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Adding one more to the list: A new species of *Eniochobothrium* (Cestoda: Lecanicephalidea) from the Oman cownose ray in South Africa

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ABSTRACT

A new species of *Eniochobothrium* Shipley and Hornell, 1906 was recovered from the Oman cownose ray (*Rhinoptera jayakari* Boulenger) from the body of water off the south-eastern coastline of the KwaZulu-Natal Province, South Africa. *Eniochobothrium acostae* n. sp. is described on morphological and molecular grounds. The new species is placed within *Eniochobothrium* (viz., *Eniochobothrium gracile* Shipley and Hornell, 1906, *Eniochobothrium qatarense* Al Kawari, Saoud and Wanas, 1994, *Eniochobothrium euaxos* Jensen, 2005) by possessing key generic characteristics such as the absence of a vagina, expansion of the anterior region of the strobila forming a trough and presence of a thick-walled cirrus sac. Molecular phylogenetic analyses of the partial 28S rRNA and mtCOI genes confirm the generic characterisation as the newly proposed species groups together with other members of the genus. *Eniochobothrium acostae* n. sp. currently represents the largest described species of the genus; it possesses slightly fewer testes compared to most congeners, given that this feature has been provided in the original description (e.g., *E. euaxos* and *E. qatarense*). The new species of *Eniochobothrium* is the fourth valid species described to date and the first species record from South African waters.

1. Introduction

Eniochobothrium Shipley and Hornell, 1906 (Cestoda: Lecanicephalidea) is recognised for its unfamiliar and unique morphological features (Jensen, 2005). Members of this genus are apolytic, characterised by a scolex possessing four acetabula in the form of suckers, an expansion of the anterior region of the strobila consisting of non-reproductive proglottids forming a trough, and a reproductive strobilar region with testes anterior to the ovary (Jensen, 2005). According to Shipley and Hornell (1906), species of *Eniochobothrium* possess a distinctive morphological characteristic, where scoleces of specimens easily detach from the strobila. Jensen (2005) stated that this peculiarity highlights the fragile connection between the anterior trough region of the stobila and the scolex. According to the latter author, preliminary data suggests that the trough might serve as the primary attachment structure rather than the scolex. Another characteristic of *Eniochobothrium* is the presence or absence of a vagina in the female reproductive system which requires clarification (Jensen, 2005). In total, three members of *Eniochobothrium* are considered valid (viz., *E. gracile* Shipley and Hornell, 1906, *E. qatarense* Al Kawari, Saoud and Wanas, 1994, and *E. euaxos* Jensen, 2005) (Caira et al., 2022). A fourth species, *Eniochobothrium trygonis* Chincholikar and Shinde, 1978, that has previously been placed in this genus, was later declared a *species inquirenda* by Al Kawari et al. (1994) due to its proglottid anatomy and scolex morphology, which differs from the generic characteristics of *Eniochobothrium* (Shipley and Hornell, 1906).

The first thorough phylogenetic analyses of the interrelationships among lecanicephalidean cestodes were conducted by Jensen et al. (2016). These authors greatly increased the spectrum of available lecanicephalidean taxa to a total of 61 species in 25 genera, including three undescribed genera (New genus 11, 12, and 13), providing sequences of the complete 18S rRNA, 16S rRNA, partial 28S rRNA and partial mtCOI genes. Eight primary lineages resulting from their phylogenetic analyses were recognised at the family level (Jensen et al., 2016). The existing families (i.e. Cephalobothriidae, Lecanicephalidae, Polypocephalidae and Tetragonocephalidae) were maintained, while Aberrapecidae, Eniochobothriidae, Paraberrapecidae, and Zanobatocestidae were established as new families for the remaining lineage clusters (Jensen et al., 2016). These authors also provided a key to the families based on morphological characteristics and revealed

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monophyly of the order Lecanicephalidea via molecular sequence data (Jensen et al., 2016). The family Eniochobothriidae was revealed as one of the most molecularly divergent families within the order. Its unusual morphology with anterior proglottids expanding laterally to form a trough and absence of a vagina justified its status as an independent family (Jensen et al., 2016).

Schaeffner and Smit's (2019) checklist on elasmobranch parasites of South Africa confirmed that species from the order Lecanicephalidea are not recorded from southern African waters. As a part of a larger project on marine parasites from southern Africa, a new species of *Eniochobothrium* was discovered parasitising the Oman cownose ray, *Rhinoptera jayakari* Boulenger (Myliobatoformes: Rhinopteridae). The new species is recognised on the basis of unique morphological features as well as on molecular grounds. It represents the first record of an eniochobothriid and lecanicephalidean from elasmobranchs in southern Africa. This study also provides molecular phylogenetic analyses of the group based on sequences obtained from two molecular markers (partial 28S rRNA and mtCOI genes).

2. Materials and methods

2.1. Collection of specimens and fixation of material

In March 2020, three specimens of the Oman cownose ray, *R. jayakari*, were recovered from shark nets along the south-eastern coastline of the KwaZulu-Natal Province, South Africa (28.5306° S, 30.8958° E) by the KwaZulu-Natal Sharks Board (KZNSB). Sampling permits of batoids for research were issued by the South African Department of Agriculture, Forestry and Fisheries (Permit number RES 2020/20 issued to the KZNSB). Ethical approval was provided by the North-West University (NWU) Animal Care, Health and Safety, Research Ethics Committee (Ethics number: NWU-01777-20-A9). Batoid specimens were previously frozen and subsequently dissected at the KZNSB facility having three-fourths of the material (valve and content) fixed in 10% formalin for morphological studies while the fourth part was placed in pure ethanol for molecular studies. In the laboratory, gravid, mature and immature worms were hand-picked from the spiral intestines and sifted and placed in 70% ethanol for morphological analyses and in molecular grade ethanol (96%) for molecular analyses.

2.2. Morphological study

Worms stored in 70% ethanol were re-hydrated, stained in Delafield's haematoxylin, dehydrated in a graded series of ethanol, cleared in clove oil and permanently mounted in Canada balsam on microscope slides. Mounted specimens were observed and measured using a Nikon ECLIPSE 80i (compound) and Nikon ECLIPSE Ni (compound/DIC/phase contrast) microscopes (Nikon Instruments, Tokyo, Japan). Drawings were made with a drawing attachment tube. Measurements consist of the range, followed in parentheses by the mean, standard deviation, the number of measurements (n) made, and the total number of observations (*n*) when more than one measurement was taken per worm. All measurements are in micrometres unless otherwise indicated. The terminology for morphological characteristics follows Al Kawari et al. (1994) except for the presence of a vagina, thereby following Jensen's (2005) amendment of the generic diagnosis of *Eniochobothrium* of "vagina absent (possibly present in *E. qatarense*)". Type specimens were deposited in the helminthological collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic (IPCAS), the National Museum, Bloemfontein, South Africa (NMB), and the Natural History Museum, Geneva, Switzerland (MHNG).

Two specimens were used for scanning electron microscopy (SEM) (one whole specimen and one partial specimen lacking a scolex). The specimens were fully dehydrated with pure ethanol, placed in a 50/50 solution of pure ethanol and hexamethyldisilazane (HMDS), followed by pure HMDS and allowed to air dry in a fume hood. The dried specimens were mounted on an aluminium stub with double-sided adhesive carbon tape, sputter-coated with a layer of carbon in an Emscope TB 500 sputter coater (Quorum Technologies, Ltd., Laughton, U.K.) followed by gold-palladium sputter coating, using an EIKO IB-2 ion coater (EIKO Engineering, Ltd., Yamazaki Hitachinaka, Japan). The coated specimens were examined in an FEI Nova NanoSEM 450 scanning electron microscope (Fei Company, Eindhoven, Netherlands) at 5 kV.

2.3. Molecular characterisation

Four whole individual specimens preserved in molecular grade ethanol (96%) were used for DNA extraction (isolate 1 and 2 – large specimens; isolate 3 and 4 – small specimens). A conspecific specimen mounted on a slide was kept as a paragenophore (sensu Pleijel et al., 2008). Extraction of genomic DNA was performed using 200 µl of a 5% solution of Chelex in deionised water and 2 µl of proteinase K, incubated for 4 h at 56°C and boiled at 90°C for 8 min, and then centrifuged at 15, 000 rpm for 10 min. The partial 28S rRNA (D1-D3 region) and the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) genes were amplified. Polymerase Chain Reactions (PCR) were performed using 3 µl of extraction supernatant, 10 µl of Dream Taq Master Mix (2x) (ThermoFischer Scientific™) and 1.6 µl of each primer (10 µM) adding to a total reaction mixture of 20 µl. Partial 28S rRNA was amplified following the cycling conditions of Brabec et al. (2012): denaturation (94°C for 5 min), 40 cycles of amplification (94°C for the 30s, 55°C for 30s, and 72°C for 2 min), and 7 min extension hold at 72°C using the primers LSU5 (Littlewood et al., 2000) and 1500R (Olson et al., 2003). Mitochondrial COI was amplified using the primers PBI-cox1F_PCR and PBI-cox1R_PCR (Scholz et al., 2013); cycling conditions followed Inqaba Biotechnical Industries Pty Ltd. (Pretoria, South Africa) (general PCR protocol): denaturation (94°C for 5 min), 35 cycles of amplification (94°C for the 30s, 50°C for 30s, and 68°C for 1 min), and 10 min of extension hold at 68°C. The PCR amplicon was run on 1% agarose gel using loading buffer and gel red. The PCR product for 28S rRNA was purified and sequenced at Inqaba Biotechnical Industries Pty Ltd. using the PCR primers and the internal primers L1200R (Lockyer et al., 2003) and ZX-1 (Van der Auwera et al., 1994) and PBI-cox1F_seq and PBI-cox1R_seq (Scholz et al., 2013) for mtCOI. Contiguous sequences were assembled using Geneious version 7.1.3 (Kearse et al., 2012).

2.4. Phylogenetic analyses

Six partial sequences (three sequences of 28S rRNA [isolates 1, 2, and 3] and three sequences of mtCOI genes [isolates 2, 3, and 4]) were newly generated in this study. Unfortunately, the PCR for 28S did not amplify isolate 4, while the PCR for COI did not amplify isolate 1. The new sequences were aligned with sequences of related taxa obtained from GenBank. *Paragrillotia similis* Linton, 1909 (KF685909) and *Triaenophorus stizostedionis* Miller, 1945 (KR780900) were used as outgroups for the 28S rRNA analysis and aligned with 49 selected sequences of lecanicephalideans (Table 1). *Hexacanalis folifer* Cielocha and Jensen, 2011 (KU249130) was used as an outgroup for the mtCOI analysis and aligned with the three new sequences, along with *Eniochobothrium* n. sp. 1 (KU249111), *Eniochobothrium* n. sp. 2 (KU249108), *Eniochobothrium* n. sp. 3 (KU249109) and *E. euaxos* (KU249110). Sequences from both data sets (28S rRNA and mtCOI) were aligned using default parameters of MUSCLE implemented in Geneious 7.1.3, with the extremes of the alignments trimmed. The alignments were 561 base pairs (bp) (mtCOI) and 1655 bp (28S rRNA) long. An alignment including only the 28S sequences of *Eniochobothrium* spp. was created in order to verify the values of genetic divergence (seven taxa, 1558 bp long). Phylogenetic analyses were run under maximum likelihood (ML) and Bayesian inference (BI) criteria, applying the evolutionary model GTR + I + G. ML analysis was performed using the program RAxML version 8 (Guindon and Gascuel, 2003). The model parameters and bootstrap support values

Table 1
List of partial 28S rRNA sequences of lecanicephalidean species included in the phylogenetic analyses, including information on hosts, localities and the studies in which sequences were provided. New sequences obtained for the present study are highlighted in bold.

Parasite taxon	Family	Host	Locality	GenBank ID	Reference
Aberrapex n. sp. 1	Aberrapecidae	*Aetomylaeus bovinus*	Senegal	KU249052	Jensen et al. (2016)
Adelobothrium aetiobatidis	Cephalobothriidae	*Aetobatus ocellatus*	Australia	KU249060	Jensen et al. (2016)
Adelobothrium n. sp. 1	Cephalobothriidae	*Aetobatus ocellatus*	Solomon Islands	KU249063	Jensen et al. (2016)
Adelobothrium n. sp. 2	Cephalobothriidae	*Aetobatus narutobiei*	Vietnam	KU249062	Jensen et al. (2016)
Anteropora comicus	Polypocephalidae	*Narcine maculata*	Malaysia	KU249094	Jensen et al. (2016)
Anteropora joannae	Polypocephalidae	*Taeniura lymma*	Malaysia	KF685864	Caira et al. (2014)
Anteropora klosmamorphis	Polypocephalidae	*Narcine maculata*	Malaysia	KU249095	Jensen et al. (2016)
Anteropora leelongi	Polypocephalidae	*Hemiscyllium ocellatum*	Australia	KF685857	Caira et al. (2014)
Anteropora patulobothridium	Polypocephalidae	*Taeniura lymma* 1	Malaysia	KU249092	Jensen et al. (2016)
Anteropora pumilionis	Polypocephalidae	*Himantura* cf. *pastinacoides*	Malaysia	KU249093	Jensen et al. (2016)
Cephalobothrium aetobatidis	Cephalobothriidae	*Aetobatus ocellatus*	Thailand	KU249066	Jensen et al. (2016)
Cephalobothrium n. sp. 1	Cephalobothriidae	*Aetobatus ocellatus*	Australia	KU249058	Jensen et al. (2016)
Cephalobothrium n. sp. 5	Cephalobothriidae	*Aetobatus ocellatus*	Solomon Islands	KU249059	Jensen et al. (2016)
Cephalobothrium n. sp. 6	Cephalobothriidae	*Aetobatus ocellatus*	Vietnam	KU249064	Jensen et al. (2016)
***Eniochobothrium acostae* n. sp. isolate 1**	Eniochobothriidae	*Rhinoptera jayakari*	South Africa	**ON972441**	Present study
***Eniochobothrium acostae* n. sp. isolate 2**	Eniochobothriidae	*Rhinoptera jayakari*	South Africa	**ON972440**	Present study
***Eniochobothrium acostae* n. sp. isolate 3**	Eniochobothriidae	*Rhinoptera jayakari*	South Africa	**ON972442**	Present study
Eniochobothrium euaxos	Eniochobothriidae	*Rhinoptera neglecta*	Australia	KF685859	Caira et al. (2014)
Eniochobothrium n. sp. 1	Eniochobothriidae	*Rhinoptera* cf. *steindachneri*	USA	KF685860	Caira et al. (2014)
Eniochobothrium n. sp. 2	Eniochobothriidae	*Rhinoptera* sp.	Senegal	KU249055	Jensen et al. (2016)
Eniochobothrium n. sp. 3	Eniochobothriidae	*Rhinoptera neglecta*	Australia	KU249056	Jensen et al. (2016)
Flapocephalus n. sp. 1	Polypocephalidae	*Pastinachus atrus*	Australia	KF685861	Caira et al. (2014)
Flapocephalus n. sp. 1	Polypocephalidae	*Pastinachus atrus*	Indonesia	KU249087	Jensen et al. (2016)
Floriparicapitus plicatilis	Lecanicephalidae	*Glaucostegus typus*	Australia	KU249074	Jensen et al. (2016)
Floriparicapitus n. sp. 2	Lecanicephalidae	*Glaucostegus thouin*	Indonesia	KU249075	Jensen et al. (2016)
Hexacanalis folifer	Lecanicephalidae	*Gymnura zonura*	Indonesia	KU249073	Jensen et al. (2016)
Hornellobothrium najaforme[a]	Polypocephalidae	*Aetobatus ocellatus*	Australia	KF685865	Caira et al. (2014)
Hornellobothrium n. sp. 1	Polypocephalidae	*Aetobatus ocellatus*	Australia	KU249090	Jensen et al. (2016)
Hornellobothrium n. sp. 2	Polypocephalidae	*Aetobatus ocellatus*	Australia	KU249089	Jensen et al. (2016)
Lecanicephalum sp. 1	Lecanicephalidae	*Dasyatis marmorata*	Senegal	KU249076	Jensen et al. (2016)
Lecanicephalum sp. 2	Lecanicephalidae	*Dasyatis guttata*	Belize	KU249077	Jensen et al. (2016)
Lecanicephalum n. sp. 1	Lecanicephalidae	*Dasyatis* sp.	Taiwan	KU249078	Jensen et al. (2016)
Paraberrapex manifestus	Paraberrapecidae	*Squatina californica*	Mexico	KF685868	Caira et al. (2014)
Paragrillotia similis[a]	Lacistorhynchidae	*Ginglymostoma cirratum*	USA	KF685909	Caira et al. (2014)
Polypocephalus helmuti	Polypocephalidae	*Rhinoptera neglecta*	Australia	KF685869	Caira et al. (2014)
Polypocephalus sp. 3	Polypocephalidae	*Taeniura lymma*	Malaysia	KU249088	Jensen et al. (2016)
Seussapex karybares[b]	Polypocephalidae	*Himantura uarnak*	Australia	KF685867	Caira et al. (2014)
Seussapex n. sp. 2	Polypocephalidae	*Himantura uarnak*	Malaysia	KU249100	Jensen et al. (2016)
Seussapex n. sp. 3	Polypocephalidae	*Himantura leoparda*	Australia	KU249101	Jensen et al. (2016)
Stoibocephalum arafurense	Lecanicephalidae	*Rhina ancylostoma*	Australia	KU249080	Jensen et al. (2016)
Stoibocephalum campanulatum	Lecanicephalidae	*Rhina ancylostoma*	Australia	KU249082	Jensen et al. (2016)
Stoibocephalum koenneckeorum	Lecanicephalidae	*Rhynchobatus* cf. *laevis*	Australia	KU249079	Jensen et al. (2016)
Tetragonocephalum passeyi	Tetragonocephalidae	*Himantura leoparda*	Australia	KF685871	Caira et al. (2014)
Tetragonocephalum sp. 1	Tetragonocephalidae	*Urogymnus asperrimus*	Australia	KF685872	Caira et al. (2014)
Tetragonocephalum n. sp. 2	Tetragonocephalidae	*Himantura jenkinsii*	Australia	KU249085	Jensen et al. (2016)
Tetragonocephalum n. sp. 3	Tetragonocephalidae	*Himantura leoparda*	Australia	KU249086	Jensen et al. (2016)
*Triaenophorus stizostedionis**	Triaenophoridae	*Sander vitreus*	USA	KR780900	Brabec et al. (2015)
Tylocephalum sp. 1	Cephalobothriidae?	*Rhinoptera bonasus*	USA	KU249084	Jensen et al. (2016)
Tylocephalum sp. 3	Cephalobothriidae?	*Rhinoptera* cf. *steindachneri*	USA	KU249083	Jensen et al. (2016)
Zanobatocestus major	Zanobatocestidae	*Zanobatus schoenleinii*	Senegal	KU249053	Jensen et al. (2016)
Zanobatocestus minor	Zanobatocestidae	*Zanobatus schoenleinii*	Senegal	KU249054	Jensen et al. (2016)

*Outgroup taxa.
[a] , as '*Hornellobothrium* n. sp. 1' in Caira et al. (2014).
[b] , as 'New Genus 6 n. sp. 1' in Caira et al. (2014).

(1000 repetitions) were estimated using RAxML. BI trees were generated using MrBayes 3.2 (Ronquist and Huelsenbeck, 2003) running two independent Markov Chain Monte Carlo (MCMC) runs of four chains for 10^7 generations and sampling tree topologies every 10^3 generations. Burn-in periods were set to the first 25,000 generations. MrBayes and RaxML analyses were carried out on the computational resource CIPRES (Miller et al., 2010). Genetic divergence was calculated for 28S rRNA and mtCOI sequences using the uncorrected *p*-distances model in the MEGA7 software (Kumar et al., 2016). Phylogenetic trees were visualised and edited in FigTree v1.4.4 (Rambaut, 2020).

3. Results

3.1. *Eniochobothrium acostae* n. sp. Oosthuizen, Smit & Schaeffner, 2022 (Figs. 1 and 2)

Our observation of *E. acostae* n. sp. indicated the presence of two morphotypes infecting the same host individual, namely large and small. These are highly variable worms ranging greatly in size and morphological characteristics (suppl. Table 1). The diagnosis is based on both morphotypes (40 whole mounts of 25 immature worms, six mature and nine gravid worms [all lacking scoleces] and two immature specimens [one with scolex] prepared for SEM).

Adult worms apolytic (all lacking scoleces), 1318–6007 (3534 ± 1428; $n = 15$) long; maximum width either at level of trough or posteriormost proglottid 155–921 (540 ± 243; $n = 15$); total number of

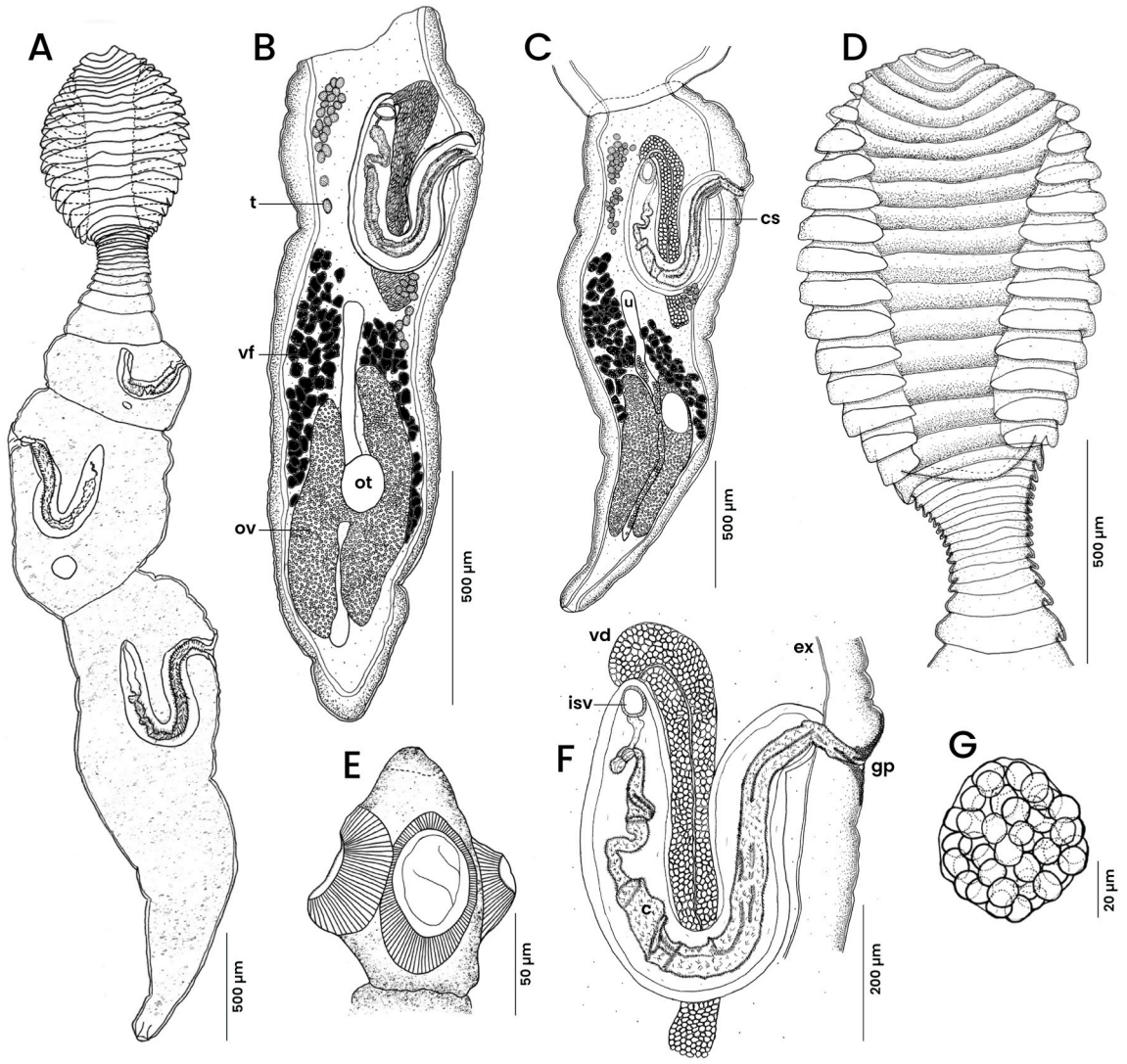

Fig. 1. Line drawings of *Eniochobothrium acostae* n. sp. from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, outline of entire cestode; B, mature proglottid; C, early gravid proglottid; D, trough formed by non-reproductive proglottids of the anterior strobila; E, scolex; F, terminal genitalia; G, cocoon with eggs. Abbreviations: c (cirrus); cs (cirrus sac); ex (excretory canal); gp (genital pore); isv (internal seminal vesicle); ot (ootype); ov (ovary); t (testes); u (uterus); vd (vas deferens); vf (vitelline follicle).

proglottids 22–47 (36 ± 7; *n* = 14) (Figs. 1A and 2A). Strobila divided into anterior trough region consisting of non-reproductive proglottids, expanding laterally to form U-shaped trough and posterior reproductive region consisting of reproductive proglottids with internal reproductive organs in mature development stages (Fig. 1A). Scolex from scanning electron microscopy 91 (*n* = 1) long by 79 (*n* = 1) wide, bearing four acetabula (Fig. 2B). Acetabula in form of sessile suckers, 47–61 (54 ± 10; *n* = 1; *2*) long by 34–36 (35 ± 1; *n* = 1; *2*) wide (Figs. 1E and 2B). Apical modification of scolex proper in form of narrowed extension with small terminal apical aperture (Fig. 2B), with small, mostly glandular, inextensible and irreversible apical organ. Apical organ 14 (*n* = 1) long by 20 (*n* = 1) wide (Figs. 1E and 2B). Cirrus covered with small, triangular microtriches visible at opening of the genital pore (Fig. 2C). Cephalic peduncle not observed. Non-reproductive and reproductive proglottids craspedote, non-laciniate (Figs. 1A and 2A). Trough 402–980 (730 ± 172; *n* = 15) long by 125–736 (451 ± 215; *n* = 14) wide, consisting of 12–22 (18 ± 3; *n* = 14) non-reproductive proglottids (Fig. 1A, D, 2A, D). Reproductive region of strobila 933–5471 (2811 ± 1320; *n* = 15) long by 151–921 (525 ± 242; *n* = 15) wide, consisting of 10–26 (17 ± 5; *n* = 15) reproductive proglottids (Figs. 1A and 2A). Immature proglottids 9–25 (16 ± 5; *n* = 15) in number, initially wider than long, becoming longer than wide; posteriormost immature proglottid 164–1150 (619 ± 323; *n* = 15) long by 370–844 (565 ± 154; *n* = 15) wide (Figs. 1A and 2A). Mature proglottids 0 or 1 in number, longer than wide, 591–2172 (1062 ± 638; *n* = 6) long by 151–667 (317 ± 202; *n* = 6) wide (Fig. 1A and B). Gravid proglottids 0 or 1 in number, 1208–2511 (1805 ± 475; *n* = 9) long by 418–921 (654 ± 148; *n* = 9) wide (Fig. 1A, C). Total number of testes 16–34 (21 ± 7; *n* = 6), arranged in two distinct groups of aporal and poral testes extending from anterior part to middle of proglottid in both mature and gravid proglottids (Fig. 1B and C). Aporal testes extend from anterior part of proglottid to corresponding group of vitelline follicles, 11–27 (15 ± 6; *n* = 6) in number; poral testes 3–7 (5 ± 2; *n* = 6) in number (Fig. 1B and C). Testes 15–30 (24 ± 5; *n* = 6) long by 6–27 (18 ± 7; *n* = 6) wide, anterior to ovary, in several irregular columns in dorso-ventral view (Fig. 1B and C). Vas deferens with glandular wall observed at level of cirrus sac, entering cirrus sac at distal end visible along lateral margin of proglottid, just posterior to margin of U-shape of cirrus sac, 206–765 (376 ± 227; *n* = 6) long by 18–115 (52 ± 36; *n* = 6) wide (Fig. 1B–C, F). External seminal vesicle absent. Internal seminal vesicle present, small, 31–72 (53 ± 16; *n* = 6) long by 15–64 (37 ± 18; *n* = 6) wide (Fig. 1B–C, F). Cirrus sac U-shaped, thick-walled, 251–935 (468 ± 267; *n* = 6) long by 41–111 (71

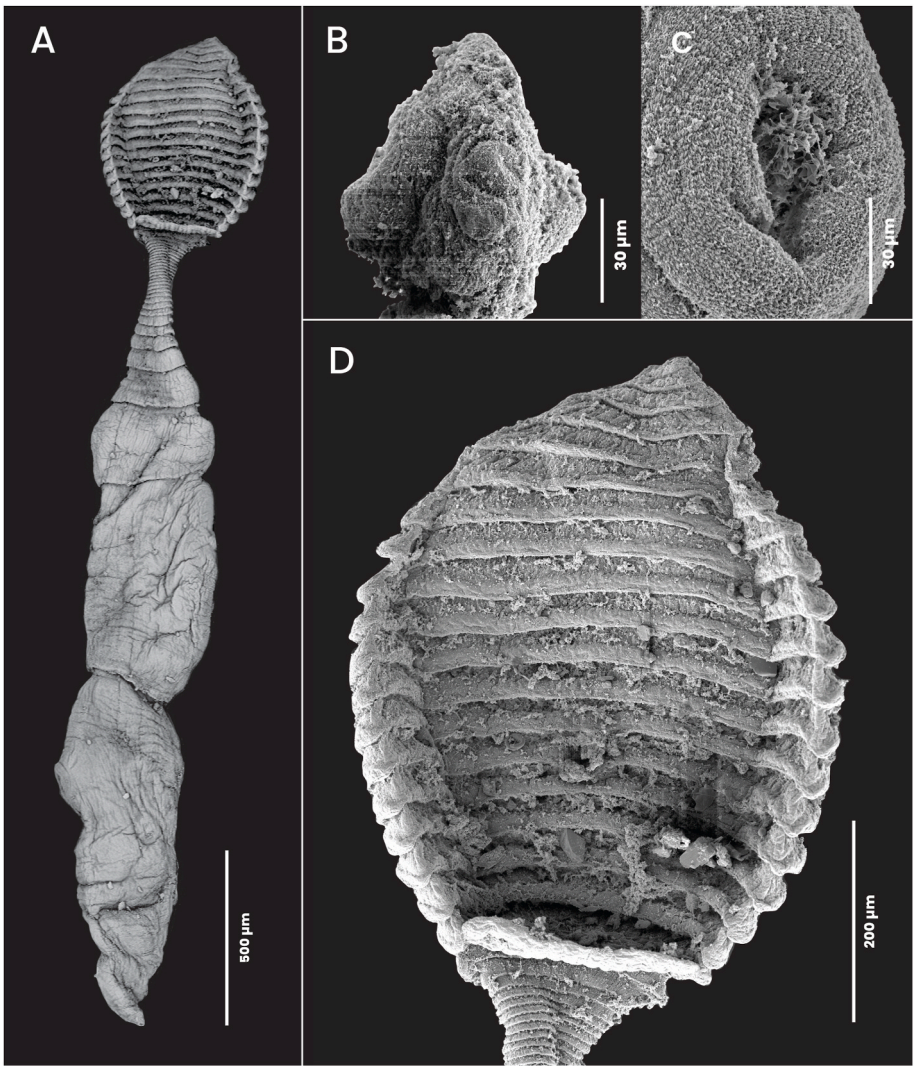

Fig. 2. Scanning electron micrographs of an immature specimen of *Eniochobothrium acostae* n. sp. from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, entire strobila; B, scolex; C, genital pore; D, trough formed by non-reproductive proglottids of the anterior strobila.

± 24; $n = 6$) wide, containing a long, inverted cirrus (Fig. 1A–C, F). Cirrus armed, 222–889 (403 ± 275; $n = 6$) long by 21–46 (30 ± 10; $n = 6$) wide (Fig. 1B–C, F). Ovary H-shaped in dorso-ventral view, 197–650 (374 ± 177; $n = 6$) long by 49–159 (85 ± 48; $n = 6$) wide (Fig. 1B and C). Ootype between bases of ovarian lobes, large, ovoid, 61–161 (91 ± 41; $n = 6$) long by 32–103 (55 ± 34; $n = 6$) wide (Fig. 1B and C). Vagina absent. Genital pores lateral, irregularly alternating, 69–86% (75 ± 6; $n = 6$) of proglottid length from posterior margin (Fig. 1A–C). Uterus medial, saccate, extending from posterior margin of ovary to near posterior margin of cirrus sac, 213–799 (465 ± 284; $n = 5$) long by 37–54 (43 ± 7; $n = 5$) wide; uterine duct not observed; uterine pore absent (Fig. 1B and C). Vitellaria arranged in two lateral bands with multiple columns, extending from middle of cirrus sac to level of ovarian isthmus; vitelline follicles 7–37 (18 ± 13; $n = 6$) long by 4–24 (11 ± 10; $n = 6$) wide (Fig. 1B and C). Two lateral pairs of excretory vessels present (Fig. 1B–C, F). Eggs in cocoons; total number of cocoons 41–79 (62 ± 14; $n = 5$) (Fig. 1C, G). Each cocoon contains 30–42 (35 ± 6; $n = 5$) eggs; free cocoons 56–71 (62 ± 5; $n = 6$) long by 44–52 (48 ± 3; $n = 6$) wide (Fig. 1G). Eggs subspherical, thin-walled, 14–15 (14 ± 1; $n = 6$) long by 11–12 (12 ± 1; $n = 6$) wide (Fig. 1G).

3.2. Taxonomic summary

Type host: Oman cownose ray, *Rhinoptera jayakari* Boulenger (Myliobatiformes, Rhinopteridae).

Type locality: South-western Indian Ocean off Scottburgh (28°78′0″S, 30°76′0″E), KwaZulu-Natal Province, South Africa.

Additional locality: South-western Indian Ocean off Richards Bay (28°78′07″S, 32°03′83″E), KwaZulu-Natal Province, South Africa.

Site *of infection:* Spiral intestine.

Prevalence *and intensity of infection:* Prevalence 67% (2 out of 3 *R. jayakari*); intensity >70 worms per host.

Specimens *deposited:* Holotype in NMB (NMB P-883); paratypes in IPCAS (IPCAS C-916), MNHG (MHNG-PLAT-0138936–0138937) and NMB (NMB P-884–898). The specimen used for SEM is retained in the parasite collection of the Water Research Group, North-West University.

Representative *DNA sequences:* Partial sequences of 28S rRNA 1229–1389 bp in length (GenBank accession numbers: **ON972441**; **ON972440**; **ON972442**); partial sequences of mtCOI 536–555 bp in length (GenBank accession numbers: **ON964522**, **ON964530**, **ON964533**). Paragenophore in NMB (NMB P-882).

ZooBank *registration*: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub: F0C0720A-66CE-40F4-A621-4BCD1EC233B7. The LSID for the new name *Eniochobothrium acostae* n. sp. is urn:lsid:zoobank.org:act: B6CB02C9-B528-49AA-894B-

4F41D24333B2.

Etymology: The species name is dedicated to Dr. Aline Angelina Acosta for her contributions to the systematics of parasitic platyhelminths.

3.3. Remarks

Eniochobothrium acostae n. sp. closely resembles congeners within *Eniochobothrium*, namely *E. gracile*, *E. qatarense* and *E. euaxos*, in morphological characteristics. However, the new species presents the largest specimen recorded in total body length (without scolex) exceeding that of *E. qatarense* (including scolex) by more than 300 μm

Table 2
Metrical comparison of species of *Eniochobothrium* (Shipley and Hornell, 1906). Abbreviations: L (length); W (width); TN (total number); P (proglottid); At (anterior); Pt (posterior).

Species	*Eniochobothrium acostae* n. sp.	*Eniochobothrium euaxos*	*Eniochobothrium qatarense*	*Eniochobothrium gracile*
Study	Present study	Jensen (2005)	Al Kawari, Saoud and Wanas (1994)	Shipley and Hornell (1906)
Body (L) (scolex absent)	1.318–6.007 (3.534)	1.524–3.247 (2.232)	–	±3.500–5.000
Body (L) (scolex present)	–	1.724–2.406 (2.112)	3.250–5.650	–
Max (W)	155–921 (540)	218–353 (274)	600–850	–
Scolex (L)	91	88–101 (94)	100–120	–
Scolex (W)	79	76–80 (78)	90–130	–
Acetabula (L)	47–61 (54)	34–40 (37)	40–70	–
Acetabula (W)	34–36 (35)	25–29 (28)	40–70	–
Apical organ (L)	14	36–42 (39)	–	–
Apical organ (W)	20	21–25 (23)	–	–
Rostrum (L)	–	–	14–26	–
Rostrum (W)	–	–	±33*	–
TN neck P	0	0	0	3
TN P	22–47 (36)	29–39 (33)	39–43	±42–44
At trough region (TN P)	12–22 (18)	18–25 (22)	18–20	±18
At trough region (L)	402–980 (730)	523–777 (659)	690–900	–
At trough region (W)	125–736 (451)	218–353 (274)	370–520	–
Pt reproductive region (TN reproductive P)	10–26 (17)	8–12 (10)	21–23	±24–26
Pt reproductive region (L)	933–5.471 (2.811)	970–2.573 (1.572)	±2.260–4.380#	–
Pt reproductive region (W)	151–921 (525)	523–777 (659)	600–850	–
Pt reproductive region (TN immature P)	9–25 (16)	6–11 (9)	17	±18
Pt most immature P (L)	164–1.150 (619)	77–320 (170)	–	–
Pt most immature P (W)	370–844 (565)	124–214 (171)	120–620	–
TN mature P	0 or 1	0 or 1	4–6	±6–8
Mature P (L)	591–2.172 (1.062)	312–1.070 (744)	1.370–2.300	–
Mature P (W)	151–667 (317)	189–290 (230)	620–820	–
TN gravid P	0 or 1	0 or 1	1*	–
Gravid P (L)	1.208–2.511 (1.805)	899–1.550 (1.202)	±1.375*	–
Gravid P (W)	418–921 (654)	233–344 (301)	±561*	–
TN testes	16–34 (21)	35–48	35–43	–
TN aporal testes	11–27 (15)	–	27–32	–
TN poral testes	3–7 (5)	–	8–11	–
Testes (L)	15–30 (24)	10–37 (24)	20–40	–
Testes (W)	6–27 (18)	10–34 (23	20–40	–
Cirrus sac (L)	251–935 (468)	242–467 (371)	630–1.170	–
Cirrus sac (W)	41–111 (71)	42–73 (62)	90–140	–
Cirrus (L)	222–889 (403)	–	720–900	–
Cirrus (W)	21–46 (30)	–	50–70	–
Ovary (L)	197–650 (374)	90–396 (240)	360–570	–
Ovary (W)	49–159 (85)	89–176 (128)	110–180	–
Genital pore from Pt end	69–86% (75)	70–84% (76)	±78–87%*	–
Vitelline follicles (L)	7–37 (18)	8–37 (19)	±38–57 (45)*	–
Vitelline follicles (W)	4–24 (11)	11–44 (28)	±23–38 (30)*	–
TN cocoons	41–79 (62)	–	–	–
Cocoon (L)	56–71 (62)	104–123 (115)	–	–
Cocoon (W)	44–52 (48)	80–92 (86)	–	–
TN eggs	30–42 (35)	40–51 (45)	±10	–
Egg (L)	14–15 (14)	8–15 (11)	17–24	–
Egg (W)	11–12 (12)	11–21 (14)	12–18	–
Ootype (L)	61–161 (91)	–	40–60	–
Ootype (W)	32–103 (55)	–	40–60	–
Re-ceptaculum diameter	–	–	110–180	–
Vas deferense (L)	206–765 (376)	–	–	–
Vas deferense (W)	18–115 (52)	–	–	–
External seminal vesicle (L)	–	–	360–490	–
Internal seminal vesicle (L)	31–72 (53)	–	250–310	–
Internal seminal vesicle (W)	15–64 (37)	–	±38*	–
Uterus (L)	213–799 (465)	–	±604*	–
Uterus (W)	37–54 (43)	–	±38*	–

*, metrical information of *Eniochobothrium qatarense* calculated from illustrations of Al Kawari et al. (1994) for vitelline follicle (L) (n = 5) and (W) (n = 5) (Fig. 3), uterus (L) and (W) (Fig. 3), internal seminal vesicle (W) (Figs. 3 and 4), rostrum (W) (Fig. 1), gravid proglottid (L) and (W) (Fig. 4) and distance of genital pore from posterior end (Figs. 3 and 4).

#, calculated posterior reproductive length of E. qatarense subtracting metrical values of scolex, anterior trough region and mature proglottid from the total body length provided in Al Kawari et al. (1994).

(Table 2). *Eniochobothrium acostae* n. sp. can be further distinguished from *E. euaxos* in possessing only postporal testes on the poral side of the proglottid while the distribution of testes in *E. euaxos* is both posterior and anterior of the genital pore. In addition, *E. acostae* n. sp. has slightly fewer testes than *E. euaxos* (16–34 vs. 35–48) and smaller cocoons (Table 2). A morphological differentiation based on metrical features is impeded between *E. acostae* n. sp. and *E. gracile* due to a scarcity of morphological information provided in the original description (Shipley and Hornell, 1906). However, *E. acostae* n. sp. can be differentiated from *E. gracile* in lacking the region described and illustrated as a "short neck of three segments" (Shipley and Hornell, 1906). In the description of *E. gracile*, Shipley and Hornell (1906) referred to the apex of the scolex as the rostrum, whereas *E. acostae* n. sp. possesses a rather noticeable apical organ. *Eniochobothrium acostae* n. sp. differs from *E. gracile* in the number of mature proglottids (0 or 1 vs. ± 6–8, respectively). In addition, *E. acostae* n. sp. differs from *E. qatarense* in possessing slightly fewer testes (16–34 vs. 35–43) and fewer mature proglottids (0 or 1 vs. 4–6), respectively. *Eniochobothrium acostae* n. sp. has slightly smaller eggs than *E. qatarense* (14–15 vs. 17–24, respectively). In contrast, cocoons of *E. acostae* n. sp. contain 30–42 eggs, whereas *E. qatarense* is described as possessing "egg balls" (sensu Al Kawari et al., 1994) containing approximately ten eggs. *Eniochobothrium acostae* n. sp. can also be distinguished from *E. qatarense* in having a larger ootype (61–161 μm vs. 40–60 μm, respectively) and a much smaller internal seminal vesicle (31–72 μm vs. 250–310 μm, respectively) (Table 2). In the description of *E. qatarense*, the apex of the scolex is referred to as "a weak proximal pyramidal rostellum" (sensu Al Kawari et al., 1994), while *E. acostae* n. sp. has an apical organ. Additional differences in metrical features between *E. acostae* n. sp. and congeners are listed in Table 2.

Even though we observed the presence of two morphotypes, the molecular data of smaller and larger morphotypes verified that these belong to the same species (isolate 1 and 2 – large; isolate 3 and 4 – small) (Figs. 3 and 4). Regrettably, the only obtained scolex of *E. acostae* n. sp., which has been examined with scanning electron microscopy, was lost after the picture was taken, emphasising just how fragile the connection between the scolex and the anterior trough region of the strobila really is.

3.4. Phylogenetic relationships

Both ML and BI topologies for 28S and COI recovered the same relationships among the taxa included in the phylogenetic analyses. Figs. 3 and 4 show the ML phylogram for each analyses (28S and COI, respectively). The phylogenetic analyses of the 28S showed the clade encompassing *Eniochobothrium* spp. (including the new sequences of *E. acostae* n. sp.) as a monophyletic group (supported by BI analyses [0.99]), supporting the Eniochobothriidae that was proposed by Jensen et al. (2016) to accommodate this genus. The relationship among *Eniochobothrium* spp. is well supported (see Fig. 3). ML analyses recovered a support value of 73 between isolates 2 and 3 of *E. acostae* n. sp. Nevertheless, the partial sequences of the 28S of the three isolates of *E. acostae* n. sp. are identical (*p*-distance 0%, 0 difference in bp). The new South African species appeared more closely related to *Eniochobothrium* n. sp. 1. The Eniochobothriidae appeared more closely related to the Lecanicephalidae and the Cephalobothriidae (Fig. 3). The newly generated partial mtCOI sequences of *E. acostae* n. sp. were compared to *E. euaxos* and the taxa *Eniochobothrium* n. sp. 1, 2 and 3. The phylogenetic analysis of the mtCOI sequences of *Eniochobothrium* spp. showed

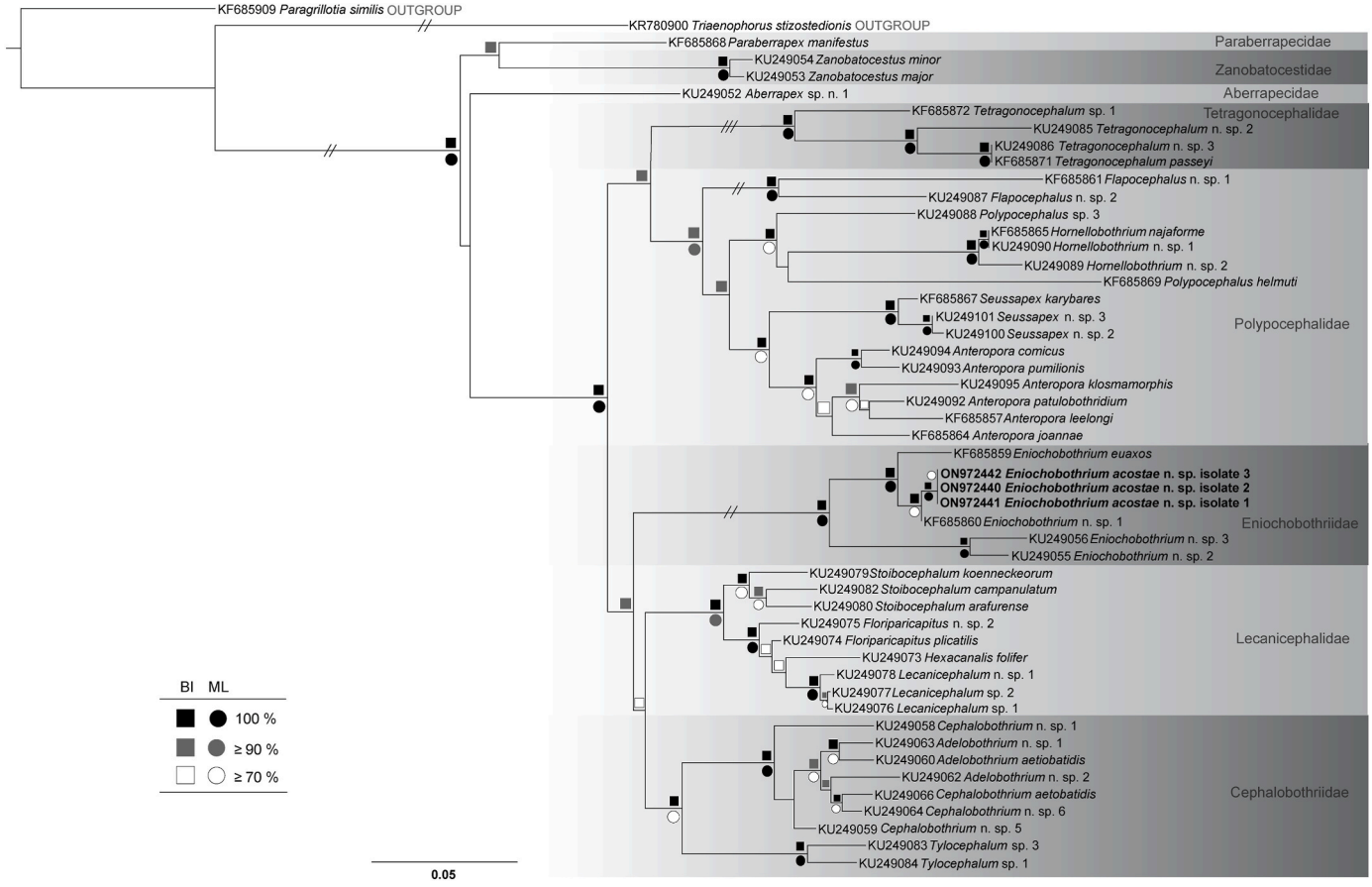

Fig. 3. Maximum likelihood phylogram based on partial sequences of the large subunit 28S rRNA gene. Nodal support is shown as posterior probability and bootstrap. GenBank accession number precedes species name. Branch length scale bar indicates the number of substitutions per site. (//) Branch length reduced to one time the scale bar; (///) branch length reduced to two times the scale bar. Squares represent Posterior Probability values while circles represent Bootstrap values.

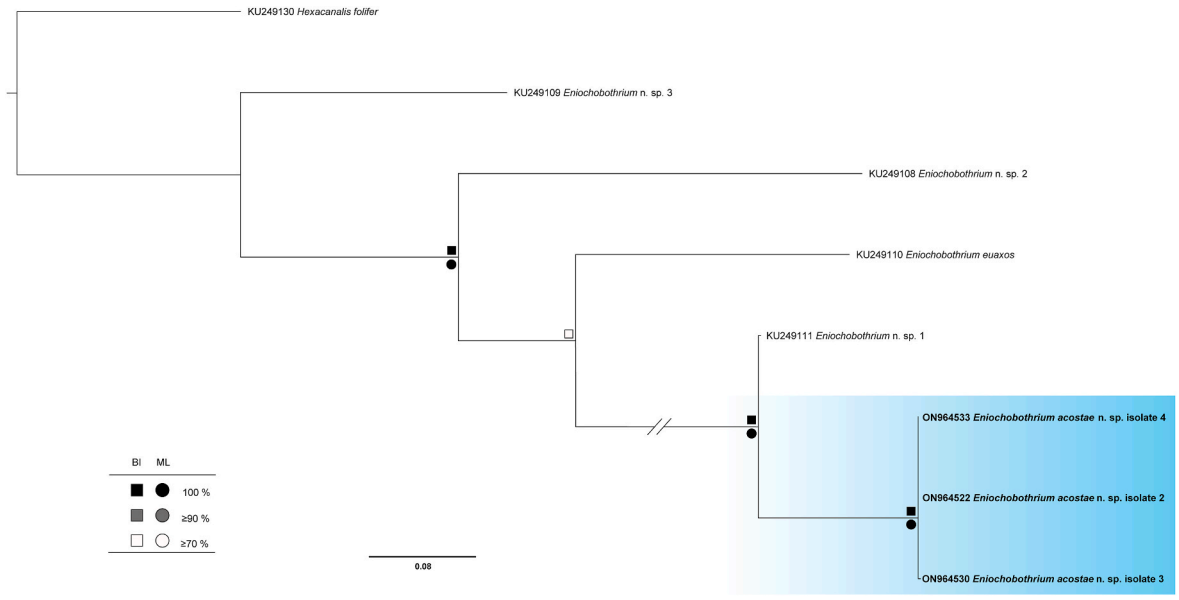

Fig. 4. Maximum likelihood phylogram based on partial sequences of the mitochondrial cytochrome oxidase subunit I (mtCOI) gene. Nodal support is shown as posterior probability and bootstrap. GenBank accession number precedes species name. Branch length scale bar indicates the number of substitutions per site. (//) Branch length reduced to one time the scale bar. Squares represent Posterior Probability values while circles represent Bootstrap values.

the new species as sister to *Eniochobothrium* n. sp. 1, with strong support, which mirrors the results of the 28S rRNA analysis (Fig. 4).

The estimates for evolutionary divergences for 28S rRNA were compared using the newly generated and the available sequences of *Eniochobothrium* spp. from GenBank. The *p*-distances were 2.1–2.2% (27–29 bp) between *E. acostae* n. sp. and *E. euaxos*; 0.2% (3 bp) between *E. acostae* n. sp. and *Eniochobothrium* n. sp. 1; and 5.9–7.3% (77–91 bp) between *E. acostae* n. sp. and *Eniochobothrium* n. sp. 2 and 3. The estimates for evolutionary divergences for mtCOI were compared using partial sequences of *E. acostae* n. sp. with the four available sequences of *Eniochobothrium* and one sequence of *H. folifer* used as an outgroup retrieved from GenBank, with *p*-distances varying from 0 to 22.4%. The *p*-distance values between *E. acostae* n. sp. and *E. euaxos* were 18.1–18.6% (96–102 bp); *E. acostae* n. sp. and *Eniochobothrium* n. sp. 1 were 8.7–8.9% (44–46 bp); and between *E. acostae* n. sp. and *Eniochobothrium* n. sp. 2 and 3 were 19.2–19.9% (92–106 bp).

4. Discussion

According to Jensen (2005), *Eniochobothrium* is presently one of two genera restricted to parasitising a single batoid genus. All species of *Eniochobothrium* currently considered valid have been described as adults from the batoid genus *Rhinoptera*. *Eniochobothrium euaxos* was described from the Australian cownose ray, *Rhinoptera neglecta* Ogilby, from Dundee Beach, Fog Bay, Australia; *E. gracile* has been reported from the flapnose ray *Rhinoptera javanica* Müller and Henle, from Dutch Bay, Sri Lanka; and *E. qatarense* infects *R. javanica* (as *Rhinoptera adspersa* Müller and Henle) from the Arabian Gulf, Qatar. Additional host-parasite associations have been reported for three undescribed species of *Eniochobothrium*: *Eniochobothrium* n. sp 1 (Caira et al., 2014) from the Pacific cownose ray, *Rhinoptera* cf. *steindachneri* Evermann and Jenkins, from Ship Island, Mississippi, USA; *Eniochobothrium* n. sp. 3 (Jensen et al., 2016) from *R. neglecta*, from Dundee Beach, Fog Bay, Australia; and *Eniochobothrium* n. sp. 2 (Jensen et al., 2016) from *Rhinoptera* sp., from St. Louis, Senegal. In his Masters Thesis (2007), Garrett Call reports two species, *Eniochobothrium overstreeti* and *Eniochobothrium sedlockae*, from *Rhinoptera bonasus* Mitchill, yet these species and corresponding species names have never been officially described (Unpublished data; https://kuscholarworks.ku.edu/handle/1808/5536).

Ebert et al. (2021) verified that there is only a single species of *Rhinoptera* in South African waters, *R. jayakari*. Previous records (see Compagno et al., 1989; Compagno, 1986, 1999; Ebert et al., 2015; Heemstra and Heemstra, 2004; Smith, 1952, 1961; Wallace, 1967) mentioned the occurrence of *R. javanica*. Furthermore, all the voucher material from South African specimens is deposited under *R. javanica*. Jensen et al. (2017) estimated that 24 lecanicephalidean species parasitise rhinopterid hosts globally. Thus far, four of the eight rhinopterid hosts have been examined for *Eniochobothrium*, with each species hosting one to two unique species of *Eniochobothrium*. We therefore estimate that the actual species diversity of *Eniochobothrium* in this host group most likely ranges between eight and 16 species worldwide. Even considering *E. acostae* n. sp. from *R. jayakari*, only 63% of the potential rhinopterid hosts (five out of eight species) have been examined for the presence of *Eniochobothrium* species. *Rhinoptera adspersa* (Indo-West Pacific: off India, Malaysia, and East Indies), *Rhinoptera brasiliensis* Müller (southern tip of Brazil to western Florida) and *Rhinoptera marginata* Geoffroy Saint-Hilaire (western coast of Africa and Mediterranean Sea) still await parasitological examination.

The phylogenetic analyses of the 28S rRNA presented herein support the allocation of the new species within *Eniochobothrium*, which formed a strongly supported clade with its congeners (Fig. 3). In the present study, the analyses of 28S rRNA sequences of selected lecanicephalideans corroborate the results of Jensen et al. (2016). These authors presented a concatenated analysis of lecanicephalidean sequences of four genes (complete 28S rRNA, partial 18S rRNA, partial mtCOI, and partial 16S rRNA). Their combined phylogram of the concatenated analyses recovered eight lecanicephalidean clades, similar to the analyses presented herein. The eight clades of Jensen et al. (2016) correspond to the prior existing families Lecanicephalidae, Polypocephalidae, Tetragonocephalidae, and Cephalobothriidae, and their proposed families Aberrapecidae, Eniochobothriidae, Paraberrapecidae and Zanobatocestidae. The proposal of the family Eniochobothriidae for species of *Eniochobothrium* by Jensen et al. (2016) was supported by their phylogenetic analyses, since this clade appeared as one of the most molecularly divergent groups. Such results were also verified in the present study (Fig. 3), in which the addition of a new *Eniochobothrium* species did not alter the topology of lecanicephalidean families of the former authors. In addition, the present study highlights the importance of including both morphological and molecular analyses on newly collected specimens to aid the support of their phylogenetic position.

The tegument of lecanicephalideans is extremely intriguing and has value for taxonomic and presumably phylogenetic studies. According to Jensen (2005), lecanicephalideans possess a unique character trait involving a specific microthrix form described as "long, pointed filiform" (sensu Caira et al., 1999) found on different external surfaces. Microthrix pattern examinations of Jensen (2005) revealed that this unique character state of lecanicephalideans was not observed in any of the >80 specimens forming part of the tetraphyllideans and other outgroup taxa examined by Caira et al. (1999, 2001). The description of the microthrix morphology of *E. acostae* n. sp., as well as comparison to that of *E. euaxos*, was impeded by the freezing and thawing of the host material, which seemed to negatively affect microtriches on individual body regions. Collection of fresh material from the type host and, preferably, the type locality are needed to describe the microthrix pattern of *E. acostae* n. sp. in the future. The microthrix patterns have been examined in only one species of *Eniochobothrium* (see Jensen, 2005). Therefore, additional studies focusing on the surface ultrastructure of members of *Eniochobothrium* can add more detailed characteristics for the diagnosis and species circumscription of representatives of the Eniochobothriidae.

Lecanicephalideans have a global distribution, known from eight of the 12 marine biogeographic regions identified by Spalding et al. (2007). The Central Indo-Pacific has the highest species diversity (69%) followed by the Western Indo-Pacific (14%). Other biogeographical realms present a much lower number of reported species [Temperate Northern Pacific, Tropical Atlantic, and Temperate Northern Atlantic (5% each), Eastern Indo-Pacific, Tropical Eastern Pacific, Temperate South America, Temperate Australasia (1% each)]. Up until now, lecanicephalideans have not been reported from the marine regions of the Arctic, Southern Ocean, and Temperate Southern Africa (Jensen et al., 2017). *Eniochobothrium acostae* n. sp. is the first species of the order Lecanicephalidea reported from southern Africa. Partial sequences of 28S rRNA and mtCOI genes are provided for the new species, adding relevant data for the genus and thus aiding future studies. Phylogenetic analyses support the validity of Eniochobothriidae for species of *Eniochobothrium* by Jensen et al. (2016). When taking into consideration that only 63% of the potential rhinopterid hosts have been examined for the presence of *Eniochobothrium* species, it is clear that a considerable number of representatives might still remain unknown and await future discovery and description.

Note

Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers: **ON972441**, **ON972440**, **ON972442**, **ON964522**, **ON964530**, **ON964533**.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2022.08.011.

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