Biodiversity assessment of marine cestode lineages infecting the Oman cownose ray, *Rhinoptera jayakari* Boulenger off the KwaZulu-Natal Province, South Africa

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ABSTRACT

South Africa is home to a remarkable variety of marine fauna, with a notable diversity among chondrichthyans. However, only a small fraction of these species has been thoroughly studied for parasites. The existing knowledge about chondrichthyan parasites in South Africa is limited and incomplete. To comprehensively understand the extent of diversity among chondrichthyan parasites, dedicated biodiversity initiatives are crucial. These efforts should prioritise underrepresented hosts and regions. Expanding our understanding of cartilaginous fishes and their parasites can also contribute significantly to future conservation endeavours. Conservation strategies should not only focus on the threatened host species but also consider parasites as important targets. This holistic approach will not only safeguard endangered host species but also protect a broad spectrum of parasites, preventing their potential extinction alongside their host species and contributing to the overall preservation of marine biodiversity. Acknowledging the lack of taxonomic biodiversity research for cestode-elasmobranch systems in the waters of southern Africa, this study aims to establish a basis for future research in this field. Utilising a combination of morphological and molecular methods alongside taxonomic assessments, the research aims to address this significant knowledge gap in taxonomic biodiversity focussing on assessing the biodiversity of cestode lineages infecting the endangered Oman cownose ray (*Rhinoptera jayakari* Boulenger) off KwaZulu-Natal Province, South Africa. The biodiversity assessment of marine cestode lineages infecting *R. jayakari*, revealed a diversity of five cestodes species. These species include a new species, *Eniochobothrium acostae* Oosthuizen, Naidoo, Smit & Schaeffner, 2022, described on morphological and molecular grounds, currently representing the largest described species of the genus. In addition, two additional locality records of *Rhinoptericola* species, *Rhinoptericola mozambiquensis* Herzog & Jensen, 2022 and *Rhinoptericola butlerae* Beveridge & Campbell, 1988, were discovered, expanding the known geographic distribution of *R. mozambiquensis* and *R. butlerae* to South Africa. The latter species is also introduced from a novel host species. A morphological study of *Tetrarhynchobothrium* cf. *unionifactor* (Shipley & Hornell, 1904) expands the known geographical range of this species and introduces a potential novel host species. Lastly, a potential new species of *Duplicibothrium* Williams & Campbell, 1978 has been discovered which exhibits distinct differences from its congeners, especially the length of the cephalic peduncle, and further expands the known geographic distribution of the genus to include the waters off South Africa. The description of these species was accomplished through the usage of both morphological methods (such as light microscopy and scanning electron microscopy) and molecular methods (involving molecular characterisation and phylogenetic analyses). Furthermore, each species described was systematically differentiated from all other valid species within respective genera. In cases where molecular material was accessible, the generated sequences were cross-referenced with all relevant available sequences, thereby affirming the authenticity of the identified species.

Keywords

Marine parasites, cestodes, taxonomy, elasmobranchs, new species, *Rhinoptera*, species diversity, South-western Indian Ocean, intraspecific variability, biodiversity.

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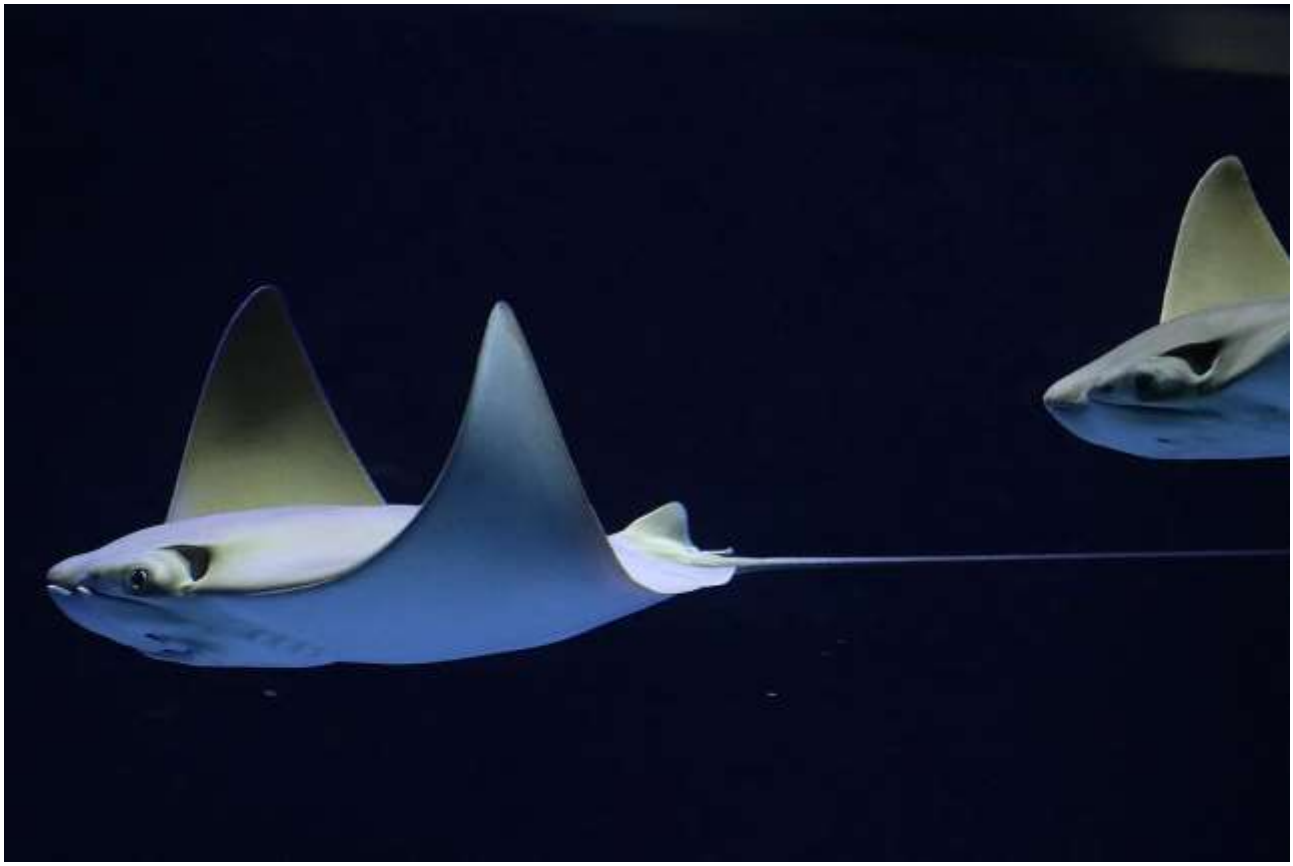
CHAPTER 1

GENERAL INTRODUCTION



Credit: Zoo-Leipzig (www.zoo-leipzig.de)

CHAPTER 1: GENERAL INTRODUCTION

Marine parasitology has emerged as a critical field of study due to the recognition of parasites as integral components of ecosystems, indirectly influencing multiple trophic levels and ecosystem dynamics (Lafferty *et al.,* 2008; Dobson *et al.,* 2008). Despite their importance, parasites often remain neglected in biodiversity assessments, with each animal serving as a host to at least one parasite species, hinting at a potentially greater diversity of parasites compared to non-parasitic species (Poulin & Morand, 2000; Rohde, 2001).

Chondrichthyans hold an unique place in the evolutionary timeline, dating back approximately 455 million years, making them among the oldest vertebrate lineages (Grogan & Lund, 2004). Their historical endurance, combined with their ecological importance, emphasises the need for a comprehensive understanding of their parasites (Grogan & Lund, 2004). Parasites, often overlooked, play crucial roles in marine ecosystems, influencing the complexity of food webs and contributing to the overall health of interconnected systems (Mouritsen & Poulin, 2005; Poulin *et al.,* 2016). Conservation efforts historically focused on endangered species are evolving to encompass their associated parasites, recognising the interdependence between hosts and parasites in maintaining ecosystem health (Spencer *et al.,* 2016; Dougherty *et al.,* 2016). The ancient co-evolution between elasmobranch hosts and parasites, spanning more than 270 million years (Dentzien-Dias *et al.,* 2013), highlights the need to incorporate parasites into conservation strategies to prevent co-extinction and maintain ecological balance (Spencer *et al.,* 2016).

The taxonomic group Chondrichthyes is characterised as a monophyletic group, consisting of two sister taxa with the subclasses Elasmobranchii (sharks, rays, and skates) and Holocephali (chimaeras) (Grogan & Lund, 2004). Species from these subclasses harbour a diverse array of metazoan and protozoan parasites, which form both permanent and temporary associations with their hosts (Caira & Healy, 2004; Schaeffner & Smit, 2019). The parasitic invertebrates that infect elasmobranchs belong to six distinct phyla: Acanthocephala, Arthropoda, Annelida, Nematoda, Mollusca, and Platyhelminthes (Caira & Healy, 2004). Although a broad classification categorises these parasites as endoparasites or ectoparasites, the complexity of their interactions with chondrichthyan hosts adds distinction to their ecological roles (Novelo *et al.,* 2018). Ectoparasites, found on the external body parts of the host, include arthropods, leeches, monogeneans, and molluscs (Novelo *et al.,* 2018). This diverse group of ectoparasites contributes to the external dynamics of host-parasite relationships. On the other hand, endoparasites inhabit interior cavities of their hosts, contributing to the internal dynamics of host-parasite relationships. The endoparasitic community includes most helminths (Novelo *et al.,* 2018). Both ectoparasites and endoparasites exhibit a high diversity within elasmobranchs, with platyhelminths, especially cestodes, dominating this parasitic fauna (Caira & Healy, 2004).

In South Africa, only a fraction of potential chondrichthyan hosts have been investigated for parasites and respective parasite records might represent only the 'tip of the iceberg'. The parasite records available from Chondrichthyans in South Africa are based on information collected from 90 species, including 24 unidentified ones (Schaeffner & Smit, 2019). Less than 50% of the known South African chondrichthyan species have been screened for parasites and reports from certain orders (viz., Echinorhinifromes, Squatiniformes, Heterodontiformes) which include species such as the bramble, angel and bullhead sharks are currently absent (Schaeffner & Smit, 2019). Parasite infections of batoids, holocephalans and the remaining selachiid orders remain almost entirely unexplored, with available information primarily focusing on the most charismatic selachiid orders (viz., Carcharhiniformes, Lamniformes) representing the ground- and mackerel sharks, respectively (Schaeffner & Smit, 2019). Current knowledge of the marine parasite fauna of chondrichthyans from South Africa is dominated by parasitic copepods (Schaeffner & Smit, 2019). The second largest group of chondrichthyan parasites is tapeworms (or cestodes) (Schaeffner & Smit, 2019). The cestode diversity is higher than all other groups of metazoan parasites combined with 10 of the 19 known orders of cestodes that infect chondrichthyans (Caira *et al.,* 2012). However, information on chondrichthyan cestodes in South Africa is based on only 12 independent studies with 32 species of 8 orders (Schaeffner & Smit, 2019; Oosthuizen *et al.,* 2021; 2022; Van Der Spuy *et al.,* 2020; 2022). Only 20 of 204 chondrichthyans species have been examined for cestodes. Based on what is known globally, it is expected that the cestode diversity of South African chondrichthyans is at least equal to or even larger than the diversity of chondrichthyan hosts present. In addition to copepods and cestodes, 19 species belonging to six parasite groups (i.e. trypanosomes, ciliates, monogeneans, trematodes, nematodes, isopods) were reported from South Africa but information on the remaining 12 parasite groups is absent (i.e. amoebae, sporozoans, myxozoans, acanthocephalans, hirudineans, remaining parasitic crustaceans) (Schaeffner & Smit, 2019). Therefore, we expect that a significant proportion of the actual diversity is yet to be discovered from this diversity-rich region.

The cestodes that parasitise the two elasmobranch groups Selachimorpha (sharks) and Batoidea (rays and skates) display a remarkable variety of forms (Caira & Jensen, 2014). Cestodes parasitising elasmobranchs represent nine orders, of which 202 genera and 1034 species are now recognised (Caira & Jensen, 2017). Within the last two decades, about 250 new species and 50 new genera have been described leading to cestode orders fluctuating in diversity from less than 10 to hundreds of species. Additionally, every cestode order, apart from the Tetraphyllidea, is monophyletic sharing a common ancestor (Caira & Jensen, 2014). Cestodes of elasmobranchs, ranging from 500 µm to 60 cm in length, display moderate size, with species of some orders exclusively parasitising sharks, others only batoids, and some can be found in both groups (Caira & Jensen, 2017). Almost all orders are present in the spiral intestine of their hosts (Caira & Jensen, 2014). Furthermore, most species inhabit marine environments with very few exceptions that occur

in freshwater, and usually, strict host specificity is observed at species level, except for members of the Trypanorhyncha (Caira & Jensen, 2014).

The life cycle of tapeworms infecting elasmobranchs typically involves a complex series of stages often beginning with the release of tapeworm eggs into the marine environment through the faeces of infected elasmobranchs (Caira & Reyda, 2005). These eggs then hatch into larvae called oncospheres, which are ingested by intermediate hosts such as teleosts, molluscs, crustaceans, and even marine mammals (Jensen & Bullard, 2010). Once inside the intermediate host, the larvae develop into cysticercoids, a stage that remains dormant until the intermediate host is consumed by a definitive host, typically an elasmobranch (Caira & Reyda, 2005). Upon ingestion, the cysticercoids develop into adult tapeworms in the intestine of the definitive host, completing the life cycle (Caira & Healy, 2004; Jensen & Bullard, 2010). However, the life cycle of tapeworms infecting elasmobranchs remains complex and poorly understood, as highlighted by various studies (Chambers *et al.,* 2000; Chervy, 2002; Palm, 2004; Caira & Reyda, 2005; Jensen, 2005; Jensen & Bullard, 2010). While more than 1000 tapeworm species are documented to infect elasmobranchs, complete life cycles have been elucidated for <10 of these species due to the challenge of distinguishing between larval and adult stages based solely on morphology (Bennett *et al*., 2019). While a complete life cycle remains unknown for most species, it is generally believed that elasmobranch tapeworms parasitise two to three intermediate host species before reaching their elasmobranch definitive host (Caira & Jensen, 2014). According to Palm (2004), some cases even suggest the involvement of a fourth intermediate or paratenic host. Trophic transmission facilitates the movement of life cycle stages between hosts (Caira & Reyda, 2005). Identification of cestode larvae to species level poses challenges, but exceptions exist, particularly within the Trypanorhyncha where the rhyncheal apparatus aids in species-level identification (Palm, 2004). The study conducted by Jensen and Bullard (2010) demonstrated advancements in molecular methods that enhance larval identification, uncovering specific larval forms linked to their adult counterparts. Nevertheless, additional research is required to thoroughly elucidate the complexities of tapeworm life cycles in elasmobranchs.

In South Africa, a country characterised by a long coastline spanning more than 2,500 km and encompassing the South-eastern Atlantic and Western Indian Ocean basins, marine parasitology takes on a unique significance (Welsh, 2000). The coastal waters of South Africa boast a rich diversity of elasmobranchs, with 204 species of chondrichthyan documented in the region, making it the third most diverse region globally (Ebert & van Hees, 2015). According to Ebert and van Hees (2015), the 204 known chondrichthyan species consist of 119 sharks, 79 rays, and 8 chimeras. This extensive array of species represents approximately 17% of the total chondrichthyan diversity documented globally (Ebert & van Hees, 2015). The biogeographical region known as Temperate Southern Africa, as defined by Spalding *et al.* (2007), is characterised by the dominance of two major ocean currents – the Benguela Current and the Agulhas Current. These currents divide the region

into two distinct marine provinces, namely the Benguela and Agulhas provinces (Spalding *et al.,* 2007). The Agulhas province encompasses the eastern and a major portion of the southern coastline of South Africa. Here, the marine waters experience the influence of the fast-flowing, warm Agulhas Current, originating from the equatorial Indian Ocean and flowing southward along the eastern African coastline towards Cape Point (Briggs, 1995; Van As *et al.,* 2012). On the other hand, the Benguela province extends over the marine expanse of Namibia and the western coastline of South Africa within the south-eastern Atlantic Ocean. This province is characterised by the impact of the slow-flowing, cold Benguela Current, moving northwards from the Antarctic towards Angola (Briggs, 1995). The distribution of chondrichthyan fauna in the marine environment exhibits notable variations, as highlighted by various studies (Compagno, 1999; Ebert & van Hees, 2015). These variations are closely related to the biogeographical provinces, with most chondrichthyans displaying a limited geographical presence in either the Agulhas or Benguela provinces (Ebert & van Hees 2015). Interestingly, the Agulhas province is known for its significantly higher chondrichthyan diversity, hosting 175 species that are predominantly of tropical and warm-temperate origin, in contrast, the Benguela province, situated in the south-eastern Atlantic, exhibits a comparatively lower diversity, with only 96 temperate chondrichthyan species (Ebert & van Hees, 2015). However, 62 of the chondrichthyan species extend their distribution across both the Agulhas and Benguela biogeographical provinces and the region demonstrates notable species endemism, with approximately 13% of chondrichthyan species confined to the waters of southern Africa (Ebert & van Hees, 2015).

Despite the enormous chondrichthyan diversity, the knowledge of parasites of this host group in South Africa is limited, particularly in batoid rays, indicating a critical gap in understanding the parasite fauna of these hosts (Schaeffner & Smit, 2019). In the most recent assessment of Caira and Jensen (2017), the global diversity of cestodes infecting elasmobranchs is estimated to be 5,126 species. However, a considerable number have yet to be identified, with an estimated 1,259 species of cestodes of elasmobranchs that await discovery on a global scale (Caira *et al.,* 2017). Notably, the estimation by Randhawa and Poulin (2010) suggests an even greater richness in species. Through an analysis of cestode records from 317 elasmobranch species, the latter authors proposed that approximately 3,600 species from elasmobranch hosts are still awaiting description in the future. Despite global estimates suggesting a vast diversity of elasmobranch-infecting cestodes, with thousands of species yet to be discovered, the specific knowledge of these parasites in the South African context remains limited (Schaeffner & Smit, 2019). Cestode records are available for representatives of most of the main groups of elasmobranchs, however only few members of species-rich orders have to date been screened for parasites (i.e. Squaliformes, Rhinopristiformes, Rajiformes, Myliobatiformes).

Batoids, a diverse group of cartilaginous fishes, are classified into four orders: Myliobatiformes, Torpediniformes, Pristiformes, and Rhinopristiformes. Within the order Myliobatiformes, the genus *Rhinoptera* Cuvier, 1829, stands out as a notable representative of stingrays belonging to the family Myliobatidae, commonly recognised as cownose rays due to their anterior lobes resembling a cow's nose (Fisher *et al.,* 2013). Found in warm coastal waters and estuaries, these rays are known for their distinctive wing-shaped pectoral fins (Fisher *et al.,* 2013). Species of *Rhinoptera* are characterised by their large size, extended lifespan, slow maturation, and limited reproductive capacity, making them susceptible to overexploitation (Hoenig & Gruber, 1990; Musick, 1999). Full maturity is not reached until approximately 70% of the maximum size is reached, taking six to seven years for males (>85 cm disc width) and seven to eight years for females (85-88 cm disc width) (Fisher *et al.,* 2013; Smith & Merriner, 1987). Typically, females give birth to one generation per year, with pupping occurring in late June to early July following an 11-12 month gestation period (Fisher *et al.,* 2013; Smith & Merriner, 1986), although twins have been documented (Fisher *et al.,* 2014). Slow population growth and bycatch contribute to the decreasing population status of most ray species (Carlson *et al.,* 2020). Rays, as mesopredators, play a vital role in the structure and functioning of food webs (Carlson *et al.,* 2020). Changes in their abundance can lead to trophic cascades that impact all trophic levels down to primary producers (Pauly *et al.,* 1998). Overfishing of elasmobranch species has been linked to negative ecological consequences (Barley *et al.,* 2017; Myers *et al.,* 2007). Several cownose ray species have undergone assessment on the IUCN Red List of Threatened species: the Lusitanian cownose ray (*Rhinoptera marginata* Geoffroy Saint-Hilaire, 1817) is Critically Endangered, the Javanese Cownose ray (*Rhinoptera javanica* Müller & Henle, 1841) and the Oman cownose ray (*Rhinoptera jayakari* Boulenger, 1895) are listed as Endangered, the Pacific cownose ray (*Rhinoptera steindachneri* Evermann & Jenkins, 1891) is Near Threatened, the Australian cownose ray (*Rhinoptera neglecta* Ogilby, 1912) is Data Deficient, and the Atlantic cownose ray (*Rhinoptera bonasus* Mitchill, 1815) and the Brazilian cownose ray (*Rhinoptera brasiliensis* Müller, 1836) are categorised as Vulnerable (Palacious *et al.,* 2023).

The Oman cownose ray, *R. jayakari*, exhibits a widespread distribution primarily in the Indo-West Pacific region, extending from South Africa to the Philippines, north to the Ryukyu Islands, and south to eastern Indonesia (Palacious *et al.,* 2023). This benthopelagic species is found in tropical and temperate seas and estuaries (Neer & Thompson, 2005). Recent verification by Ebert *et al.* (2021) indicates that *R. jayakari* is the only species of *Rhinoptera* present in South African waters. Given this finding, understanding the feeding habits of *R. jayakari* is crucial, as it could offer valuable insights into their ecological role (Ehemann *et al*., 2019). However, it's crucial to emphasise that the feeding behaviour of *R. jayakari* remains unexamined in all documented regions, including the waters of Southern Africa. Existing studies predominantly focus on regions where *R. bonasus* and *R. steindachneri* are prevalent, such as the lower Chesapeake Bay, the Gulf of Mexico, Florida, and Brazil, offering insights into the feeding habits of cownose rays, which are predominantly known to

prey upon benthic invertebrates like crustaceans, molluscs, and small fish (Bayliff, 1951; Bornatowski *et al*., 2014; Omori & Fisher, 2017; Ehemann *et al*., 2019; Enríquez-García *et al*., 2023). According to Omori and Fisher (2017), cownose rays have been observed using specialised dental plates to crush and consume hard-shelled prey items like crabs and bivalves. This dietary preference, known as durophagy, indicates a preference for benthic organisms and suggests a potential role in shaping the trophic dynamics of the ecosystem (Omori & Fisher, 2017).

The dietary preferences and feeding habits of *R. jayakari*, will not only provide insights into its ecological role but also have significant implications for understanding transmission strategies of tapeworms across different hosts and locations (Ehemann *et al*., 2019). While the regions mentioned in the studies above have provided valuable insights into the feeding behaviour of two representatives of *Rhinoptera*, gaps in our understanding still exist, particularly in Southern African waters. Despite this, recent research has unveiled a fascinating aspect of *R. jayakari*'s ecology—its role as a host for various cestodes. Cestodes from three different orders have to date been described from *R. jayakari* and include *Duplicibothrium bilai* Stephan, Bueno & Caira, 2023; *Eniochobothrium acostae* Oosthuizen, Naidoo, Smit & Schaeffner, 2022 (as part of the present study, see Chapter 2); *Nanoduplicibothrium megaphallum* Stephan, Bueno & Caira, 2023; *Rhinoptericola mozambiquensis* Herzog & Jensen, 2022; and *Amiculucestus herzogae* Jensen & Caira, 2022. Interestingly, all the species currently known from this host were described in either 2022 or 2023, from either Mozambique or South Africa. Two other valid species (viz., *Amiculucestus penghuensis* Jensen & Caira, 2022; *Eniochobothrium vegrande* Jensen & Caira, 2022) have been reported from *Rhinoptera* cf. *jayakari* from Taiwan, although further research is still needed to verify the host identity.

Given these sparse accounts, it becomes obvious that very little knowledge on the cestode parasites of cartilaginous fishes in South Africa in general and specifically almost nothing about those infecting the endangered Oman cownose ray is present. Therefore, this study aims to address this knowledge gap by investigating the species diversity of cestodes that infect the Oman cownose ray off KwaZulu-Natal, South Africa. Through morphological and molecular methods and taxonomic assessments, the study seeks to contribute to the understanding of elasmobranch parasites, emphasising their role in maintaining the health and balance of marine ecosystems. The findings are expected to provide insights into the intricate relationships between elasmobranch hosts and their parasites, providing valuable information for future conservation strategies in the region.

1.1. Aim of the study

The aim of this study was to determine the species diversity of cestodes infecting the Oman cownose ray, *Rhinoptera jayakari,* off the KwaZulu-Natal coast, South Africa.

1.2. Objectives of the study

- Identification of cestodes in the Oman cownose ray using morphological and molecular methods (depending on available material).
- Assessment of taxonomic status of cestode lineages and description of species.

1.3. Dissertation outline

The first chapter serves as a comprehensive introduction to the current study, with the aim of providing the reader with a short overview and background of the research, encompassing the problem statement, the research gaps, and the specific aim and objectives of the study. Chapters 2 to 5 constitute the core result chapters. Within each of these chapters, a structure is maintained, consisting of an abstract summarising the chapter's work, an introduction, materials and methods, morphological descriptions of either newly discovered or previously identified species, and a discussion. The sixth chapter serves as the concluding section of the dissertation, presenting recommendations for future studies. Chapter 2 has already been published in an international peer-reviewed journal (Oosthuizen *et al.,* 2022) and is incorporated into the thesis in its original published format, adhering to specific journal editing requirements (refer to Appendix 1 for the journals' instructions to authors). The unpublished chapters (1, 3, 4, 5, and 6) adhere to the prescribed NWU Harvard style of referencing, with each chapter accompanied by its own reference list.

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CHAPTER 2

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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2.1. 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* 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 morphological characterisation as the new species groups together with other members of the genus. *Eniochobothrium acostae* 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 species known to date and the first species record from South African waters.

Keywords: Marine parasites, cestode, elasmobranchs, species diversity, integrative taxonomy, south-western Indian Ocean.

2.2. 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 monophyly of the order Lecanicephalidea via molecular sequence data (Jensen et al., 2016), although generic interrelationships within families and monophyly of genera could not be supported. 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).

2.3. Materials and Methods

2.3.1. 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 RES2020/20 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 sift and placed in 70% ethanol for morphological analyses and in molecular grade ethanol (96%) for molecular analyses.

2.3.2. 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*)". The following abbreviations were used: 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. 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.

2.3.3. Molecular characterisation

Four whole individual specimens preserved in molecular grade ethanol (96%) were used for DNA extraction. A conspecific specimen mounted on a slide was kept as a paragenophore (sensu Pleijel et al., 2008). Extraction of genomic DNA was performed using 200µl of a 5% solution of Chelex in deionised water and 2µl of proteinase K, incubated for 4 hours at 56°C and boiled at 90°C for 8 minutes, and then centrifuged at 15,000 rpm for 10 minutes. The partial 28S rRNA (D1-D3 region) and mtCOI (mitochondrial cytochrome oxidase 1) genes were amplified. Polymerase Chain Reactions (PCR) were performed using 3µl of extraction supernatant, 10µl of Dream Taq DNA Polymerase (ThermoFischer Scientific™) and 1.6µl of each primer adding to a total reaction mixture of 20µl. 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 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3'; Littlewood et al., 2000) and 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'; Olson et al., 2003). Mitochondrial COI was amplified using the primers PBI-cox1F_PCR (5'- CATTTTGCTGCCGGTCARCAYATGTTYTGRTTTTTTGG-3') and PBI-cox1R_PCR (5'- CCTTTGTCGATACTGCCAAARTAATGCATDGGRAA-3') (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 (5'-GCATAGTTCACCATCTTTCGG-3'; Lockyer et al., 2003) and ZX-1 (5'-ACCCGCTGAATTTAAGCATAT-3'; van der Auwera et al., 1994) and PBI-cox1F_seq (5'-CATTTTGCTGCCGGTCA-3') and PBI-cox1R_seq (5'-TAATGCATDGGRAAAAAAC-3') (Scholz et al., 2013) for mtCOI. Contiguous sequences were assembled using Geneious version 7.1.3 (Kearse et al., 2012).

2.3.4. Phylogenetic analyses

Six newly generated partial sequences (three sequences of 28S rRNA and three sequences of mtCOI genes) 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 2.1). *Hexacanalis folifer* Cielocha and Jensen, 2011 (**KU249130**) was used as outgroup for the mtCOI analysis and aligned with the three new sequences, along with *Eniochobothrium* sp. n. 1 (**KU249111**), *Eniochobothrium* sp. n. 2 (**KU249108**), *Eniochobothrium* sp. n. 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 bp (mtCOI) and 1,655 bp (28S rRNA) 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), and BI using MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Bootstrap support values for ML were determined by performing 10^3 repetitions. Markov Chain Monte Carlo chains were run 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).

Table 2.1

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 sp. n. 1	Aberrapecidae	*Aetomylaeus bovinus*	Senegal	**KU249052**	Jensen et al. (2016)

Table 2.1 continued.

Parasite taxon	Family	Host	Locality	GenBank ID	Reference
Adelobothrium aetiobatidis	Cephalobothriidae	*Aetobatus ocellatus*	Australia	**KU249060**	Jensen et al. (2016)
Adelobothrium sp. n. 1	Cephalobothriidae	*Aetobatus ocellatus*	Solomon Islands	**KU249063**	Jensen et al. (2016)
Adelobothrium sp. n. 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 sp. n. 1	Cephalobothriidae	*Aetobatus ocellatus*	Australia	**KU249058**	Jensen et al. (2016)
Cephalobothrium sp. n. 5	Cephalobothriidae	*Aetobatus ocellatus*	Solomon Islands	**KU249059**	Jensen et al. (2016)
Cephalobothrium sp. n. 6	Cephalobothriidae	*Aetobatus ocellatus*	Vietnam	**KU249064**	Jensen et al. (2016)
***Eniochobothrium acostae* isolate 1**	Eniochobothriidae	*Rhinoptera jayakari*	South Africa	**ON972441**	Present study
***Eniochobothrium acostae* isolate 2**	Eniochobothriidae	*Rhinoptera jayakari*	South Africa	**ON972440**	Present study
***Eniochobothrium acostae* isolate 3**	Eniochobothriidae	*Rhinoptera jayakari*	South Africa	**ON972442**	Present study

Table 2.1 continued.

Parasite taxon	Family	Host	Locality	GenBank ID	Reference
Eniochobothrium euaxos	Eniochobothriidae	*Rhinoptera neglecta*	Australia	**KF685859**	Caira et al. (2014)
Eniochobothrium sp. n. 1	Eniochobothriidae	*Rhinoptera* cf. *steindachneri*	USA	**KF685860**	Caira et al. (2014)
Eniochobothrium sp. n. 2	Eniochobothriidae	*Rhinoptera* sp.	Senegal	**KU249055**	Jensen et al. (2016)
Eniochobothrium sp. n. 3	Eniochobothriidae	*Rhinoptera neglecta*	Australia	**KU249056**	Jensen et al. (2016)
Flapocephalus sp. n. 1	Polypocephalidae	*Pastinachus atrus*	Australia	**KF685861**	Caira et al. (2014)
Flapocephalus sp. n. 1	Polypocephalidae	*Pastinachus atrus*	Indonesia	**KU249087**	Jensen et al. (2016)
Floriparicapitus plicatilis	Lecanicephalidae	*Glaucostegus typus*	Australia	**KU249074**	Jensen et al. (2016)
Floriparicapitus sp. n. 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 sp. n. 1	Polypocephalidae	*Aetobatus ocellatus*	Australia	**KU249090**	Jensen et al. (2016)
Hornellobothrium sp. n. 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 sp. n. 1	Lecanicephalidae	*Dasyatis* sp.	Taiwan	**KU249078**	Jensen et al. (2016)
Paraberrapex manifestus	Paraberrapecidae	*Squatina californica*	Mexico	**KF685868**	Caira et al. (2014)

Table 2.1 continued.

Parasite taxon	Family	Host	Locality	GenBank ID	Reference
Paragrillotia similis[*]	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 sp. n. 2	Polypocephalidae	*Himantura uarnak*	Malaysia	**KU249100**	Jensen et al. (2016)
Seussapex sp. n. 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 sp. n. 2	Tetragonocephalidae	*Himantura jenkinsii*	Australia	**KU249085**	Jensen et al. (2016)
Tetragonocephalum sp. n. 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)

Table 2.1 continued.

Parasite taxon	Family	Host	Locality	GenBank ID	Reference
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).

2.4. Results

2.4.1. Eniochobothrium acostae Oosthuizen, Smit & Schaeffner, 2022 (Figs. 2.1–2.2)

Diagnosis (based on 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), 1,318–6,007 (3,534 ± 1,428; *n* = 15) long; maximum width either at level of trough or posteriormost proglottid 155–921 (540 ± 243; *n* = 15); total number of proglottids 22–47 (36 ± 7; *n* = 14) (Figs. 2.1A, 2.2A). 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. 2.1A). Scolex from scanning electron microscopy 91 (*n* = 1) long by 79 (*n* = 1) wide, bearing four acetabula (Fig. 2.2B). Acetabula in form of sessile suckers, 47–61 (54 ± 10; *n* = 1; *2*) long by 34–36 (35 ± 1; *n* = 1; *2*) wide (Figs. 2.1E, 2.2B). Apical modification of scolex proper in form of narrowed extension with small terminal apical aperture (Fig. 2.2B), with small, mostly glandular, inextensible and irreversible apical organ. Apical organ 14 (*n* = 1) long by 20 (*n* = 1) wide (Figs. 2.1E, 2.2B). Cirrus covered with small, triangular microtriches visible at opening of the genital pore (Fig. 2.2C). Cephalic peduncle not observed. Non-reproductive and reproductive proglottids craspedote, non-laciniate (Figs. 2.1A, 2.2A). 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 (Figs. 2.1A, D, 2.2A, D). Reproductive region of strobila 933–5,471 (2,811 ± 1,320; *n* = 15) long by 151–921 (525 ± 242; *n* = 15) wide, consisting of 10–26 (17 ± 5; *n* = 15) reproductive proglottids (Figs. 2.1A, 2.2A). Immature proglottids 9–25 (16 ± 5; *n* = 15) in number, initially wider than long, becoming longer than wide; posteriormost immature proglottid 164–1,150 (619 ± 323; *n* = 15) long by 370–844 (565 ± 154; *n* = 15) wide (Figs. 2.1A, 2.2A). Mature proglottids 0 or 1 in number, longer than wide, 591–2,172 (1,062 ± 638; *n* = 6) long by 151–667 (317 ± 202; *n* = 6) wide (Fig. 2.1A, B). Gravid

proglottids 0 or 1 in number, 1,208–2,511 (1,805 ± 475; *n* = 9) long by 418–921 (654 ± 148; *n* = 9) wide (Fig. 2.1A, 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. 2.1B–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. 2.1B–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. 2.1B–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. 2.1B–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. 2.1B–C, F). Cirrus sac U-shaped, thick-walled, 251–935 (468 ± 267; *n* = 6) long by 41–111 (71 ± 24; *n* = 6) wide, containing a long, inverted cirrus (Fig. 2.1A–C, F). Cirrus armed, 222–889 (403 ± 275; *n* = 6) long by 21–46 (30 ± 10; *n* = 6) wide (Fig. 2.1B–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. 2.1B–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. 2.1B–C). Vagina absent. Genital pores lateral, irregularly alternating, 69–86% (75 ± 6; *n* = 6) of proglottid length from posterior margin (Fig. 2.1A–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. 2.1B–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. 2.1B–C). Two lateral pairs of excretory vessels present (Fig. 2.1B–C, F). Eggs in cocoons; total number of cocoons 41–79 (62 ± 14; *n* = 5) (Fig. 2.1C, 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. 2.1G). Eggs subspherical, thin-walled, 14–15 (14 ± 1; *n* = 6) long by 11–12 (12 ± 1; *n* = 6) wide (Fig. 2.1G).

2.4.2. 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 1,229–1,389 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* 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.

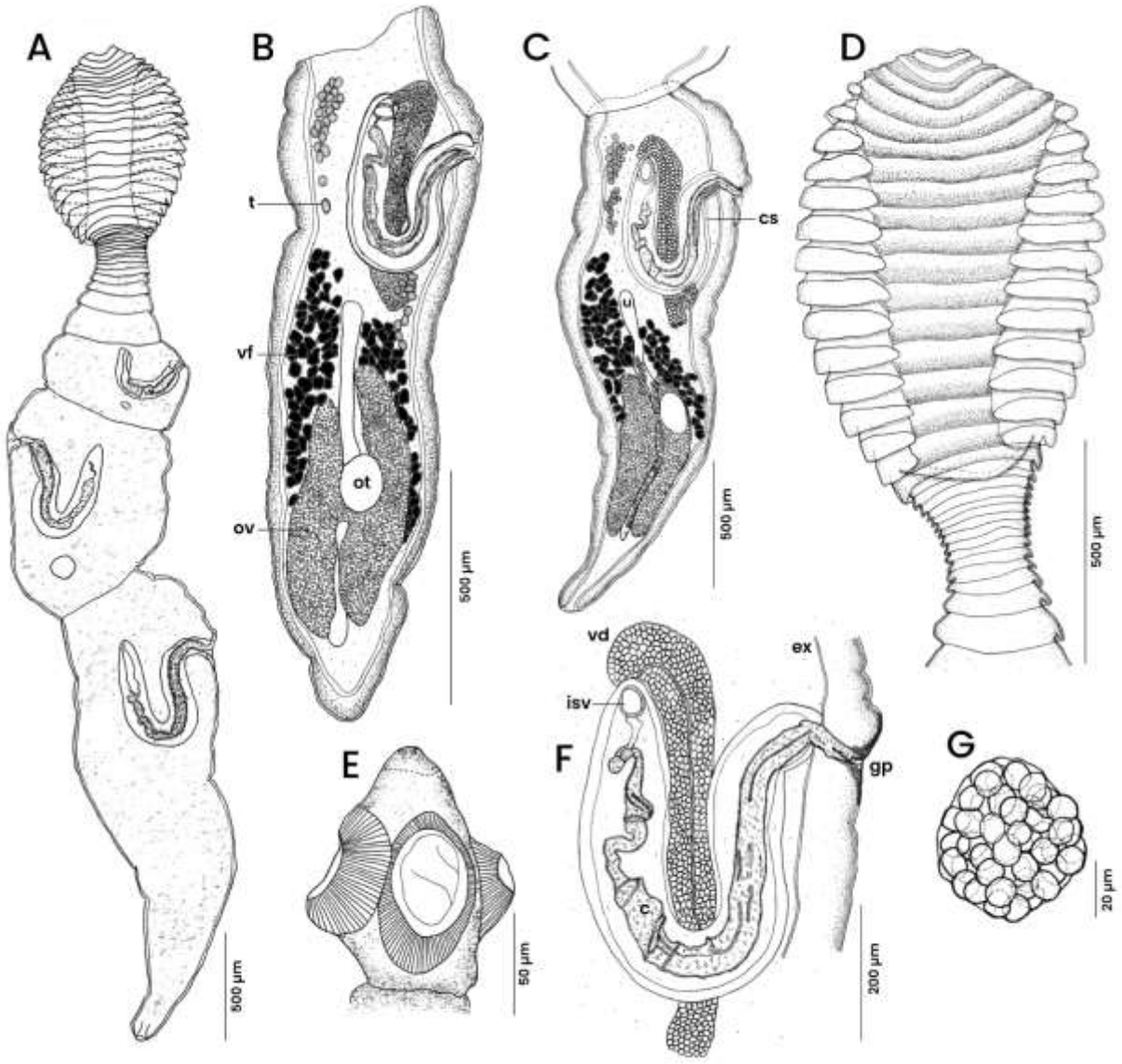

Fig. 2.1 Line drawings of *Eniochobothrium acostae* from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, outline of entire cestode; B, mature proglottid; C, early gravid proglottid; D, trough formed by non-reproductive proglottids of the anterior strobila; E, scolex; F, terminal genitalia; G, cocoon with eggs.

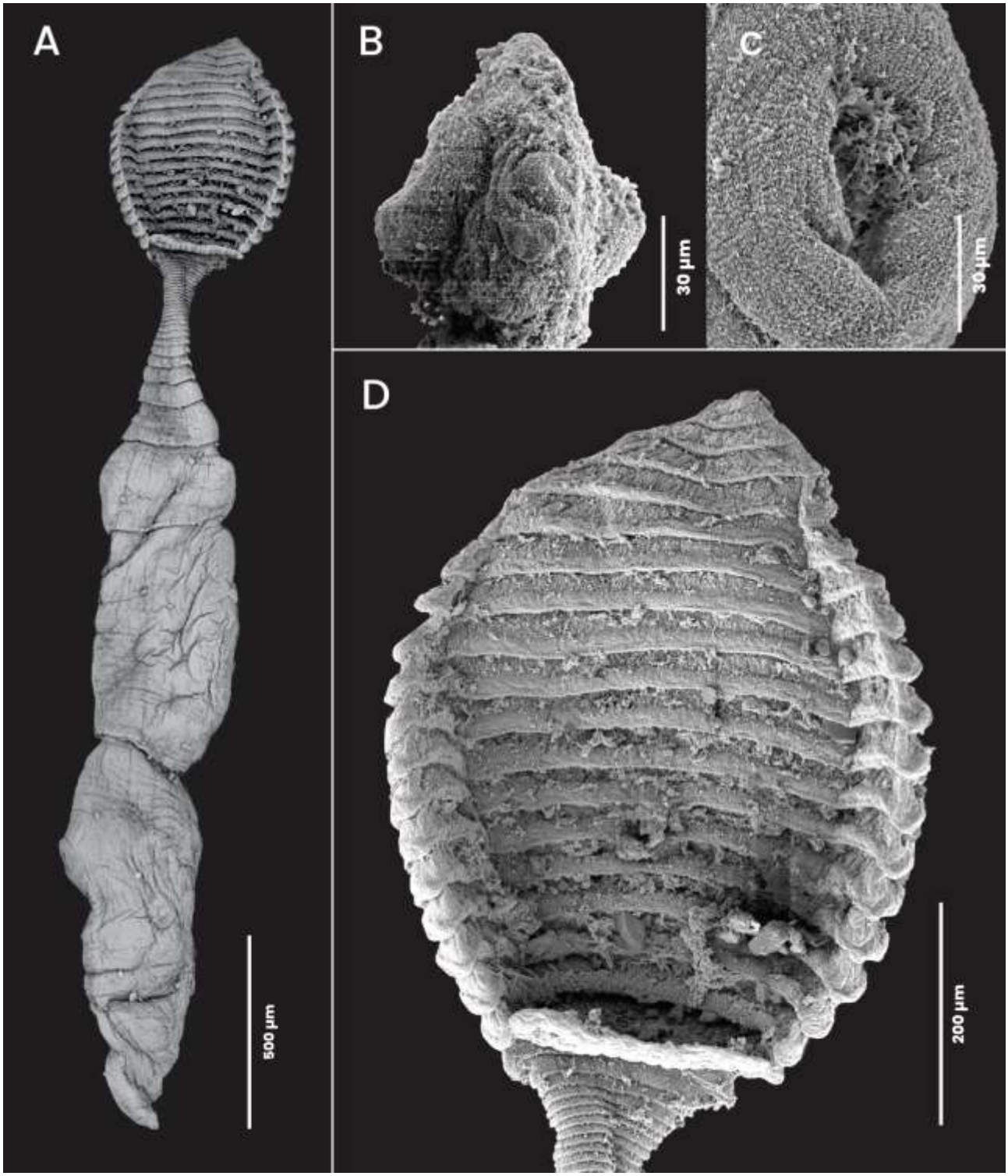

Fig. 2.2 Scanning electron micrographs of an immature specimen of *Eniochobothrium acostae* from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, entire strobila; B, scolex; C, genital pore; D, trough formed by non-reproductive proglottids of the anterior strobila.

2.4.3. Remarks

Eniochobothrium acostae 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 2.2). *Eniochobothrium acostae* 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* has slightly fewer testes than *E. euaxos* (16–34 vs. 35–48) and smaller cocoons (Table 2.2). A morphological differentiation based on metrical features is impeded between *E. acostae* and *E. gracile* due to a scarcity of morphological information provided in the original description (Shipley and Hornell, 1906). However, *E. acostae* 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* possesses a rather noticeable apical organ. *Eniochobothrium acostae* differs from *E. gracile* in the number of mature proglottids (0 or 1 vs. ± 6–8, respectively). In addition, *E. acostae* 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* has slightly smaller eggs than *E. qatarense* (14–15 vs. 17–24, respectively). In contrast, cocoons of *E. acostae* 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* 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 2.2). 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* has an apical organ. Additional differences in metrical features between *E. acostae* and congeners are listed in Table 2.2.

Furthermore, our observation of *E. acostae* indicated the presence of two morphotypes (infecting the same host individual); highly variable worms ranging greatly in size and morphological characteristics (suppl. Table S1). However, 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. 2.3, 2.4). Regrettably, the only obtained scolex of *E. acostae*, 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.

Table 2.2

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*	*Eniochobothrium euaxos*	*Eniochobothrium qatarense*	*Eniocobothrium 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

Table 2.2 continued.

Species	*Eniochobothrium acostae*	*Eniochobothrium euaxos*	*Eniochobothrium qatarense*	*Eniocobothrium gracile*
Study	Present study	Jensen, (2005)	Al Kawari, Saoud and Wanas, (1994)	Shipley and Hornell, (1906)
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	–

Table 2.2 continued.

Species	*Eniochobothrium acostae*	*Eniochobothrium euaxos*	*Eniochobothrium qatarense*	*Eniocobothrium gracile*
Study	Present study	Jensen, (2005)	Al Kawari, Saoud and Wanas, (1994)	Shipley and Hornell, (1906)
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)	–	–	–

Table 2.2 continued.

Species	*Eniochobothrium acostae*	*Eniochobothrium euaxos*	*Eniochobothrium qatarense*	*Eniocobothrium gracile*
Study	Present study	Jensen, (2005)	Al Kawari, Saoud and Wanas, (1994)	Shipley and Hornell, (1906)
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, 4), rostrum (W) (Fig. 1), gravid proglottid (L) and (W) (Fig. 4) and distance of genital pore from posterior end (Figs. 3, 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).

2.4.4. Phylogenetic relationships

Both ML and BI phylogenetic analyses yielded similar topologies (see Figs. 2.3, 2.4). The newly generated partial sequences of the 28S rRNA of *E. acostae* grouped with *E. euaxos* from Australia, and three unidentified species, namely *Eniochobothrium* sp. n. 1 of Caira et al. (2014) collected in the USA, *Eniochobothrium* sp. n. 2 of Jensen et al. (2016) collected in Australia and *Eniochobothrium* sp. n. 3 of Jensen et al. (2016) collected in Senegal. The clade composed of *Eniochobothrium* spp. is strongly supported. The new South African species appeared more closely related to *Eniochobothrium* sp. n. 1. The phylogenetic analysis of the 28S rRNA sequences of selected lecanicephalideans showed that taxa grouped according to their family level classification (Fig. 2.3). Representatives of the Paraberrapecidae, Zanobatocestidae and Aberrapecidae appeared as early divergent clades in the tree. A major well supported clade divided into five subclades grouped lineages of the Tetragonocephalidae, Polypocephalidae, Eniochobothriidae, Lecanicephalidae and Cephalobothriidae. The newly generated partial mtCOI sequences of *E. acostae* were compared to *E. euaxos* and taxa of *Eniochobothrium* sp. n. 1, 2 and 3. The phylogenetic analysis of the mtCOI

sequences of *Eniochobothrium* spp. showed the new species grouping together with *Eniochobothrium* sp. n. 1, with strong support, which mirrors the results of the 28S rRNA analysis (Fig. 2.4).

The estimates for evolutionary divergences for 28S rRNA were compared using the sequences of *Eniochobothrium* spp. with 46 other sequences of lecanicephalideans and one sequence of *P*. *similis* and *T*. *stizostedionis* that were used as outgroup taxa retrieved from GenBank, with genetic divergences (*p*-distance) varying from 0 to 21.8%. The *p*-distances were 2.2–2.4% (29–31 bp) between *E. acostae* and *E. euaxos*, 0.4–0.5% (6 bp) between *E. acostae* and *Eniochobothrium* sp. n. 1, and 5.7–7% (74–86 bp) between *E. acostae* and *Eniochobothrium* sp. n. 2 and 3. Supplementary Table S2 provides information on the genetic divergence values among *E. acostae* and sequences used in the phylogenetic analysis for partial 28S rRNA (this table was amended to only include congeners of the new species). The estimates for evolutionary divergences for mtCOI were compared using partial sequences of *E. acostae* 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* and *E*. *euaxos* was 18.1–18.6% (96–102 bp), between *E. acostae* and *Eniochobothrium* sp. n. 1 8.7–8.9% (44–46 bp), and between *E. acostae* and *Eniochobothrium* sp. n. 2 and 3 19.2–19.9% (92–106 bp)

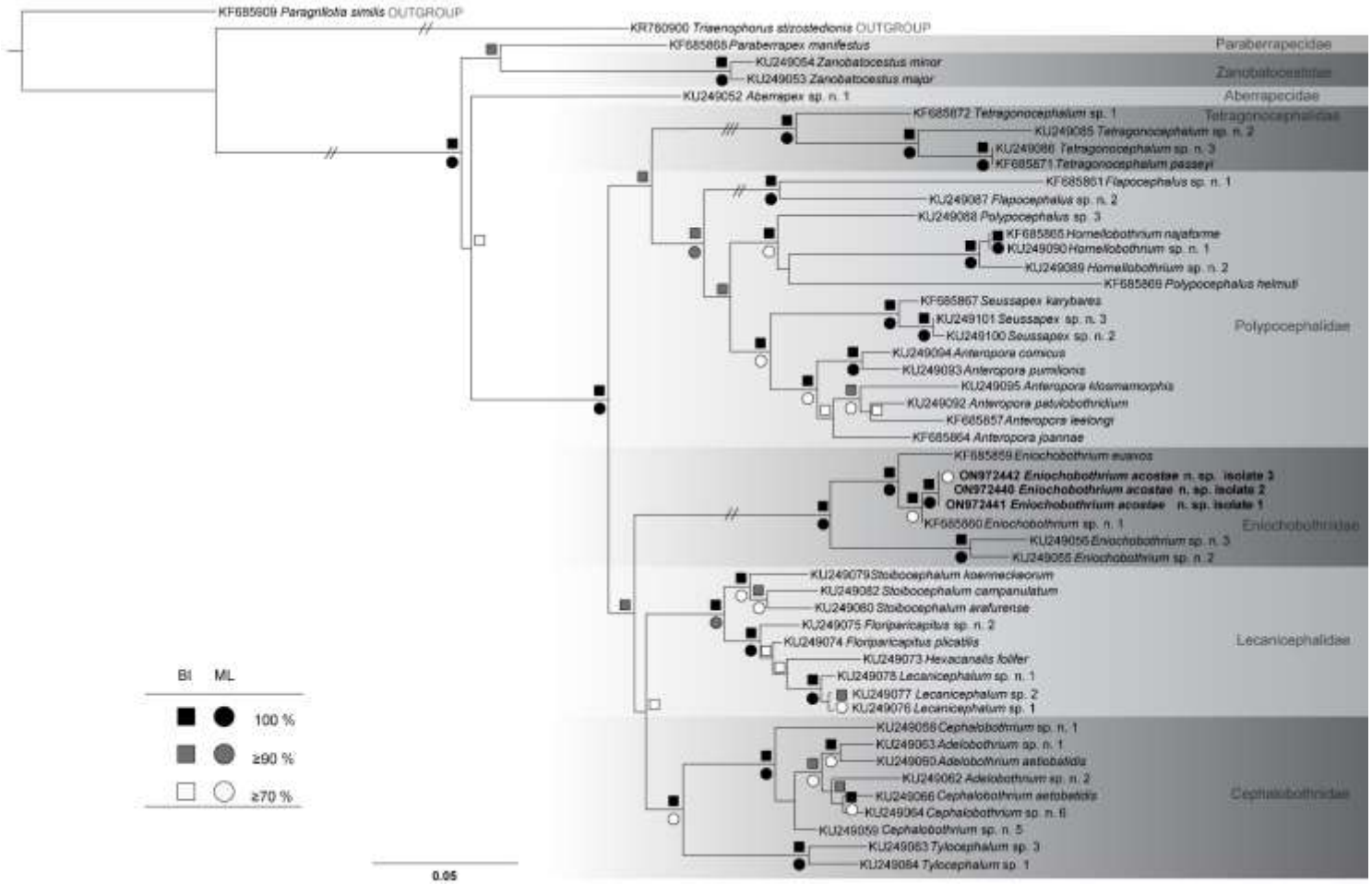

Fig. 2.3 Maximum likelihood phylogram based on partial sequences of the large subunit 28S rRNA gene. Nodal support is shown as posterior probability and bootstrap. GenBank accession number precedes species name. *Paragrillotia similis* Linton, 1909 (**KF685909**) and *Triaenophorus stizostedionis* Miller, 1945 (**KR780900**) were used as outgroup. 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.

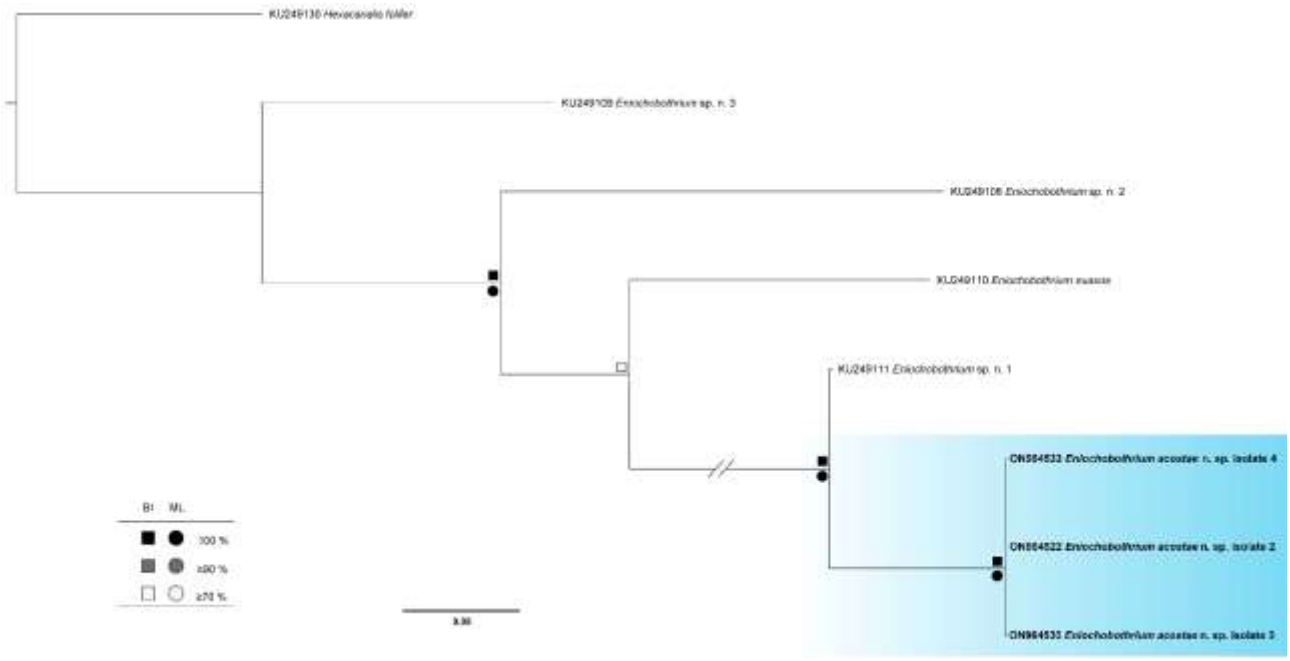

Fig. 2.4 Maximum likelihood phylogram based on partial sequences of the mitochondrial cytochrome oxidase subunit I (mtCOI) gene. Nodal support is shown as posterior probability and bootstrap. GenBank accession number precedes species name. *Hexacanalis folifer* Cielocha and Jensen, 2011 (**KU249130**) was used as an outgroup. Branch length scale bar indicates the number of substitutions per site. (/ /) Branch length reduced to one time the scale bar.

2.5. 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. 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 and van Hees, 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*. Thus far only two of the eight batoids from the family Rhinopteridae have been examined for *Eniochobothrium*, with each host species hosting one to two unique species of *Eniochobothrium*. It is estimated that the actual species diversity of *Eniochobothrium* in this host group ranges from eight to 16 species worldwide. Even

considering *E. acostae* from *R. jayakari*, only 38% of the potential host species have been examined for the presence of *Eniochobothrium* species. *Rhinoptera adspersa* (Indo-West Pacific), *Rhinoptera bonasus* Mitchill (western Atlantic and Caribbean Sea), *Rhinoptera brasiliensis* Müller (southern tip of Brazil to western Florida), *Rhinoptera marginata* Geoffroy Saint-Hilaire (western coast of Africa and Mediterranean Sea) and *Rhinoptera steindachneri* Evermann and Jenkins (Eastern Pacific) still await parasitological examination.

The phylogenetic analyses presented herein support the allocation of the new species within *Eniochobothrium*, which formed a strongly supported clade with its congeners in the phylograms of both 28S rRNA and mtCOI genes (Figs. 2.3, 2.4). In the present study, the analyses of 28S rRNA sequences of selected lecanicephalideans corroborates the results of Jensen et al. (2016). These authors presented a concatenated analysis of lecanicephalidean sequences of four genes (complete large subunit 28S rRNA [*lsr*RNA], partial small subunit 18S rRNA [*ssr*RNA], partial cytochrome c oxidase subunit I [cox1], and partial large mitochondrial ribosomal RNA subunit [*rrn*L]). 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. 2.3), 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 in 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 was not observed in any of the >80 specimens forming part of the outgroup taxa examined by Caira et al. (1999, 2001). The description of the microthrix morphology of *E. acostae*, 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* 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* 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 less than half 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**.

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Supplementary data

Table S1

Metrical comparison of large and small forms of *Eniochobothrium acostae* considered as two morphotypes infecting the same host individual. Abbreviations: L (length); W (width); TN (total number); P (proglottid); At (anterior); Pt (posterior).

Characteristics	*Eniochobothrium acostae* (large morphotype)	*Eniochobothrium acostae* (small morphotype)
Total (L)	3.217–4.660 (3.939 ± 1.020; *n* = 2)	1.318–1.860 (1.623 ± 258; *n* = 4)
Max (W)	455–757 (606 ± 214; *n* = 2)	155–216 (197 ± 28; *n* = 4)
TN P	38 (38 ± 0; *n* = 2)	22–26 (25 ± 2; *n* = 3)
At trough region (L)	676–806 (741 ± 92; *n* = 2)	402–636 (509 ± 102; *n* = 4)
At trough region (W)	346–701 (524 ± 251; *n* = 2)	125– 201 (158 ± 32; *n* = 4)
TN non-reproductive P	18–21 (20 ± 2; *n* = 2)	12–15 (14 ± 2; *n* = 3)
TN reproductive P	17–20 (19 ± 2; *n* = 2)	10–12 (11 ± 1; *n* = 4)
Reproductive region (L)	2.492–3.829 (3.161 ± 945; *n* = 2)	933–1.304 (1.133 ± 167; *n* = 4)
Reproductive region (W)	414–757 (586 ± 243; *n* = 2)	151–216 (196 ± 30; *n* = 4)
TN immature P	16–19 (18 ± 2; *n* = 2)	9–11 (10 ± 1; *n* = 4)
Pt most immature P (L)	558–803 (681 ± 173; *n* = 2)	164–216 (185 ± 25; *n* = 4)
Pt most immature P (W)	414–757 (586 ± 243; *n* = 2)	96–146 (120 ± 22; *n* = 4)
TN mature P	0–1	0–1
Mature P (L)	1.494–2.172 (1.833 ± 479; *n* = 2)	591–785 (676 ± 81; *n* = 4)
Mature P (W)	455–667 (561 ± 150; *n* = 2)	151–216 (196 ± 30; *n* = 4)
TN testes	22–34 (28 ± 8; *n* = 2)	16–19 (17 ± 1; *n* = 4)
TN aporal testes	15–27 (21 ± 8; *n* = 2)	11–13 (13 ± 1; *n* = 4)
TN poral testes	7 (7 ± 0; *n* = 2)	3–6 (5 ± 1; *n* = 4)
Testes (L)	27–30 (29 ± 2; *n* = 2)	15–26 (22 ± 5; *n* = 4)
Testes (W)	19–27 (23 ± 6; *n* = 2)	6–21 (16 ± 7; *n* = 4)
Vas deferense (L)	542–765 (654 ± 158; *n* = 2)	206–263 (238 ± 24; *n* = 4)

Table S1 continued.

Characteristics	*Eniochobothrium acostae* (large morphotype)	*Eniochobothrium acostae* (small morphotype)
Vas deferense (W)	73–115 (94 ± 30; *n* = 2)	18–38 (30 ± 9; *n* = 4)
Internal seminal vesicle (L)	68–72 (70 ± 3; *n* = 2)	31–38 (35 ± 3; *n* = 4)
Internal seminal vesicle (W)	48–64 (56 ± 11; *n* = 2)	9–14 (12 ± 2; *n* = 4)
Cirrus sac (L)	641–935 (788 ± 208; *n* = 2)	251–347 (307 ± 44; *n* = 4)
Cirrus sac (W)	81–111 (96 ± 21; *n* = 2)	41–67 (58 ± 12; *n* = 4)
Cirrus (L)	581–889 (735 ± 218; *n* = 2)	222–256 (237 ± 15; *n* = 4)
Cirrus (W)	36–46 (41 ± 7; *n* = 2)	21–32 (25 ± 5; *n* = 4)
Ovary (L)	530–650 (590 ± 85; *n* = 2)	197–336 (266 ± 58; *n* = 4)
Ovary (W)	131–159 (145 ± 20; *n* = 2)	49–62 (55 ± 5; *n* = 4)
Ootype (L)	121–161 (141 ± 28; *n* = 2)	61–72 (66 ± 5; *n* = 4)
Ootype (W)	93–103 (98 ± 7; *n* = 2)	32–35 (33 ± 1; *n* = 4)
Genital pore from Pt end	69–86% (78 ± 12; *n* = 2)	71–78% (74 ± 3; *n* = 4)
Uterus (L)	742–799 (771 ± 40; *n* = 2)	213–335 (261 ± 65; *n* = 3)
Uterus (W)	48–54 (43 ± 7; *n* = 2)	37–41 (38 ± 2; *n* = 3)
Vitelline follicles (L)	32–37 (35 ± 4; *n* = 2)	7–11 (9 ± 2; *n* = 4)
Vitelline follicles (W)	24 (24 ± 0; *n* = 2)	4–6 (5 ± 1; *n* = 4)

Table S2

Nucleotide genetic divergence values among sequences of the partial 28S rRNA gene of the lecanicephalidean species included in the phylogenetic analyses. Values below the diagonal are expressed in percentage (p-distance) while values above the diagonal represent number of differences in nucleotides. Newly sequenced taxon in bold. This table was amended to only include congeners of the new species.

No.	Species	GenBank ID	1	2	3	4	5	6	7
1	Eniochobothrium *euaxos*	KF685859		27	29	31	31	87	88
2	*Eniochobothrium* sp. n. 1	KF685860	2		6	6	6	78	85
3	***Eniochobothrium acostae* isolate 3**	**ON972442**	**2**	**1**		**0**	**0**	**74**	**82**
4	***Eniochobothrium acostae* isolate 1**	**ON972440**	**2**	**0**	**0**		**0**	**78**	**86**
5	***Eniochobothrium acostae* isolate 2**	**ON972441**	**2**	**0**	**0**	**0**		**78**	**86**
6	*Eniochobothrium* sp. n. 2	KU249055	6	5	6	6	6		33
7	*Eniochobothrium* sp. n. 3	KU249056	7	7	7	7	7	3	

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CHAPTER 3

Novel insights on two species of *Rhinoptericola* Carvajal and Campbell, 1975 (Cestoda: Trypanorhyncha) from the Oman cownose ray, *Rhinoptera jayakari* Boulenger, in South Africa

CHAPTER 3: Novel insights on two species of *Rhinoptericola* Carvajal and Campbell, 1975 (Cestoda: Trypanorhyncha) from the Oman cownose ray, *Rhinoptera jayakari* Boulenger, in South Africa

3.1. Abstract

The genus *Rhinoptericola* Carvajal and Campbell, 1975 (Rhinoptericolidae) is a group of cestodes known to infect elasmobranchs, particularly those in the order Rajiformes. Recent taxonomic revisions have expanded the diversity within this genus, highlighting the need for a comprehensive understanding of the evolutionary relationships within the Rhinoptericolidae. Molecular data, specifically from the D1–D3 gene area of 28S rRNA, has revealed the monophyly of the Rhinoptericolidae, challenging traditional morphological classifications. Two additional locality records of *Rhinoptericola* species, *Rhinoptericola mozambiquensis* Herzog & Jensen, 2022 and *Rhinoptericola butlerae* Beveridge & Campbell, 1988, were discovered. Both congeners parasitise the Oman cownose ray, *Rhinoptera jayakari* Boulenger, off the south-eastern coast of KwaZulu-Natal, South Africa. Morphological and molecular analyses were conducted to characterise these tapeworms. Scanning electron microscopy (SEM) was used to study the detailed morphology of the specimens. Morphological observations revealed minor distinctions in the metabasal armature of *R. mozambiquensis* and minor variations in the basal armature of *R. butlerae* compared to previous descriptions of these two species. Molecular analyses supported morphological identity of these species despite the observed morphological differences. The study expands the known geographic distribution of *R. mozambiquensis* and *R. butlerae* to South Africa, highlighting the dynamic nature of species distribution. Ongoing research efforts in less-explored regions, such as South Africa, are essential for a comprehensive understanding of trypanorhynch tapeworms and their interactions with elasmobranch hosts.

3.2. Introduction

Rhinoptericola Carvajal & Campbell, 1975 is a genus of cestodes in the family Rhinoptericolidae Carvajal & Campbell, 1975. The genus is known to infect particularly rajiform hosts. In a recent revision of the family, the diversity of *Rhinoptericola* species greatly increased from a single member to a total of eight members considered as valid (Herzog & Jensen, 2022). *Rhinoptericola* currently encompasses the type species *Rhinoptericola megacantha* Carvajal & Campbell, 1975, four species transferred to the genus [viz., *Rhinoptericola aetobatidis* n. comb. (Shipley & Hornell, 1906), *Rhinoptericola butlerae* n. comb. (Beveridge & Campbell, 1988), *Rhinoptericola panamensis* n.

comb. (Schaeffner, 2016), *Rhinoptericola jensenae* n. comb. (Schaeffner & Beveridge, 2012)] and three new species (*Rhinoptericola schaeffneri* Herzog & Jensen, 2022, *Rhinoptericola mozambiquensis* Herzog & Jensen, 2022, *Rhinoptericola hexacantha* Herzog & Jensen, 2022). In addition to *Rhinoptericola*, Palm (2010) described a new genus and species, *Nataliella marcelli* Palm, 2010, from Hawaiian fishes, which also belongs to the family Rhinoptericolidae.

Members of the genus *Rhinoptericola* are characterised by four bothria, a heteroacanthous typical armature, and the presence of pre-bulbar organs, however, lacking gland cells in the bulbs (Palm, 2010). According to Herzog and Jensen (2022), four members of the genus (viz., *R. hexacantha*, *R. jensenae*, *R. mozambiquensis*, and R. *schaeffneri*) have billhooks in their basal armatures that are dorsoventrally flattened with mucronate tips, which have previously only been described from species in two trypanorhynch genera (*Mobulocestus* Campbell & Beveridge, 2006 and *Hemionchos* Campbell & Beveridge, 2006) parasitising devil rays. An unique combination of characteristics was also observed in the proglottid anatomies of six species of *Rhinoptericola* (unknown for *R. panamensis* and *R. aetobatidis*), including the possession of vitelline follicles that are interrupted ventrally and dorsally by the ovary, testes that are in two columns overlapping the anterior part of the ovary, a seminal receptacle, an unarmed cirrus sac, an uterus with a bifurcated posterior end, and separate pores for male and female genitalia, and the absence of internal and external seminal vesicles (Herzog & Jensen, 2022).

According to Herzog and Jensen (2022), a global, synergistic approach is being undertaken to re-examine and reclassify the trypanorhynch tapeworms in this family. This is because the taxonomy of the Rhinoptericolidae family has long been based solely on morphological characteristics, but recent molecular data have revealed that these characteristics do not always align with evolutionary relationships.

Phylogenetic analyses have been used to examine the relationships within the Rhinoptericolidae. The study by Herzog and Jensen (2022) used both morphological and molecular data to infer evolutionary relationships among trypanorhynch tapeworms in this family. For the first time, a monophyletic Rhinoptericolidae was revealed by Herzog and Jensen (2022) using phylogenetic analysis of the D1–D3 gene region of 28S rRNA, which included seven of the current nine species of rhinoptericolids and a significant proportion of the other Trypanobatoida. The assessment of the level of intraspecific vs interspecific variation in the 28S rRNA for mature trypanorhynchs across the various hosts and geographical locations from which they have been reported, indicated a reasonably consistent margin for *Rhinoptericola* (Herzog & Jensen, 2022). Regarding morphological differences within the genus *Rhinoptericola*, there seem to be smaller species (viz., *R. hexacantha*, *R. jensenae*, *R. mozambiquensis*, and *R. schaeffneri*) with billhooks in the basal armature and larger species (viz., *R. aetobatidis*, *R. butlerae*, *R. megacantha*, and *R. panamensis*) with macrohooks in

the basal armature, however, molecular phylogenetic data indicated that these two groups of species are not mutually monophyletic (Herzog & Jensen, 2022).

Beveridge *et al.* (2017) noted that before the gaps in our current understanding of trypanorhynchs can be filled, new genera and a significant number of new species still need to be described. This is especially true given that currently, only a fraction of elasmobranch species have been screened for this group of cestodes. The effectiveness of using a synergistic, global approach is clearly indicated in the study by Herzog and Jensen (2022) where the type species, *R. megacantha*, formerly only known from the American cownose ray (*Rhinoptera bonasus* Mitchill, 1815) from the Chesapeake Bay and Venezuela, and the Ticon cownose ray (*Rhinoptera brasiliensis* Müller, 1836) from the Gulf of Mexico and Brazil, is now known from another species of cownose ray (*Rhinoptera marginata* Geoffroy St. Hilaire, 1817) from Senegal and a species of stingray (*Hypanus say* Lesueur, 1817) from South Carolina, indicating that the species of this genus have a transatlantic distribution.

A review of South African elasmobranch parasites by Schaeffner and Smit (2019) highlighted the lack of research on this specific group of cartilaginous fishes and revealed that species from the tapeworm family Rhinoptericolidae have not yet been discovered from the waters of southern African, despite the high diversity of host species in this area. This scenario changed in a recent review on the Rhinoptericolidae by Herzog and Jensen (2022) in which a new species, *R. mozambiquensis* from cownose rays from Mozambique was described. As part of an ongoing project on marine parasites from South Africa, two rhinoptericolid, *R. mozambiquensis* and *R. butlerae,* were recorded parasitising the Oman cownose ray, *Rhinoptera jayakari* Boulenger 1895 (Myliobatiformes: Rhinopteridae). This study provides two additional rhinoptericolid records and their respective comparison to congeners within the genus based on morphological and molecular data.

3.3. Materials and methods

3.3.1. Collection of specimens and fixation of material

Three male specimens of *R. jayakari*, ranging between 92 and 167 cm in total length, were collected in March 2020 along the south-eastern coastline of KwaZulu-Natal, South Africa by the KwaZulu-Natal Sharks Board (KZNSB). This project was approved by the North-West University's Faculty of Natural and Agricultural Research Sciences Ethics Committee with the ethics number NWU-01777-20-A9. The South African Department of Agriculture, Forestry and Fisheries issued a permit for the collection and possession of sharks for research purposes (permit number RES2020/20 issued to the KZNSB).

As part of the South African bather protection programme, the three deceased specimens were collected in shark nets along the coasts of Richards Bay and Scottburgh before being stored at -

20°C in the Sharks Board Institute's laboratory. In the dissection process after defrosting the batoids, the spiral intestines were removed by a mid-ventral incision followed by 10% formalin fixation of approximately three-fourths of each intestine along with the contents. After two weeks, the intestines and contents were exchanged to 70% ethanol. Molecular grade ethanol (96%) was used to fix the remainder of the intestinal tract. In the laboratory, preserved spiral intestines and contents were examined for cestodes with a stereomicroscope and specimens were manually picked and placed in both 70% and 96% ethanol for morphological and molecular analyses.

3.3.2. Morphological study

A graded ethanol series was used to hydrate the specimens, which were subsequently stained with Delafield's haematoxylin, and dehydrated to 70% ethanol. As soon as the specimens were stained, they were placed in 1% hydrogen chloride to clear excess stain, followed by gradation of ethanol to completely dehydrate them. Clove oil was used to clear the tissue of these specimens, followed by Canada balsam to permanently mount each specimen on a microscope slide.

Two specimens of each of the two morphologically different species observed were used for scanning electron microscopy (SEM). A small soft brush was used to carefully clean specimens of remaining host tissue in 70% ethanol.

The selected specimens for scanning electron microscopy (SEM) were fully dehydrated with pure ethanol, immersed in a 50/50 ethanol/hexamethyldisilazane (HMDS) solution, followed by pure HDMS, and air dried. After drying, specimens were mounted on carbon tape on aluminium stubs and sprayed with carbon (Emscope TB 500, Quorum Technologies, Ltd., Laughton, U.K.), followed by gold/palladium coatings of between 20 nm and 30 nm using an EIKO IB-2 ion coater (EIKO Engineering, Ltd., Yamazaki Hitachinaka, Japan). The specimens were viewed using a FEI Nova NanoSEM 450 scanning electron microscope (FEI Company, Eindhoven, Noord-Bravant, The Netherlands) at 5 kV for micrographic examination.

Morphological observations and measurements of the mounted specimens were performed using a Nikon ECLIPSE Ni light microscope (Nikon, Tokyo, Japan) once the microscope slides were completely dry. The line drawings were made using a drawing attachment tube. In the description, measurements are presented as the range, followed by the mean, standard deviation, and the number of specimens (n) examined. Unless otherwise stated, all measurements are in micrometres. Campbell and Beveridge (1994) provide the terminology for morphological characteristics, except that the attachment organs are called bothria following Jones *et al.* (2004). The following abbreviations were used: pbo (pars bothrialis), pva (pars vaginalis), pbu (pars bulbosa). Hook terminology is in accordance with Palm (2004) and Beveridge and Campbell (2007).

3.3.3. Molecular characterisation

Terminal proglottids of three individual specimens preserved in molecular grade ethanol (96%) were used for DNA extraction (isolate 1 – *R. mozambiquensis*; isolates 2 and 3 – *R. butlerae*). Genomic DNA was extracted using 200µl of a 5% solution of Chelex in deionised water and 2µl of proteinase K, incubated for 4 hours at 56°C and boiled at 90°C for 8 min and centrifuged at 15,000 rpm for 10 min. The partial 28S rRNA (D1-D3 region) genes were amplified. Polymerase Chain Reactions (PCR) were performed using 3µl of extraction supernatant, 10µl of Dream Taq Master Mix (ThermoFischer Scientific™) and 1.6µl of each primer accumulating to a reaction mixture of 20µl. The partial 28S rRNA was amplified using the cycling conditions of Brabec *et al.* (2012): denaturation of DNA (94°C for 5 min), 40 cycles of amplification (94°C for 30s, 55°C for 30s, and 72°C for 2 min), and 7 min extension hold at 72°C using the primers LSU5 and 1500R (Littlewood *et al.,* 2000; Olson *et al.,* 2003, respectively). The PCR amplicons were run on 1% agarose gel using loading buffer and gel red. The PCR product was purified and sequenced at Inqaba Biotechnical Industries Pty Ltd. (Pretoria, South Africa) using the PCR primers and the internal primers 1200R and ZX-1 for 28S rDNA (Lockyer *et al.,* 2003; van der Auwera *et al.,* 1994, respectively). Contiguous sequences were assembled using Geneious version 7.1.3 (Kearse *et al.,* 2012).

3.3.4. Phylogenetic analyses

Three partial sequences of 28S rRNA (isolates 1, 2, and 3) were generated in this study. The 28S rRNA sequences were aligned with sequences of related taxa obtained from GenBank. *Nataliella marcelli* was used as an outgroup and aligned with 32 available sequences of the genus *Rhinoptericola*. The 28S rRNA sequences were aligned using default parameters of MUSCLE implemented in MEGA7 software (Kumar *et al.,* 2016) with the extremes of the alignment trimmed, resulting in an alignment with 966 base pairs (bp). Phylogenetic analyses were run under Maximum Likelihood (ML) and Bayesian Inference (BI) criteria, applying the nucleotide evolution model GTR + G selected by MEGA7. ML analyses were carried out using RAxML version 8 (Guindon & Gascuel, 2003). The model parameters and bootstrap support values (1000 repetitions) were estimated using RAxML. The BI trees were generated using MrBayes 3.2 (Ronquist & 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. Both ML and BI analyses were carried out on the computational resource CIPRES (Miller *et al.,* 2010). Genetic divergence was calculated for 28S rRNA sequences using the uncorrected *p*-distance model in MEGA7 software (Kumar *et al.*, 2016). Phylogenetic trees were edited and visualised in FigTree v1.4.4 (Rambaut, 2020).

3.4. Results

3.4.1. Rhinoptericola mozambiquensis Herzog & Jensen, 2022 (Figs. 3.1–3.2)

Diagnosis (based on 22 immature fragmented specimens, 2 immature specimens were used for SEM; 4 tentacles were detached from a scolex and placed in glycerol).

Worms apolytic; immature fragmented worms 1.43–3.00 (1.80 ± 0.30, n = 20) mm long (Fig. 3.1A). Scolex acraspedote, elongate, 1.30–1.70 (1.52 ± 0.10, n = 20) mm long; maximum width at level of pars bothrialis (Fig. 3.1A). Pars bothrialis 270–352 (309 ± 22, n = 20) long, 242–412 (336 ± 48, n = 20) wide (Fig. 3.1A). Four bothria with free anterior and posterior margins, 243–325 (277± 22, n = 20) long, 91–138 (106 ± 13, n = 20) wide (Fig. 3.1A); bothrial pits absent (Fig. 3.1A). Pars vaginalis 761–1,298 (938 ± 114, n = 20) long, 129–224 (170 ± 27, n = 20) wide (Fig. 3.1A); tentacle sheaths sinuous. Pars bulbosa 513–668 (605 ± 36, n = 20) long, 189–267 (232 ± 17, n = 20) wide. Bulbs elongate, muscular (Fig. 3.1A), 504–638 (586 ± 33, n = 20) long, 68–91 (80 ± 6, n = 20) wide; bulb length to width ratio 6.2–9.0 (7.4 ± 1.0, n = 20): 1.0; prebulbar organ present; retractor muscle 12–20 (16 ± 3, n = 20) wide, attaches at the base of bulb (Fig. 3.1A). Pars postbulbosa mostly absent or short, 16–33 (23 ± 7, n = 5) long when present (Fig. 3.1A). Scolex ratio (pbo: pva: pbu) 1.0: 2.2–4.5: 1.7–2.4 (n = 20).

Tentacles up to 1,087 long, with basal swelling (Figs. 3.1A–F, 3.2B); diameter at base 19–31 (26 ± 3, n = 20), 23–34 (31 ± 3, n = 20) at basal swelling, 18–29 (26 ± 3, n = 20) in distal region of tentacles. Armature heteroacanthous typical; hooks solid, heteromorphous. Metabasal armature consists of 7–8 principal hooks per row, forming half spirals. Principal rows begin on bothrial surface of tentacle, terminate on antibothrial surface (Figs. 3.1B–D, 3.2A–B, D); small space between hooks files 1 and 1' on bothrial surface, 4–8 (6 ± 1, n = 9) apart (Figs. 3.1B, 3.2D). Hooks 1(1') large, uncinate, with broad base (Figs. 3.1B, F–G), 11–15 (13 ± 2, n = 6) long, base 9–11 (10 ± 1, n = 6) long; hooks 2(2') falcate, with shorter anterior base extensions (Figs. 3.1B, F–G, 3.2C), 11–17 (14 ± 2, n = 5) long, base 9–10 (10 ± 1, n = 5) long; hooks 3(3') falcate with small anterior base extension (Figs. 3.1B–C, F–G, 3.2C–D, F), 9–23 (18 ± 4, n = 11) long, base 6–9 (8 ± 1, n = 11) long; hooks 4(4') falcate, with small anterior base extension (Figs. 3.1F–G, 3.2C, F), 10–17 (15 ± 2, n = 8) long, base 6–8 (7 ± 1, n = 8) long; hooks 5(5') falcate, with small anterior base extensions (Figs. 3.1C–G, 3.2C), 11–18 (16 ± 2, n = 10) long, base 4–8 (7 ± 1, n = 12) long; hooks 6(6') falcate with small anterior base extension (Fig. 3.1C–G), 7–8 (7 ± 1, n = 7) long, base 3–4 (4 ± 1, n = 7) long; hooks 7(7') falcate, with small anterior base extension (Fig. 3.1C–G), 7–9 (8 ± 1, n = 6) long, base 3–4 (4 ± 1, n = 6) long; hooks 8(8') falcate, with small anterior base extensions (Fig. 3.1C–D, G), 6–7 (7 ± 1, n = 3) long, base 3 (3, n = 3) long.

Basal armature prominent (Figs. 3.1B–F, 3.2A–B), 78–104 (89 ± 10, n = 5) long consisting out of 6–7 indistinct hook rows from the tentacle base to the first principal row of the metabasal armature. Hooks in the first three initial rows, 6–12 (9 ± 2, n = 8) long, base 3–8 (5 ± 2, n = 8) long, solid, uncinate with hooks sometimes extending beyond the base and sometimes slight anterior base extensions to falcate (Figs. 3.1B–F, 3.2A–B). Triangular base hooks in rows 4–7 on bothrial surface 4–13 (10 ± 3, n = 7) long, base 6–8 (7 ± 1, n = 7) long, solid, dorsoventrally flattened, with curved tips extending beyond base (Figs. 3.1B, 3.2A). Internal, external and antibothrial surfaces with falcate billhooks 9–15 (12 ± 2, n = 11) long, base 4–6 (5 ± 1, n = 11) long, erect, flattened dorsoventrally, hollow or solid, with mucronate curved backwards tips (Figs. 3.1C–F, 3.2A–B). Strobila consists of only a few immature segments and therefore cannot be described.

3.4.2. Taxonomic summary

Study of Herzog and Jensen (2022)

Type host: Rhinoptera jayakari Boulenger, 1895 (Rhinopteridae: Myliobatiformes).

Type locality: Mozambique Channel, Mozambique: Tofo (23°47′33.02″S, 35°31′16.38″E), Inhambane.

Present study

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

Additional locality: South-western Indian Ocean off Scottburgh (28°78'0"S, 30°76'0"E) and 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* examined); intensity of >40 worms per host.

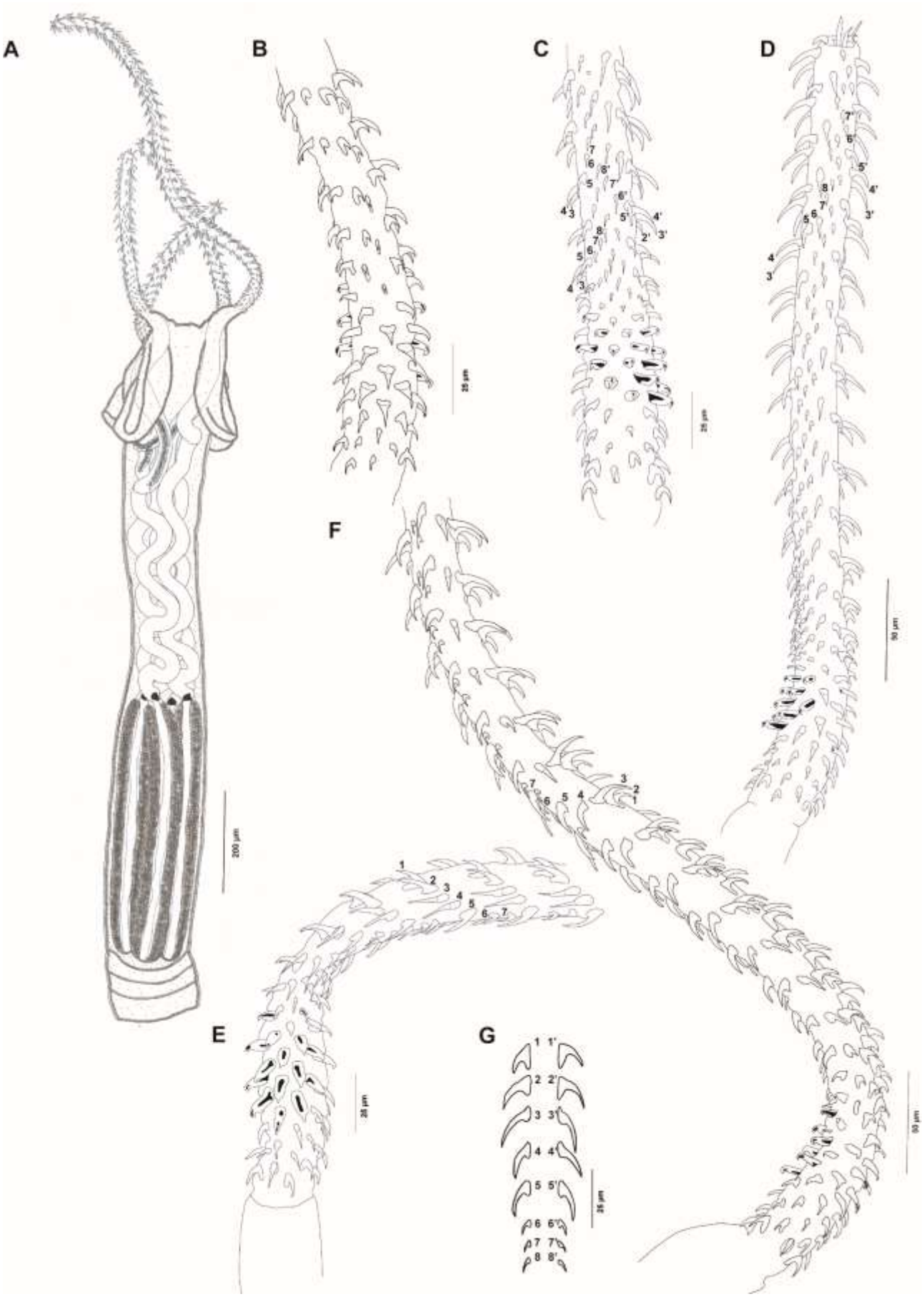


Fig. 3.1 Line drawings of *Rhinoptericola mozambiquensis* from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, scolex, lateral view; B,

basal and metabasal tentacular armature, bothrial surface; C, basal and metabasal tentacular armature, antibothrial surface; D, basal and metabasal tentacular armature, internal surface (basal) turning to antibothrial surface (apical); E, basal and metabasal tentacular armature, external surface; F, basal and metabasal tentacular armature, internal surface (basal) turning to external surface (apical); G, profile of hooks.

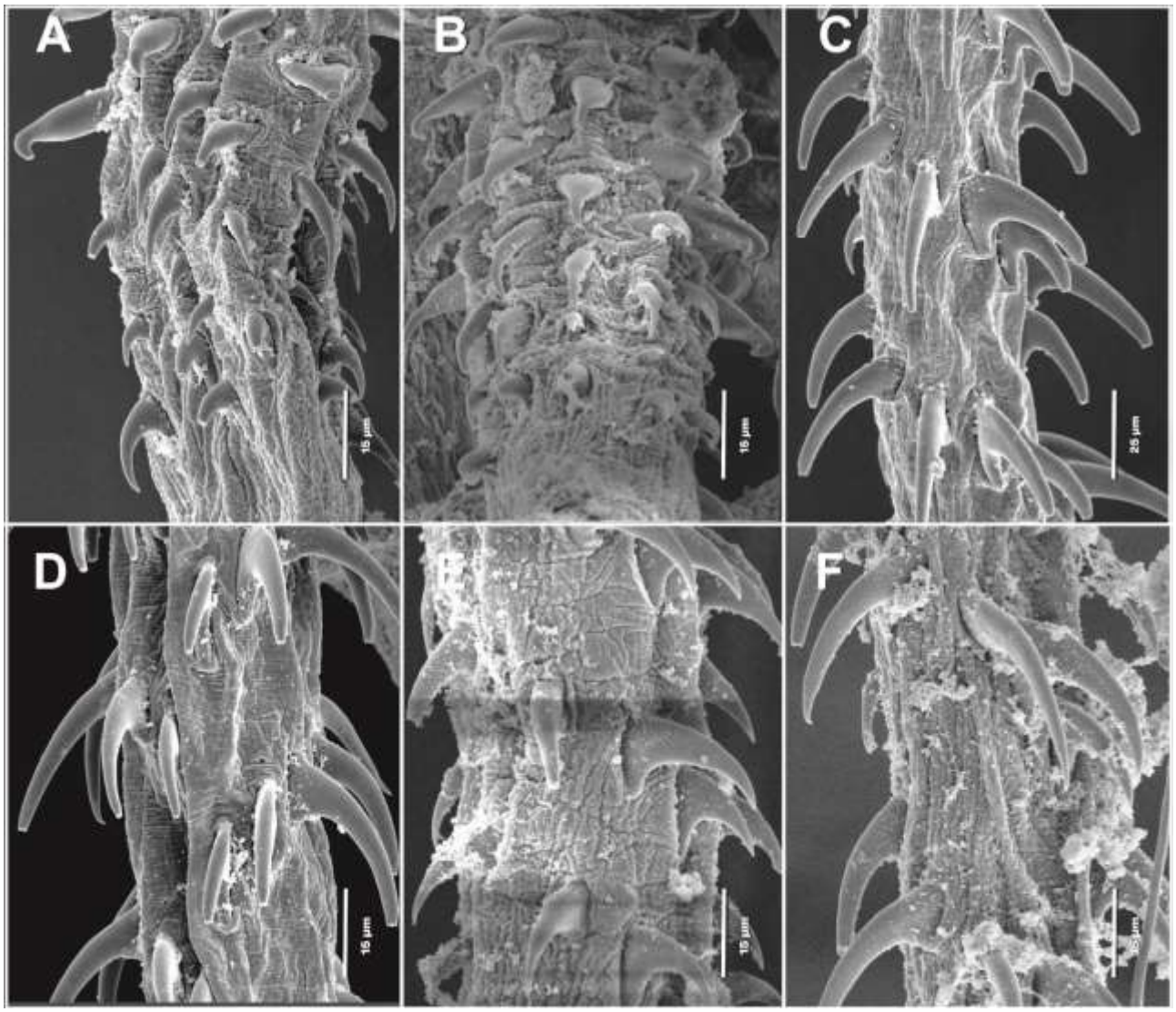


Fig. 3.2 Scanning electron micrographs of the tentacular armature of immature specimens of *Rhinoptericola mozambiquensis* from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, basal tentacular armature, bothrial surface; B, basal tentacular armature, antibothrial surface; C, metabasal tentacular armature, external surface; D, metabasal tentacular armature, bothrial surface; E, basal tentacular armature, internal surface; F, metabasal tentacular armature, external surface.

3.4.3. Remarks

In their recent study, Herzog and Jensen (2022) delineate the distinctive features of *R. mozambiquensis*, setting it apart from other species within the genus *Rhinoptericola*. The key characteristics that distinguish *R. mozambiquensis* include seven hooks per principal row, decreasing to six hooks per principal row towards the tentacle's distal end (Herzog & Jensen, 2022). The basal armature is characterised by billhooks lacking short forward protrusions on their lower surface, accompanied by either billhooks with such protrusions or triangular hooks with tips extending well beyond the hook base (Herzog & Jensen, 2022). Our examination of the South African specimens aligns with the description of *R. mozambiquensis* provided by Herzog and Jensen (2022), displaying similar basal armature traits and metrical measurements of body parts and regions (Table 3.1). However, our investigation reveals subtle variations, attributed to intraspecific variability. Specifically, our study observes *R. mozambiquensis* with seven to eight hooks per principal row at the initial stage of the metabasal armature, diminishing to seven hooks per principal row towards the distal end (Fig. 3.1C–D; Table 3.2). This differs from Herzog and Jensen's (2022) description, which notes a consistent reduction from seven to six hooks per principal row distally. Furthermore, Herzog and Jensen (2022) emphasise the exclusive association of *R. mozambiquensis* with a single host species, *R. jayakari*, and its confined geographical distribution to the waters off Mozambique, which distinguishes the species from its congeners. Contrary to this, our study expands the known geographical range of *R. mozambiquensis* to include the waters off South Africa, thereby contributing novel insights into the distribution of this species.

3.4.4. Rhinoptericola butlerae **Beveridge & Campbell, 1988** (Figs. 3.3–3.4)

Diagnosis (based on 10 immature fragmented specimens, 2 immature specimens were used for SEM; 4 tentacles were detached from a scolex and placed in glycerol).

Worms apolytic, immature fragmented worms 6.6–13.2 (9.1 ± 2.2, n = 7) mm long, proglottids acraspedote (Fig. 3.3A). Scolex elongate, 5.3–6.2 (5.8 ± 0.4, n = 7) mm long; maximum width at level of pars bothrialis (Fig. 3.3A). Pars bothrialis 591–788 (708 ± 80, n = 7) long, 815–1,163 (960 ± 127, n = 7) wide. Four bothria with free anterior and posterior margins 543–766 (671± 78, n = 7) long, 232–349 (283 ± 36, n = 7) wide (Fig. 3.3A). Pars vaginalis 2,928–3,665 (3,220 ± 234, n = 7) long, 441–609 (487 ± 60, n = 7) wide (Fig. 3.3A); tentacle sheaths sinuous. Pars bulbosa 2,388–2,959 (2,625 ± 233, n = 7) long, 520–930 (643 ± 141, n = 7) wide. Bulbs elongate, muscular (Fig. 3.3A), 2,362–2,859 (2,563 ± 191, n = 7) long, 209–264 (225 ± 20, n = 7) wide; bulb length to width ratio 9.0–13.0 (12.0 ± 1.0, n = 7): 1.0; prebulbar organ present; retractor muscle in bulbs 38–70 (50 ± 12, n = 7) wide, attaches at base of bulb (Fig. 3.3A). Pars postbulbosa short, 34–168 (106 ± 51, n = 6) long (Fig. 3.3A). Scolex ratio (pbo: pva: pbu) 1.0: 4.0–5.0: 3.0–4.1 (n = 7).

Tentacles up to 2,476 long, with basal swelling (Figs. 3.3A–D, 3.4D, I); diameter at base 76–119 (94 ± 13, n = 7), 95–124 (105 ± 10, n = 7) at basal swelling; 82–103 (91 ± 8, n = 7) in distal region of tentacles. Armature heteroacanthous typical; hooks solid, heteromorphous. Metabasal armature consists of 7 principal hooks per row, forming half spirals. Principal rows begin on the internal surface of tentacle, terminate on external surface (Figs. 3.3B, D, 3.4B–C); small space between hooks files 1 and 1' on internal surface, 23–44 (34 ± 8, n = 7) apart (Fig. 3.3B–C). Hooks 1(1') large, uncinate, with broad base (Figs. 3.3B–C, E, 3.4A), 65–91 (77 ± 7, n = 10) long, base 47–84 (67 ± 11, n = 10) long; hooks 2(2') falcate, with shorter anterior base extensions (Figs. 3.3B–C, E, 3.4A, C), 61–84 (77 ± 8, n = 8) long, base 26–49 (39 ± 7, n = 8) long; hooks 3(3') falcate with small anterior base extension (Figs. 3.3C, E, 3.4A, C), 61–108 (82 ± 16, n = 8) long, base 23–41 (32 ± 7, n = 8) long; hooks 4(4') falcate, with small anterior base extension (Figs. 3.3C, E, 3.4A, C), 56–91 (72 ± 12, n = 8) long, base 17–36 (27 ± 6, n = 8) long; hooks 5(5') falcate, with small anterior base extensions (Figs. 3.3C–E, 3.4A–C), 37–67 (51 ± 10, n = 8) long, base 16–31 (25 ± 5, n = 8) long; hooks 6(6') falcate to uncinate with small anterior base extension (Figs. 3.3C–E, 3.4A–C), 35–58 (47 ± 8, n = 8) long, base 19–32 (25 ± 5, n = 8) long; hooks 7(7') falcate to uncinate with small anterior base extension (Figs. 3.3D–E, 3.4A–C), 28–54 (41 ± 1, n = 8) long, base 19–30 (23 ± 4, n = 8) long.

Basal armature prominent (Figs. 3.3B–D, 3.4D, H–J), 421–536 (462 ± 43, n = 6) long consisting out of 12 indistinct hook rows with 84–98 (93 ± 5, n = 6) hooks from the tentacle base to the first principal row of the metabasal armature. Single blunt microhook present on bothrial surface before first initial rows (Figs. 3.3C, 3.4I). Hooks in the first three initial rows, 17–58 (31 ± 12, n = 13) long, base 12–35 (21 ± 7, n = 13) long, solid, uncinate with hooks sometimes extending beyond the base (Figs. 3.3B–D, 3.4D, H–J). Hooks in rows 4–7 large, falcate to triangular or spiniform, hollow or solid, 21–86 (51 ± 19, n = 13) long, base 7–26 (19 ± 6, n = 13) long (Figs. 3.3B–D, 3.4D, H, J). Hooks in rows 8–9, falcate sometimes with tips recurved or triangular, flattened dorsoventrally, hollow or solid, 16–43 (31 ± 9, n = 13) long, base 9–28 (15 ± 6, n = 13) long (Figs. 3.3B–D, 3.4D, F, K). Hooks in rows 9–10, with three or four macrohooks, external surface with two macrohooks, uncinate, flattened dorsoventrally, receded with tips recurved, hollow, 37–42 (40 ± 2, n = 4) long, base 14–20 (17 ± 3, n = 4) long; internal surface with one smaller macrohook, uncinate, flattened dorsoventrally, receded and blunt, hollow, 27–37 (32 ± 7, n = 3) long, base 13–16 (15 ± 2, n = 3) long (Figs. 3.3B–D, 3.4D–G, K). Antibothrial surface with one macrohook anteriorly with an open region devoid of hooks posteriorly to macrohook's base, uncinate, dorsoventrally flattened, receded, hollow, 35–49 (42 ± 10, n = 3) long, base 22–23 (23 ± 1, n = 3) long (Fig. 3.3C). Hooks in rows 11–12 small, thin, solid, spiniform to falcate, 16–47 (31 ± 11, n = 7) long, base 7–14 (12 ± 3, n = 7) long (Figs. 3.3B–D, 3.4B, D–E).

3.4.5. Taxonomic summary

Study of Beveridge and Campbell (1988)

Type host: Hemitrygon fluviorum (Ogilby, 1908) (Dasyatidae: Myliobatiformes).

Additional hosts: Hemitrygon bennetti (Müller & Henle, 1841), *Himantura tutul* Borsa, Durand, Shen, Alyza, Solihin & Berrebi, 2013, *Maculabatis gerrardi* (Gray, 1851), *Pastinachus ater* (Macleay, 1883), and *Pastinachus solocirostris* Last, Manjaji & Yearsley, 2005 (Dasyatidae: Myliobatiformes); *Rhinoptera javanica* Müller & Henle, 1841 and *Rhinoptera neglecta* Ogilby, 1912 (Rhinopteridae: Myliobatiformes); *Chiloscyllium punctatum* Müller & Henle, 1838 (Hemiscyliidae: Orectolobiformes).

Type locality: Coral Sea, Australia: Deception Bay, Queensland.

Additional localities: Arafura Sea, Australia: East of Wessel Islands, Northern Territory. Gulf of Carpentaria, Australia: Weipa, Queensland. Timor Sea, Australia: Dundee Beach, Northern Territory, Fog Bay. Java Sea, Indonesia: Gusungnge near Pagatan market, South Kalimantan; and Pagatan market, South Kalimantan. Makassar Strait, Indonesia: Muara Pasir, East Kalimantan. South China Sea, Malaysia: Mukah, Sarawak. South China Sea, Viet Nam: Cat Ba, Haiphong Province, Gulf of Tonkin; and Long Hai, Ba Ria Province

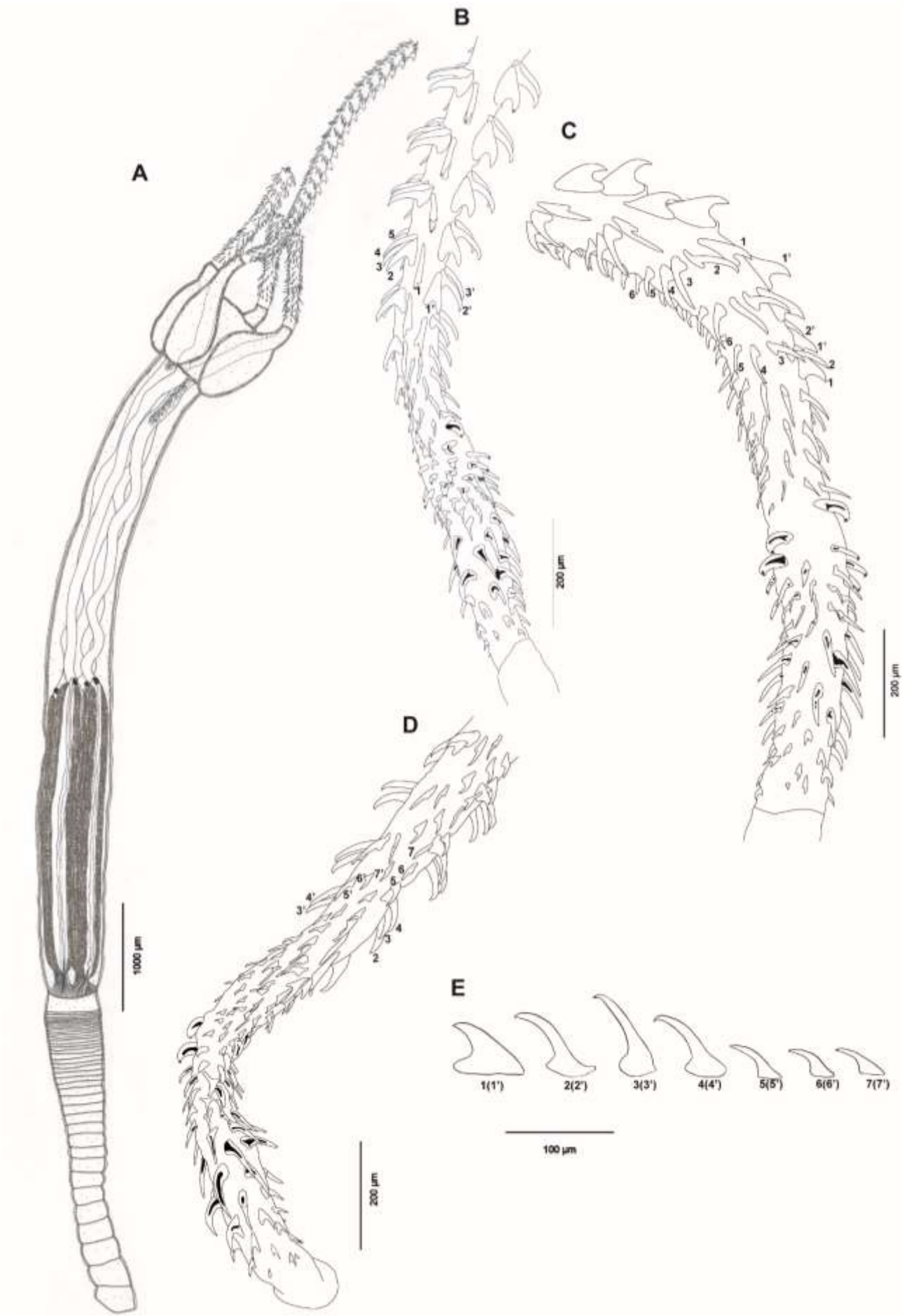
Present study

Additional host: Oman cownose Ray, *Rhinoptera jayakari* Boulenger (Myliobatiformes, Rhinopteridae).

Additional locality: South-western Indian Ocean off Scottburgh (28°78'0"S, 30°76'0"E) and 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* examined); intensity of >25 worms per host.


A
B
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D
E
1000 µm
200 µm
200 µm
200 µm
100 µm
1(1')
2(2')
3(3')
4(4')
5(5')
6(6')
7(7')

Fig. 3.3 Line drawings of *Rhinoptericola butlerae* from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, scolex, dorsoventral view; B, basal and metabasal tentacular armature, internal surface; C, basal and metabasal tentacular armature, bothrial surface; D, basal and metabasal tentacular armature, antibothrial surface (basal) turning to external surface (apical); E, profile of hooks.

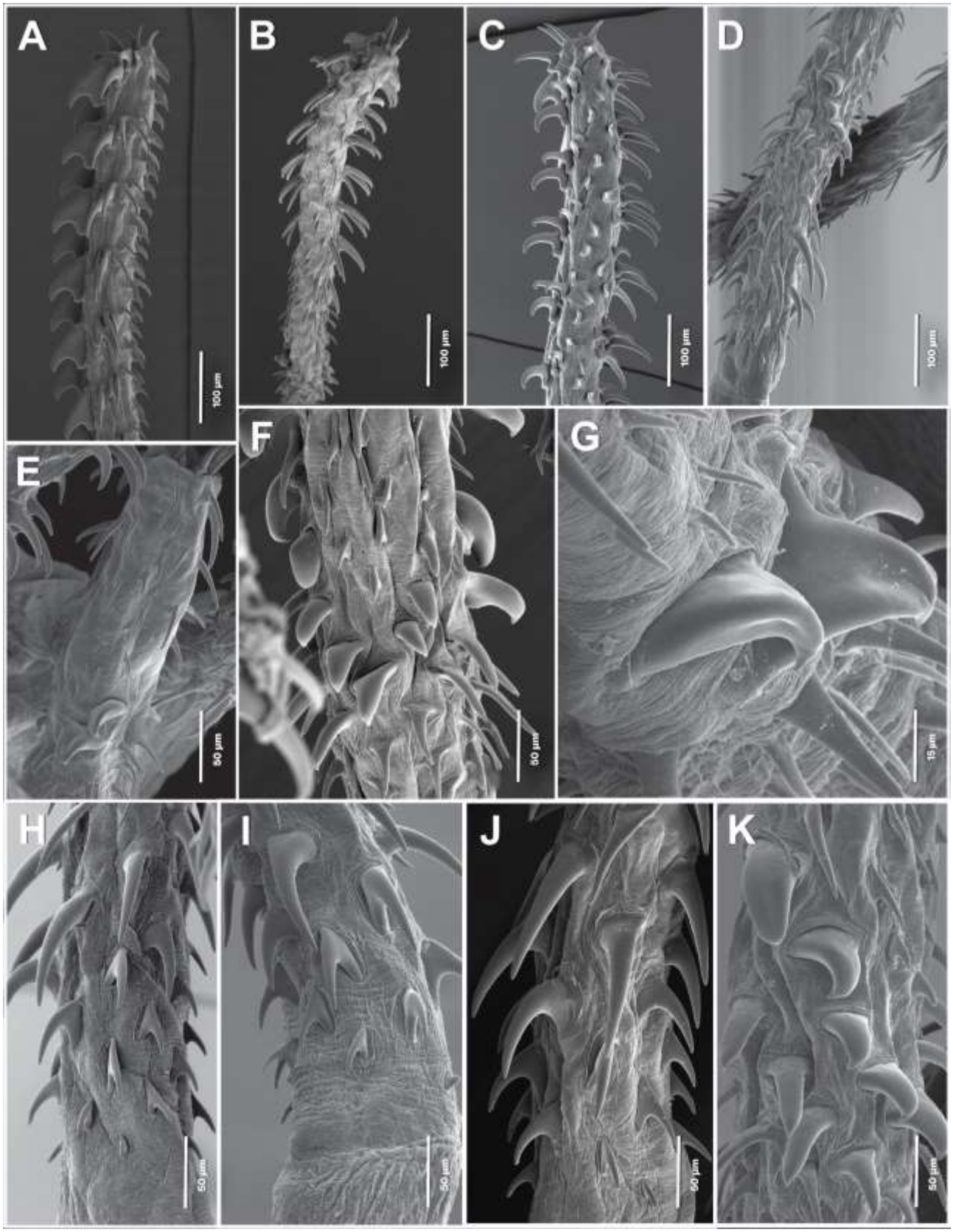

Fig. 3.4 Scanning electron micrographs of the tentacular armature of immature specimens of *Rhinoptericola butlerae* from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, metabasal tentacular armature, bothrial surface; B, metabasal tentacular armature, external surface; C, metabasal tentacular armature, external

surface; D, basal tentacular armature, internal surface; E, basal tentacular armature, external surface; F, detail view of basal tentacular armature, antibothrial surface; showing G, detail view of basal tentacular armature, external surface, showing two macrohooks; H, basal tentacular armature, antibothrial surface; I, basal tentacular armature, bothrial surface, showing microhook at the start of the basal armature; J, basal tentacular armature, internal surface; K, detail view of basal tentacular armature, internal surface.

3.4.6. Remarks

According to Herzog and Jensen (2022), *R. butlerae* exhibits distinct characteristics, such as a characteristic basal armature with three to four macrohooks, more than 80 hooks in the basal armature, and seven hooks per principal row in the metabasal region. Our examined specimens share all these characteristics mentioned above, as well as approximately the same metrical measurements of body parts and regions (Table 3.1). Despite this conformity, a slight morphological disparity from the typical *R. butlerae* morphology is observed in our specimens, which we attribute to interspecific variation. Notably, this includes the presence of a small microhook at the initiation of the basal armature on the bothrial tentacular surface, distinguishing it from the known characteristics of *R. butlerae* (Figs. 3.3C, 3.4I).

Originally described by Beveridge and Campbell in 1988 from *Hemitrygon fluviorum* Ogilby, 1908 in Australia, *R. butlerae*'s known host associations have since broadened considerably. Additional hosts include *Hemitrygon bennetti* (Müller & Henle, 1841), *Himantura tutul* Borsa, Durand, Shen, Alyza, Solihin & Berrebi, 2013, *Maculabatis gerrardi* (Gray, 1851), *Pastinachus ater* (Macleay, 1883), and *Pastinachus solocirostris* Last, Manjaji & Yearsley, 2005 in the family Dasyatidae (Myliobatiformes); *Rhinoptera javanica* Müller & Henle, 1841 and *Rhinoptera neglecta* Ogilby, 1912 in the family Rhinopteridae (Myliobatiformes); and *Chiloscyllium punctatum* Müller & Henle, 1838 in the family Hemiscyliidae (Orectolobiformes). Our study introduces a novel host association, as *R. butlerae* is found to be associated with *R. jayakari* in the family Rhinopteridae (Myliobatiformes). Although the distribution of *R. butlerae* currently spans from Australia to Vietnam, our research extends its geographic range to include the waters of South Africa, suggesting a potential distribution throughout the Indian Ocean.

Table 3.1

Metrical comparison of body parts and regions of two species from the genus *Rhinoptericola* Carvajal & Campbell, 1975. All measurements are stated in millimetres. Abbreviations: a (absent); Bu (bulb); f (fragmented); g (gravid); i (immature); L (length); m (mature); Pbo (pars bothrialis); Pbu (pars bulbosa); Ppbu (pars postbulbosa); Pva (pars vaginalis); s (short); S (scolex); T (total); W (width); W/L R (width/ length ratio).

Species	*R. mozambiquensis*	*R. mozambiquensis*	*R. butlerae*	*R. butlerae*
Study	Present study	Herzog & Jensen (2022)	Present study	Herzog & Jensen (2022)
TL	1.43–3.00 (1.80) [i & f]	2.60–4.80 (3.70) [m]	6.60–13.20 (9.12) [i & f]	15.50–18.90 [m]
SL	1.30–1.70 (1.52)	1.12–1.86 (1.39)	5.32–6.20 (5.84)	4.53–5.90 (5.10)
SW	0.24–0.41 (0.34)	0.20–0.36 (0.28)	0.82–1.16 (0.96)	0.70–1.00 (0.80)
Pbo L	0.27–0.35 (0.31)	0.19–0.38 (0.25)	0.59–0.79 (0.71)	0.42–0.71 (0.60)
Pbo W	0.24–0.41 (0.34)	0.22–0.36 (0.28)	0.82–1.16 (0.96)	0.70–1.00 (0.80)
Pbu L	0.51–0.67 (0.61)	0.38–0.58 (0.46)	2.40–3.00 (2.63)	1.80–2.48 (2.10)
Pbu W	0.20–0.27 (0.23)	0.16–0.24 (0.19)	0.52–0.93 (0.64)	0.56–1.06 (0.70)
Pva L	0.76–1.3 (0.94)	0.72–1.37 (0.93)	2.93–3.67 (3.22)	2.48–3.42 (2.60)
Pva W	0.13–0.22 (0.17)	0.12–0.23 (0.16)	0.44–0.61 (0.49)	0.35–0.79 (0.56)
BuL	0.50–0.64 (0.59)	0.34–0.57 (0.45)	2.36–2.86 (2.56)	1.64–2.50 (2.05)
BuW	0.07–0.09 (0.08)	0.05–0.10 (0.07)	0.21–0.26 (0.23)	0.19–0.31 (0.23)
Bu W/L R	1.0: 6.2–9.0 (7.4)	1.0: 4.4–11.0 (6.7)	1.0: 9.0–13.0 (12.0)	1.0: 5.8–11.3 (8.9)
Ppbu L	0.02–0.03 (0.02) [s or a]	0.01–0.02 [s or a]	0.03–0.17 (0.11) [s]	0.08–0.03 (0.02) [s]

Table 3.2

Comparison of hooks in principal hook rows of two species from the genus *Rhinoptericola* (Carvajal & Campbell, 1975). Metrical data stated in micrometers. Abbreviations: B (base); H (hook); L (length).

Species	*R. mozambiquensis*	*R. mozambiquensis*	*R. butlerae*	*R. butlerae*
Study	Present study	Herzog & Jensen (2022)	Present study	Herzog & Jensen (2022)
H1(1') L	11–15 (13)	8–15 (13)	65–91 (77)	65–126 (86)
H1(1') B	9–11 (10)	6–11 (9)	47–84 (67)	53–102 (72)
H2(2') L	11–17 (14)	14–21 (18)	61–84 (77)	66–125 (97)
H2(2') B	9–10 (10)	6–11 (8)	26–49 (39)	31–66 (47)
H3(3') L	9–23 (18)	14–24 (20)	61–108 (82)	62–119 (92)
H3(3') B	6–9 (8)	5–9 (6)	23–41 (32)	27–42 (34)
H4(4') L	10–17 (15)	14–21 (17)	56–91 (72)	55–99 (71)
H4(4') B	6–8 (7)	4–6 (6)	17–36 (27)	19–39 (26)
H5(5') L	11–18 (16)	13–19 (17)	37–67 (51)	36–75 (56)
H5(5') B	4–8 (7)	5–7 (6)	16–31 (25)	14–26 (20)
H6(6') L	7–8 (7)	6–8 (7)	35–58 (47)	24–69 (39)
H6(6') B	3–4 (4)	3–5 (3)	19–32 (25)	10–31 (20)
H7(7') L	7–9 (8)	6–8 (7)	28–54 (41)	20–64 (39)
H7(7') B	3–4 (4)	3–5 (4)	19–30 (23)	18–31 (26)
H8(8') L	6–7 (7)	-	-	-
H8(8') B	3 (3)	-	-	-
H9(9') L	-	-	-	-
H9(9') B	-	-	-	-

3.4.7. Phylogenetic relationships

Both ML and BI analyses yielded similar tree topologies (see Fig. 3.5). The newly generated sequences of *Rhinoptericola mozambiquensis* (isolate 1) and *Rhinoptericola butlerae* (isolates 2 and 3) grouped in the genus *Rhinoptericola* together with two other specimens of *R. mozambiquensis* collected in Mozambique, and four specimens of *R. butlerae* collected in Viet Nam, Australia, and Indonesia, forming strongly supported clades. The phylogenetic analyses of the 28S showed the clade encompassing Rhinoptericolidae spp. (including the new sequences of *R. mozambiquensis* and *R. butlerae*) as a monophyletic group (supported by BI analyses [100]), supporting the Rhinoptericolidae that was proposed by Herzog and Jensen (2022). *Rhinoptericola mozambiquensis*, *R. schaeffneri*, and *R. jensenae* (all three smaller species with total lengths <6.8mm) created a monophyletic group (supported by BI analyses [92]). The relatively large species, *R. butlerae* and *R. megacantha* (total lengths <10mm) created a monophyletic group (supported by BI analyses [100]) sister to *R. hexacantha* (small species with total length <6.5mm; supported by BI analyses [99]). ML analyses recovered a support value of 97 between isolates 2 and 3 of *R. butlerae*. However, the partial sequences of the 28S of the two isolates of *R. butlerae* are almost identical (p-distance 0.1%, 1 bp difference). The relationship between *Rhinoptericola* spp. is well supported (see Fig. 3.5). The estimates for evolutionary divergences for 28S rRNA were compared using the newly generated and the available sequences of *Rhinoptericola* spp. from GenBank. The p-distances were 0.1% (1 bp) between *R. mozambiquensis* (isolate 1) and *R. mozambiquensis* (OL412738 and OL412739); 2.5% (24–25 bp) between *R. mozambiquensis* (isolate 1) and *R. jensenae* (OL412712, OL412713, and OL412714); and 4.9% (41 bp) between *R. mozambiquensis* (isolate 1) and *R. schaeffneri* (OL412737). The p-distances were 0.1–1.2% (0–20 bp) between *R. butlerae* (isolate 2 and 3) and *R. butlerae* (OL412711, OL412709, OL412708, OL412710); 5.6–5.7% (56–57 bp) between *R. butlerae* (isolate 2 and 3) and *R. hexacantha* (OL412736); and 1.9–2.2% (18–23 bp) between *R. butlerae* (isolate 2 and 3) and *R. megacantha* (OL412732; OL412731, OL412720; OL412718, OL412717, OL412715, OL412716; OL412719; OL412721, OL412722, OL412723, OL412724, OL412725, OL412726, OL412727, OL412728, OL412729, OL412730, OL412733, OL412734, OL412735).

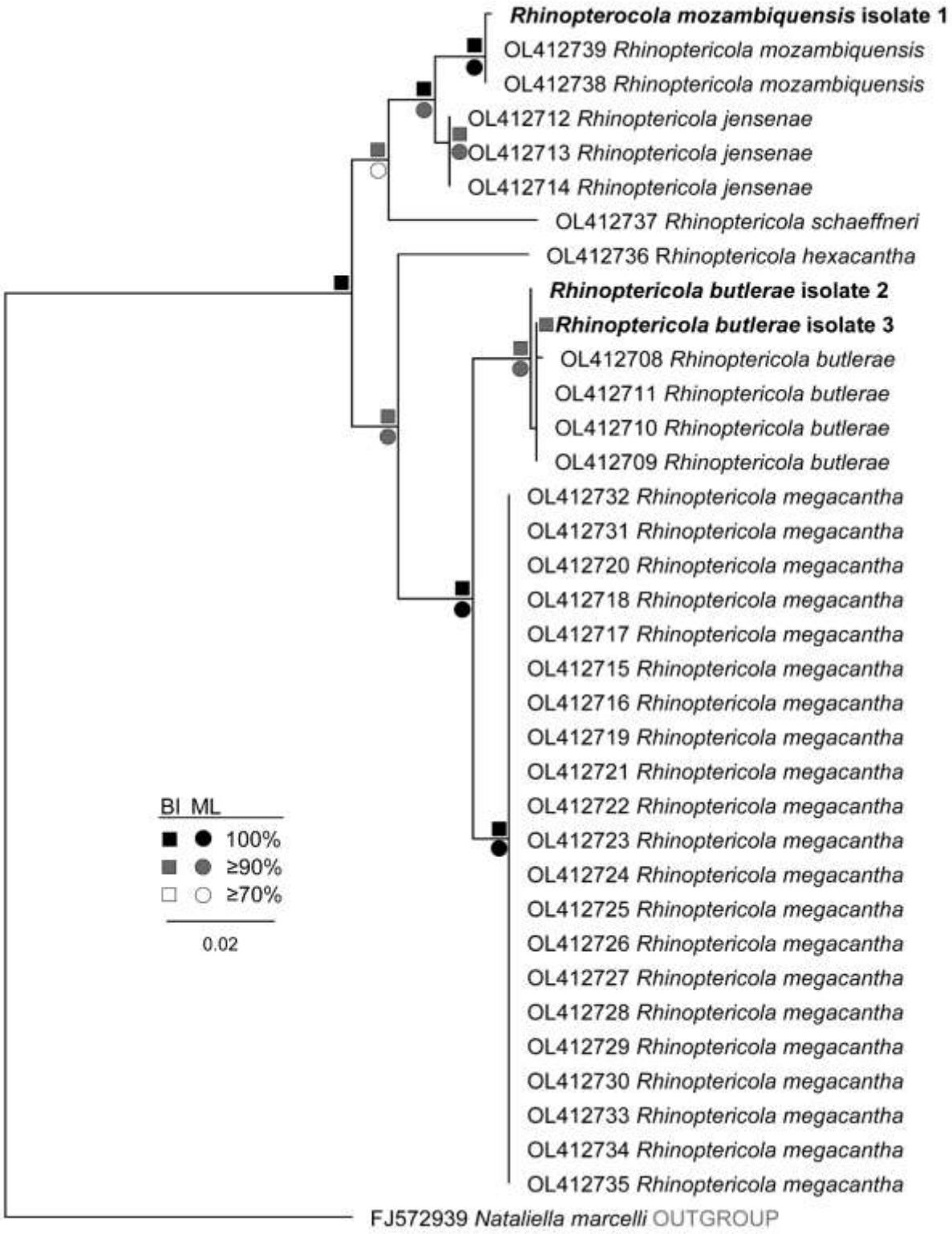

Fig. 3.5 Maximum likelihood phylogram based on sequences of the partial 28S rDNA. Nodal support is shown as posterior probability and bootstrap percentages. GenBank accession number precedes species name. *Nataliella marcelli* Palm, 2010 was used as an outgroup taxa. Branch length scale bar indicates number of substitutions per site.

3.5. Discussion

The recent taxonomic revision of the Rhinoptericolidae by Herzog and Jensen (2022) sheds light on the diversity within the genus *Rhinoptericola* and its evolutionary relationships. Their discovery of three new species, including *R. mozambiquensis*, from cownose rays in Mozambique, and the reclassification of existing species based on morphological and molecular data highlight the dynamic nature of tapeworm taxonomy.

The morphological characteristics of *Rhinoptericola* species, such as the presence of four bothria, a heteroacanthous armature, and the absence of gland cells in the bulbs, provide a basis for the initial classification of these tapeworms. However, the recent inclusion of molecular data in phylogenetic analysis has revealed that traditional morphological characteristics do not always align with evolutionary relationships. This is a crucial insight, emphasising the need for a comprehensive, synergistic approach that combines both morphological and molecular data for accurate taxonomy and systematics. The discovery of billhooks in the basal armature of certain *Rhinoptericola* species, a feature previously only observed in other trypanorhynch genera parasitising devil rays, raises questions about the evolutionary history and ecological interactions of these tapeworms. The phylogenetic analysis using the D1–D3 gene area of 28S rRNA represents a significant advance in understanding the evolutionary relationships within the Rhinoptericolidae. The establishment of a monophyletic Rhinoptericolidae supports the classification proposed by Herzog and Jensen (2022) and highlights the importance of molecular data in elucidating evolutionary connections.

The investigation of *R. butlerae* and its closely related species, *R. mozambiquensis*, has revealed intriguing insights into both inter- and intraspecific variation within these taxa, particularly in comparison to the updated description provided by Herzog and Jensen (2022). A minor, but notable morphological distinct characteristic identified in *R. butlerae* in the present study is the presence of a small microhook at the initiation of the basal armature, a feature not documented in Herzog and Jensen's (2022) description. Furthermore, our investigation reveals variations in the metabasal armature of *R. mozambiquensis*. In contrast to the previously reported consistent count of seven hooks per principal row, diminishing to six hooks per principal row distally, our study demonstrates that individuals exhibit seven to eight hooks per principal row at the initial stage, gradually diminishing to seven hooks per principal row towards the distal end. Remarkably, molecular analyses indicate that, despite these observed morphological differences, the species remain genetically identical (Fig. 3.5). This variation underscores the complexity of species characterisation, emphasising the importance of a comprehensive approach that integrates both morphological and molecular perspectives for a nuanced understanding of biological diversity.

The study also underscores the importance of ongoing research to fill in the gaps in our understanding of trypanorhynch tapeworms. Beveridge *et al.* (2017) rightly pointed out the need to describe new genera and a significant number of new species. The discovery of *R. mozambiquensis* and the additional records of *R. butlerae* from the waters of South Africa exemplify the continuous exploration necessary for a comprehensive understanding of the diversity and distribution of tapeworms. The morphological and molecular analyses of *R. mozambiquensis* and *R. butlerae* provide valuable insights into their taxonomy and relationships within the genus. The distinct characteristics observed in these species, such as the presence of macrohooks and unique features in the basal armature, contribute to their differentiation from other *Rhinoptericola* species. The expansion of the geographical distribution of *R. butlerae* to include the waters of South Africa exemplifies the dynamic nature of species distribution and highlights the importance of regional studies in understanding parasite ecology.

3.6. Conclusion

This research expands the known geographical range of both *R. butlerae* and *R. mozambiquensis* to include the waters off South Africa. The Oman cownose ray, *R. jayakari*, is identified as a new host for *R. butlerae* in this study, expanding our knowledge of the host species associated with this parasite. Furthermore, additional morphological characteristics for both *Rhinoptera* species are revealed through the findings of this study. The taxonomic revision of the Rhinoptericolidae, especially within the genus *Rhinoptericola*, exemplifies the integration of morphological and molecular approaches in tapeworm taxonomy. The discovery of new species, the clarification of evolutionary relationships, and the expansion of geographic distributions contribute to a more comprehensive understanding of the diversity and ecology of these marine parasites. Ongoing research efforts, especially in regions with limited exploration, are crucial for filling gaps in our knowledge of trypanorhynch tapeworms and their interactions with elasmobranch hosts.

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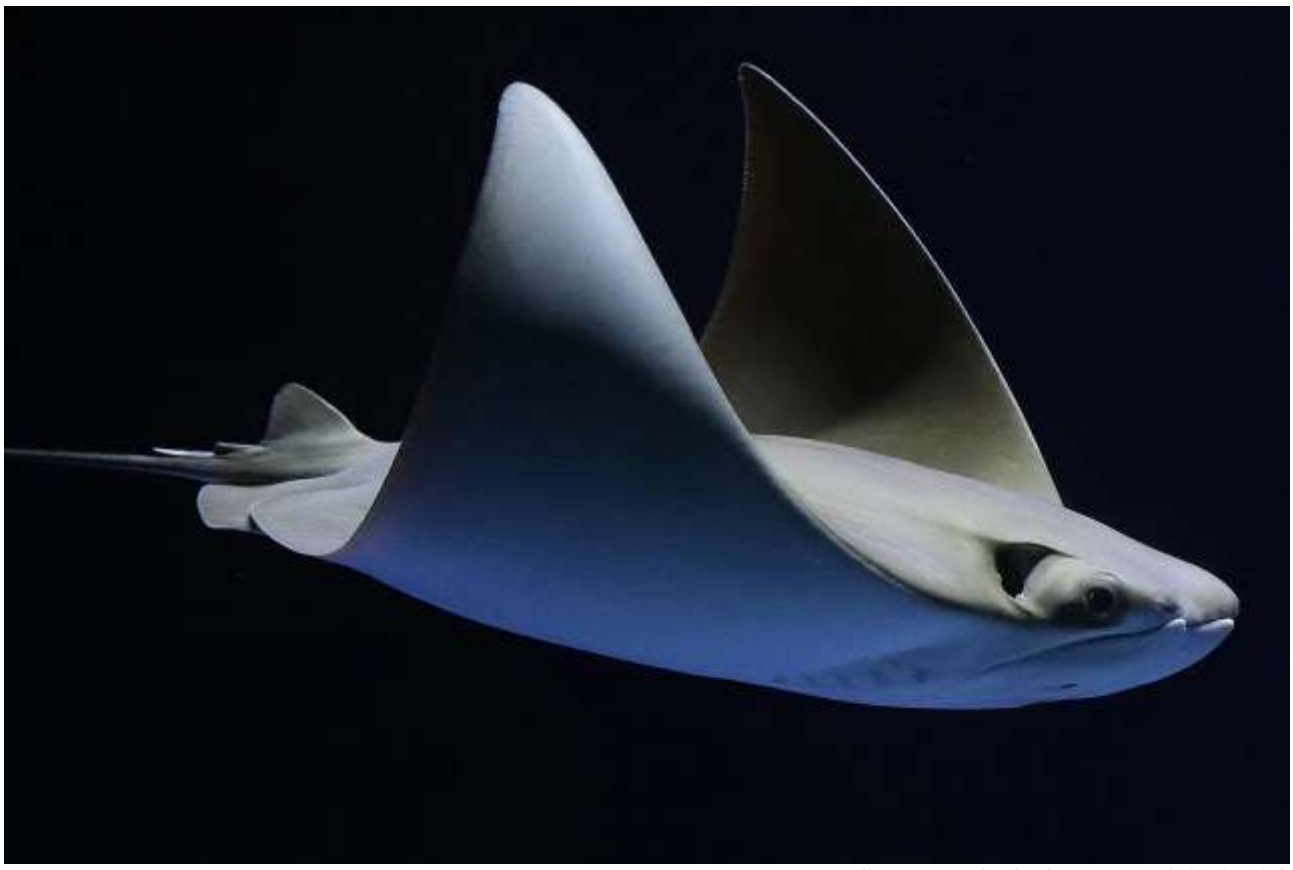
CHAPTER 4

New host and distribution records for *Tetrarhynchobothrium* cf. *unionifactor* (Shipley and Hornell, 1904) (Cestoda: Trypanorhyncha) from the Oman cownose ray, *Rhinoptera jayakari* Boulenger, in South Africa



Credit: Zoo-Leipzig (www.zoo-leipzig.de)

CHAPTER 4: New host and distribution records for *Tetrarhynchobothrium* cf. *unionifactor* (Shipley and Hornell, 1904) (Cestoda: Trypanorhyncha) from the Oman cownose ray, *Rhinoptera jayakari* Boulenger, in South Africa

4.1. Abstract

Since its proposal by Dollfus in 1969 the taxonomic validity of the family Tetrarhynchobothriidae has been a subject of prolonged debate. The controversy centres around the classification of its type genus, *Tetrarhynchobothrium* Diesing, 1850 and the family's overall existence, with conflicting opinions from various cestode specialists. However, in 2008, Beveridge contributed insights by redescribing *Tetrarhynchobothrium tenuicolle* Diesing, 1850 and elucidating the tentacular armature of *Didymorhynchus southwelli* Beveridge & Campbell, 1988 reinforcing the family's distinctiveness based on a homeoacanthous metabasal armature. This article presents a morphological study of *Tetrarhynchobothrium* cf. *unionifactor* (Shipley & Hornell, 1904) parasitising the Oman cownose ray in South Africa. The study expands the known geographical range of *T. unionifactor* and introduces a novel host species, *Rhinoptera jayakari* Boulenger, 1895. The morphological examination aligns with previous descriptions but reveals subtle variations.

4.2. Introduction

The validity of the family Tetrarhynchobothriidae, initially proposed by Dollfus in 1969, has been a subject of extensive taxonomic debate (Beveridge, 2008). Dollfus (1969) created the family to accommodate a single genus, *Tetrarhynchobothrium* Diesing, 1850, which he characterised by a distinctive homeoacanthous metabasal armature. However, Schmidt (1986) challenged this classification, considering *Tetrarhynchobothrium* as a synonym of *Eutetrarhynchus* Pinter, 1913, within the Eutetrarhynchidae Guiart, 1927, a family with members characterised by a heteroacanthous armature (Beveridge, 2008). Within the *Tetrarhynchobothrium*, a total of five members are currently considered valid, namely *Tetrarhynchobothrium tenuicolle* Diesing, 1850, *T. striatum* (Wagener, 1854) Dollfus, 1929, *T. australe* Beveridge & Campbell, 1988, *T. rossi* (Southwell, 1912) Beveridge & Campbell, 1988, and *T. unionifactor* (Shipley & Hornell, 1904).

Despite the controversy, Beveridge and Campbell (1988) resurrected the Tetrarhynchobothriidae and added two new genera, *Didymorhynchus* Beveridge & Campbell, 1988 and *Zygorhynchus* Beveridge & Campbell, 1988. The family, once again, faced suppression by Palm (2004) who, based on cladistic analyses, challenging its existence within the Eutetrarhynchoidea (Beveridge, 2008). The uncertainty

stems from incomplete descriptions of species, notably the type-species, *T. tenuicolle*, and the monotypic genus *Didymorhynchus*, lacking a description of the tentacular armature (Beveridge, 2008).

Tetrarhynchobothrium, the type-genus, also continually faced conflicting opinions regarding its validity (Beveridge, 2008). Despite Palm's (2004) suppression, efforts to clarify the family persist. Therefore, Beveridge (2008) contributed additional morphological data, by redescribing *T. tenuicolle* from new material and elucidating the tentacular armature of *Didymorhynchus southwelli* Beveridge & Campbell, 1988. The existing descriptions and revisions largely affirm earlier generic characteristics, implying that retaining the Tetrarhynchobothriidae as a distinct family from the Eutetrarhynchidae is justifiable based on its homeomorphous and homeoacanthous metabasal armature, which comprised of uncinate hooks (Beveridge, 2008). Despite these challenges, it has been possible to establish a functional definition of the genus (Beveridge, 2008). The genus is distinguished by two bothria, a homeoacanthous armature, a prominent pars postbulbosa, and a cirrus sac housing two seminal vesicles (Beveridge, 2008).

Despite ongoing debates surrounding the validity of the family, the distinctive homeoacanthous metabasal armature of *Tetrarhynchobothrium* serves as a potential taxonomic marker (Beveridge, 2008). Further detailed descriptions of species within the Tetrarhynchobothriidae are crucial for resolving taxonomic uncertainties and establishing family-specific characters (Beveridge, 2008). As research continues, the family's distinctiveness, particularly in comparison to the Eutetrarhynchidae, may become more evident, shedding light on the intricate relationships within the superfamily Eutetrarhynchoidea.

Host specificity and geographical distribution are notable aspects of *Tetrarhynchobothrium* species (Beveridge, 2008). Adult worms of this genus are known from a diverse array of batoid hosts, contributing to their ecological significance (Beveridge, 2008). The type species *T. tenuicolle*, is only known from the thornback ray (*Raja clavata* Linnaeus, 1758) from Bosnia-Herzegovina, *T. striatum* is known from the common eagle ray (*Myliobatis aquila* Linnaeus, 1758) from Italy and France, and the common stingray (*Dasyatis pastinaca* Linnaeus, 1758) from France, *T. australe* is known from the New Zealand eagle ray (*Myliobatis tenuicaudatus* Hector, 1877) from South Australia, *T. rossi* is known from the bluespotted stingray (*Neotrygon kuhlii* Müller & Henle, 1841) from Sri Lanka, and from the scaly whipray (*Brevitrygon imbricata* Bloch & Schneider, 1801) from Sri Lanka, and *T. unionifactor* is known from the flapnose ray (*Rhinoptera javanica* Müller & Henle, 1841) from Sri Lanka. In summary, the existing data indicates a genus associated with specific ray species in different regions, including instances of transoceanic distribution.

Schaeffner and Smit (2019) conducted a review of elasmobranch parasites in South Africa, emphasising the limited research on cartilaginous fishes as hosts for internal parasites. These authors highlighted the absence of tapeworm species from the Tetrarhynchobothriidae family in southern African waters, despite the rich diversity of host species in the region. During an ongoing project focused on marine parasites in South Africa, a new record of *Tetrarhynchobothrium*, representing *T. unionifactor*, was discovered parasitising the Oman cownose ray, *Rhinoptera jayakari* Boulenger, 1895 (Myliobatiformes: Rhinopteridae). This study provides an additional tetrarhynchobothrid record, presenting a morphological comparison with congeners within the genus.

4.3. Materials and methods

4.3.1. Collection of specimens and fixation of material

Three male specimens of *R. jayakari*, ranging between 92 and 167 cm in total length, were collected in March 2020 along the south-eastern coastline of KwaZulu-Natal, South Africa by the KwaZulu-Natal Sharks Board (KZNSB). This project was approved by the Ethics Committee of the Faculty of Natural and Agricultural Research Sciences of North-West University with the ethics number NWU-01777-20-A9. The South African Department of Agriculture, Forestry and Fisheries issued a permit for the collection and possession of sharks for research purposes (permit number RES2020/20 issued to the KZNSB).

As part of the South African bather protection programme, the three deceased specimens were collected in shark nets along the coasts of Richards Bay and Scottburgh and stored at -20°C in the Sharks Board Institute’s laboratory. In the dissection process after defrosting the batoids, the spiral intestines were removed by a mid-ventral incision followed by 10% formalin fixation of approximately three-fourths of each intestine along with the contents. After two weeks, the intestines and contents were exchanged to 70% ethanol. Molecular grade ethanol (96%) was used to fix the remainder of the intestinal tract. In the laboratory, preserved spiral intestines and contents were examined for cestodes with a stereomicroscope and specimens were manually picked and placed in both 70% and 96% ethanol for morphological and molecular analyses.

4.3.2. Morphological study

A graded ethanol series was used to hydrate the specimens, which were subsequently stained with Delafield's haematoxylin, and dehydrated back to 70% ethanol. As soon as the specimens were stained, they were put in 1% hydrogen chloride to clear excess stain, followed by gradation of ethanol to completely dehydrate them. Clove oil was used to clear the tissue of these specimens, followed by Canada balsam to mount each specimen permanently on a microscope slide.

Morphological observations and measurements of the mounted specimens were performed using a Nikon ECLIPSE Ni light microscope (Nikon, Tokyo, Japan) once the microscope slides were completely dry. The line drawings were made using a drawing attachment tube. In the description, measurements are presented as the range, followed by the mean, standard deviation, and the number of specimens (n) examined. Unless otherwise stated, all measurements are in micrometers. Campbell and Beveridge (1994) provide the terminology for morphological characteristics, except that the attachment organs are called bothria following Jones *et al.* (2004). The following abbreviations were used: pbo (pars bothrialis), pva (pars vaginalis), pbu (pars bulbosa) and ppbu (pars postbulbosa). Hook terminology follows Palm (2004) and Beveridge and Campbell (2007).

4.4. Results

4.4.1. Tetrarhynchobothrium cf. *unionifactor* (Shipley & Hornell, 1904) (Fig. 4.1)

Diagnosis (based on 2 scolexes).

Scolex acraspedote, elongate, 1.77–2.00 (1.88, n = 2) mm long; maximum width at level of pars bothrialis (Fig. 4.1A). Pars bothrialis 413–416 (415, n = 2) long, 397–651 (524, n = 2) wide (Fig. 4.1A). Two oval bothria, 384–477 (431, n = 2) long, 137–162 (150, n = 2) wide (Fig. 4.1A). Pars vaginalis 1,208–1,291 (1,250, n = 2) long, 310–375 (343, n = 2) wide (Fig. 4.1A); tentacle sheaths sinuous, large spherical gland cells present, terminating anterior to bulbs (Fig. 4.1A). Pars bulbosa 561–706 (634, n = 2) long, 332–341 (337, n = 2) wide. Bulbs elongate, muscular (Fig. 4.1A), 560–701 (631, n = 2) long, 94–111 (103, n = 2) wide; bulb length to width ratio 6.0–6.3 (6.1, n = 2): 1.0; prebulbar organ present; retractor muscle attaches at the base of bulb, gland cells prominent attached to retractor muscle within bulb (Fig. 4.1A). Pars postbulbosa short, 28–34 (31, n = 2) long. Scolex ratio (pbo: pva: pbu:ppbu) 1.0: 2.9–3.1: 1.4–1.7: 0.1 (n = 2).

Tentacles up to 495 long, without basal swelling (Fig. 4.1A); diameter at base 38–40 (39, n = 2), 33–35 (34, n = 2) in distal region of tentacles (Figs. 4.1A–B). Armature heteroacanthous; hooks hollow, homeomorphous (Fig. 4.1B). No distinctive basal armature present, but hooks at base of tentacle slightly smaller (Fig. 4.1B). Hooks uncinate; gradation in hook size from bothrial to anti-bothrial surface. Hooks on bothrial surface of tentacle, 6–7 (6 ± 0.6, n = 7) long, base 5–7 (6 ± 0.6, n = 7) long, gradually decreasing in diameter around tentacle; hooks on antibothrial surface 4–5 (5 ± 0.5, n = 7) long, base 2–4 (3 ± 0.6, n = 7) long; 8–9 hooks per half circle (Fig. 4.1B).

4.4.2. Taxonomic summary

Study of Beveridge and Campbell (1988)

Type host: 'Cotype' (metacestode) from *Pinctada margaritifera* (Linnaeus, 1758) (syn. *Margaritifera vulgaris*).

Additional specimens (adults): Two scoleces plus fragments of strobila from *Rhinoptera javanica* Mueller & Henle, 1841 Dutch Bay, Sri Lanka.

Present study:

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

Additional locality: South-western Indian Ocean off Scottburgh (28°78'0"S, 30°76'0"E) and 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 33% (1 out of 3 *R. jayakari* examined); intensity of 2 worms.

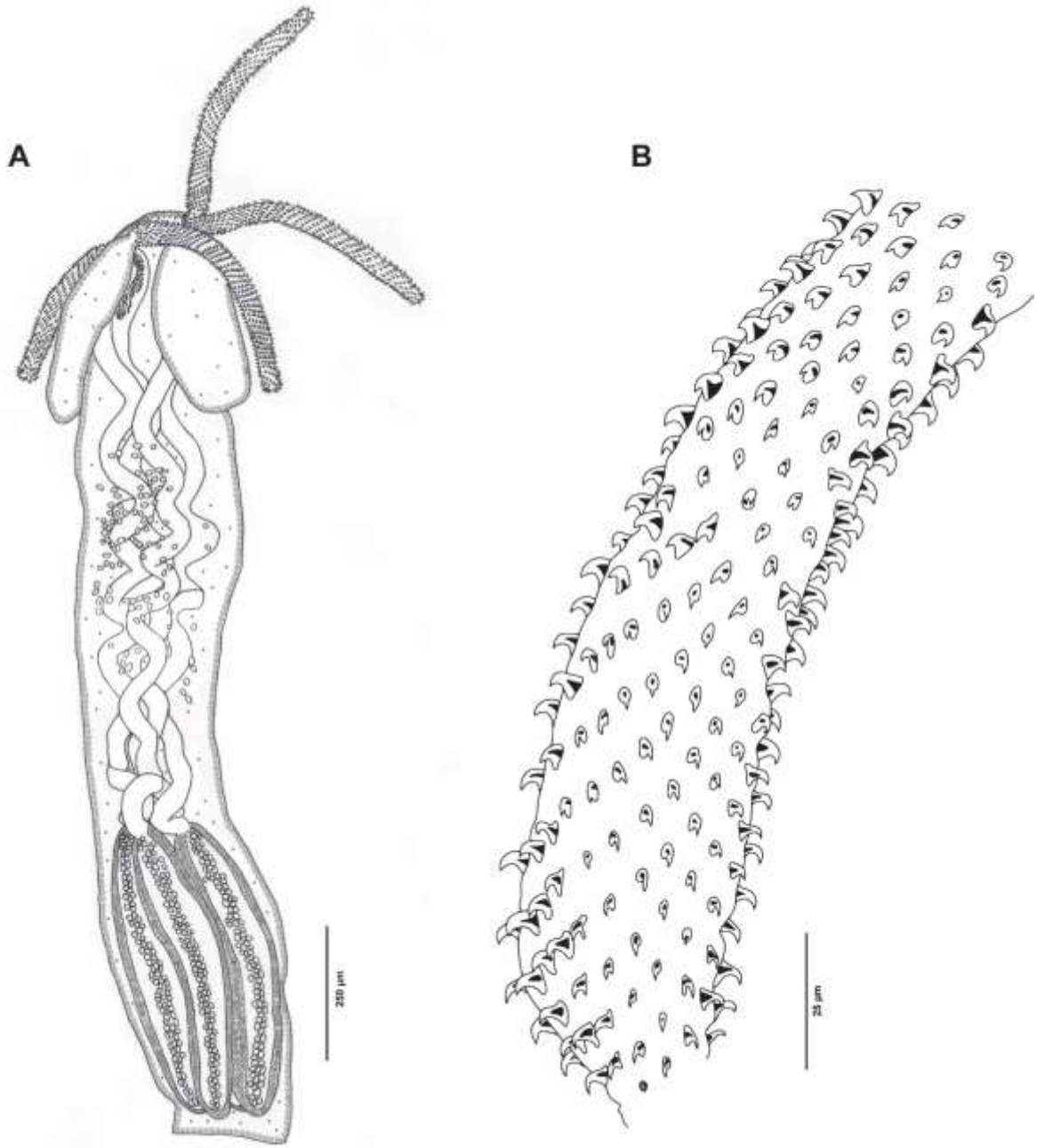


Fig. 4.1. Line drawings of *Tetrarhynchobothrium* cf. *unionifactor* from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, scolex, lateral view; B, basal and metabasal tentacular armature, external surface.

4.4.3. Remarks

The examination of the two South African specimens revealed features that align closely with the characteristics described for *T. unionifactor* by Beveridge and Campbell (1988). The observed features include two bothria and a tentacular armature with a homeoacanthous armature and eight to nine hooks per half circle on the tentacle. The gradation in hook size around the tentacle is also

consistent with the re-description provided by Beveridge and Campbell (1988). However, it should be noted that our specimens lack a strobila, making confirmation of the characteristics related to the cirrus sac containing two seminal vesicles impossible.

However, our investigation revealed subtle variations in the metrical measurements of scolex parts and regions, as indicated in Table 4.1. These differences may be due to limitations in our sample size or could be attributed to intraspecific variability. Subtle, yet notable variations include the width of the bulbs [94–111 (103) vs. 70, respectively] in our specimens, which is larger than in the specimens of Beveridge and Campbell's (1988) study. Furthermore, a distinction was observed in tentacular armature and hook sizes on different surfaces between *T. unionifactor* in the current study and the findings by Beveridge and Campbell (1988). In our study, the diameter at the base and the distal region of the tentacles is slightly larger [38–40 (39) at base, 33–35 (34) at distal region vs. 36 at base, 28–30 in distal region, respectively]. The overall hook measurements were slightly larger in the study of Beveridge and Campbell (1988) on both the bothrial and antibothrial surface than in the present study [bothrial surface 7–10 (8) in length with a base of 8–10 (9) vs. 6–7 (6) in length with a base of 5–7 (6); antibothrial surface 5.5–6.5 (6) in length with a base of 5.5–6.5 (5.8) vs. 4–5 (5) in length with a base of 2–4 (3), respectively]. These discrepancies in hook sizes on different surfaces indicate an intriguing variation that could be attributed to factors such as geographical location, host choice, or may resemble intraspecific variability. More research is needed to clarify the precise factors that contribute to these observed differences in the tentacular armature of *T. unionifactor.*

The known geographical distribution of adult *T. unionifactor*, reported from *R. javanica*, encompasses the waters of Sri Lanka. There is a potential occurrence, if deemed valid, of a single specimen bearing resemblance to *T. unionifactor* from Singapore (BMNH 1968.11.4.5), collected from an unidentified ray species, *Dasyatis* sp. (Beveridge & Campbell, 1988). However, the examination of a single specimen resembling *T. unionifactor* from Singapore revealed significant differences, such as a larger scolex, bulbs, and pars bothrialis size, indicating potential variability in scolex measurements within the species (Beveridge & Campbell, 1988).

Our current investigation significantly extends the recognised geographical range of *T. unionifactor* to now encompass the waters off South Africa. Notably, our study identifies a novel host species, *R. jayakari*, further enriching our understanding of the distribution patterns and host associations of *T. unionifactor* in the southwestern Indian Ocean.

Table 4.1

Metrical comparison of scolex parts of *Tetrarhynchobothrium unionifactor* (Shipley & Hornell, 1904). All measurements are stated in micrometers. Type hosts and localities are given in bold. Abbreviations: a (absent); Bu (bulb); L (length); Pbo (pars bothrialis); Pbu (pars bulbosa); Ppbu (pars postbulbosa); Pva (pars vaginalis); s (short); S (scolex); W (width); W/L R (width/ length ratio).

Species	*T.* cf. *unionifactor*	*T. unionifactor*
Study	Present study	Beveridge & Campbell (1988)
Country	South-western Indian Ocean: **South Africa**	Northern Indian Ocean: **Sri Lanka**
Host species	Rhinopteridae: ***Rhinoptera jayakari***	Rhinopteridae: ***R. javanica***
SL	1.770–2.000 (1.880)	1.600–2.300 (1.950)
SW	397–651 (524)	380
Pbo L	413–416 (415)	410–420
Pbo W	397–651 (524)	–
Pbu L	561–706 (634)	–
Pbu W	332–341 (337)	–
Pva L	1.210–1.290 (1.250)	1.220–1.610 (1.420)
Pva W	310–375 (343)	–
BuL	560–701 (631)	500–590 (550)
BuW	94–111 (103)	70
Bu W/L R	1.0: 6.0–6.3 (6.1)	-
Ppbu L	28–34 (31) [s]	14 [s or a]

4.5. Discussion

The history of *T. unionifactor* is convoluted, marked by a series of name changes and taxonomic uncertainties. The journey begins when Herdman (1903) first introduced the name "*Tetrarhynchus unionifactor*." This designation was derived from the discovery of a *Tetrarhynchus* larva, a species that was yet to be determined as known or unknown. In 1904, Shipley and Hornell officially described *Tetrarhynchus unionifactor* based on larvae from the pearl oyster, *Margaritifera vulgaris* Schumacher, 1817. Shipley and Hornell (1906) recovered adults, which they believed to be *Tetrarhynchus unionifactor*, from the stomach and intestines of the flapnose ray, *R. javanica*. They modified their original description of *Tetrarhynchus unionifactor* to include features of the adult worms. However, Southwell (1929) introduced the new combination "*Tentacularia unionifactor*" and listed "*Tetrarhynchus unionifactor* Shipley & Hornell, 1904" as a synonym. Southwell (1929) considered

that adults from *R. javanica* belong to the trypanorhynch genus *Tentacularia* Bosc, 1797, while larvae from *M. vulgaris* were thought to represent a distinct species within the lecanicephalidean genus *Tylocephalum* Linton, 1890. This may have been the first instance of the combination "*Tylocephalum unionifactor*."

However, Schmidt (1986) classified *Tylocephalum unionifactor* under the genus *Lecanicephalum* Linton, 1890, forming the new combination "*Lecanicephalum unionifactor*." Schmidt (1986) cited the authority for the name as "Herdmann & Hornell, 1903," which, however, does not exist. It is speculated that Schmidt may have referred to the publication "Pearl Production" by Herdman and Hornell (1904) or one of the earlier usages of the name in the literature from 1903. Beveridge and Campbell (1988) redescribed the species, relocating it to the genus *Tetrarhynchobothrium*. Furthermore, Beveridge and Campbell (1988) expressed scepticism about the conspecificity of the larvae from the pearl oyster and the adults from *R. javanica*, stating that the type specimen cannot be clearly identified. The adults, they suggest, represent a distinct species, and they propose using the name "*Tetrarhynchobothrium unionifactor*" until further detailed studies can be conducted on new material from the type-locality (Beveridge & Campbell, 1988).

However, the convoluted history is not limited to the parasite species; the same complexity extends to the definitive host species. Ebert *et al.* (2021) recently conducted a study that revised the distribution of *Rhinoptera* Cuvier, 1829 species, particularly updating information on *R. jayakari* and *R. javanica*. Ebert *et al.* (2021) verified the presence of only a single species of *Rhinoptera* in South African waters, identified as *R. jayakari*. This determination was made by closely examining regional catalogues and guides, as well as analysing museum voucher material from South Africa. This challenged previous reports (see Compagno *et al.,* 1989; Compagno, 1986, 1999; Ebert *et al.,* 2015; Heemstra & Heemstra, 2004; Smith, 1952, 1961; Wallace, 1967) that suggested the presence of *R. javanica* in South African waters. *Rhinoptera jayakari*, initially reported from the Gulf of Oman to the Red Sea along the northwestern Indian Ocean, has been a subject of taxonomic scrutiny. Ahmad *et al.* (2013) provided some identification characters in a field guide, but confirmation of the specimen's identity remained elusive. Reports from Indian waters, including the Gulf of Mannar (Joshi *et al.,* 2016) and Kerala coast (Bineesh *et al.,* 2016), further documented the presence of *R. jayakari*, emphasising its distribution in the Indian Ocean. However, *R. javanica* has a known range in the Indian and Western Pacific Oceans, extending from Oman to the Philippines and north to Japan (Akhilesh *et al.,* 2014). The challenge arises when attempting to differentiate between *R. jayakari* and *R. javanica*, as species-specific information is often lacking, and morphological characteristics alone may not be sufficient for accurate identification (Akhilesh *et al.,* 2014). According to Pradeep *et al.* (2018) for precise confirmation of the identity of *Rhinoptera* specimens, it is recommended to employ molecular marker-based taxonomic annotation through Mitochondrial COI gene sequencing.

Distinguishing between these two species becomes important when considering a potential infection with the parasite *T. unionifactor*. The geographical distribution of adult *T. unionifactor*, initially reported from *R. javanica*, was documented in the waters of Sri Lanka (Shipley & Hornell, 1906). Ebert *et al.* (2021) verified that *R. jayakari* in Southern Africa represents an independent species closely related to *R. javanica*, thus raising the possibility of it being a suitable host for the parasite in the southwestern Indian Ocean. This study introduces *R. jayakari* as a novel host species, suggesting that this parasite is host specific to closely related *Rhinoptera* and that its distribution in this new host expands well into the southwestern Indian Ocean.

The present specimens resemble *T. unionifactor* morphologically, exhibiting key features described by Beveridge and Campbell (1988). The presence of two bothria, gland cells in the bulbs and a homeoacanthous armature, along with eight to nine hooks per half circle on the tentacle, closely aligns with the characteristics of *T. unionifactor*. However, our specimens lack a strobila, preventing confirmation of characteristics related to internal structures of proglottids, such as the cirrus sac containing two seminal vesicles. Subtle variations in the metrical measurements of scolex parts and regions were also noted, possibly influenced by specimen sample size or intraspecific variability. Despite these variations, the morphological similarity led us to tentatively designate our specimens as *T.* cf. *unionifactor*, highlighting the need for further investigations to confirm this identification. The variations in scolex measurements within *T. unionifactor*, as observed in the Singapore specimen, also highlights the importance of comprehensive descriptions and the challenges posed by limited material. It becomes clear that additional collections and studies are essential to confirm the observed differences and provide a more refined understanding of the species.

4.6. Conclusion

This research reports on the presence of *T.* cf. *unionifactor* in the oman cownose ray, *R. jayakari*, thereby extending the known geographical range of the parasite to include the waters off South Africa. The morphological features observed in the specimens closely match the description outlined by Beveridge and Campbell (1988). However, limitations in the study, such as the absence of a strobila in the specimens, hinder the confirmation of specific internal structures, which emphasises the need for further investigation. Subtle variations in metrical measurements imply potential intraspecific variability or the impact of specimen sample size. Therefore, sampling efforts encompassing a greater number of specimens and host species, are crucial to validate the observed differences and attain a more comprehensive understanding of *T. unionifactor* and its host associations, geographical distribution, and factors that can potentially influence morphological variations.

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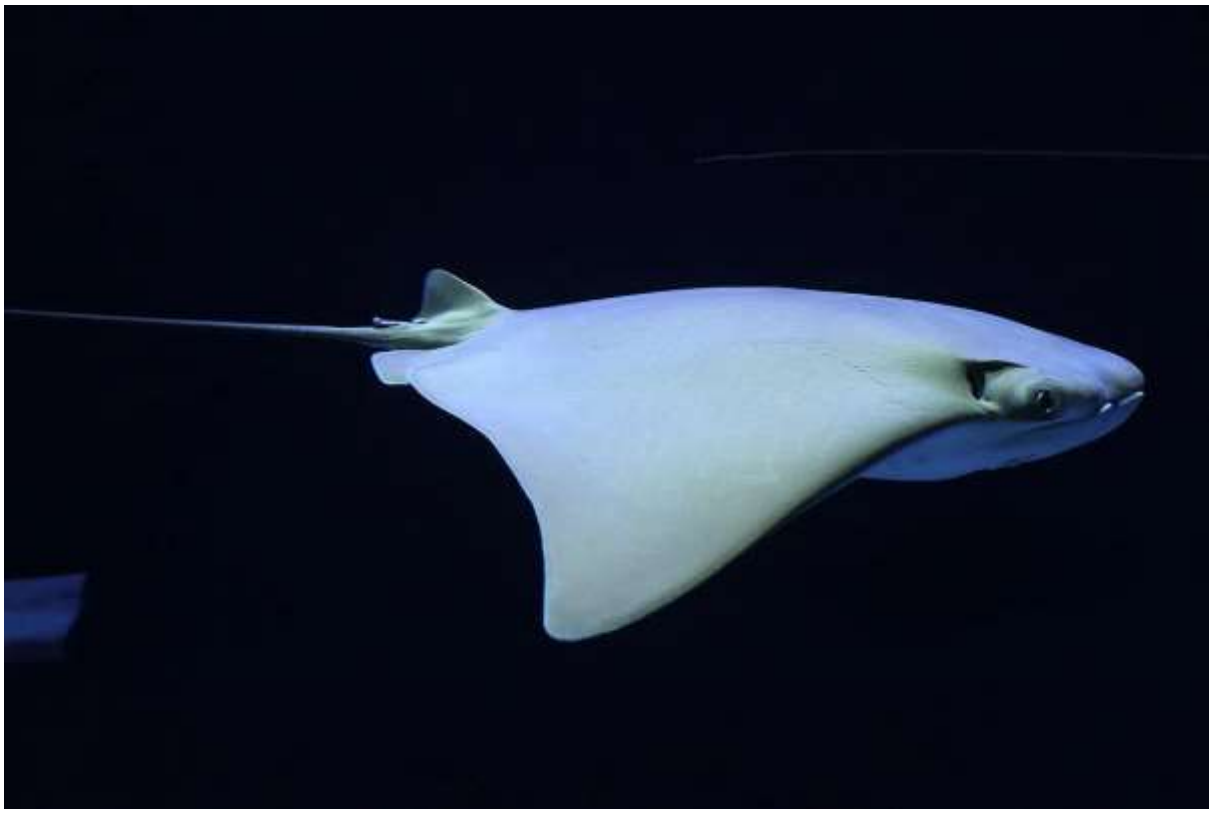
CHAPTER 5

A putative new species of *Duplicibothrium* (Cestoda: Tetraphyllidea) from the Oman cownose ray, *Rhinoptera jayakari* Boulenger, in South Africa



Credit: Zoo-Leipzig (www.zoo-leipzig.de)

CHAPTER 5: A putative new species of *Duplicibothrium* (Cestoda: Tetraphyllidea) from the Oman cownose ray, *Rhinoptera jayakari* Boulenger, in South Africa

5.1. Abstract

This study reports the discovery of a potential new species of *Duplicibothrium* Williams & Campbell, 1978 (Cestoda: Serendipeidae), parasitising the Oman cownose ray, *Rhinoptera jayakari* Boulenger, in South African waters, expanding the known geographical range of the genus. The family Serendipeidae currently comprises 15 valid species in four genera, including three provisionally identified species, exclusively associated with cownose rays of the genus *Rhinoptera*. Species of *Duplicibothrium* have been identified in various *Rhinoptera* hosts worldwide, and the discovery in South Africa contributes to the knowledge on the cosmopolitan distribution of these tapeworms. Morphological analysis reveals similarities with congeners but highlights limitations due to the compromised condition of the available material, hindering confirmation of key characteristics. Despite these challenges, *Duplicibothrium* sp. exhibits distinct differences from its congeners, especially the length of the cephalic peduncle, that warrants further investigation with better-preserved specimens to confirm its status as a new species. This study expands our knowledge of tapeworm diversity by unveiling a possible new *Duplicibothrium* species in the waters off South Africa, highlighting the importance of ongoing research for a comprehensive understanding of parasite diversity in this neglected zoogeographical region and the need to focus on host species that have not yet been the focus of parasitological studies.

5.2. Introduction

The exploration of the cestode family Serendipeidae Brooks & Evenhuis, 1995 within the order Tetraphyllidea has taken an intriguing turn, marked by recent findings and taxonomic revisions (Stephan *et al.,* 2023). The family, initially recognised as a distinctive clade within Tetraphyllidea, encompasses nine described species exclusively found in cownose rays of the genus *Rhinoptera* Cuvier, 1829. The morphological similarities in proglottid anatomy among its three genera *Duplicibothrium* Williams & Campbell, 1978, *Glyphobothrium* Williams & Campbell, 1977, and *Serendip* Brooks & Barriga, 1995 have long captivated researchers (Brooks & Evenhuis, 1995). Stephan and Caira (2022) identified three new species of *Duplicibothrium* from cownose rays, uncovering previously unknown aspects of diversity within the Serendipeidae and shedding light on unexplored species in cownose rays. Notably, the scolex morphology of one of these species aligns more closely with that of *Serendip*, introducing a captivating twist to the understanding of these

genera (Stephan & Caira, 2022). However, the phylogenetic analysis conducted by the same authors encompassing specimens collected worldwide with the unconventional scolex morphology of *Duplicibothrium*, positions the species robustly within a *Duplicibothrium* clade. This unexpected result raised questions about the established mutual monophyly of *Serendip* and *Duplicibothrium*.

Stephan *et al.* (2023) expanded on the previous work of Stephan and Caira (2022), by formally describing three species, previously known only by provisional names, for which adequate material was made available. Notably, these species include *Duplicibothrium* n. sp. 2, as identified by Jensen and Bullard (2010), and *Duplicibothrium* n. sp. 4 and *Duplicibothrium* n. sp. 5, identified in their previous work (Stephan & Caira, 2022). Additionally, a revision of the generic classification within the family was undertaken, aligning it with morphological characteristics and emerging phylogenetic insights (Stephan *et al.,* 2023). The revision of the generic classification led to the establishment of a new genus, *Nanoduplicibothrium* Stephan, Bueno & Caira, 2023, to accommodate a morphologically cohesive and molecularly divergent subset of species originally assigned to *Duplicibothrium*. The genus is characterised by bothridia fused lengthwise in two pairs lacking a distinct row of posterior loculi and a cephalic peduncle. It contains the smallest members of the Serendipeidae (Stephan *et al.,* 2023). Within this newly established genus, Stephan *et al.* (2023) described two novel species, namely *Nanoduplicibothrium leanneae* Stephan, Bueno & Caira, 2023, from *Rhinoptera bonasus* Mitchill off the coast of South Carolina and *Nanoduplicibothrium megaphallum* Stephan, Bueno & Caira, 2023 from *Rhinoptera jayakari* Boulenger off the shores of Mozambique. Two species previously assigned to *Duplicibothrium* were transferred to the new genus as *Nanoduplicibothrium paulum* (Ruhnke, Curran & Holbert, 2000) and *Nanoduplicibothrium jillae* (Stephan & Caira, 2022) (Stephan *et al.,* 2023). In addition, Stephan *et al.* (2023) described a new species, *Duplicibothrium bilai* Stephan, Bueno & Caira, 2023, identified from *R. jayakari* off the coast of Mozambique.

In the phylogenetic analysis conducted by Stephan *et al.* (2023), the authors confirmed the monophyly of *Nanoduplicibothrium*, *Duplicibothrium*, and *Serendip* and expanded the understanding of host associations within the Serendipeidae. Notably, the sequence data used for the D1–D3 region of the 28S rDNA gene from an undescribed *Serendip* species collected off Panama revealed host associations beyond Rhinopteridae to include a species of Myliobatidae (Stephan *et al.,* 2023). However, despite these advancements, the phylogenetic placement of *Glyphobothrium*, the fourth genus in the family, remains elusive. Stephan *et al.* (2023) agrees with the position of Caira *et al.* (2017), asserting that Glyphobothriidae Monks, Pulido & Gardner, 2015, created to encompass *Duplicibothrium* and *Glyphobothrium*, is considered a junior synonym of Serendipeidae. The monophyly of *Serendip*, *Duplicibothrium*, and *Nanoduplicibothrium* is substantiated by Stephan *et al.* (2023), but the unresolved phylogenetic affinities of *Glyphobothrium* suggest that further molecular analyses are needed to unravel its true placement within the family.

In terms of global diversity, the establishment of *Nanoduplicibothrium* increases the total number of genera within the Serendipeidae to four, namely *Duplicibothrium*, *Serendip*, *Nanoduplicibothrium*, and *Glyphobothrium*. Serendipeidae currently comprises a total of 12 valid species and three provisionally identified species that have not yet been formally described. Stephan *et al.* (2023) reported that 15 species in this family are exclusively associated with cownose rays of the genus *Rhinoptera*. However, the same authors later discovered a new species, *Serendip* n. sp. 1, in the rough eagle ray, *A. asperrimus*, from Panama, a finding which they concluded expands the potential host species beyond Rhinopteridae to include Myliobatidae, challenging the previous notion of limited diversity in serendipeids. This suggests that the global diversity of this cestode family may be more extensive than previously believed. However, the detailed description of this newfound species is pending until additional material is collected to facilitate the comprehensive characterisation of its morphological features.

During an ongoing project dedicated to marine parasites in South Africa, a novel occurrence of *Duplicibothrium* was identified, parasitising the Oman cownose ray, *R. jayakari*. This investigation introduces an additional record that offers a morphological comparison with other members within the genus.

5.3. Materials and methods

The same methodology was followed as presented in Chapter 4, except that Clopton's (2004) terminology is used to describe shapes.

5.4. Results

5.4.1. Duplicibothrium sp. (Fig. 5.1)

Diagnosis (based on 10 immature fragmented specimens, 2 immature specimens were used for SEM).

Worms weakly craspedote, euapolytic, 3,536–19,968 (10,561 ± 5,621; n = 8) long; maximum width at level of scolex. Scolex 346–479 (414 ± 49; n = 7) long, 323–870 (466 ± 201; n= 7) wide, consisting of 4 bothridia arranged in 2 fused dorso-ventral pairs (Figs. 5.1A–B) and elongate cephalic peduncle (Fig. 5.1A). Bothridia pyriform, 346–479 (414 ± 49; n = 7) long, 323–870 (459 ± 187; n= 8) wide, attached posteriorly, free anteriorly, loculated. Loculi number on individual bothridia not observed. Cephalic peduncle 1,746–13,154 (9,056 ± 3,643; n = 7) long, 41–169 (91 ± 42; n = 10) wide. Strobila with few immature segments,7–46 (21 ± 13; n =7) in number.

5.4.2. 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 33% (1 out of 3 *R. jayakari* examined); intensity of 10 worms per host.

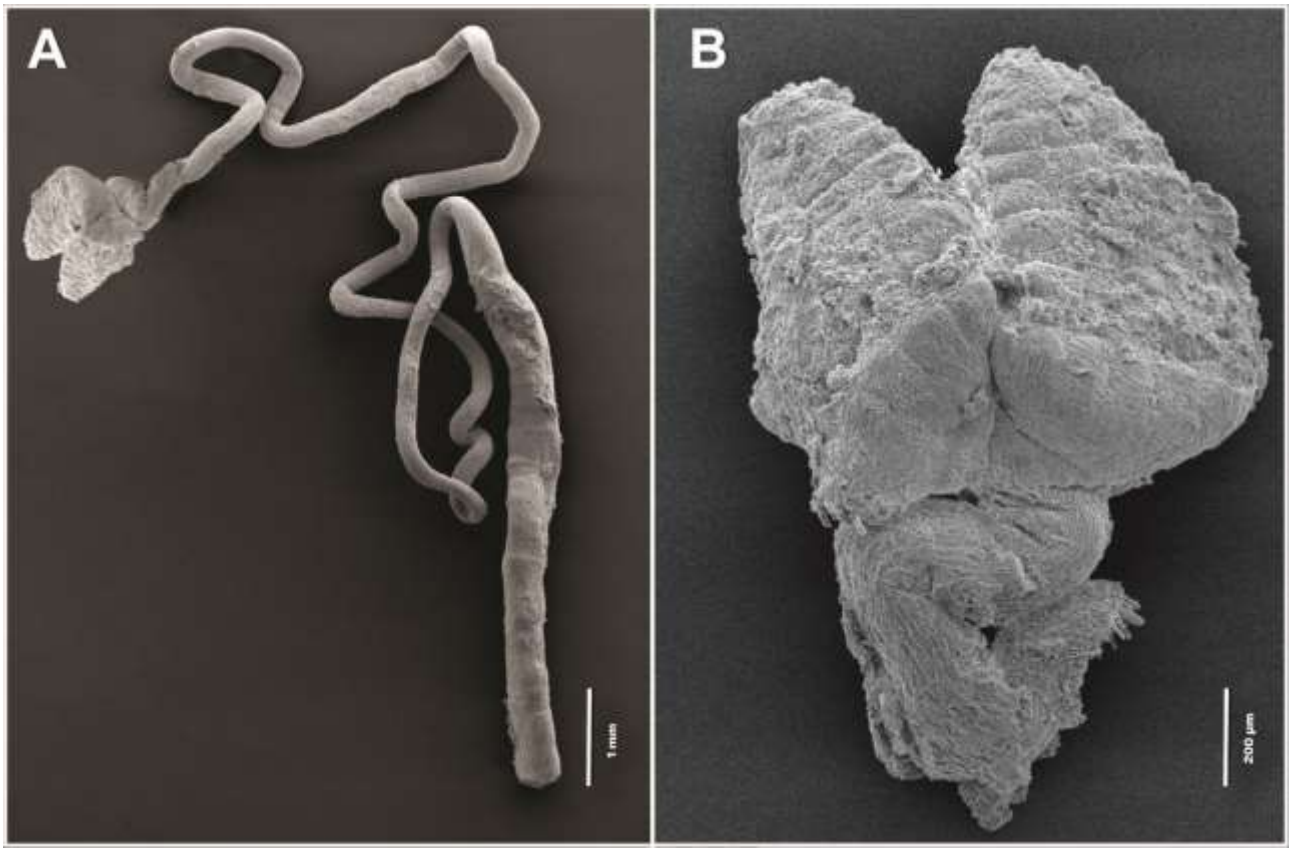


Fig. 5.1 Scanning electron micrographs of *Duplicibothrium* sp. from the South-western Indian Ocean off Scottburgh and Richards Bay, KwaZulu-Natal Province, South Africa. A, incomplete worm; B, scolex.

5.4.3. Remarks

Duplicibothrium sp. closely resembles the five valid congeners within *Duplicibothrium* (viz., *D. cairae* Ruhnke, Curran & Holbert, 2000; *D. minutum* Williams & Campbell, 1978; *D. jeannettae* Stephan & Caira, 2022; *D. colossum* Stephan & Caira, 2022; *D. bilai*), by possessing a strobila that is weakly craspedote and euapolytic, a scolex with four bothridia, fused together lengthwise in two pairs, each bothridium divided into loculi, and the presence of a cephalic peduncle. Due to the condition of the available material, the presence of some characteristics could not be confirmed. This includes the presence of an apical sucker on the bothridium, the total number and specific arrangement of loculi on the bothridia, the scutellate covering on the cephalic peduncle, the surface ultrastructural features, and the characteristics of the mature proglottids.

However, the South African *Duplicibothrium* sp. differs from *D. minutum* by possessing both transverse and longitudinal septa instead of only transverse septa, as visible in SEM micrographs (Fig. 5.1). *Duplicibothrium* sp. is also distinguished from *D. colossum* in having bothridia that are longer than wide, rather than wider than long. Furthermore, *Duplicibothrium* sp. has a smaller bothridium width than *D. colossum* [323–870 (459) vs. 684–1.208 (945)], but a significantly larger bothridium width than all other congeners (see Table 5.1). *Duplicibothrium* sp. also has a smaller cephalic peduncle width than *D. collosum* [41–169 (91) vs. 246–425 (322)]. The morphological feature that distinguishes the present specimens from the five congeners currently valid in this group is the length of the cephalic peduncle (see Table 5.1). The largest species, *D. colossum*, has a cephalic peduncle length of 2,633–4,643 (3,501) while the cephalic peduncle of *Duplicibothrium* sp. reaches up to 13 mm in length, with a mean of 10 mm. As a consequence, this distinctive characteristic is believed to be sufficient for the erection of a new species. However, the current material is compromised in terms of morphological features due to the freezing process of the hosts, which ultimately prevents the delivery of a comprehensive species description at this time.

Table 5.1

Metrical comparison of body parts and regions of species from the genus *Duplicibothrium* Williams & Campbell, 1978. All measurements are stated in micrometres. Type hosts and localities are given in bold. Abbreviations: Bo (bothridia); Cep (cephalic peduncle); f (fragmented); i (immature); L (length); Loc (loculi); m (mature); Prog (proglottid); s (short); S (scolex); T (total); W (width).

Species	*Duplicibothrium* sp.	*D. bilai*	*D. cairae*	*D. colossum*	*D. jeannettae*	*D. minutum*
Study	Present study	Stephan, Bueno & Caira (2023)	Ruhnke, Curran & Holbert (2000)	Stephan & Caira (2022)	Stephan & Caira (2022)	Williams & Campbell (1978)
Country	South-western Indian Ocean: **South Africa**	Western Indian Ocean: **Mozambique**	Northern Pacific Ocean: **Mexico**	Northern Atlantic Ocean: **Senegal**	Northern Atlantic Ocean: **Senegal**	Northern Atlantic Ocean: **Virginia, Rhode Island**
Host species	Rhinopteridae: ***R. jayakari***	Rhinopteridae: ***R. jayakari***	Rhinopteridae: ***R. steindachneri***	Rhinopteridae: ***R. marginata***	Rhinopteridae: ***R. marginata***	Rhinopteridae: ***R. bonasus***
TL	3.536–19.968 (10.561) [i & f]	4.033–5.940 (4.730) [m]	3.600–9.800 (5.400) [m]	11.200–29.400 (19.400) [m]	3.300–4.600 (4.000) [m]	2.500–6.500 [m] [strobila only]
SL	346–479 (414)	-	171–461 (309)	-	-	220–346 (289)
SW	323–870 (466)	-	250–566 (357)	-	-	140–280 (207)
T Loc	-*	37	27–33	13	31	6–8
BoL	346–479 (414)	287–384 (327)	195–399 (292)	425–688 (537)	366–484 (422)	274–350 (286)
BoW	323–870 (459)	174–260 (206)	120–312 (183)	684–1.208 (945)	180–229 (207)	70–118 (92)
Cep L	1.746–13.154 (9.056)	1.407–2.127 (1.732)	700–2.900 (1.800)	2.633–4.643 (3.501)	774–1.920 (1.298)	940–3.400 (1.600)
Cep W	41–169 (91)	118–148 (130)	-	246–425 (322)	103–134 (116)	76–110 (93)
T Prog	7–46 (21)	11–18 (14)	20–35 (25)	85–139 (109)	11–22 (17)	6–14 (10)

*, number of loculi could not be assessed due to the state of bothridia which were significantly impacted by the freezing and defrosting processes.

5.5. Discussion

When we take a closer look at the genus *Duplicibothrium*, all currently recognised species have been described as adults from species of the batoid genus *Rhinoptera*. *Duplicibothrium bilai* was described from the Oman cownose ray, *R. jayakari* from the Western Indian Ocean, Mozambique; *D. cairae* has been reported from the Pacific cownose ray, *Rhinoptera* cf. *steindachneri* Evermann and Jenkins, from the Northern Pacific Ocean, Mexico; *D. colossum* and *D. jeannettae* infects the Lusitanian cownose ray, *R marginata* Geoffroy Saint-Hilaire, 1817 (as *R. peli* Bleeker, 1863) from the Northern Atlantic Ocean, Senegal; and *D. minutum* infects the cownose ray, *R. bonasus* Mitchill, 1815 from the Northen Atlantic Ocean, Virginia and Rhode Island (see Stephan, Bueno & Caira, 2023; Ruhnke, Curran & Holbert, 2000; Stephan & Caira, 2022; Stephan & Caira, 2022; Williams & Campbell, 1978). Additional host-parasite associations have been reported for two undescribed species of *Duplicibothrium*: *Duplicibothrium* n. sp. 1, from the Brazilian cownose ray, *R. brasiliensis* Müller, 1836 from the Northern Pacific Ocean, Mexico; and *Duplicibothrium* n. sp. 6 (in Stephan & Caira, 2022), from the Australian cownose ray, *R. neglecta* Ogilby, 1912 from the Southern Pacific Ocean, Australia. Thus far, six of the seven rhinopterid hosts have been examined for *Duplicibothrium*. However, the flapnose ray, *R. javanica* Müller & Henle, 1841 (Indo-West Pacific: Oman to the Philippines, north to Ryukyu, south to eastern Indonesia) still awaits parasitological examination. It is important to acknowledge that the species identified as *Echeneibothrium javanicum* Shipley & Hornell, 1906 exhibits notable similarities with *Duplicibothrium*. This resemblance has been previously highlighted by Euzet (1994) and Ruhnke *et al.* (2000). Consequently, there is a likelihood that *R. javanica* will be included in the host list for *Duplicibothrium* once specimens from this host undergo a more detailed examination (Stephan & Caira, 2022). If this scenario unfolds, one to two species of *Duplicibothrium* will be identified in association with all seven valid species of *Rhinoptera*.

The discovery of *Duplicibothrium* sp. from the waters of South Africa exemplifies the continuous exploration necessary for a comprehensive understanding of tapeworm diversity and distribution. The distinct characteristics observed in this species, such as the presence of a unique cephalic peduncle length contribute to its differentiation from other *Duplicibothrium* species. However, as previously mentioned, due to the condition of the material, additional samples will be required to do a comprehensive species description. This study expands the geographical distribution of *Duplicibothrium* to include the waters of South Africa, further adding to its cosmopolitan distribution records.

5.6. Conclusion

This study reveals the presence of a possible new species of *Duplicibothrium* in the Oman cownose ray, *R. jayakari*, thereby expanding the geographical distribution of the genus to include the waters off South Africa. The observed morphological features in the specimens closely align with the revised description provided by Stephan *et al.* (2023). However, certain critical characteristics cannot be confirmed due to limitations with the available material, such as the absence of a strobila in the specimens. Despite these challenges, the notably long cephalic peduncle length of *Duplicibothrium* sp. raises the possibility that it represents a new species, pending a comprehensive description.

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CHAPTER 6

SUMMATIVE CONCLUSION

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CHAPTER 6: SUMMATIVE CONCLUSION

6.1. Introduction

As part of an ongoing project on the diversity of marine parasites from South Africa, the diversity of marine cestode lineages infecting the Oman cownose ray, *Rhinoptera jayakari* Boulenger of South Africa was investigated. This was accomplished through the usage of both morphological methods (such as light microscopy and scanning electron microscopy) and molecular methods (involving molecular characterisation and phylogenetic analyses). The present study offers insights into the taxonomic status of cestodes from three orders, belonging to the genera *Eniochobothrium* Shipley and Hornell, 1906 (Lecanicephalidea); *Rhinoptericola* Carvajal & Campbell, 1975 (Trypanorhyncha); *Tetrarhynchobothrium* Diesing, 1850 (Trypanorhyncha); and *Duplicibothrium* Williams & Campbell, 1978 (Tetraphyllidea). Furthermore, the research improves our understanding of the intricate host-parasite relationships between these cestodes and *R. jayakari* in South Africa's KwaZulu-Natal province. It also draws significant comparisons between the cestodes described in this study and those found in other zoogeographical regions. This study introduces five previously undocumented cestode species from the waters off KwaZulu-Natal, South Africa.

6.2. Research findings and recommendations

6.2.1. Cestode diversity in the Oman cownose ray

The Oman cownose ray (*Rhinoptera jayakari* Boulenger), belonging to the genus *Rhinoptera* Cuvier within the diverse order Myliobatiformes, is the only representative of the family Rhinopteriidae in southern African waters (Ebert *et al.,* 2021). Despite its wide distribution, all cestodes infecting this host have only been documented in the past two years from two locations, Mozambique and South Africa (see Stephan *et al.,* 2023; Oosthuizen *et al.,* 2022; Herzog & Jensen, 2022; Jensen & Caira, 2022). Before 2021, South Africa's records of cestodes from the order Myliobatiformes were limited to three species from six hosts, all in the family Dasyatidae (Schaeffner & Smit, 2019). However, due to recent descriptions, five cestode species representing three families (viz., Eniochobothriidae, Rhinoptericolidae, Serendipeidae) from the orders Lecanicephalidea, Trypanorhyncha and Tetraphyllidea are now known to infect *R. jayakari* in southern African waters.

Lecanicephalideans exhibit a worldwide distribution and have been documented in eight of the 12 marine biogeographic regions identified by Spalding *et al*. (2007). Interestingly, these

parasitic organisms have not been recorded in the marine regions of the Arctic, Southern Ocean, and Temperate Southern Africa (Jensen *et al.,* 2017). However, the investigation of *R, jayakari* from South Africa not only added a new species of *Eniochobothrium* Shipley & Hornell, 1906 but also expanded the known distribution of lecancephalideans to include a ninth biogeographical region as well as an additional host record. Currently, *Eniochobothrium* comprises four species: *E. gracile* Shipley & Hornell, 1906, *E. qatarense* Al Kawari, Saoud & Wanas, 1994, *E. euaxos* Jensen, 2005 and *E. acostae* Oosthuizen, Naidoo, Smit & Schaeffner, 2022 (described as part of the present study). However, only 38% of batoids from the Rhinopteridae family (three out of eight) have been examined for *Eniochobothrium* species. Species of this genus is known to exclusively parasitise members of the genus *Rhinoptera*, suggesting an expected species diversity of *Eniochobothrium* in this host group ranging from eight to 16 species worldwide, with each host harbouring one to two unique species. Until recently, *Eniochobothrium* was the sole genus in the family Eniochobothriidae, recognised as one of the most molecularly divergent families within the order (Jensen *et al.,* 2016). However, a study by Jensen and Caira (2022), published just 18 days after Oosthuizen *et al.* (2022), explored the tapeworm diversity in the family Eniochobothriidae. Investigating cestodes from cownose rays globally, these authors (2022) conducted a comprehensive study on six species of *Rhinoptera* and discovered eight new species in the family Eniochobothriidae and a new genus, *Amiculucestus* Jensen & Caira, 2022. The family includes two new *Eniochobothrium* species (*E. overstreeti* Jensen & Caira, 2022 and *E. vegrande* Jensen & Caira, 2022) from *R. brasiliensis* and *Rhinoptera* cf*. jayakari*, respectively. Furthermore, they described four species of the new genus: *Amiculucestus calli* Jensen & Caira, 2022 from *R. brasiliensis*, *A. australiensis* Jensen & Caira, 2022 from *R. neglecta*, *A. herzogae* Jensen & Caira, 2022 from *R. jayakari*, and *A. penghuensis* Jensen & Caira, 2022 from *Rhinoptera* cf*. jayakari*. Jensen and Caira (2022) reported that the number of recognised species in Eniochobothriidae has reached nine, and their estimate suggests that the global count of eniochobothriids likely does not exceed 27 species. The precise classification of *E. acostae* within Eniochobothriidae is currently unclear, necessitating additional phylogenetic analysis and material for a definitive determination of its specific genus placement. Despite this uncertainty, *E. acostae* brings the total number of described species in the Eniochobothriidae to ten.

Among the Cestoda, the order Trypanorhyncha stands out as the largest group, encompassing 81 genera and a current count of 315 recognised species (Beveridge *et al.,* 2017). Notably, in the waters of South Africa, 12 species of trypanorhynchs have been identified (Schaeffner & Smit in 2019, Oosthuizen *et al.,* 2021). Within the order, the family Rhinoptericolidae has recently gained significant attention. This interest stems from a

noteworthy expansion in the genus *Rhinoptericola* Carvajal & Campbell, 1975, evolving from a single member to a total of eight species, as determined in a recent revision of the family (Herzog & Jensen, 2022). The genus is recognised for parasitising hosts mainly belonging to the rajiform group (Herzog & Jensen, 2022). Only until recently was the first rhinoptericolid described in southern African waters from *R. jayakari* in Mozambique (Herzog & Jensen, 2022). In the present study two rhinoptericolid species [viz., *Rhinoptericola mozambiquensis* Herzog & Jensen, 2022 and *Rhinoptericola butlerae* (Beveridge & Campbell, 1988)] where discovered parasitising *R. jayakari* in the waters of South Africa, expanding the known geographical range for both species and identifying a new host species for *R. butlerae.* Herzog and Jensen (2022) stated that there is currently a worldwide, collaborative effort to reassess and reclassify trypanorhynch tapeworms within the Rhinoptericolidae family. This initiative is prompted by the historical reliance on morphological traits for the taxonomy of this family, which recent molecular data indicate may not consistently reflect evolutionary relationships. This was apparent in the current study, as morphological examinations revealed slight differences in the metabasal armature of *R. mozambiquensis* and minor variations in the basal armature of *R. butlerae* compared to previous descriptions of these species (see Herzog & Jensen, 2022; Beveridge & Campbell, 1988). However, despite these observed morphological distinctions, molecular analyses supported the identity of these species. Furthermore, the synergistic approach employed by Herzog and Jensen (2022) has revealed a transatlantic distribution for species within this genus. Current research expands the recognised geographical distribution of *R. mozambiquensis* and *R. butlerae* to include South Africa, emphasising the dynamic nature of species distribution patterns.

The third trypanorhynch species reported from *R. jayakari* in the waters of South Africa in the present study is from the genus *Tetrarhynchobothrium* Diesing, 1850. A total of five members are currently valid in this genus. Adult worms from this genus are described from a diverse array of batoid hosts parasitising six different genera (viz., *Raja* Linnaeus*, Myliobatis* Linnaeus*, Dasyatis* Rafinesque*, Neotrygon* Castelnau*, Brevitrygon* Last, Naylor & Manjaji-Matsumoto*,* and *Rhinoptera*) contributing to their ecological significance. The two specimens form the present study morphologically resemble *T. unionifactor* (Shipley & Hornell, 1904) found in *R. javanica* Müller & Henle, in the waters off Sri Lanka (Shipley & Hornell, 1906). Ebert *et al.* (2021) confirmed that *R. jayakari* in Southern Africa constitutes an independent species closely related to *R. javanica*, which consequently raises the possibility that *R. jayakari* may serve as a suitable host for the parasite in the southwestern Indian Ocean. Therefore, the current study introduces *R. jayakari* as a new host species, suggesting that the parasite is specifically adapted to closely related *Rhinoptera* hosts. This means that an additional cestode family, Tetrarhynchobothriidae, might be known from *R. jayakari*.

The cestode family Serendipeidae within the order Tetraphyllidea currently encompasses 15 valid species in four genera, with an additional three provisionally identified species (Stephan *et al.,* 2023). Cestodes in this family were believed to only be associated with cownose rays belonging to the genus *Rhinoptera* (Stephan *et al.,* 2023). However, Stephan *et al.* (2023) made a significant discovery by identifying a new but unnamed species, *Serendip* n. sp. 1, within the rough eagle ray, *Aetomylaeus asperrimus* Gilbert, 1898, in Panama, expanding the potential host range from Rhinopteridae to also include Myliobatidae. Consequently, this discovery implies that the global diversity within this cestode family may be more extensive than previously thought. The present study identified a potential new species of *Duplicibothrium* that infects *R. jayakari*. The distinctive elongated cephalic peduncle observed in this cestode morphologically separates it morphologically apart from other members of the genus, suggesting that it may be a new species that awaits formal description.

This study documents five cestodes parasitising the Oman cownose ray, *R. jayakari*, off KwaZulu-Natal, South Africa. In particular, it marks the first recorded instances of eniochobothriid and tetrarhynchobothriid species in southern African waters, as well as the first reports of rhinoptericolid and serendipeid cestodes in South African waters. This is particularly intriguing given that South Africa is recognised as a biodiversity hotspot for chondrichthyan fishes and ranks as the third most biodiverse country globally (Weigmann, 2016). Furthermore, the discovery of *E. acostae*, *R. butlerae*, and *T. unionifactor* in *R. jayakari* as a previously unexplored host expands our understanding of the parasites associated with this host species. The study also reveals additional morphological characteristics for *R. butlerae* and *R. mozambiquensis*. While the molecular data indicated no differences, the morphological discrepancies are attributed to intraspecific variability, highlighting the difficulties of species characterisation based on morphological grounds. This emphasises the importance of an integrative approach that incorporates both morphological and molecular methods for a refined understanding of biological diversity.

6.2.2. Challenges and future recommendations

While documenting the parasite fauna and discovering new species in South Africa holds great potential to advance scientific research, several challenges hinder these efforts. The first of these obstacles is the scarcity of experts and systematists capable of comprehensively assessing the diversity of parasitic groups, including protists, myxozoans, helminths, and hirudineans. Another significant challenge arises from the difficulty in sampling chondrichthyans for parasitological studies. This is particularly daunting as 29% of South African chondrichthyans, including 13% of endemic species, are listed as threatened on the

IUCN Red List. Furthermore, the assessment of internal parasites requires lethal sampling of host individuals, making it challenging to obtain permits for such parasitological work. An alternative approach to overcome this dilemma involves fostering collaborations with organisations and participating in scientific trawls. This collaborative strategy may offer a more feasible avenue to advance our understanding of parasitic fauna in the region while navigating the complexities associated with threatened chondrichthyans and regulatory constraints. Furthermore, the absence of surface ultrastructural features in all the documented cestode specimens from this study can be attributed to the fact that the batoids from the KwaZulu-Natal province were collected after hosts' suffocation in shark nets and subsequent freezing of specimens by the KwaZulu-Natal Sharks Board. This ultimately led to the disintegration of most parasites. As a consequence, numerous species obtained from this region either lacked parasites or provided only fragments of indistinguishable species, making it difficult to describe the anatomy of the strobila or external body features. To address this, future collections should prioritise obtaining fresh host samples, enabling immediate dissection and organ screening for parasites. Although molecular analyses were attempted and succeeded for three species, the disintegration of the parasite material hindered the collection of molecular samples for others. The study could have benefited significantly from a comprehensive SEM investigation and molecular analyses, which should be prioritised in future research efforts.

6.3. Conclusion

Temperate Southern Africa, a 'hotspot' for elasmobranch diversity, harbours a hidden diversity of cestodes. Exploring additional hosts and localities, is likely to unveil numerous new species, further enriching an already biodiverse region. Despite the discovery of a new species, together with new host and distribution records, the present study only scratches the surface of information from a single host species. Numerous elasmobranch hosts remain unexplored for marine parasites, highlighting the need for comprehensive assessments. The absence of molecular and phylogenetic analyses supporting morphological characterisation leaves a significant gap in understanding phylogenetic relationships among congeners and the accurate identification of morphologically similar species. Given the current human-induced threats to elasmobranch populations worldwide, this study highlights the importance of a synergistic approach for future research. The discovery of new species, clarification of evolutionary relationships, and expansion of geographic distributions contribute to a more comprehensive understanding of the diversity and ecology of marine parasites. Continuous research efforts, especially in regions with limited exploration, are imperative for filling

knowledge gaps on this unique host-parasite system in general and tapeworms and their interactions with elasmobranch hosts in particular.

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APPENDICES

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Pettersson, E.U., Ljunggren, E.L., Morrison, D.A., Mattsson, J.G., in press. Functional analysis and localisation of a delta-class glutathione S-transferase from Sarcoptes scabiei. Int. J. Parasitol.

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the accession number should always be capitalised. (See the example). When published they will appear in normal type.

Example: ′ GenBank accession nos. **AI631510**, **AI631511**, **AI632198**, and **BF223228**), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank accession no. **AA361117**)′.

Additionally, any multiple alignments of nucleotide or protein data must be submitted to a recognised database and must also receive a unique accession number. The accession number can appear in the text in the relevant section of the Results, as: ′ Alignment files are available by anonymous FTP from FTP.EBI.AC.UK in directory/pub/databases/embl/align or via the EMBLALIGN database via SRS at http://srs.ebi.ac.uk; under accession(s)′. The usual method for submitting alignments is by the World Wide Web to the European Bioinformatics Institute (via WebinAlign: http://www.ebi.ac.uk). Microarray data, in MIAME-compliant format, should be submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) or GEO (http://www.ncbi.nlm.nih.gov/geo/). Accession identifiers relating to the data should be provided in the manuscript text.

Policy on bioinformatics papers. In silico analysis: The following guidelines apply to papers that exclusively use in silico analysis or rely heavily on this approach for analysis and conclusions. Such papers should address a significant biological issue or issues. Bioinformatic data should be supported by novel or published biological data. Work would typically use information from a number of databases and even from a number of parasite or host species and use a number of analytical methods. Types of ′ metaanalysis′ are encouraged either across a wide range of parasites or, say, at a number of points in a metabolic or signalling pathway or an immune cascade. In silico analysis may be especially suitable for review articles.

Guidelines for reporting of protein identifications using mass spectrometry

The following information should be provided for protein or peptide identifications using mass spectrometry:

1. The program, and version number, used to create peak lists and the parameters used in the creation of the list.
2. The program, and version number, of the program used for database searching. Parameters used for searching should be specified, including, but not limited to, precursor-

ion mass tolerance, fragmention mass tolerance, modifications allowed for, missed cleavages and enzymes used in protein cleavage.

3. The name and version number of the sequence database used in searches. If a custom-made database is used then complete information on the origin of the sequences and database size should be disclosed. Given the dependence of scoring on database size, the use of a small database, or one excluding contaminants, should be justified.
4. A short description of the methods use to interpret the significance of search results, including any statistical analysis, confidence thresholds and other values specific to judging the certainty of the identification.
5. For large-scale experiments a false-positive determination should be reported. This may be the result of randomised database searches or other approaches.
6. Each protein identification should include the accession number, score generated by the search algorithm used, sequence coverage and the number of unique peptide sequences assigned in the protein identification.
7. Single peptide identifications should include an annotated MS/MS spectrum showing fragment assignments together with the peptide sequence, precursor mass, charge and error.
8. Identifications arising from peptide mass fingerprinting should include an annotated mass spectrum. The number of matched peaks, the number of unmatched peaks and the sequence coverage should also be reported along with all parameters and thresholds used to analyse the data. This includes mass accuracy, resolution, calibration methods, contaminant exclusions along with the scoring scheme used and measure of the false-positive rate.

Author inquiries

For inquiries relating to IJPPAW please contact the Managing Editor of IJPPAW at: IJPPAW@elsevier.com. You can track accepted articles at https://www.elsevier.com/trackarticle and set up e-mail alerts to inform you of when an article's status has changed. Also accessible from here is information on copyright, frequently asked questions and more. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher.

APPENDICS 2: ETHICS APPROVAL

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Senate Committee for Research Ethics
Tel: 018 299-4849
Email: nkosinathi.machine@nwu.ac.za

ETHICS APPROVAL LETTER OF STUDY

Based on the review by the **Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)**, the Committee hereby clears your study as no ethical risk. This implies that the FNASREC grants permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Biodiversity assessment of marine cestode lineages infecting dominant batoid species off the KwaZulu-Natal Province, South Africa
Study Leader/Supervisor: Prof NJ Smit
Student: G Oosthuizen

Ethics number:

N	W	U	-	0	1	7	7	7	-	2	0	-	A	9
Institution				Study Number						Year			Status	

Status: S – Submission; R – Re-Submission; P – Provisional Authorisation; A – Authorisation

Application type: Single **Risk Category:** **No Risk**

Commencement date: 01/11/2020
Expiry date: 01/02/2022

General conditions:
The following general terms and conditions apply:

- *The commencement date indicates the date when the study may be started.*
- *In the interest of ethical responsibility, the NWU-SCRE and FNASREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the FNASREC or that information has been false or misrepresented;*
    - *submission of the annual (or otherwise stipulated) monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *FNASREC can be contacted for further information or any report templates via Roelof.Burger@nwu.ac.za 018 299 4269*

The FNASREC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the FNASREC or the NWU-SCRE for any further enquiries or requests for assistance.

Yours sincerely,

Prof Roelof Burger
Chairperson Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)

File reference: 9.1.5.4.2

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