retractable; retracted scales resembling pits (openings) in most specimens (Figure 6). Rhynchus a muscular sucker, oval bearing six (6) tentacles (Figure 5A). Mouth medial, equatorial, ventral, almost midway of uterus surrounded by pharynx (Figure 5D). Pharynx muscular, elliptical, equatorial at pre-vitelline region, mostly medial, occasionally overlapping ovary, 47.4–122 (79.7) long, 54.4–146 (83.6). Caecum tube-like, 312–725 (486) long, 59.5–74.6 (67.9), extends anteriorly from the pharynx, posteriorly reaching pre-ovarian region; tapered in anterior part, posterior extremity of caecum rounded. Gonads in posterior half of body (Figure 4A).

Testes two, oval in dorso-ventral view, smooth, oblique, post-equatorial, partially overlapped to spaced in some specimens 0–29 (4.15) long; both testes larger than ovary; anterior testis 98.8–316 (172) long, 82.2–249 (153) wide, posterior testis, 109–221 (144) long, 85.2–214 (136) wide (Figure 4A). Cirrus-sac elongate 423–687 (538) long, 68.9–192 (96.2) wide, sinistrally in hindbody, almost uniform in diameter throughout entire length, parallel-sided, reaching posterior margin of posterior testis; it encloses ellipsoidal seminal vesicle in its proximal part measuring 69.3–258 (115) long, 45.6–117 (69.8) wide. *Pars prostatica* elliptical, uniform, straight. Ejaculatory duct large in diameter opens on genital lobe inside genital atrium (Figure 4C). Genital lobe large, 171–342 (286) in diameter. Genital pore subterminal opens ventrally into genital atrium (Figure 4C).

Ovary oval, located at beginning of dextral vitellaria, smooth outline, post-equatorial, pre-testicular, generally smaller than testes, 91.6–252 (141) long, 86.1–198 (113) wide. Oviduct uniform, descending from postero-dextral side of ovary. Laurer's canal not visible, probably obscured by uterus. Mehlis' gland developed, far posterior from ovary, overlapping posterior testis. Vitelline duct joins latero-dextral side of Mehlis' gland, then forming widened part prior to connecting dextral vitelline follicles (Figure 4B). Vitellarium containing two lateral fields of vitelline follicles clustered on each side, commences at level of posterior testes running anteriorly up to ovarian level; one field slightly longer than other; vitelline follicles varying from oval to irregular in shape, each measuring 41.1–67 (55) long, 27–63.2 (40.4) wide; dextral vitelline follicles numbering 12–13, sinistral ones 12–16; transverse vitelline duct between ovary and anterior testis connects vitelline follicles from

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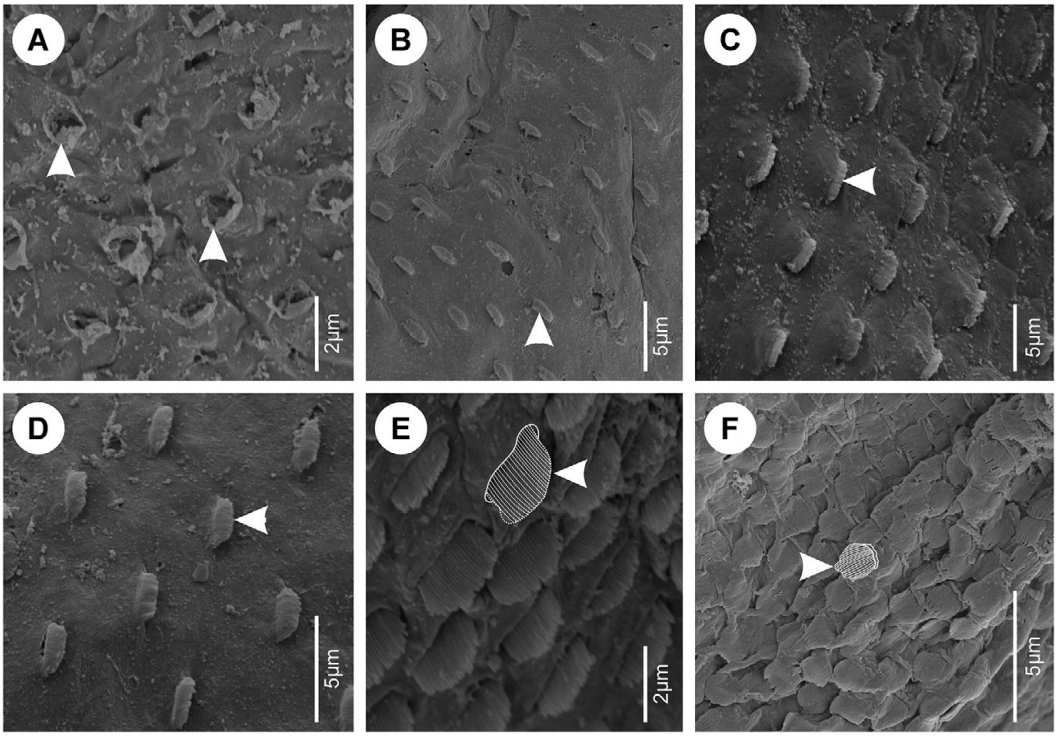

Figure 6. Scanning electron micrographs of surface topology of *R. nolwe* n. sp. and *R. siphonyaka* n. sp. **A**. Tegument with pits (openings) when scales are retracted; **B**. Spines semi-rectracted; **C, D**. Spines extruded above the tegument (arrowhead); **E**. Extruded scales (broken line) indicated with arrowhead; **F.** Spine with a ring in the base (represented with broken line) is apparent when it is completely extruded above the tegument.

each side. Uterus filled with eggs extends from beyond genital lobe at 44.5–250 (136) from posterior extremity; it fills most available space between genital pore and posterior testis, passing medially between vitelline fields reaching almost anterior third 55% of body, distinctly beyond anterior extent of pharynx (Figure 4A). Eggs numerous, operculate, appear golden yellow in colour, 18.6–21.9 (20.1) long, 11.8–16.8 (14.1) wide (Figures 4A; 5C). Metraterm not observed, probably obscured by uterus.

Excretory pore subterminal; excretory vesicle saccular.

Taxonomic summary

Type-host: sawtooth barracuda, *Sphyraena putnamae* Jordan & Seale (Carangaria: Sphyraenidae)
Type locality: Maputo Bay, western shore of Inhaca Island
Site of infection: Intestines
Infection parameters: Prevalence 48.57% (17 of 35 fish infected); Intensity 1–5.
Specimens deposited: Holotype – ovigerous adult specimen deposited at the Iziko South African Museum, Cape Town, South Africa (SAMC-A096881); Paratypes: five specimens deposited at the Iziko South African Museum, Cape Town, South Africa (SAMC-A096882 - 6); six paratype (NHM 2024.9.23.6 - 11) and one hologenophore (NHM 2024.9.23.12) specimens deposited at the Natural History Museum, London, UK.
Representative DNA sequences: 28S rDNA – PQ453500-PQ453503; ITS rDNA – PQ453487-PQ453490.
Zoobank: urn:lsid:zoobank.org:act:06ED6D67-A681-4DCF-A882-E4F52DA5FA8B
Etymology: The species '*nolwe*' refers to the name of the Nolwe sandy bank along the western and southern coasts facing Inhaca Island, where the fish host was collected.

Remarks

Rhipidocotyle nolwe n. sp. resembles *R. siphonyaka* n. sp. in having the digestive structures arranged in the pre-vitelline field. However, *R. siphonyaka* n. sp. is distinguished from *R. nolwe* n. sp. by a mouth and pharynx positioned in the pre-uterine field, a longer body length and cirrus-sac reach, and shorter pre-vitelline distance and post-testicular distance according to the visual key for the metrical criteria (See Supplementary Table S2).

Species of *Rhipidocotyle* known from *Sphyraena* spp. – namely, *R. longicirrus*, *R. barracudae*, *R. sphyraenae* and *R. bartolli* – have a shorter body length versus longer body length in *R. nolwe* n. sp. (Nagaty 1937; Manter 1940; Yamaguti 1959; Bray and Justine 2011). Two species, *Rhipidocotyle longleyi* and *R. khalili*, are distinct from *R. nolwe* n. sp. by their longer body length (Manter 1934; Nagaty 1937). *Rhipidocotyle khalili* and *R. bartolli* share the same host species with new species but are distinct from *R. nolwe* n. sp. by the larger egg size.

American *Rhiphidocotyle* are distinct from *R. nolwe* n. sp. by their shorter pre-vitelline distance and placement of the ovary and intestinal caeca in the post-vitelline field. Furthermore, the pharynx and mouth opening are placed posteriorly to vitellaria (Chandler 1935, 1941; Manter 1934, 1940; McFarlane 1936; Hopkins 1954; Vicente and Santos 1973). The pre-vitelline distance is also shorter in the Asian species, therefore differing from the new species (Chauhan 1943; Velasquez 1959; Yamaguti 1959; Nahhas *et al.* 2006).

Among European *Rhipidocotyle*, *R. nicolli* Bartoli, Bray & Gibson, 2006 is the only species having a uterus extending to the pre-vitelline field as in *R. nolwe* n. sp., but the former differs in possessing a longer body length (Bartoli *et al.* 2006). *Rhipidocotyle galeata* (Rudolphi, 1819), *Rhipidocotyle genovi* Dimitrov, Kostadinova & Gibson, 1996, *Rhipidocotyle minima* (Wagener, 1852),

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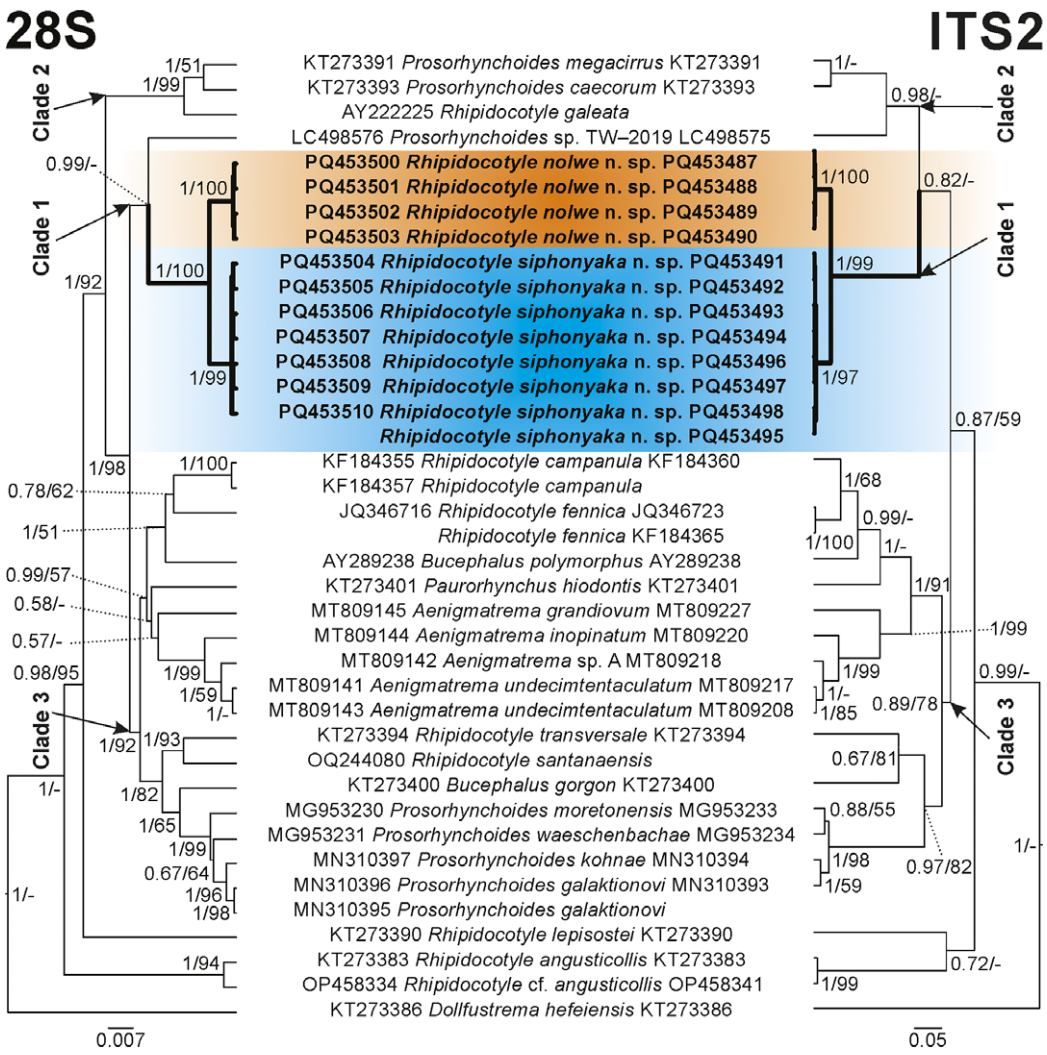

Figure 7. Topology based on ITS and 28S rDNA using Bayesian Inference (BI) approaches indicating the evolutionary history of *Rhipidocotyle siphonyaka* n. sp. and *R. nolwe* n. sp. (bold blocks) in relation to other species of Bucephalidae with *Dollfustrema hefeiense* Liu in Zhang *et al.* 1999 used as outgroup. Support for BI and maximum likelihood indicated at nodes (BI/ML), nodes with less than 50% bootstrap support indicated with "-".

Rhipidocotyle triglae (van Beneden, 1870), and *Rhipidocotyle viperae* (van Beneden, 1870) are distinct from *R. nolwe* n. sp. by possessing a shorter body length, larger egg size, and a short sac-like intestinal caecum which is positioned in the post-vitelline field (Nagaty 1937; Dimitrov *et al.* 1996; Bartoli *et al.* 2006).

African *Rhipidocotyle* – namely, *R. eckmanni*, *R. ernsti*, *R. heptatheleta*, *R. lamberti*, *R. paruchini*, *R. senegalensis*, *R. tonimahnkei*, *R. Khalili*, and *R. ghanensis* – differ from the *R. nolwe* n. sp. by the larger size of the eggs (Nagaty 1937; Fischthal and Thomas 1968, 1972; Stunkard 1974; Gavrilyuk-Tkachuk 1979; Reimer 1985).

Specifically, *R. eckmanni* from the Red Sea is distinct from *R. nolwe* n. sp. by its shorter body length, shorter post-testicular distance, and longer pre-mouth distance. Additionally, it has a saccular caecum placed in the post-vitelline field at the level of the reproductive organs (Nagaty 1937). *Rhipidocotyle ghanensis* from Ghana is distinct from *R. nolwe* n. sp. by its larger rhynchal length, longer pre-mouth distance, shorter pre-vitelline distance, and the placement of the intestinal caecum in the post-vitelline field (Fischthal and Thomas 1968). *Rhipidocotyle senegalensis* differs from *R. nolwe* n. sp. by its shorter body length and width, longer rhynchal length and pre-uterine distance, and shorter pre-vitelline and post-testicular distance (Fischthal and Thomas 1972).

Mozambican *Rhipidocotyle*, *R. tonimahnkei* and *R. ernsti* have shorter pre-vitelline and pre-uterine distances versus longer pre-vitelline and pre-uterine distances in *R. nolwe* n. sp. Besides *R. tonimahnkei* having a uterus extending to a pre-vitelline field similar to that of *R. nolwe* n. sp., it is distinct by its digestive and female reproductive structures placed in the post-vitelline field (Reimer 1985). *Rhipidocotyle ernsti* is also distinct from *R. nolwe* n. sp. by its fusiform body, larger width, longer cirrus-sac reach, and shorter pre-vitelline and pre-uterine distances (Reimer 1985).

Molecular analysis

For 28S rDNA, 11 sequences were generated from the 13 specimens included: four for *R. nolwe* n. sp. (1249–1263bp) and seven for *R. siphonyaka* n. sp. (1255–1263bp). Each species was represented by a single haplotype with no intraspecific variation. The 28S rDNA alignment included 37 sequences and was 1391bp long with 982bp

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conserved, 360bp variable, and 211bp parsimony informative sites. Using included 28S rDNA data, intraspecific distances of up to 0.17 % (2bp) were observed, and an interspecific range of 0.64–8.98% (8–116bp) was calculated. The haplotypes for *R. nolwe* n. sp. and *R. siphonyaka* n. sp. differed by 1.27–1.28% (16bp), falling within the interspecific range and supporting their distinctness. Both species were also separated from other taxa by 3.95–11.24% (49–141bp) and 3.71–11.9% (46–149bp) for *R. nolwe* n. sp. and *R. siphonyaka* n. sp., respectively (See Supplementary Table S3).

For ITS rDNA, sequences were generated from all 13 specimens included: four for *R. nolwe* n. sp. (1195–1222bp) and nine for *R. siphonyaka* n. sp. (601–1227bp). Each species was represented by a single ITS rDNA haplotype with no intraspecific variation. The alignment (trimmed to primer 5.8S-2) with other ITS rDNA data included 35 sequences and was 827 long with 331bp conserved, 399bp variable, and 292bp parsimony informative sites.

Using included ITS rDNA data, intraspecific distances of up to 0.47% (3bp) were observed, and an interspecific range of 1.98–26.05% (11–158bp) was calculated. The ITS rDNA haplotypes for *R. nolwe* n. sp. and *R. siphonyaka* n. sp. differed by 4.55–4.76% (27bp), falling within the adjusted interspecific range and supporting their distinctness. Both species were also separated from other taxa by 15.7–26.19% (81–143bp) and 14.51–25.05% (75–137bp) for *R. nolwe* n. sp. and *R. siphonyaka* n. sp., respectively (See Supplementary Table S4).

Both 28S and ITS rDNA topologies were similar, with *R. nolwe* n. sp. and *R. siphonyaka* n. sp. grouping as closely related sister taxa in a well-supported clade, distinct from all other bucephalids. From the included data, *Rhipidocotyle angusticollis* Chandler, 1941, together with *Rhipidocotyle* cf. *angusticollis*, grouped basally followed by *Rhipidocotyle lepisostei* Hopkins, 1954. The remainder of the included data grouped into three major clades, the first with the new species from the present study (Clade 1), the second (Clade 2) with *P. megacirrus*, *P. caecorum*, and *R. galeata*, and the third with the remainder of the included data (Clade 3). The relation of these major clades varied between markers, with Clade 2 sister to Clade 1 based on ITS rDNA, whereas Clade 1 was sister to Clade 3 based on 28S rDNA. The position of the unidentified *Prosorhynchoides* sp. (TW-2019; LC498575-6) by Shirakashi *et al.* (2020) also varied between markers, grouping with the study species (Clade 1) based on 28S rDNA and with Clade 2 based on ITS rDNA.

Discussion

Taxonomy

Within sphyraenids, the great barracuda *S. barracuda* (Edwards) is the only host species harbouring three species of *Rhipidocotyle* (see Bray and Justine 2011), with the obtuse barracuda *Sphyraena obtusata* Cuvier infected with four *Aenigmatrema* species (Corner *et al.* 2020). The sawtooth barracuda has a wide distribution essentially spanning the entirety of the Atlantic, Pacific, and Indian Oceans, also including the Caribbean and Red Sea (Bray and Justine 2011; Bogorodsky *et al.* 2014; Gottfried *et al.* 2017). Compared to the widely reported distribution of the host, it appears that it has not been well sampled for trematode fauna, particularly in African waters, and so, conceivably, *R. siphonyaka* n. sp. and *R. nolwe* n. sp. might be supported across all or most of its definitive host's range.

Two morphological characteristics of the material studied here warrant further discussion: the six (6) small tentacles and the tegument with scales. Large tentacles associated with a rhynchus are characteristic of three genera within Bucephalidae – namely, *Bucephalus* with seven, *Alcicornis* MacCallum, 1917 with seven to 21, and *Aenigmatrema* Corner, Cribb & Cutmore, 2020 with 11 tentacles (Overstreet and Curran 2002). Specifically, the observed tentacles, resembling papillae, are less developed and fewer in number compared to those present in *Aenigmatrema*, *Alcicornis*, and *Bucephalus*. The presence of small tentacles (papillae-like) corroborates with Manter (1940), who stated that the 'hood' surmounting the sucker of *Rhipidocotyle* assumes various forms, and it may bear papillae, which are sometimes more or less extensible, similar to the tentacles of *Bucephalus*, to which they are probably homologous. Yamaguti (1959) described and illustrated a row of seven double papillae on the rhynchus of *R. sphyraenae*; Moreover, Nagaty (1937) also noticed the presence of papillae in *R. khalili*; Velasquez (1959) for *R. eggletoni* and *R. laruei*; Bartoli *et al.* (2006) for *R. nicolli*; and Nahhas *et al.* (2006) for *R. pseudorhombi*. Some reports such as that of Rudolphi (1819) and Stossich (1887) on *R. galeata* in the northern Atlantic and Mediterranean Oceans also noticed a rhynchus baring six or seven papillae. However, papillae in *Rhipidocotyle* species seem to lack any clear pattern in terms of number, size, and arrangement and are sometimes referred to as lobes or protuberances.

Nagaty (1937) referred to the presence of scales in the family Bucephalidae. Several reports on tegument with scales in *Rhipidocotyle* were solely based on light microscopy examination – namely, Yamaguti (1959) for *R. sphyraenae*; Bartoli *et al.* (2006) for *R. minima*; and Velasquez (1959) for *R. laruei*. They were first observed with SEM in *R. angusticollis* by Shalaby and Hassanine *et al.* (1996). The scales on the tegument of *Rhipidocotyle* species are undefined when utilising light microscopy analysis but are well visible as transverse rows and overlapping each other when using SEM examination (Shalaby and Hassanine *et al.* 1996). In the present study, both retracted and extruded states of the scales were observed in specimens of the same taxa. Based on SEM images obtained from the present material, it is apparent that the scales are retracted into the tegument during contraction of the body; therefore, small pits are noticeable when the scales are completely retracted (as seen in the sequence in Figure 6).

Intermediate and definitive hosts

The species-rich nature of bucephalid fauna is strongly connected to the bivalves acting as the first intermediate host, infected fishes as the second intermediate host, and primarily a piscivorous fish as the definitive host, where the metacercaria is ingested along with the second intermediate host through feeding (Muñoz *et al.* 2014). There are a few notable exceptions to the latter (i.e., apogonids (Bott and Cribb 2005), cleaner wrasse (Jones *et al.* 2004), and fang blennies (Roberts-Thomson and Bott 2007)). Members of Bucephalidae infect a range of first intermediate hosts as broad as that of their range of definitive hosts. They have previously been reported from at least 18 bivalve families, the greatest number of host families for any family of digenean (Cribb *et al.* 2001).

The life cycles of *Rhipidocotyle* spp. in naturally infected marine bivalves have been reported for *R. transversale* Chandler, 1935 and *R. lintoni* Hopkins, 1954 in *Lyonsia hyalina* (Conrad) (Lyionsiidae) (Stunkard 1976). A study on marine *Rhipidocotyle* in Australia reported infections by bucephalids in Isognomonidae, Spondylidae, Tellinidae, and Ostreidae bivalves. The bivalves of the family Ostreidae (genus *Saccostrea*) and Tellinidae (genus *Exotica*) were heavily infected (Bott *et al.* 2005).

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Members of the genus *Saccostrea* are most diverse and abundant in the banks of the west, south, and north coasts of the Inhaca Island (Paula *et al.* 1998; Mafambissa *et al.* 2022, 2024). Additionally, according to Marcogliese (2023), *Rhipidocotyle* spp. also use anodontid mussels (*Anodontia*: Family Lucinidae) as their first intermediate host. For our study area, the extent of a large diversity of bivalves of either group *Saccostrea* and *Anodontia* (Paula *et al.* 1998; Branch *et al.* 2000) could support the larval stages of *Rhipidocotyle*, enabling them to thrive.

The life cycles of *Rhiphidocotyle* spp. have been mostly studied in freshwater bivalves of the genera *Eurynia* Rafinesque and *Lampsilis* Rafinesque for *R. papillosa* Chauhan, 1943 and *R. septapapillata* Krull, 1934 (Woodhead 1929; Kniskern 1952); *Anodonta* for cercariae of *R. fennica* Gibson, Taskinen & Valtonen, 1992 and *R. campanula* (Dujardin, 1845) (Gibson *et al.* 1992; Taskinen and Valtonen 1995; Taskinen *et al.* 1997); and *Nitia* Pallary and *Vulsella* Röding for *R. campanula* (Baturo 1977; Fol and Abdel-Gaber 2018). All freshwater bivalves infected by *Rhipidocotyle* belonged to Unionidae, the most species-rich bivalve family, widely distributed across Europe, Asia, North America, and Africa (Lopes-Lima *et al.* 2017).

Regarding the definitive hosts, *Rhipidocotyle* spp. are commonly described or/and reported from teleost of Scombridae (n=10); Carangidae (n=8); Sphyraenidae (n=6); Triglidae (n=3); Sciaenidae (n=3); Psettodidae (n=2); and Hexagrammidae, Belonidae, Acropomatidae, Atherinopsidae, Lotidae, Trachinidae, Clupeidae, Serranidae, Sillaginidae, Cynoglossidae, Ariidae, Trachichthyidae, Antennariidae, Chanidae, Labridae, Gempylidae, and Paralichthyidae infected with one (1) species each (see Ahyong *et al.* 2024).

However, in Africa, species of *Rhipidocotyle* have been described from seven (7) teleost families Carangidae (n=3), Scombridae (n=1), Trachichthyidae (n=1), Sciaenidae (n=1), Antennariidae (1), Psettodidae (n=1), and Chanidae (n=1), arranged in six orders (Nagaty 1937; Reimer 1985; Fischthal and Thomas 1968, 1972). Only *R. khalili* has been reported more than twice in a very narrow range of hosts such as *Sphyraena japonica* Bloch & Schneider, *S. obtusata*, and *S. putnamae* (Yamaguti 1953; Madhavi 1974; Reimer 1985; Bray and Justine 2011). This denotes that *Rhipidocotyle* spp. in Africa appear to be host-specific, at least to the level of host family.

Molecular taxonomy

The genetic data generated here support the morphological distinctness of *R. siphonyaka* n. sp. and *R. nolwe* n. sp. from one another and from other bucephalid taxa, while also illustrating their relatedness. The haplotypes from both species formed well-supported clades, distinct from other taxa and supported by genetic distances while grouping as sister taxa in well-supported clades in all analyses. The presence of sister taxa in the same host species is not uncommon in bucephalids (see Bott *et al.* 2013; Corner *et al.* 2020). For example, the Bucephalinae species *Prosorhynchoides galaktionovi* Hammond, Cribb, Nolan & Bott, 2020 and *Prosorhynchoides kohnae* Hammond, Cribb, Nolan & Bott, 2020 were both described from the same host, *Tylosurus crocodilus* (Péron & Lesueur), but are morphologically and genetically distinct taxa. Similarly, *Prosorhynchoides moretonensis* Hammond, Cribb & Bott, 2018 and *Prosorhynchoides waeschenbachae* Hammond, Cribb & Bott, 2018 infects *Tylosurus gavialoides* (Castelnau), and four *Aenigmatrema* (*Aenigmatrema grandiovum* Corner, Cribb & Cutmore, 2020, *Aenigmatrema inopinatum* Corner, Cribb & Cutmore, 2020, *Aenigmatrema undecimtentaculatum* Corner, Cribb & Cutmore, 2020, and an unidentified *Aenigmatrema* sp.) infect *S. obtusata.* In all three of these cases, the taxa from the same host cluster together as sister taxa (Clade 3). Interestingly, *Aenigmatrema* from *S. obtusata* are distant from the present material, which is from a congeneric host. Corner *et al.* (2020) previously proposed that bucephalids adopted sphyraenid fishes as definitive hosts on two separate occasions based on their results. Following this, the present results suggest a third such adoption.

The relation of the new species to other included taxa needs further investigation as this was not constant between the gene regions used. The topologies presented here mimic those of previous studies including similar bucephalid taxa, with Bucephalinae appearing to be polyphyletic (Nolan *et al.* 2015; Hammond *et al.* 2018; Shirakashi *et al.* 2020). As such, *R. siphonyaka* n. sp. and *R. nolwe* n. sp. do not group with other *Rhipidocotyle* in a monophyletic clade, but rather independently. The grouping of the unidentified *Prosorhynchoides* sp. (TW-2019; LC498576) by Shirakashi *et al.* (2020) was also not consistent, grouping with the study taxa (Clade 1) in the 28S rDNA topology and basal in Clade 2 based on ITS rDNA. The support for the 28S rDNA grouping was only well supported by BI analyses, with the ITS rDNA groupings less well-supported; thus, the 28S rDNA topology is likely more reliable. Unfortunately, the sequence of *Prosorhynchoides* sp. by Shirakashi *et al.* (2020) was generated using metacercaria; thus, the generic identification may be incorrect. But due to the polyphyletic nature of this group, most generic designations therein may likely change in the future.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000476.

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Competing interest. None.

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Author contributions. JCD: conceptualisation, sampling, morphological (LM and SEM) analysis, and writing (original draft); QMD: genetic analysis, genetic data curation, SEM analysis, review, and editing; AAO: funding acquisition, review, editing, and approval of final draft.

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