Diversity of digenean trematodes of *Clinus superciliosus* (Linnaeus, 1758) from the coast of South Africa

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ABSTRACT

To date, 87 digenean species from 63 genera of 24 families have been described or reported from marine fishes in South Africa. However, when considering the range of available hosts along this biodiversity-rich coastline, this is not all there is to be found. From these, two species have been found in the super klipfish *Clinus superciliosus* from Port Elizabeth: *Coitocaecum capense* and *Helicometra fasciata*. The present study aimed to further explore the trematode diversity of this endemic fish host by incorporating more and dispersed sampling localities. Specimens of *C. superciliosus* collected from Saldanha Bay, Cape Town harbour, Hermanus, Tsitsikamma National Park, Jeffreys Bay and Chintsa, were subjected to helminthological examination. Digenean trematodes found were characterised by molecular (28S rDNA, ITS1-5.8S-ITS2, ITS2, *cox*1) and morphological analyses. This revealed the presence of metacercariae belonging to four species: *Cardiocephaloides physalis*, *Cardiocephaloides* sp., *Dollfustrema* sp., *Stephanostomum* sp.; and adult specimens of ten species: *Co. capense*, *Coitocaecum* sp. 1, *Coitocaecum* sp. 2, *Coitocaecum* sp. 3, *Helicometra* sp. 1, *Helicometra* sp. 2, Hemiuridae gen. sp. 1, Hemiuridae gen. sp. 2, *Proctoeces* sp. and Zoogonidae gen. sp. Thus, *C. superciliosus* is now known to host digeneans from seven families (Acanthocolpidae, Bucephalidae, Fellodistomidae, Hemiuridae, Opecoelidae, Strigeidae and Zoogonidae).

This is the first report of *C. superciliosus* as second intermediate host to digenean species of the families Acanthocolpidae, Bucephalidae and Strigeidae, as well as definitive host to species of the families Fellodistomidae, Hemiuridae and Zoogonidae. Except for *Co. capense*, all other species are reported from this fish host for the first time. This study also provides the first molecular characterisation of adult digeneans from South Africa as well as the molecular characterisation of digenean species from this fish host. The astonishing diversity of digeneans found from a single fish species, supports the theories that the marine parasite diversity in South Africa is vastly understudied, thereby highlighting the opportunity for future explorative taxonomic and biodiversity research along this coastline.

Key words: Digenea, Trematodes, Fish parasites, Marine, South Africa, Intertidal fishes, Genetics, Morphology

Table of contents

PREFACE

This dissertation follows the article format as prescribed by North-West University. Table I below provides the contribution of each author to the various manuscripts and their consent for use as part of this dissertation.

Table I. Contributions of each author to the various chapters of this dissertation and their consent for the work to be used as part of this dissertation.

Author	Chapter	Contribution	Consent
A. Vermaak	1-6	Responsible for study design, fieldwork, taking photographs of specimens and hosts, molecular laboratory work, morphological laboratory work, data analyses, digitalising drawings, drafting and editing of the dissertation and manuscripts.	
N.J. Smit	1-6	Responsible for study design, provided support in the field, gave input on writing and editing of the dissertation and manuscripts.	
O. Kudlai	1-6	Responsible for study design, haplotype network analyses (Chapter 3), provided support and input with genetic analyses and morphological work, providing of literature sources, editing of the dissertation and manuscripts.	

The manuscripts have been/are aimed to be submitted to the following journals:

Chapter 3 – Folia Parasitologica (accepted)

Chapter 4 – Systematic Parasitology or journal of similar level.

Chapter 5 – Parasitology Research or journal of similar level.

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 5

Chapter 1

General Introduction

Chapter 1. General Introduction

Digenean trematodes (Subclass Digenea Carus, 1863)

There are more parasitic organisms in the world, than non-parasitic organisms and nearly all free-living organisms are infected with one or various types of parasites (Roberts & Janovy, 2009). This has occurred throughout evolutionary times, as the body of each organism offers a variety of habitats for a diversity of parasites to thrive (Roberts & Janovy, 2009). The phylum Platyhelminthes is a large phylum of flatworms, many of which are parasitic. These worms are normally characterised by a dorsoventrally flattened and bilaterally symmetrical soft body as well as having clear anterior and posterior ends (Roberts & Janovy, 2009). Within the superclass Neodermata of the phylum Platyhelminthes, lies the class Trematoda, consisting of two subclasses – Aspidogastrea Faust & Tang, 1936 (alternatively referred to as Aspidocotylea or Aspidobothrea) and Digenea Carus, 1863 (Cribb, 2005; Kudlai *et al*., 2018). Aspidogastrea are cosmopolitan parasites of molluscs and live in vertebrate final hosts, such as fishes or turtles (Alves *et al*., 2015). Digenea are the second most abundant group of cosmopolitan parasitic worms, succeeding nematodes (Roberts & Janovy, 2009). The various life stages of these worms are able to parasitise virtually every organ of their vertebrate hosts' bodies, especially that of marine fishes.

Digeneans can have a large variety of body shapes and sizes, but most are dorsoventrally flattened and elongate-oval (Kudlai *et al*., 2018; Lucius *et al*., 2017). Although, some can be as thick as they are wide and range from long, elongated bodies to round bodies (Cribb, 2005). Digeneans normally have an oral sucker surrounding the mouth, as well as a ventral sucker on more distal parts of the body. Additionally, these trematodes can have various structures present on their outer tegumental layers, including spines, ridges, pits, sensory papillae and even some vesicles (Cribb, 2005; Kudlai *et al*., 2018). Although these organisms might seem simple, they possess intricate nervous, musculature and excretory systems, some of which can differ between the varying life stages (Cribb, 2005; Galaktionov & Dobrovolskij, 2003). Almost all digeneans are hermaphroditic and are able to self-fertilise, but they tend to rather find other adult trematodes in the host by means of chemoattractants such as cholesterol and then cross-fertilise (Galaktionov & Dobrovolskij, 2003; Klimpel *et al*., 2019). Only a few species are able to reproduce parthenogenetically, as most require cross-fertilisation to produce viable offspring (Roberts & Janovy, 2009).

The Greek name Digenea (*di*=two, *génos*=ancestry, generation) refers to the alteration between the various generations that reproduce either sexually (adults) or asexually (larval stages) (Lucius *et al*., 2017). Their complex life cycle generally includes two intermediate and one definitive host (Fig. 1). The first intermediate host typically being a mollusc or in rare cases, an annelid; the second intermediate host can include a variety of vertebrate and invertebrate hosts; whilst the definitive host is nearly always a vertebrate (Galaktionov & Dobrovolskij, 2003; Ginetsinskaya, 1988; Kostadinova & Pérez-del-Olmo, 2014). Firstly, the egg is released from the adult trematode inside the definitive host (Klimpel *et al*., 2019). The egg contains a developing embryo or sometimes even a fully developed miracidium, which is encapsulated (Klimpel *et al*., 2019; Roberts & Janovy, 2009). In some species, it is seen that the miracidia already hatch within the uterus of the parent (Roberts & Janovy, 2009). Though, for most species, the eggs hatch in water outside the definitive host or when eaten by a suitable first intermediate host (Lucius *et al*., 2017). Except for the key availability of water, there are a couple of factors that have an effect on the hatching of digenean eggs, including oxygen concentration, pH, water temperature, light, osmotic pressure or the presence of host enzymes that initiate hatching (Roberts & Janovy, 2009).

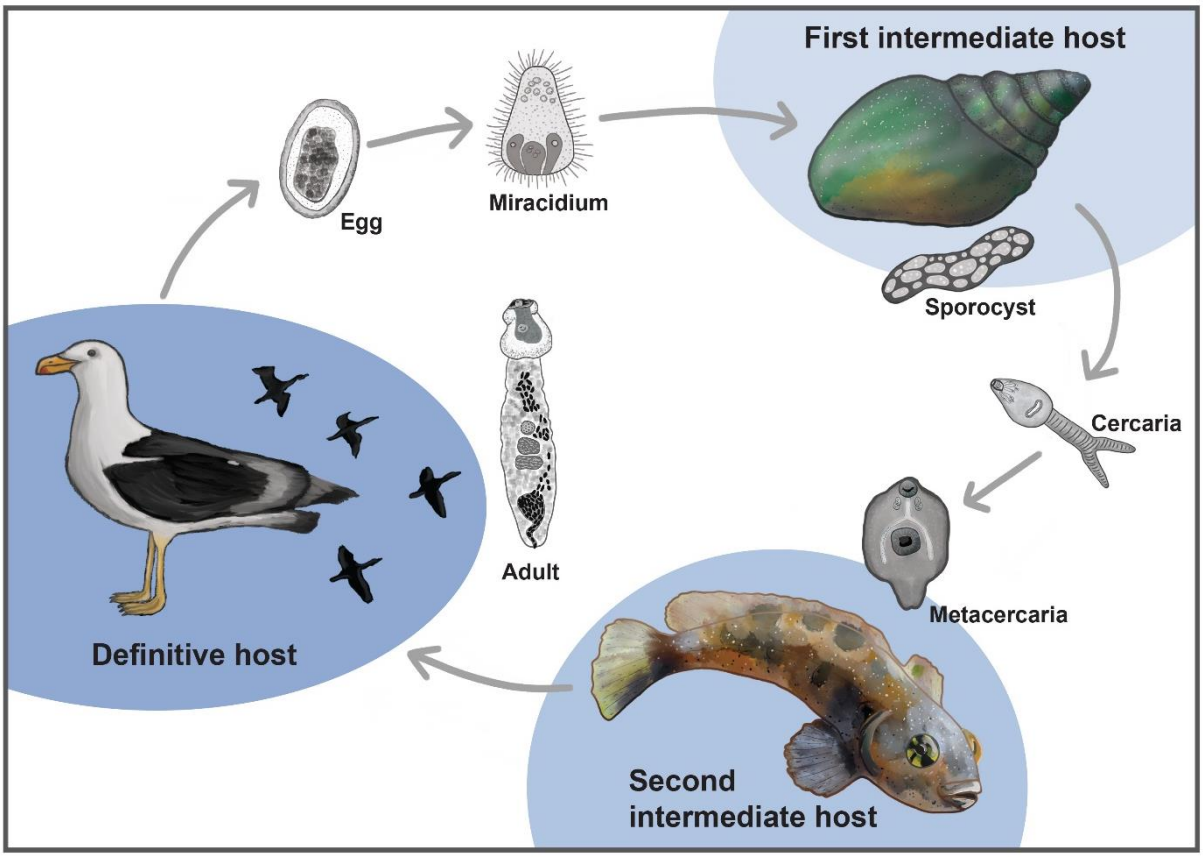

Figure 1. Schematic diagram of the life cycle of digenean trematodes of the genus *Cardiocephaloides.* (Not drawn to scale)

From the egg emerges the miracidium – this small, normally ciliated larval stage is either consumed by the first intermediate host or penetrates the tissues of this host, shedding its ciliated tegument (Cribb, 2005; Ginetsinskaya, 1988). Once in the correct site within the first intermediate host, typically the hepatopancreas or gonads of the molluscan host, the miracidium metamorphosises into a mother sporocyst (Klimpel *et al*., 2019; Kudlai *et al*., 2018). The main goal of the sporocyst is to support the developing embryos that it contains and is sometimes referred to as a germinal sac (Roberts & Janovy, 2009). The embryos in the sporocyst can then develop into either daughter sporocysts or rediae, which can further develop into daughter rediae (Cribb, 2005; Klimpel *et al*., 2019). The daughter rediae or daughter sporocysts produce cercariae, which emerge from the first intermediate host and start their search for either a definitive host or second intermediate host, depending on the species (Cribb, 2005; Kudlai *et al*., 2018). This free-living stage employs various strategies to detect and enter their next host including (i) active movement in search of the host, (ii) passive gliding in the water and (iii) waiting poses. Different host-recognition mechanisms such as photodetection, chemo-orientation, detecting a thermal gradient, reacting to water turbulence and touch (Ginetsinskaya, 1988; Haas, 2003; Roberts & Janovy, 2009) play an important role in predator evasion and dispersal of cercariae in the environment.

Thereafter, the cercariae can either penetrate or be eaten by a second intermediate host or they can encyst on vegetation and objects in the water or float freely until being ingested by a definitive host (Galaktionov & Dobrovolskij, 2003; Klimpel *et al*., 2019). The encysted cercariae then develop into metacercariae (or adolescariae in the environment outside a host) and wait to excyst when ingested by the definitive host (Lucius *et al*., 2017; Roberts & Janovy, 2009). However, some species from the genus *Alaria* Greville, 1830 first develop into an intermediate form called mesocercariae and the life cycle of others, such as blood flukes, do not incorporate a metacercarial stage (Roberts & Janovy, 2009). Once in the definitive host, the metacercariae, or cercariae of some species, migrate to the site within the host where they are most suited to live and develop into adult trematodes that are sexually mature (Galaktionov & Dobrovolskij, 2003; Roberts & Janovy, 2009). When considering this multitude of physiological and chemical environmental changes that digeneans have to overcome as each stage emerges from one host to another, it is clear that these trematodes are tremendously well adapted to a parasitic lifestyle (Roberts & Janovy, 2009). Digeneans have a higher host specificity for their molluscan hosts than for their vertebrate hosts, therefore it can be assumed that they first parasitized molluscs before including vertebrates in their life cycle (Roberts & Janovy, 2009).

Diversity of digenean trematodes in marine fishes in South Africa

Digenea comprises of about 18,000 species that have been described from an array of hosts globally (Bray, 2008; Kostadinova & Pérez-del-Olmo, 2014). According to Cribb and Bray (2010), about 5,000 of these species have been described from fishes, yet further yearly descriptions indicate that their numbers might in fact be much higher. Due to their economic and medical importance, trematodes of the subclass Digenea have been widely studied and thus large amounts of literature is available on these parasitic worms. Except for a multitude of taxonomic, phylogenetic and medical studies, these trematodes have been incorporated in various research fields including, but not limited to, parasitic biological tagging in the monitoring and assessment of fish populations and stock (Reed *et al*., 2012; Weston *et al*., 2015; Ukomadu, 2017 (unpublished PhD thesis)), their effect on host organisms (Gordon *et al*., 1998; Johnson *et al*., 2001; Santoro *et al*., 2007)), physiology and biology (Justine *et al*., 1993; Krupenko *et al*., 2016; Miquel *et al*., 2000; Niewiadomska & Mocozoń, 1982), ecology (Valtonen & Gibson, 1997), life cycle (Bartoli *et al*., 2000; Cribb *et al*., 1998; Dias *et al*., 2003; Nolan & Cribb, 2004), life cycle strategies (van Beest *et al*., 2019) as well as the influence of environmental factors on these trematodes (Thieltges & Rick, 2006), to list just a few.

The majority of taxonomic studies done on digenean trematodes of marine fishes from South Africa occurred mainly during the 20th century, with only limited, scattered reports during the 21st century (Fig. 2). The very first marine parasite from South Africa was described nearly 200 years ago, however the first digenean trematode from this region, *Probolitrema richiardii* (López, 1888) Looss, 1902 (described as *Probolitrema capense*), was only described 116 years ago by Looss (1902) from a shark of the family Scyliorhinidae Gill, 1862 caught in Cape Town (Looss, 1902). A period of thirty-six years lapsed before Fantham (1938) recorded *Lecithostaphylus retroflexus* (Molin, 1859) Odhner, 1911 (Zoogonidae) from a Hottentot fish, *Spondyliosoma blochii* (Valenciennes, 1830), collected in Cape Town (Fantham, 1938). Thereafter, another 20 years passed before the third digenean was described from South Africa – during 1956 Prudhoe described *Pseudaephnidiogenes rhabdosargi* (Prudhoe, 1956) Yamaguti, 1971 (as *Aephnidiogenes rabdosargi*) (Aephnidiogenidae) from the Goldlined seabream *Rhabdosargus sarba* (Forsskål, 1775) collected from Durban, Natal (Prudhoe, 1956). Between 1976 and 1979, a group of researchers from the Soviet Union reported or described 16 digenean species from marine fishes during a global expedition (Parukhin, 1976; Gavrilyuk-Tkachuk, 1979). Later, Parukhin (1989) reported or described an additional 14 digenean species from South African marine fishes. In 1983, Gibson reported the presence of *Kenmackenzia gigas* (Nardo, 1827) (Sclerodistomidae) from fishes collected in Cape Town.

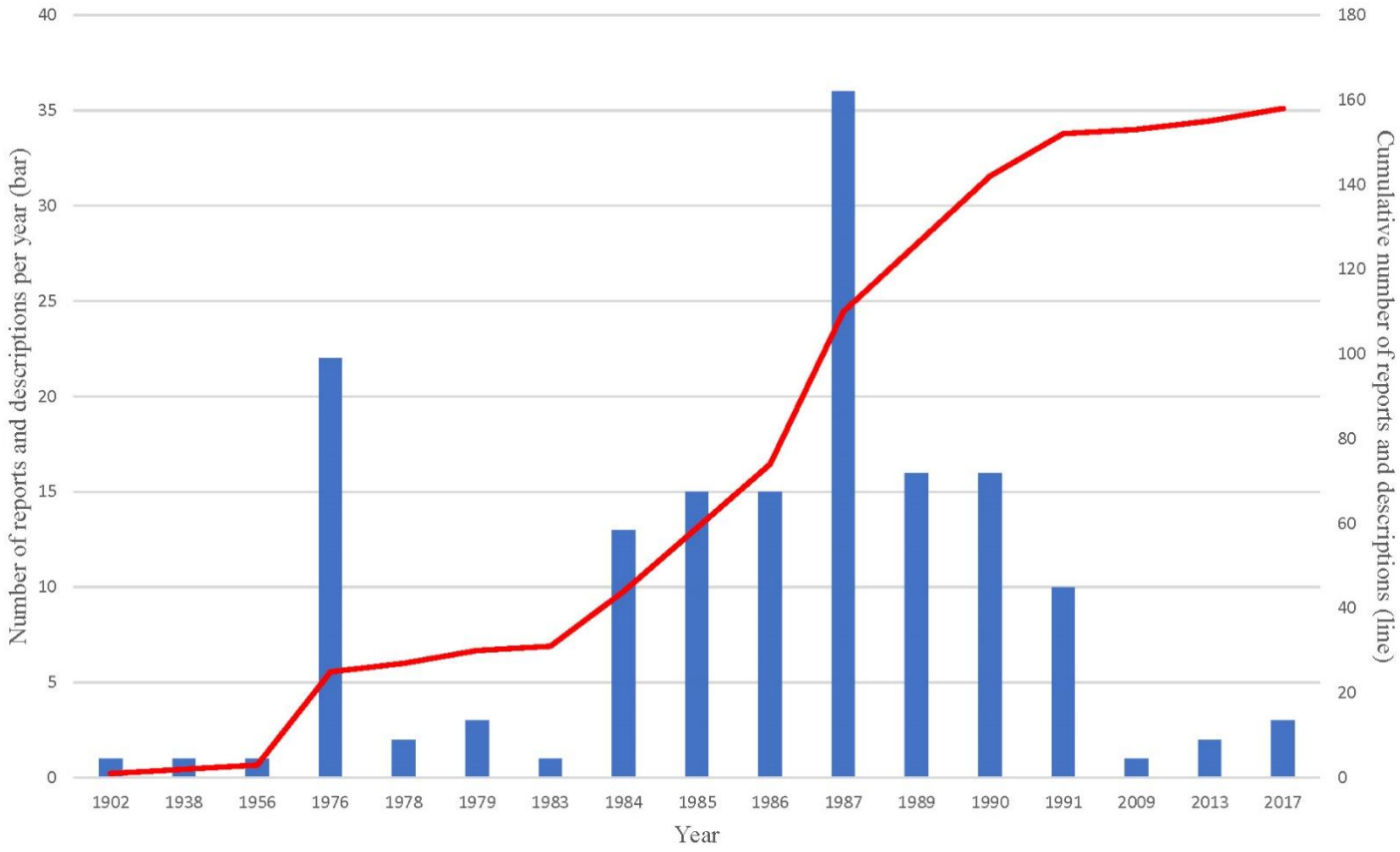

Figure 2. Number of annual reports and descriptions of digeneans from marine fishes in South Africa. The blue bars represent the number of reports and descriptions per year, whereas the red line illustrates the cumulative number of reports and descriptions over time

During his exploration of digenean trematodes from marine fishes in South Africa, Bray contributed immensely to the available literature on trematodes from this region, with a series of papers from 1978 to 1991. Within this paper series, he recorded a total of 53 digeneans from fishes found along the South African coast, including 17 newly described species (Bray, 1978, 1984, 1985, 1986a, 1986b, 1978a, 1987b, 1990, 1991). Of these, one species of the **Aephnidiogenidae**: *Pseudaephnidiogenes rossi* Bray, 1985 ex *Caffrogobius nudiceps* (Valenciennes, 1837); one species of the **Cryptogonimidae**: *Aphallus rubalo* Bray, 1986 ex *Cheimerius nufar* (Valenciennes, 1830); four species of the **Enenteridae**: *Enenterum elsti* Bray, 1978, *E. prudhoei* Bray, 1978, *E. stinkvis* Bray, 1986 and *E. tongaatense* Bray, 1986 all collected from *Neoscorpis lithophilus* (Gilchrist & Thompson, 1908); two species of the **Hemiuridae**: *Elytrophalloides humerus* Bray, 1990 ex *Trachinotus botla* (Shaw, 1803), *Lecithochirium parafusiforme* Bray, 1991 ex *Gymnothorax flavimarginatus* (Rüppell, 1830); one species of the **Lepocreadiidae**: *Clavogalea gaevskayae* Bray, 1985 ex *Trachinotus botla* (Shaw, 1803); five species of the **Opecoelidae**: *Pseudopecoelus ablennesi* Bray, 1987 ex *Ablennes hians*

(Valenciennes, 1846), *Dactylostomum griffithsi* Bray, 1987 ex *Cheilodactylus fasciatus* Lacepède, 1803, *Margolisia vidalensis* Bray, 1987 ex *Trachinocephalus myops* (Forster, 1801), *Allopodocotyle recifensis* Bray, 1987 ex *Pterogymnus laniarius* (Valenciennes, 1830), *Coitocaecum capense* Bray, 1987 ex *Clinus superciliosus* (Linnaeus, 1758), *C. cottoides* Valenciennes, 1836, *Cirrhibarbis capensis* Valenciennes, 1836, *Clinus rotundifrons* Barnard, 1937, *Xenopoclinus kochi* Smit, 1948, *X. leprosus* Smit, 1961 and two species of the **Zoogonidae**: *Overstreetia sodwanaensis* Bray, 1985 ex *Atherinomorus pinguis* (Lacepède, 1803), *Cephaloporus bakeri* Bray, 1985 ex *Pervagor melanocephalus* (Bleeker, 1853), were newly described (Bray, 1978, 1985, 1986a, 1986b, 1987a, 1990, 1991). Sampling for these studies was conducted in the Western Cape (Cape Town, Oudekraal), Eastern Cape (Algoa Bay, Cape Recife, Port Elizabeth, van Standens River mouth) and Natal (Cape Vidal, Durban, Kerridene, La Mercy, Mapelane, Mbibi, Richards Bay, Sodwana, St Lucia, Tongaat, Umhlanga Rocks, Umvoti, Uvongo) provinces (Bray, 1978, 1984, 1985, 1986a, 1986b, 1987a, 1987b, 1990, 1991).

Throughout the 21st century, merely five reports of digeneans from marine fishes have been made. The first of which was in 2009 by Yeld, who reported the presence of *Probolitrema richiardii* (López, 1888) Looss, 1902 (Gorgoderidae) from the Dark shyshark *Haploblepharus pictus* (Müller & Henle, 1838) (Scyliorhinidae) which was collected on the west coast of South Africa (Yeld, 2009). Le Roux (2013) reported *Tergestia laticollis* (Rudlophi, 1819) Stossich, 1899 (Fellodistomidae) and *Ectenurus lepidus* Looss, 1907 (Hemiuridae) from the Cape horse mackerel *Trachurus capensis* Castelnau, 1861 (Carangidae), collected in Port Elizabeth. During research for a PhD thesis, Ukomadu (2017) reported the presence of metacercarial trematodes of the species *Cardiocephaloides physalis* (Lutz, 1927) Sudarikov, 1959 from the South American pilchard *Sardinops sagax* (Jenyns, 1842) (Clupeidae), collected from various localities along the South African coast. The last report of a digenean trematode in South Africa was an unknown species of *Rhipidocotyle* Diesing, 1858 (Bucephalidae) from the Oilfish *Ruvettus pretiosus* Cocco, 1833 (Gempylidae), collected on the west coast of South Africa (Nunkoo *et al*., 2017). Shore sampling localities for all the above mentioned previous research endeavours are shown in Figure 3 below. Offshore sampling localities include the Indian Ocean, Agulhas Bank and South Atlantic Ocean. The most reports or descriptions were from fishes collected in Durban, Port Elizabeth, Sodwana, Cape Town and Oudekraal, respectively. Table A-A1 contains a summary of all known digenean reports or descriptions from marine fishes in South Africa.

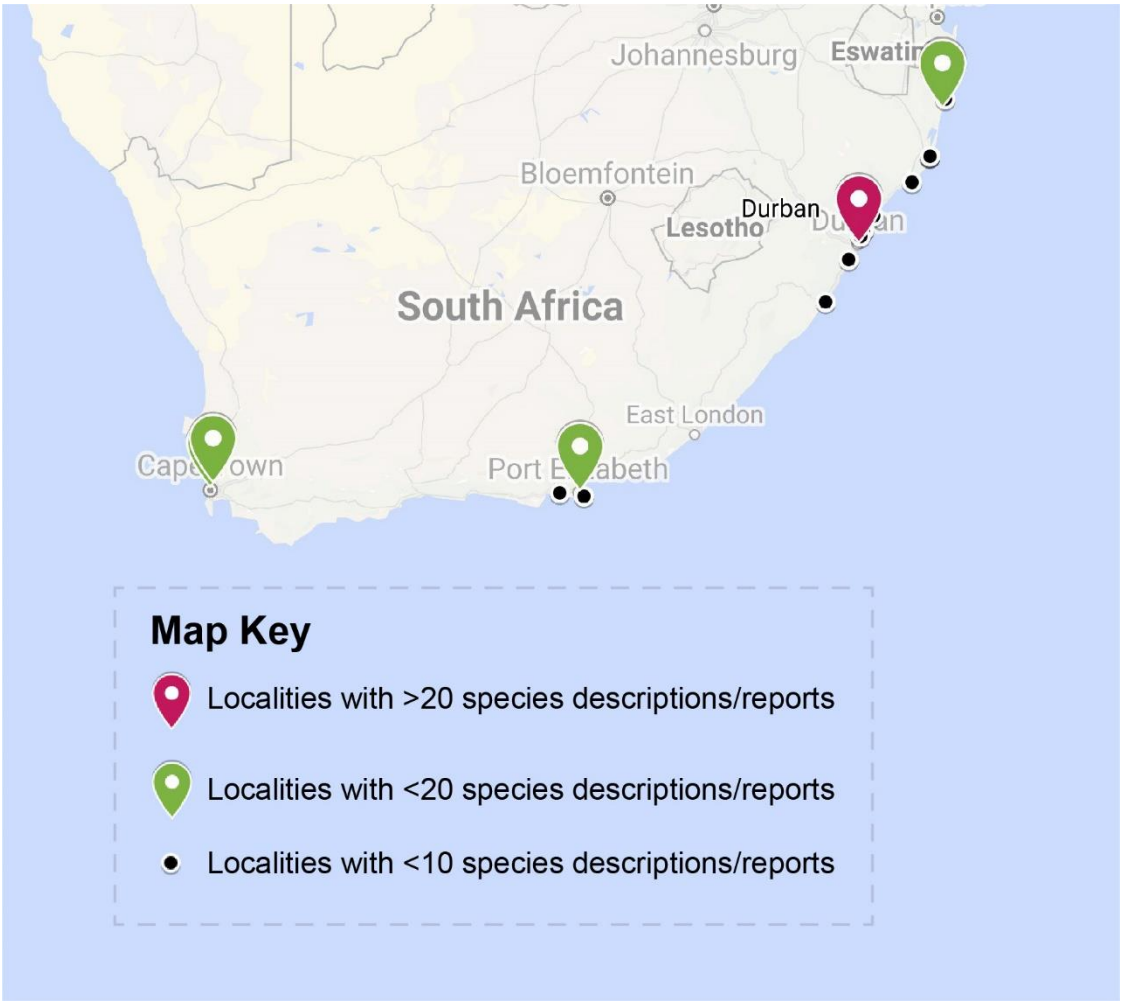

Figure 3. Map illustrating the study localities of previous research endeavours, with the amount of species descriptions and reports at each site

Collectively, a total of 87 digenean species have been reported or described from marine fishes found in South Africa. These form part of 63 genera and 24 digenean families described from 91 species of 46 marine fish families. Of these, the most abundant digenean families are the Hemiuridae and Opecoelidae respectively, as seen in Figure 4. This can be ascribed to the two papers focusing on the Hemiuridae, published by Bray (1990, 1991). He believed that species from this family have been neglected and there were some deficient descriptions that he wished to rectify, thereby greatly increasing descriptions and records for this digenean family from marine fishes in South Africa. This trematode family was also focused on by Parukhin (1976, 1989). In total, 18 species of the Hemiuridae have been reported or described from 28 species of nine families of marine fishes from South Africa. The Opecoelidae Ozaki, 1925 is the most diverse digenean family globally, consisting of more than 90 genera and about 900 species (Bray *et al*., 2016). According to Bray *et al*. (2016), this digenean family is almost exclusively found in marine and freshwater teleost fishes. Therefore, it can be expected that this family will represent

a large portion of the reports and descriptions from fish hosts in this area. Fifteen opecoelid species have been described from 23 species of 14 families of marine fishes found along the South African coast. The remainder of digenean families encountered in marine fishes from South Africa, ranges between one to seven descriptions or reports per family.

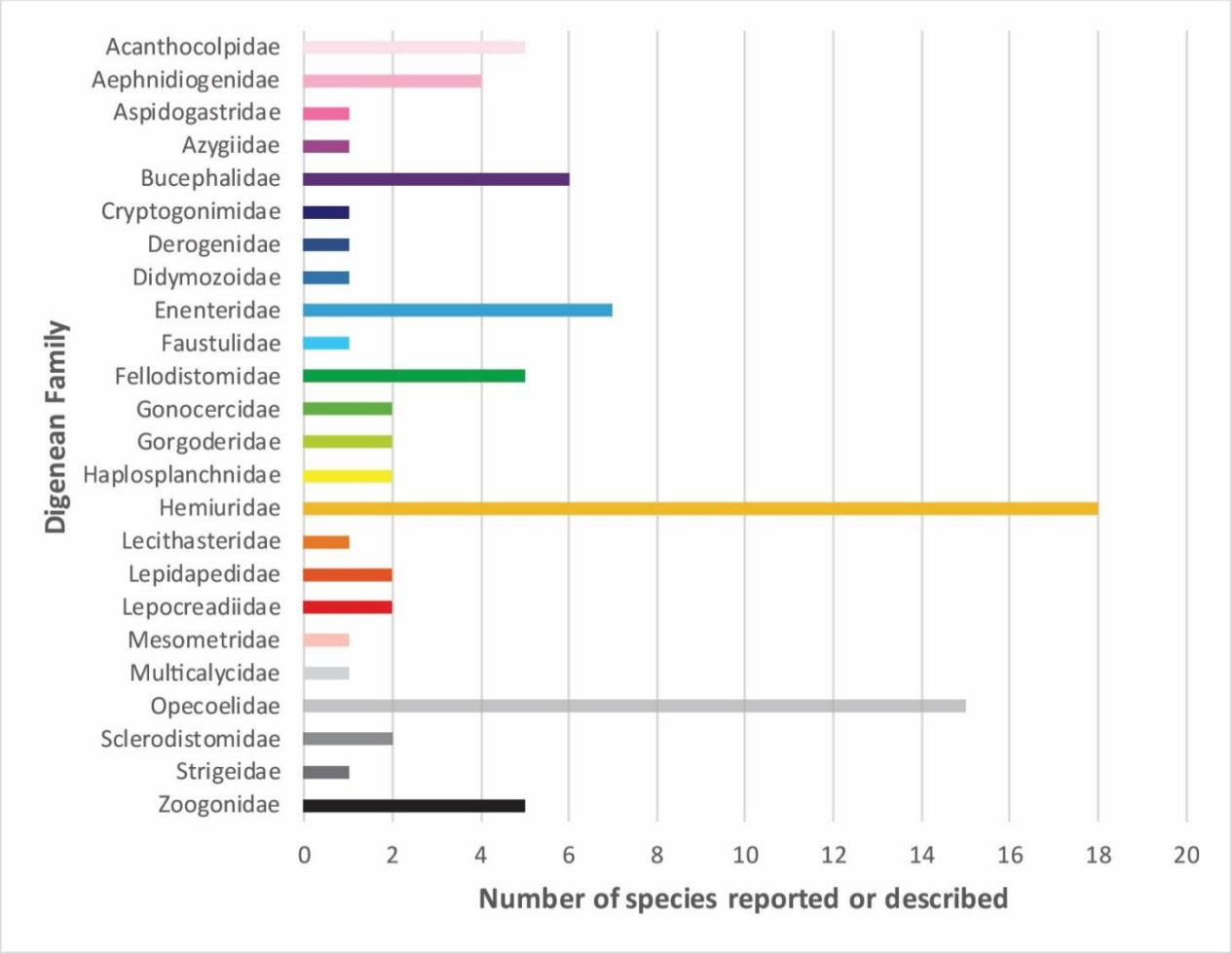

Figure 4. Number of digenean species reported or described from digenean families in South Africa

Smit and Hadfield (2015) states that there are more than 1,900 marine fish species along the South African coast, of which about 47 are endemic to this area. This high diversity can be ascribed to the unique habitats that are formed by the variety of oceanic currents in this area – the warm Agulhas current on the eastern coast, the cold, nutrient-rich Benguela current on the western coast and the mixing of attributes from both currents on the southern coast (Smit & Hadfield, 2015). According to the analyses in Figure 5, digeneans have been reported or described from 46 marine fish families from the coast of South Africa during various research endeavours between 1902 and 2017.

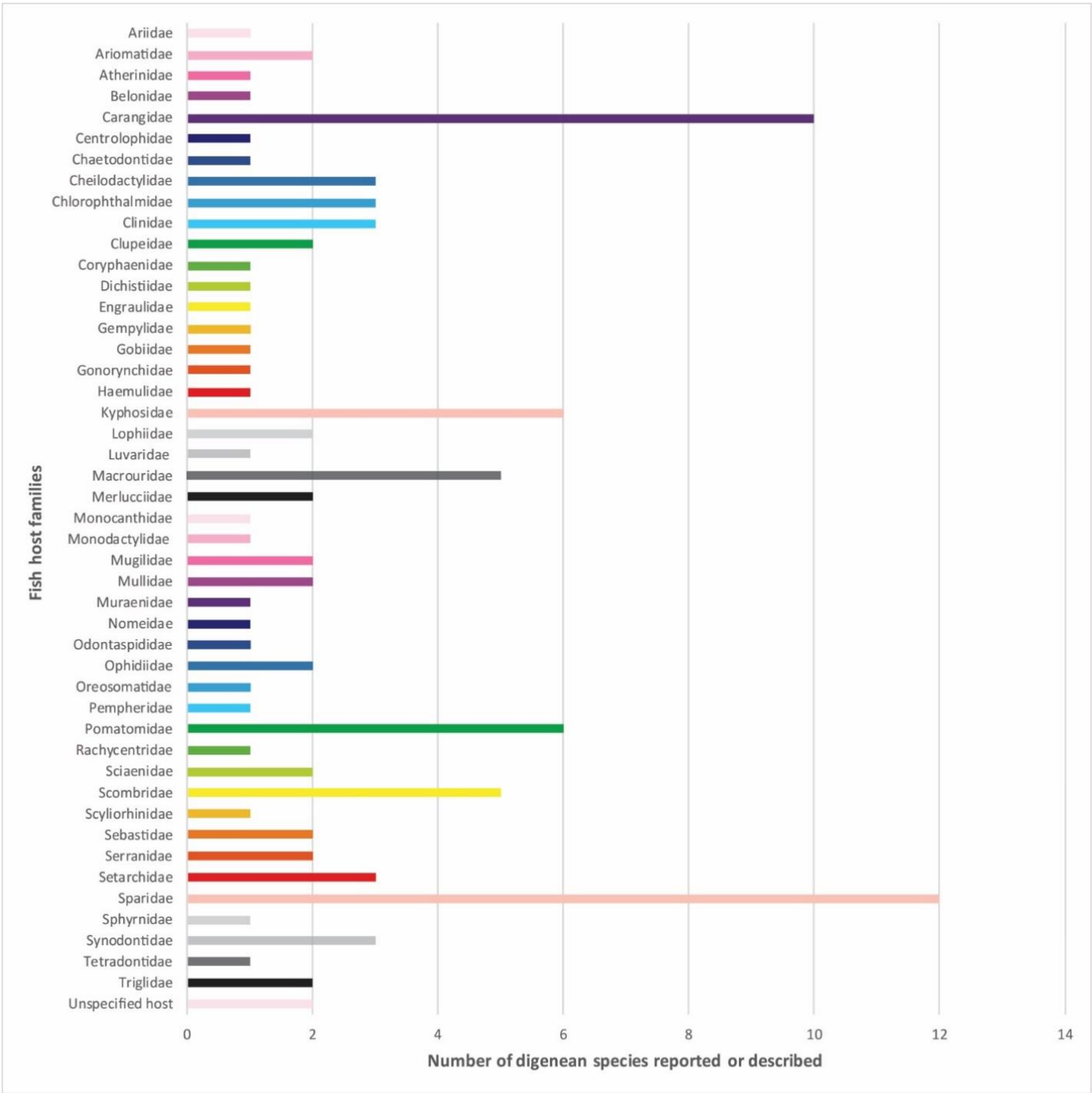

Figure 5. Number of digenean species reported or described from marine fish families in South Africa

The most digeneans were reported or described from the fish family Sparidae. Sparids such as *Sparodon durbanensis*, *Diplodus capensis* and *D. hottentotus* are commonly found in intertidal or shallow areas along the coast (Buxton & Clarke, 1991; Coetzee, 1986; Mann & Buxton, 1992; Marsh *et al*., 1978), thus they are easily obtained for helminthological examination and can explain why such a wide variety of digenean trematodes have been

recorded from this fish family. Another easily obtained group of fishes for parasitological surveys are residential intertidal fishes such as those belonging to the Clinidae. In contrast to the sparids, their parasite diversity is not as extensively studied.

Diversity of trematodes of *Clinus* spp. in South Africa

Fishes from the Clinidae are abundant in intertidal and subtidal areas along the coast of Southern Africa, and are in fact the most common intertidal, benthic fishes along the western and southern coasts of South Africa (Day, 1969; Stepien, 1992). According to FishBase, this diverse fish family consists of 88 species of 26 genera (Froese & Pauly, 2019). Of these, 43 species occur on the South African coast, many of which are endemic to this area (SAIAB, 2020). One of the most abundant species and the main focus of this study, *Clinus superciliosus* (Linnaeus, 1857) (Fig. 6), is distributed from Namibia to the Kei River mouth in South Africa (Smit *et al*., 2003; von der Heyden *et al*., 2011). This species occurs mainly in intertidal areas, but large adults (up to 30 cm in length) have been found in shallow subtidal areas along the South African coast (von der Heyden *et al*., 2011). Various research has been conducted on this recently declared species complex, including studies on its reproductive biology, diet, behaviour, morphological variations, biology and population genetic structure (Bennett *et al*., 1983; Broom, 2016 (unpublished dissertation); Crowe & Siegfried, 1978; Fishelson & Gon, 2009; Fishelson *et al*., 2006, 2007; Gibbons, 1988; Gon *et al*., 2007; Holleman *et al*., 2012; Prochazka, 1994; Veith, 1979; Veith & Cornish, 1986; von der Heyden *et al*., 2011, 2013).

Figure 6. The super klipfish, *Clinus superciliosus* (L.)

It has also been the focus of a few parasitological studies and has been reported as a host for various arthropods, annelids, digeneans, ciliates, cnidarians and haemoparasites (Table 1). The last study focusing on digeneans from this fish host was in 1987 and only studied fish from one locality, Port Elizabeth (Bray, 1987). As this study site falls within the warmer sea currents on the eastern coast of South Africa, the vast majority of this fish's distribution, which offers great variability in temperature and habitat, is unexplored with regards to trematode parasitic fauna.

Table 1. Summary data for parasites reported from *Clinus superciliosus* and *Clinus cottoides* in South Africa

Parasite species	**Fish host**	**Reference**
Phylum Arthropoda		
Gnathia africana Barnard, 1914	*S,C*	Davies & Smit, 2001
Caligus mortis Kensley, 1970	*S,C*	Kensley & Grindley, 1973
Caligus labracis Scott, 1902	*S*	Barnard, 1955
Elthusa xena van der Wal, Smit et Hadfield, 2019	*S*	van der Wal *et al*., 2019
Phylum Platyhelminthes		
Coitocaecum capense Bray, 1987	*S,C*	Bray, 1987
Helicometra fasciata (Rudolphi, 1819) Odhner, 1902	*S,C*	Bray, 1987
Phylum Myxozoa		
Haemogregarina bigemina (Laveran & Mesnil, 1901)	*S,C*	Smit & Davies, 1999
Haemogregarina curvata Hayes, Smit, Seddon, Wertheim et Davies, 2006	*C*	Hayes *et al*., 2006
Phylum Euglenozoa		
Trypanosoma nudigobii Fantham, 1919	*S,C*	Hayes *et al*., 2014
Phylum Ciliophora		
Trichodina clini Fantham, 1930	*S*	Fantham, 1930
Phylum Cnidaria		
Ceratomyxa cottoidii Reed, Basson, Van As & Dyková, 2007	*C*	Reed *et al*., 2007
Ceratomyxa dehoopi Reed, Basson, Van As & Dyková, 2007	*S*	Reed *et al*., 2007
Ceratomyxa obovalis (Fantham, 1930)	*S,C*	Bartošová-Sojková *et al*., 2018
Sphaeromyxa clini Bartošová-Sojková, Kodádková, Pecková, Kuchta & Reed, 2014	*S,C*	Bartošová-Sojková *et al*., 2014
Henneguya clini Reed, Basson, Van As & Dyková, 2007	*S,C*	Reed *et al*., 2007
Five molecularly detected undescribed species	*S,C*	Bartošová-Sojková *et al*., 2018
Phylum Annelida		
Zeylanicobdella arugamensis de Silva, 1963	*S, C*	Hayes *et al*., 2014

Abbreviations: S, Clinus superciliosus; C, Clinus cottoides

In addition to *C. superciliosus*, a small number of *Clinus cottoides* Valenciennes, 1836 have also been included in this study. This intertidal fish is endemic to South Africa and can be found in shallow coastal areas from Port Nolloth to the Kei River (Smith, 1949). Various studies regarding the biogeography, population genetics, diet, reproduction, physiology and morphology of this species has also been conducted (Beckley, 1985; Bennett *et al*., 1983; Fishelson *et al*., 2006; Gon *et al*., 2007; Prochazka, 1994; Scheepers & Gouws, 2019; Toms *et al*., 2014; von der Heyden *et al*.,2013). Similar to *C. superciliosus*, this fish species has also been found to host arthropods, digeneans, ciliates, cnidarians and haemoparasites (Table 1). Owing to the endemicity of these fish species in this area, it can be assumed that many of the parasitic species that it hosts, are not only valuable reports to the literature, but may even represent novel species to science.

Even though significant progress has been made in the study of digenean trematodes from marine fishes in South Africa, it is estimated that the true diversity might be much higher than anticipated. Yet, the last new species description of a digenean from a South African marine fish was done in 1991 (Bray, 1991). Additionally, there are merely a handful of studies that characterised digeneans molecularly, thus the availability of molecular data for digeneans from marine fishes in South Africa is severely limited, compared to the abundance of available molecular information from various other countries. Consequently, studies that incorporate both molecular and morphological techniques to study the digenean diversity in marine fishes are of great significance so as to gain knowledge on this understudied, enigmatic parasitic group from South African marine fishes.

Aims and objectives

The aim of the project was to assess the diversity of digenean trematodes of the super klipfish *Clinus superciliosus* from various localities along the South African coast.

Objectives:

- To collect specimens of *C. superciliosus* from five localities along the coast of South Africa, within the region where this fish species is endemic.
- To subject these specimens of *C. superciliosus* to helminthological examination to determine the presence of digenean trematodes.
- To generate DNA sequences for the various trematode species in order to conduct molecular identification and to assess the phylogenetic position of each species within the genus or family.
- To conduct morphological analyses of each trematode species.
- To identify each trematode isolate to the lowest possible taxonomic position by means of morphological and molecular analyses.

Layout of this dessertation

Chapter 1 provides a general introduction on digenean trematodes, previous research on these organisms in South African marine fishes, information on the various fish hosts and also provides the aims and objectives of this study. Referencing for this chapter follows the NWU Harvard style.

Thereafter, the general materials and methods are detailed in **Chapter 2**. This includes details on sampling sites, host specimen collection, trematode collection, as well as morphological and molecular analyses. The specific materials and methods used for the following chapters, are provided in the corresponding chapter. Each of the succeeding chapters consists of introduction, materials and methods, results, discussion and references sections. Referencing for this chapter follows the NWU Harvard style.

Chapter 3 focusses on metacercariae of two species of the genus *Cardiocephaloides* that infect the eyes and brain of *Clinus* spp. These species are identified to the lowest possible taxonomic position by utilising both molecular and morphological analyses. One of these species, *Cardiocephaloides physalis*, has been reported from pilchards and penguins along the South African coast. The other species, *Cardiocephaloides* sp., was found to be conspecific to the species of sporocyst isolates collected from whelks in New Zealand. A haplotype analysis also confirmed the presence of four distinct haplotypes of *Cardiocephaloides* sp. along the South African coast. Referencing for this chapter follows the formatting requirements of Folia Parasitologica (A–E1).

In **Chapter 4** species of the large family Opecoelidae have been identified to genus level by means of molecular and morphological characterisation. In total, *C. superciliosus* is definitive host to six opecoelid species: *Coitocaecum capense, Coitocaecum* sp. 1, *Coitocaecum* sp. 2, *Coitocaecum* sp. 3, *Helicometra* sp. 1 and *Helicometra* sp. 2. Referencing for this chapter follows the formatting requirements of Systematic Parasitology (A–E2).

Chapter 5 portrays the astonishing results that *C. superciliosus* from five sampling localities along the South African coast, is host to a total of 14 digenean species belonging to seven families. The trematodes are characterised molecularly. Photographs of each species are provided, as well as information regarding species of each family that have been reported or described from South African marine fishes. Referencing for this chapter follows the formatting requirements of Parasitology Research (A–E3).

Lastly, **Chapter 6** contains the summative discussion, which generally discusses the results of this project, recommendations for future studies and ends off with a conclusion.

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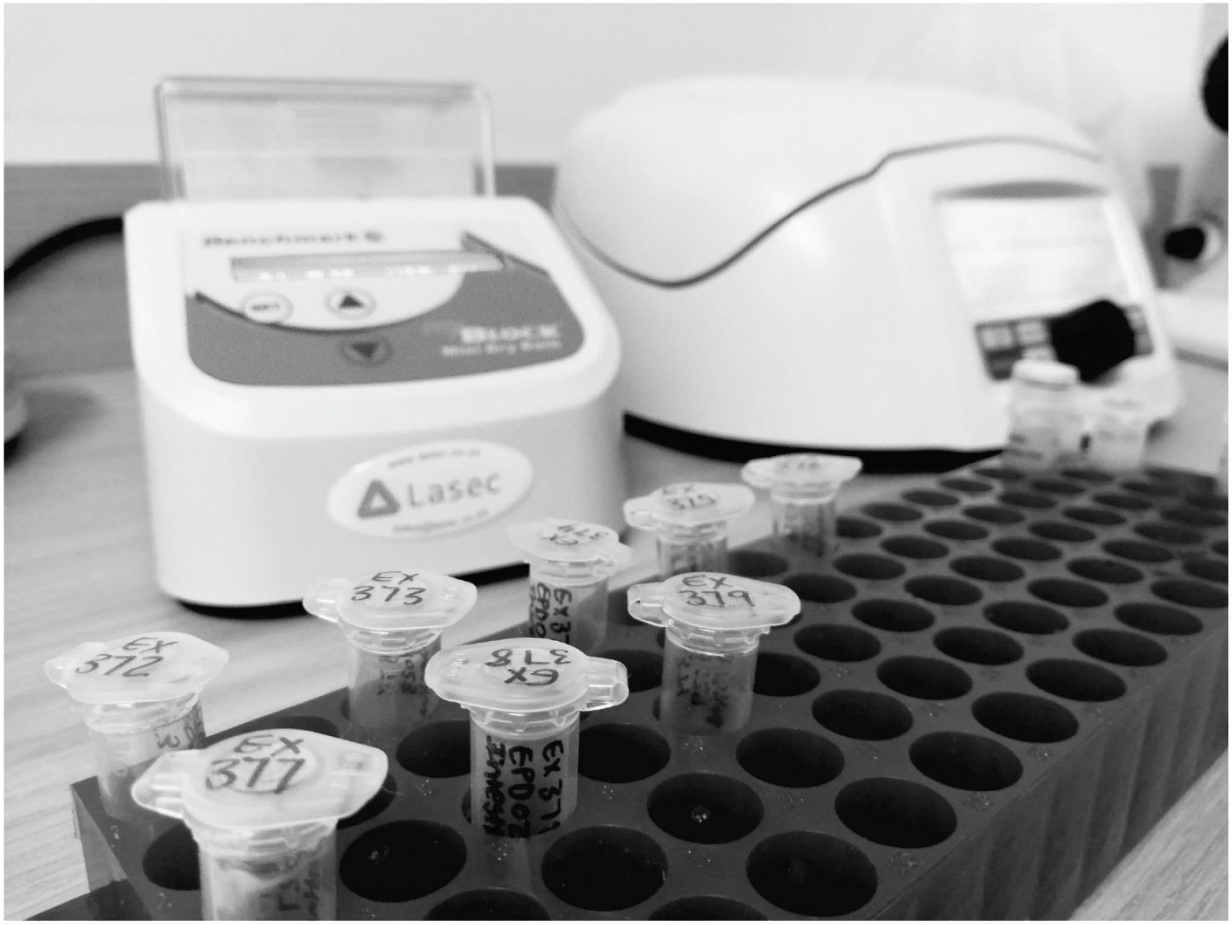
Chapter 2

Materials and Methods



Chapter 2. Materials and Methods

Sampling sites

Sampling was conducted in six distinct areas along the coast of South Africa. In the Western Cape province, sampling sites included Saldanha Bay (33° 2' 44.46" S; 18° 2' 19.06" E), Cape Town harbour (33° 54' 29.13" S; 18° 25' 5.81" E) and Hermanus (34° 25' 15.86" S; 19° 14' 37.56" E) (Fig. 1). These sites are found along the Atlantic Ocean and are thus influenced greatly by the cold, nutrient-rich Benguela current. Further sampling was conducted in the Tsitsikamma National Park (34° 1' 15.21" S; 23° 52' 43.23" E), Jeffreys Bay (34° 1 '35" S; 24° 55' 51" E) and Chintsa (32° 50' 11.54" S; 28° 7' 1.19" E) in the Eastern Cape province (Figs. 1 & 2). The Tsitsikamma National Park is the largest marine protected area in South Africa, as it encompasses nearly 80 km of the coastline and has a total area of about 360 km^2 (Cowley *et al.*, 2002; Tilney *et al.*, 1996). Except for Cape Town harbour, all sites are rocky areas or rock pools within the intertidal zone. These sites are influenced by both the cold, nutrient-rich Benguela current from the west and the warm Agulhas current from the east – the resulting conditions create a productive environment that is teeming with marine life.

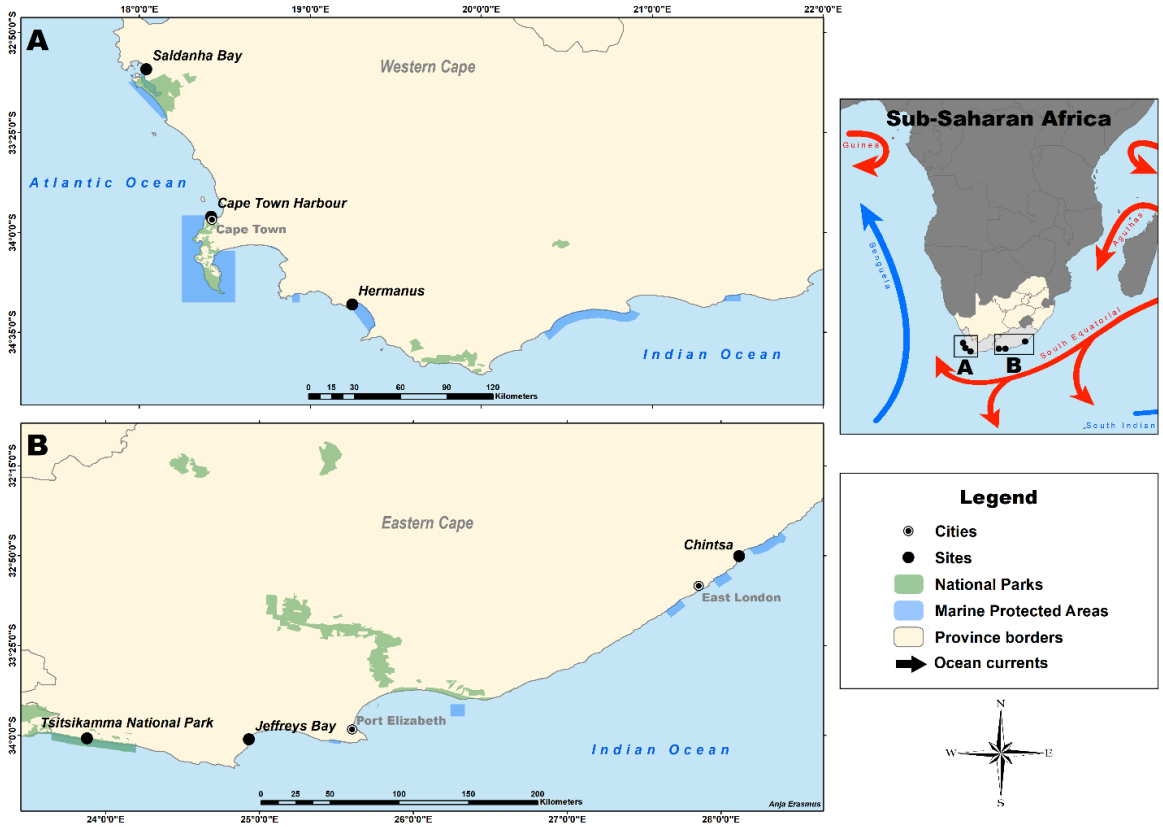


Figure 1. Map of the South African coast with sampling sites for this study. A and B indicate the areas of the map that are enlarged on the left-hand side

Figure 2. Sampling sites along the South African coast: Saldanha Bay (A), Cape Town harbour (B), Hermanus (C), Tsitsikamma National Park (D), Jeffreys Bay (E) and Chintsa (F)

According to the bioregions as classified by Lombard *et al*. (2004), Saldanha Bay and Cape Town are in the South-western Cape bioregion where the cool-temperate Namaqua bioregion and warm-temperate bioregion overlap. Hermanus, Tsitsikamma National Park and Chintsa occur in the warm-temperate Agulhas bioregion (Lombard *et al*., 2004). Spalding *et al.* (2009) further divided these ecoregions of temperate Southern Africa into two provinces – the Benguela and Agulhas. These are subdivided into the Namaqua, Agulhas Bank and the Natal ecoregions (Spalding *et al*., 2009). This classification places Saldanha Bay and Cape Town

harbour in the Namaqua ecoregion, Hermanus and the Tsitsikamma National Park in the Agulhas Bank ecoregion and Chintsa in the Natal ecoregion.

These sites also provide ample habitat for numerous molluscs that are hosts for the cercarial and metacercarial stages of digenean trematodes. The surfing whelk *Bullia digitalis* (Dillwyn, 1817) has been reported as a first intermediate host for the trematode cercariae *Cercaria hastata* Webb, 1991 (Webb, 1991), whereas the bivalve mollusc *Perna perna* (Linnaeus, 1758) has been reported as a second intermediate host for a species of *Proctoeces* Odhner, 1911 (Calvo–Ugarteburu & McQuaid, 1998). Additionally, the selected sampling sites include the distributional areas of a multitude of molluscan species from the classes Scaphopoda, Bivalvia, Polyplacophora and Gastropoda (Branch *et al.*, 2007). Two species of immature digeneans, *Lobatostoma* sp. and *Proctoeces maculatus* (Looss, 1901) Odhner, 1911, have been reported from the cephalopod host *Octopus vulgaris* Cuvier, 1797 in Durban (Bray, 1984). Therefore, sampling sites were not only selected based on their geographic dispersal and varying ecoregions within which to investigate parasite species composition and dispersal, but also because of the presence of several potential host organisms.

Specimen collection

In total, 83 specimen of *C. superciliosus* were collected by angling and with the use of traps and bait. The specimens were collected from Saldanha Bay (n=19) (permit: RES2019–103), Cape Town harbour (n=16) (permit: RES2019–103), Hermanus (n=8) (permit: RES2018/35), Tsitsikamma National Park (n=17) (permit: MALH–K2016–005a), Jeffreys Bay (n=14) (permit: permit: RES2018/35) and Chintsa (n=9) (RES2019–103). Following collection, fish were kept in temporary housing containers that maintained a constant, adequate temperature and were supplied with sufficient oxygen until they could each be examined. Fish were euthanized by spinal severance and cranial pithing and thereafter subjected to helminthological examination under a C-LEDS Nikon dissecting microscope (Nikon Instruments, Tokyo, Japan). Photographs of live specimens were taken a Zeiss Stemi 305 with camera adapter (Zeiss, Oberkochen, Germany). Any digenean trematodes present were removed from the host using fine needles, forceps and glass pipettes. The adult trematodes were then cleaned in saline and fixed by pipetting them into hot saline. The fixed trematodes were placed in their respective, labelled containers and preserved in 80% ethanol. Metacercariae were excysted, cleaned in saline and fixed and preserved in their respective, labelled containers with 96% ethanol. The contents of each tube and details thereof were written with pencil on a small piece of paper and placed in the tube. Examination, labelling and preservation was done according to Cribb & Bray (2010), additionally all other organs of the fish host were examined. The field collection data was

entered in a digital database. The collected material was then stored in the freezer collection of the Water Research Group at the North-West University, Potchefstroom campus, until further analises. Fish names follow FishBase (Froese & Pauly, 2019); trematode names follow the World Register of Marine Species (WoRMS Editorial Board, 2020). The prevalence of each species per site was determined by calculating the percentage of fish infected with that species, of the total number of fish sampled at that site (Bush *et al*., 1997). Intensity of infection is given as a range of the lowest and the highest number of a trematode species that infect a single host (Bush *et al*., 1997).

Morphological characterisation

In the laboratory, all specimens were identified to family level and divided into morphological groups. Individuals from each of these groups were selected for morphological and molecular analyses following the concept of Pleijel *et al*. (2008). For metacercariae and very small adult trematodes, a photograph was taken with the digital camera attached to a Nikon Eclipse *Ni* microscope. This photograph served as a digital voucher and was further used for morphometric analyses. Thereafter, entire worms were used for molecular analyses. From the larger adult trematodes, a small piece of the lateral part of the posterior extremity was carefully excised not to damage any important structures or affect the morphometric analyses. This small piece was placed in a separate vial with 96% ethanol and used for molecular analyses. The remainder of the worm was stained and mounted on a permanent slide for morphological analyses.

Staining was done by rinsing the worm in distilled water, stained with Mayer's haematoxylin, slightly destained with 1% hydrochloric acid, neutralised in 1% ammonium hydroxide solution and serially subjected to a gradient of ethanol concentrations (70%, 80%, 90%, 96% and 100%) to dehydrate the specimen. Trematodes were cleared in a 1:1 methyl salicylate and 100% ethanol solution, followed by 100% methyl salicylate. Each specimen was then permanently mounted in Dammar gum on a microscope slide with a cover slip. These slides were left to dry for a few days and used to collect morphometric details, make descriptive drawings and provide detailed descriptions for each species.

Measurements were taken with the NIS-Elements BR camera analysis software, with the use of a Nikon Eclipse *Ni* microscope (Nikon Instruments, Tokyo, Japan). All measurements are given in micrometres (µm) unless otherwise stated. Drawings were made with the aid of a drawing tube and digitalised with Adobe Illustrator CC v.22.0 and Photoshop CC v.25.0.

Molecular characterisation

Sequence generation

From entire small worms and the excised posterior section of large worms, the total genomic DNA was extracted by making use of a KAPA Express Extraction Kit, according to the manufacturer's instructions (Kapa Biosystems, Cape Town, South Africa) as well as a PCRBiosystems Rapid DNA Extraction Kit (PCRBiosystems available from Analytical Solutions, Randburg, South Africa). The latter was modified to add only 10 µl of lysis buffer and 5 µl of protease containing buffer to the trematode tissue sample. The procedures for reaction incubation were performed according to the manufacturers protocol, after which the reaction was diluted with only 450 µl molecular grade water instead of 900 µl as recommended by the manufacturer.

Three genetic markers were selected for amplification. Data on primers used for DNA amplification is summarised in Table 1 below. For amplification of the partial D1-D3 fragment of the 28S nuclear ribosomal DNA, a forward (Digl2) and reverse (1500R) primers were used. The entire Internal Transcribed Spacer region (ITS1-5.8S-ITS2) was amplified by using the forward primer D1 and reverse primer D2. The forward primer 3S and reverse primer ITS2.2 were used for the amplification of the ITS2 region. Two sets of forward and reverse primers (DICE1F & DICE14R; Dig_cox1Fa & Dig_cox1R) were used for the amplification of the cytochrome *c* oxidase mitochondrial DNA complex (*cox* 1). The polymerase chain reaction (PCR) steps for each gene and primer set can be seen in Figure 3.

The resultant PCR amplicons obtained were visualised by agarose gel electrophoresis - 1% agarose gel stained with EZ-Vision Bluelight DNA dye (VWR AMRESCO Life Science available from Inqaba Biotechnical Industries Pty. Ltd.). Successful amplification products were sent to a commercial sequencing company, Inqaba Biotechnical Industries Pty. Ltd. in Pretoria, South Africa for purification and sequence generation. The relevant PCR primers were used for sequencing of each gene/region, with two additional internal primers (ECD2 and 300F) for the sequencing of the partial D1-D3 fragment of the 28S nuclear ribosomal DNA. The obtained sequences were assembled and edited with Geneious v.11.1.4 bioinformatics software (Biomatters, Auckland, New Zealand).

Table 1. Primers used for DNA amplification

Locus	Primer	Sequence	Reference
28S	Digl2	5′-AAG CAT ATC ACT AAG CGG-3′	Tkach *et al*. (2001)
	1500R	5′-GCT ATC CTG AGG GAA ACT TCG-3′	Tkach *et al*. (2003)
(internal)	ECD2	5'-CTT GGT CCG TGT TTC AAG ACG GG-3'	Tkach *et al*. (2003)
(internal)	300F	5'-CAA GTA CCG TGA GGG AAA GTT G-3'	Tkach *et al*. (2003)
ITS1-5.8S-ITS2	D1	5’-AGG AAT TCC TGG TAA GTG CAA G-3’	Galazzo et al. (2002)
	D2	5’-CGT TAC TGA GGG AAT CCT GGT-3’	Galazzo et al. (2002)
ITS2	3S	5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′	Bowles *et al*. (1993)
	ITS2.2	5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′	Cribb *et al*. (1998)
cox 1	DICE1F	5′-ATT AAC CCT CAC TAA ATT WCN TTR GAT CAT AAG-3′	Van Steenkiste *et al.* (2015)
	DICE14R	5’-TAA TAC GAC TCA CTA TAC CHA CMR TAA ACA TAT GAT G-3′	Van Steenkiste *et al.* (2015)

Sequence alignment and phylogenetic analyses

The newly generated sequences were used to perform a basic local alignment search tool (BLAST) analysis with sequences deposited in GenBank (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine similarity. Novel sequences were aligned with sequences of species from the same genus or family available in GenBank according to gene/region that has been amplified, along with an outgroup sequence chosen based on the findings of available literature relevant to each digenean family. Alignments were built by using MUSCLE (Edgar, 2004) as implemented in Geneious. The best nucleotide substitution model for each alignment was statistically determined with jModelTest 2.1.1 software (Darriba *et al*., 2012; Guindon & Gascuel, 2003).

These alignments were used to construct phylogenetic trees to determine and visualise the phylogenetic position of each species. Specific details on the construction and parameters used for these trees are given in the relevant chapters. All phylogenetic trees were constructed based on Bayesian inference (BI) and maximum likelihood (ML) analyses. BI analyses were performed by using MrBayes v.3.2.7 software run on the CIPRES Science Gateway v.3.3 (available at https://www.phylo.org/) and the ML analyses were performed with PhyML v.3.0 (available at http://www.atgc-montpellier.fr/phyml/). Mega v.7 was used to calculate genetic distance matrices (uncorrected p-distance).

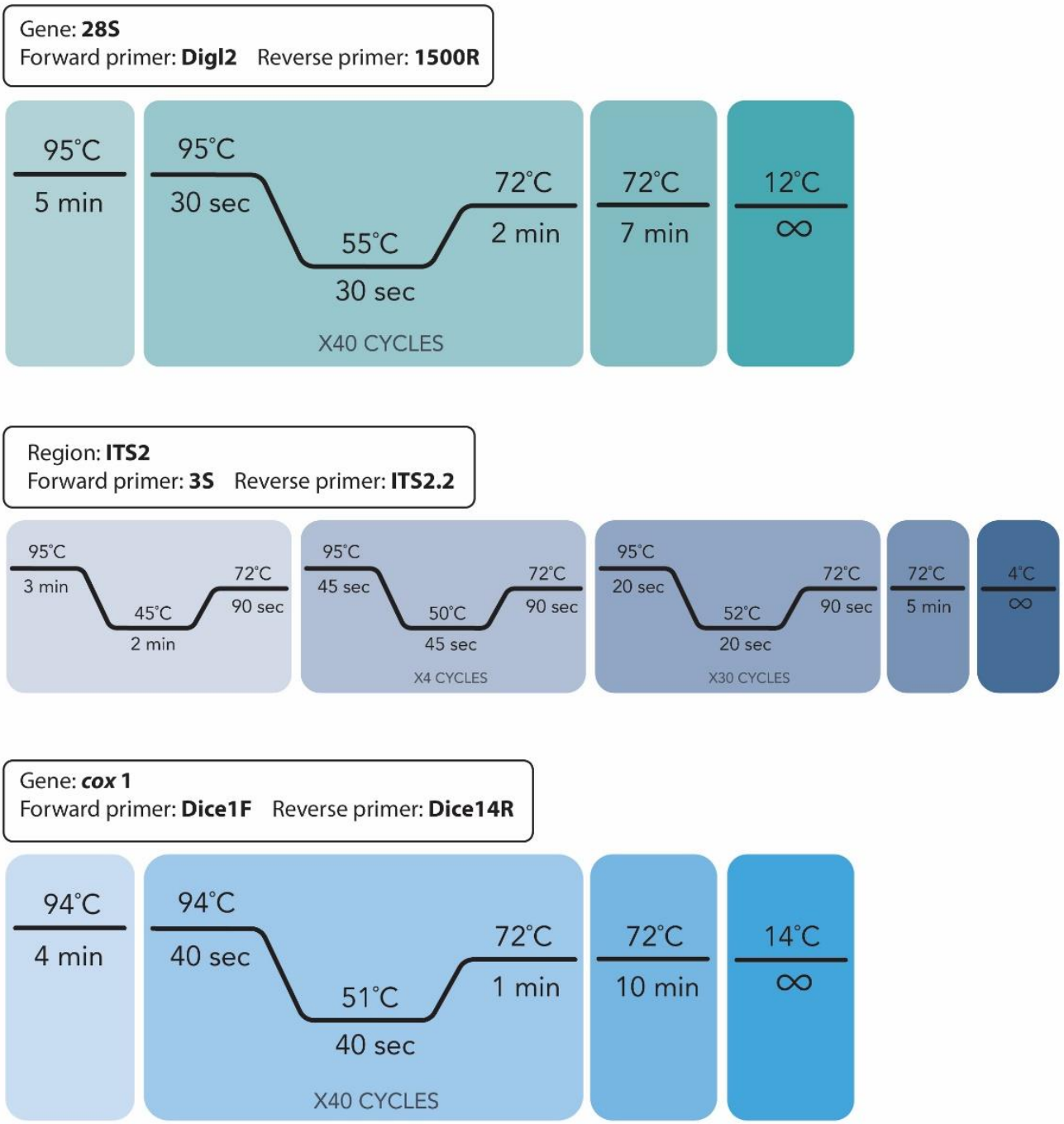


Figure 3. PCR thermocycle protocols used for DNA amplification of the three genetic markers

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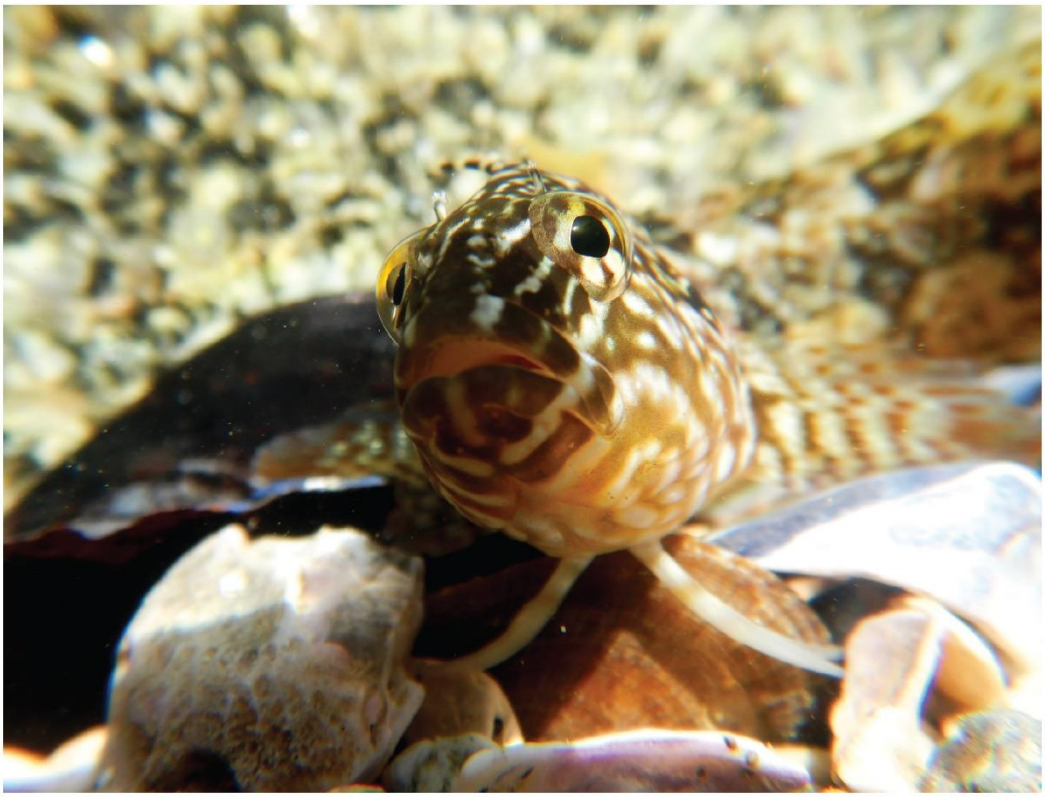
Chapter 3

Molecular and morphological characterisation of the metacercariae of two species of *Cardiocephaloides* (Digenea: Strigeidae) infecting endemic South African klipfish (Perciformes: Clinidae)



Chapter 3. Molecular and morphological characterisation of the metacercariae of two species of *Cardiocephaloides* (Digenea: Strigeidae) infecting endemic South African klipfish (Perciformes: Clinidae)

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Abstract: South African clinids are a major component of the temperate intertidal regions that are also known to participate in life cycles and transmission of several groups of parasites. However, the knowledge of trematode diversity of these fishes is incomplete. In this study, two species of *Clinus*, the super klipfish *Clinus superciliosus* (Linnaeus) and the bluntnose klipfish *Clinus cottoides* Valenciennes were collected from six localities along the South African coast and examined for the presence of trematodes. Metacercariae of *Cardiocephaloides* Sudarikov, 1959 were found in the eyes and brain of *C. superciliosus* and in the eyes of *C. cottoides*. Detailed analyses integrating morphological and molecular sequence data (28S rDNA, ITS2 rDNA-region, and COI mtDNA) revealed that these belong to two species, *Cardiocephaloides physalis* (Lutz, 1927) and an unknown species of *Cardiocephaloides*. This study provides the first report of clinid fishes serving as intermediate hosts for trematodes, reveals that the diversity of *Cardiocephaloides* in South Africa is higher than previously recorded and highlights the need for further research to elucidate the life cycles of these trematode species. The broad geographical distribution of *Cardiocephaloides* spp. was confirmed in the present study based on molecular sequence data. The host-parasite interactions between clinid fishes and metacercariae of *Cardiocephaloides* are yet to be explored.

Keywords: Trematoda, *Clinus superciliosus*, *Clinus cottoides*, marine fish parasites, DNA, morphology

Clinids (Perciformes) are common inter- and subtidal shore fishes occurring along the coast of southern Africa and many are endemic to this region (von der Heyden et al. 2011). *Clinus superciliosus* (Linnaeus), the super klipfish, and *Clinus cottoides* Valenciennes, the bluntnose klipfish, the subjects of the present study, are abundant resident intertidal fish species and have been the focus of research regarding their diet, biology, reproductive biology and population genetic structure (Bennett et al. 1983, Fishelson et al. 2007, Gon et al. 2007, Fishelson and Gon 2009, Holleman et al. 2012, and references therein).

Being associated with the rocky shore habitat, both species have the potential to play an important role in parasite distribution and life cycles, but this has not been fully investigated yet. To date, *C. superciliosus* and *C. cottoides* have been reported as hosts for four parasitic arthropods, *Gnathia africana* Barnard, 1914 (Gnathiidae) (Davies and Smit 2001), *Caligus mortis* Kensley, 1970 (Kensley and Grindley 1973), *Caligus labracis* Scott, 1902 (Caligidae) (Barnard, 1955) and *Elthusa xena* van der Wal, Smit et Hadfield, 2019 (Cymothoidae) (species reported only in *C. superciliosus*) (van der Wal et al. 2019); two species of intestinal trematodes, *Coitocaecum capense* Bray, 1987 and *Helicometra fasciata* (Rudolphi, 1819) (Opecoelidae) (Bray 1987); three species of haemoparasites, *Haemogregarina bigemina* Laveran et Mesnil, 1901 (Smit and Davies 1999), *H. curvata* Hayes, Smit, Seddon, Wertheim et Davies, 2006 (Haemogregarinidae) (species reported only in *C. cottoides*) (Hayes et al. 2006), and *Trypanosoma nudigobii* Fantham, 1919 (Trypanosomatidae) (Hayes et al. 2014); a single trichodinid species, *Trichodina clini* Fantham, 1930 (Fantham, 1930); a single species of leech *Zeylanicobdella arugamensis* de Silva, 1963 (Piscicolidae) (Hayes et al. 2014); and ten species of myxozoan parasites, of which five have been described, *Ceratomyxa cottoidii* Reed, Basson, Van As et Dyková, 2007 (Reed et al. 2007), *C. dehoopi* Reed, Basson, Van As et Dyková, 2007 (Reed et al. 2007), *C. obovalis* (Fantham, 1930) (Ceratomyxidae) (Fantham, 1919, 1930), *Sphaeromyxa clini* Bartošová-Sojková, Kodádková, Pecková, Kuchta et Reed, 2014 (Sphaeromyxidae) (Bartošová-Sojková et al. 2014), *Henneguya clini* Reed, Basson, Van As et Dyková, 2007 (Myxobolidae) (Reed et al. 2007) and five molecularly detected undescribed species reported by Bartošová-Sojková et al. (2018).

During a parasitological survey of *C. superciliosus* and *C. cottoides* along the coast of South Africa, metacercariae belonging to the genus *Cardiocephaloides* Sudarikov, 1959 were recorded in these fishes. *Cardiocephaloides* is a genus of the family Strigeidae Railliet, 1919 with merely seven currently recognised species reported from larid birds and penguins around the world (Niewiadomska 2002, Achatz et al. 2020). Species of *Cardiocephaloides* utilise a three-host life cycle with nassarid and buccinid molluscs as first intermediate hosts, sparid and scomberesocid

fish as second intermediate hosts and seabirds as definitive hosts (Niewiadomska 2002, Donald and Spencer 2016). To date, *Cardiocephaloides longicollis* (Rudolphi, 1819) is the only species for which all hosts involved in the life cycle are known (Prévot and Bartoli 1980, Osset et al. 2005, Born-Torrijos et al. 2016). One species of this genus, *Cardiocephaloides physalis* (Lutz, 1926) originally described and reported from the Magellanic penguin *Spheniscus magellanicus* (Forster) from Uruguayan and Brazilian coasts (Lutz 1926, Dubois 1937, 1938, Brandaõ et al. 2013), was also recorded in the African penguin *Spheniscus demersus* (Linnaeus) in South Africa (Randall and Bray 1983, Horne et al. 2011, Espinaze et al. 2019). This species has been reported to cause numerous mortalities of penguin chicks. Although the life cycle of *C. physalis* is unknown, there have been several reports on metacercariae of this species in the eyes of the South American pilchard *Sardinops sagax* (Jenyns) (Reed et al. 2012, Weston et al. 2015, Ukomadu 2017). Reed et al. (2012) reported metacercariae of 'tetracotyle' type from *S. sagax* and Weston et al. (2015) found what they thought to be metacercariae of the genus *Cardiocephaloides*, in particular *C. physalis*, from the same fish host. Later, metacercariae of this species collected from *S. sagax* by Ukomadu (2017) were molecularly characterised and their identification as *C. physalis* was confirmed by comparison to sequences of adult worms of *C. physalis* reported from *S. demersus* by Horne et al. (2011). Recently, Achatz et al. (2020) cautiously confirmed that *C. physalis* has a broad distribution, based on 28S sequence data of this species from South America and South Africa. However, a better substantiated conclusion required a comparison of data of more variable genes from both continents.

The aim of the present study was to provide the first molecular characterisation associated with morphological descriptions of metacercariae of *Cardiocephaloides* found in *C. superciliosus* and *C. cottoides* along the coast of South Africa. We report, for the first time, clinid fishes as intermediate hosts for trematodes, including species of *Cardiocephaloides*. This novel data contributes to our limited knowledge on the life cycles of *Cardiocephaloides* spp. and the parasite fauna of marine fishes in South Africa.

MATERIALS AND METHODS

Sample collection

Eighty-three *Clinus superciliosus* were collected from three localities along the west and south coasts of South Africa: Saldanha Bay (-33.045683°; 18.038628°), Cape Town (-33.908092°; 18.418281°), Hermanus (-34.421071°; 19.243766°), Tsitsikamma National Park (-34.020892°; 23.878674°), Jeffreys Bay (-34.026389°; 24.930833°) and Chintsa (-32.836538°; 28.116997°) (Fig. 1). In addition, six specimens of *Clinus cottoides* were collected in Jeffreys Bay. These are geographically distinct areas with varying marine habitats, anthropogenic influences and water temperatures that are affected by the cold Benguela and the warmer Agulhas currents. Sampling was carried out under the permit MALH–K2016–005a for the Tsitsikamma National Park, RES2019–103 for Saldanha Bay, Cape Town harbour and Chintsa, and RES2018/35 for Hermanus and Jeffreys Bay. Fishes were collected using baited traps and hand lines. Following euthanasia, fishes were subjected to a full helminthological examination and all trematodes found were collected according to Cribb and Bray (2010). After the trematodes were removed from the host, metacercariae were excysted with fine needles and preserved in 96% molecular grade ethanol. Metacercariae found in the eyes and brain of fishes from different localities were sorted and selected for further molecular and morphological analyses. Additionally, adult worms of *C. physalis* collected by Horne et al. (2011) from *S. demersus* and donated to us, were used to generate sequences in order to compare the sequences from adults to sequences of metacercariae collected during the present study. All animal handling, collection and dissections was approved by the North-West University AnimCare Animal Research Ethics Committee (NWU-00565-19-A5).

Morphological analysis

Photomicrographs of ethanol–preserved metacercariae selected and used for sequencing, were taken with a digital camera attached to a Nikon Eclipse *Ni* compound microscope (Nikon Instruments, Tokyo, Japan) and analysed with NIS–Elements BR Camera Analysis software; these served as digital vouchers. Other specimens were stained with Mayer's haematoxylin and mounted on permanent slides with dammar gum or alternatively stained with acetocarmine and mounted on a permanent slide in Berlese's medium. These slides along with the photomicrographs were used to collect morphometric data. All measurements were taken with NIS–Elements BR Camera Analysis software and are given in micrometres (µm), unless otherwise stated. The metrical data is presented as the range followed by the mean, while the

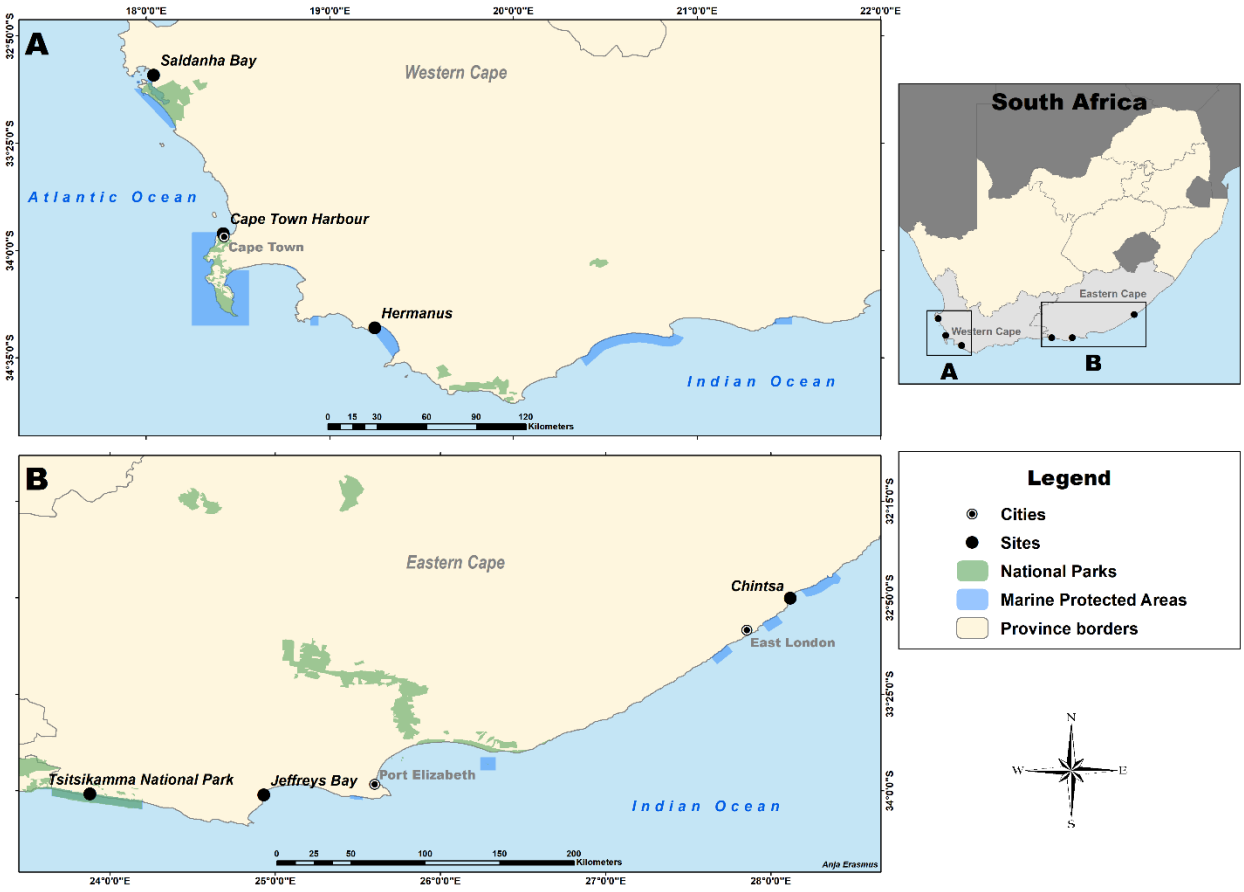

Figure 1. Map illustrating the sampling localities along the South African coast

number of specimens measured (n) is in parentheses. Voucher material has been deposited in the parasite collection of the National Museum (NMB), Bloemfontein, South Africa.

Generation of molecular data

DNA was extracted using the KAPA Express Extract Kit (KAPA Biosystems, Cape Town, South Africa) and PCRBiosystems Rapid DNA Extraction Kit (PCRBiosystems available from Analytical Solutions, Randburg, South Africa) following the manufacturers' protocol. The latter was modified to utilise only 10 µl of lysis buffer and 5 µl of protease containing buffer; recommended procedures for reaction incubation were performed according to the manufacturer's protocol, after which the reaction was diluted with 450 µl molecular grade water instead of 900 µl as recommended, in order to obtain DNA at a high concentration. DNA amplification was performed to amplify the partial D1–D3 fragment of the 28S nuclear ribosomal RNA gene, either the entire internal transcribed spacer region (ITS1-5.8S-ITS2) or the complete internal transcribed spacer 2 (ITS2) of the ribosomal gene cluster and a partial fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene. Forward and reverse

primers specific to each gene/region were used for amplification (Table 1). Amplification was performed by using various polymerase chain reaction (PCR) protocols relevant to each primer as recommended by previous studies (Galazzo et al. 2002, Tkach et al. 2003, van Steenkiste et al. 2014, Kudlai et al. 2015). The resultant PCR amplicons were visualised by agarose gel electrophoresis and sent to a commercial sequencing company for purification and sequence generation (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). The obtained sequences were assembled and edited with Geneious v. 11.1.4 bioinformatics software (Biomatters, Auckland, New Zealand). Sequences have been deposited in GenBank. Accession numbers for the sequences of one adult isolate of *C. physalis* are: MW370425 (28S), MW370433 (ITS1-5.8S-ITS2) and MW365507 (COI). Specimens preserved in ethanol have been submitted to the NMB (P 723). Accession numbers for the sequences of metacercarial isolates are provided in the relevant taxonomic summaries.

Phylogenetic analysis

In total, 39 novel sequences were generated for 22 metacercarial isolates (eight for 28S, one for ITS1-5.8S-ITS2, nine for ITS2 and 18 for COI) and for one adult isolate (28S, ITS1-5.8S-ITS2 and COI). Available sequences for representatives of the genus *Cardiocephaloides* for phylogenetic analyses were retrieved from GenBank as well as sequences for the outgroups (Table 2). Alignments incorporating these sequences and the sequences obtained during the present study, were built using MUSCLE (Edgar 2004) as implemented in Geneious v. 11.1.4. Three alignments were built according to the sequence data of each gene/region. The alignment for the 28S rRNA gene (861 nucleotides [nt]) comprised of six newly generated sequences and

Table 1. Primers used for DNA amplification and sequencing during this study

Locus	Primer	Sequence	Reference
28S	Digl2	5′-AAG CAT ATC ACT AAG CGG-3′	Tkach et al. (2001)
	1500R	5′-GCT ATC CTG AGG GAA ACT TCG-3′	Snyder and Tkach (2001)
	ECD2	5'-CTT GGT CCG TGT TTC AAG ACG GG-3'	Tkach et al. (2003)
	300F	5'-CAA GTA CCG TGA GGG AAA GTT G-3'	Littlewood et al. (2000)
ITS1-5.8S-ITS2	D1	5’-AGG AAT TCC TGG TAA GTG CAA G-3’	Galazzo et al. (2002)
	D2	5’-CGT TAC TGA GGG AAT CCT GGT-3’	Galazzo et al. (2002)
ITS2	3S	5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′	Morgan and Blair (1995)
	ITS2.2	5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′	Cribb et al. (1998)
COI	DICE1F	5′-ATT AAC CCT CAC TAA ATT WCN TTR GAT CAT AAG-3′	Van Steenkiste et al. (2014)
	DICE14R	5’-TAA TAC GAC TCA CTA TAC CHA CMR TAA ACA TAT GAT G-3′	Van Steenkiste et al. (2014)

six sequences obtained from GenBank, including an unpublished sequence for *C. physalis* (MK185719) obtained from *S. sagax* in South Africa. The alignment for the ITS2 region (421 nt) consisted of six newly generated sequences and five sequences from GenBank. The third alignment consisted of data for the COI gene (489 nt) for nine newly generated sequences and seven sequences from GenBank. The outgroups for the three alignments were selected based on the results of the phylogenetic analyses of the Diplostomoidea Poirier, 1886 published by Achatz et al. (2020).

The best nucleotide substitution model for each alignment was estimated with jModelTest 2.1 (Darriba et al. 2012) according to the Akaike information criterion. The general time reversible model with estimates of invariable sites (GTR + I) was used to construct the phylogenetic tree for the 28S rDNA; the GTR model with gamma distribution rate variation among sites (GTR + G) was used to construct the COI phylogenetic tree. The Hasegawa-Kishino-Yano model with gamma distribution rate variation among sites (HKY + G) was used for the construction of the ITS2 phylogenetic tree. All phylogenetic trees were constructed based on Bayesian inference (BI) and maximum likelihood (ML) estimate analyses. BI analyses were performed with MrBayes software that was run on CIPRES Science Gateway v. 3.3 (available at https://www.phylo.org/) and ML analyses were performed with PhyML v. 3.0 (available at http://www.atgc-montpellier.fr/phyml/). For the BI analyses of all three alignments, the Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations. The 'burn-in' was set for the first 25% of the sampled trees. Nodal support for the ML analyses of all three alignments was estimated by performing 100 bootstrap pseudoreplicates. Trees were visualised using FigTree v. 1.4.3 software (Rambaut 2012). Pairwise genetic distance matrices (p-distances) were calculated with MEGA v. 7. The unique COI haplotypes for a single species from five sampling localities along the South African coast were identified with DnaSP (Rozas et al. 2003). Haplotype networks were reconstructed using the Median-Joining method in PopART software (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz).

Table 2. Sequence data for *Cardiocephaloides* spp. used in the analyses

Species	Host	Locality	GenBank accession numbers				Haplotype	Reference
			28S	ITS1-5.8S-ITS2	ITS2	COI		
Cardiocephaloides longicollis (Rudolphi, 1819)	*Larus argentatus* Pontoppidan	Ukraine	–	–	–	MN817945	–	Achatz et al. 2020
C. longicollis	*L. argentatus*	Ukraine	–	–	–	MN817944	–	Achatz et al. 2020
C. longicollis	*L. argentatus*	Ukraine	MN820662	–	MN820662	–	–	Achatz et al. 2020
Cardiocephaloides medioconiger (Dubois et Perez-Vigueras, 1949)	*Thalasseus maximus* Boddaert	USA	–	–	–	MH581272	–	Locke et al. 2018
C. medioconiger	*T. maximus*	USA	–	–	–	MN817946	–	Achatz et al. 2020
C. medioconiger	*T. maximus*	USA	MN820664	–	MN820664	–	–	Achatz et al. 2020
Cardiocephaloides physalis (Lutz, 1927)	*Spheniscus demersus* (Linnaeus)	East coast of South Africa	MW370425	MW370433	–	MW365507	–	Present study
C. physalis	*Clinus superciliosus* (Linnaeus)	Hermanus, SA	MW370426	–	MW370417	MW365508	–	Present study
C. physalis	*C. superciliosus*	Hermanus, SA	–	–	–	MW365509	–	Present study
C. physalis	*C. superciliosus*	Hermanus, SA	MW370427	–	MW370418	MW365510	–	Present study
C. physalis	*Spheniscus magellanicus* Forster	Chile	MN820665	–	MN820665	–	–	Achatz et al. 2020
C. physalis	*S. magellanicus*	Chile	–	–	–	MN817947	–	Achatz et al. 2020
C. physalis	*Sardinops sagax* Jenyns	South Africa	MK185719	–	–	–	–	Uzonnah et al. (unpublished)
Cardiocephaloides sp.	*C. superciliosus*	Saldanha Bay, SA	–	–	–	MW365513	H1	Present study
Cardiocephaloides sp.	*C. superciliosus*	Saldanha Bay, SA	–	–	–	MW365512	H2	Present study
Cardiocephaloides sp.	*C. superciliosus*	Hermanus, SA	–	–	–	MW365511	H1	Present study
Cardiocephaloides sp.	*C. superciliosus*	Tsitsikamma National Park, SA	MW370428	MW370434	MW370419	MW365514	H4	Present study
Cardiocephaloides sp.	*C. superciliosus*	Tsitsikamma National Park, SA	MW370429	–	MW370420	–	–	Present study
Cardiocephaloides sp.	*C. superciliosus*	Tsitsikamma National Park, SA	–	–	–	MW365515	H4	Present study
Cardiocephaloides sp.	*C. superciliosus*	Tsitsikamma National Park, SA	–	–	–	MW365516	H4	Present study
Cardiocephaloides sp.	*C. superciliosus*	Jeffreys Bay, SA	–	–	–	MW365517	H2	Present study
Cardiocephaloides sp.	*C. superciliosus*	Jeffreys Bay, SA	–	–	–	MW365518	H2	Present study

Table 2. Continued

Species	Host	Locality	GenBank accession numbers				Haplotype	Reference
			28S	ITS1-5.8S-ITS2	ITS2	COI		
Cardiocephaloides sp.	*C. superciliosus*	Chintsa, SA	–	–	–	MW365519	H3	Present study
Cardiocephaloides sp.	*C. superciliosus*	Chintsa, SA	–	–	–	MW365520	H3	Present study
Cardiocephaloides sp.	*C. superciliosus*	Chintsa, SA	MW370430	–	MW370421	MW365521	H3	Present study
Cardiocephaloides sp.	*C. superciliosus*	Chintsa, SA	–	–	–	MW365522	H3	Present study
Cardiocephaloides sp.	*C. superciliosus*	Chintsa, SA	MW370431	–	MW370422	–	–	Present study
Cardiocephaloides sp.	*C. superciliosus*	Chintsa, SA	–	–	MW370423	–	–	Present study
Cardiocephaloides sp.	*Clinus cottoides* Valenciennes	Jeffreys Bay, SA	MW370432	–	MW370424	MW365523	H2	Present study
Cardiocephaloides sp.	*C. cottoides*	Jeffreys Bay, SA	–	–	–	MW365524	H2	Present study
Cardiocephaloides sp.	*C. cottoides*	Jeffreys Bay, SA	–	–	–	MW365525	H2	Present study
Cardiocephaloides sp.	*Larus occidentalis* Audubon	Mexico	MF398341	–	–	–	–	Hernández-Mena et al. 2017
Cardiocephaloides sp.	*L. occidentalis*	Mexico	–	–	–	JX977784	–	Hernández-Mena et al. 2014
Cardiocephaloides sp.	*L. occidentalis*	Mexico	–	–	JX977844	–	–	Hernández-Mena et al. 2014
Strigeidae sp.	*Cominella adspersa* (Bruguière)	New Zealand	–	KU695784[a]	–	–	–	Donald and Spencer 2016
Outgroup								
Cotylurus cornutus (Rudolphi, 1809)	*Stagnicola elodes* (Say)	Canada	–	–	–	MH369480	–	Gordy and Hanington 2019
Cotylurus marcogliesei Locke, Van Dam, Caffara, Pinto, López-Hernández et Blanar, 2018	*Lophodytes cucullatus* (Linnaeus)	Canada	MH521248	–	MH521248	–	–	Locke et al. 2018

Results

General observations

In total, 83 *Clinus superciliosus* from Saldanha Bay (19), Cape Town (16), Hermanus (8), Tsitsikamma National Park (17), Jeffreys Bay (14) and Chintsa (9) were examined for the presence of trematodes. Of these, 21 were found to be infected with metacercariae of the genus *Cardiocephaloides*. Most metacercariae were present in the eyes of fishes, but some were found in the brain of fish from Saldanha Bay (n = 8) and Chintsa (n = 1). The prevalence and intensity of infection were as follows: Saldanha Bay (21%, 1–2 in the eyes; 42%, 1–16 in the brain), Hermanus (2 of 8, 3 individuals in the eyes), Tsitsikamma National Park (29%, 1–6), Jeffreys Bay (21%, 1–2) and Chintsa (3 of 9, 1–8 in the eyes; 1 of 9, 76 individuals in the brain). No metacercariae were present in fish collected in Cape Town harbour. Out of six specimens of *C. cottoides* examined from Jeffreys Bay, the eyes of four were infected with 1–6 metacercariae of *Cardiocephaloides*.

Molecular characterisation of metacercariae

Newly generated sequences were compared with each other and with sequences of *Cardiocephaloides* spp. available in GenBank. The novel 28S, ITS2 and COI sequences of the adult *C. physalis* were identical with sequences of three metacercarial specimens obtained from the eyes of *C. superciliosus* from Hermanus and with sequences of metacercariae previously reported from *Sardinops sagax* in South Africa. All other sequences generated in this study represent another species of *Cardiocephaloides*.

Further phylogenetic analyses based on the three targeted molecular markers produced trees with a similar topology. In the phylogenetic tree based on the 28S rDNA dataset (Fig. 2A), novel sequences of *C. physalis* formed a highly supported clade with sequences of the same species retrieved from GenBank. This clade included novel sequences generated for metacercariae collected from the eyes of *C. superciliosus* in Hermanus and adult worms collected from *S. demersus* on the east coast of South Africa, as well as metacercariae collected from the eyes of South American pilchards, *S. sagax* along the coast of South Africa and adults from *Spheniscus magellanicus* from Chile. Novel sequences and sequences of *C. physalis* from GenBank were identical. Four identical sequences of the metacercarial isolates of *Cardiocephaloides* sp. collected from various localities along the South African coast, formed a separate clade at the basal position to the species of *Cardiocephaloides* included in the analyses. The interspecific divergence between *Cardiocephaloides* sp. and four other species of this genus

was 0.6–1.2% (5–10 nt), with *C. physalis* being the least divergent and *C. medioconiger* being the most divergent.

The analyses of the ITS2 dataset showed similar results (Fig. 2B). Sequences for *C. physalis* of the present study, together with an identical sequence of *C. physalis* from *S. magellanicus* collected in Chile formed a highly supported clade. Novel sequences for the unknown species of *Cardiocephaloides* collected from *C. superciliosus* and *C. cottoides* clustered together. No intraspecific divergence was observed between ITS2 sequences of this species. The interspecific divergence between *Cardiocephaloides* sp. and other species of *Cardiocephaloides* in the ITS2 analyses was 0.7–3.6% (3–15 nt), with *C. physalis* being less divergent and *Cardiocephaloides* sp. (JX977844) obtained from the western gull, *Larus occidentalis* Audubon in Mexico, being most divergent. The overall interspecific variation in the ITS2 dataset ranged between 0.7% and 3.8% (3–16 nt).

Within the analyses of the COI gene dataset, novel sequences for *C. physalis* clustered with a sequence of this species collected from *S. magellanicus* from Chile in a clade with strong support (Fig. 2C). The intraspecific divergence within this clade was 0.4–0.9% (2–4 nt) with sequences from South Africa exhibiting the highest sequence divergence. Novel sequences for the unknown species of *Cardiocephaloides* obtained in the present study formed a strongly supported clade. The intraspecific divergence between the isolates of this species was 0–0.4% (0–2 nt). Overall interspecific variation between species of *Cardiocephaloides* in the COI dataset was 8.2–13.2% (38–61 nt), with *C. physalis* and *Cardiocephaloides* sp. exhibiting the lowest interspecific divergence and *C. longicollis* and *Cardiocephaloides* sp. (JX977784) showing the highest sequence divergence.

Comparison of ITS1 sequence data between *Cardiocephaloides* sp. (MW370420) obtained in the present study, with the previously published sequences of Strigeidae sp. (*Cardiocephaloides* sp. in the analyses of Achatz et al. 2020) (KU695784 – KU695791) demonstrated that *Cardiocephaloides* sp. from the present study is conspecific to the species from New Zealand (Donald and Spencer 2016). Genetic divergence between sequences of isolates from South Africa and New Zealand was 0.2% (1 nt).

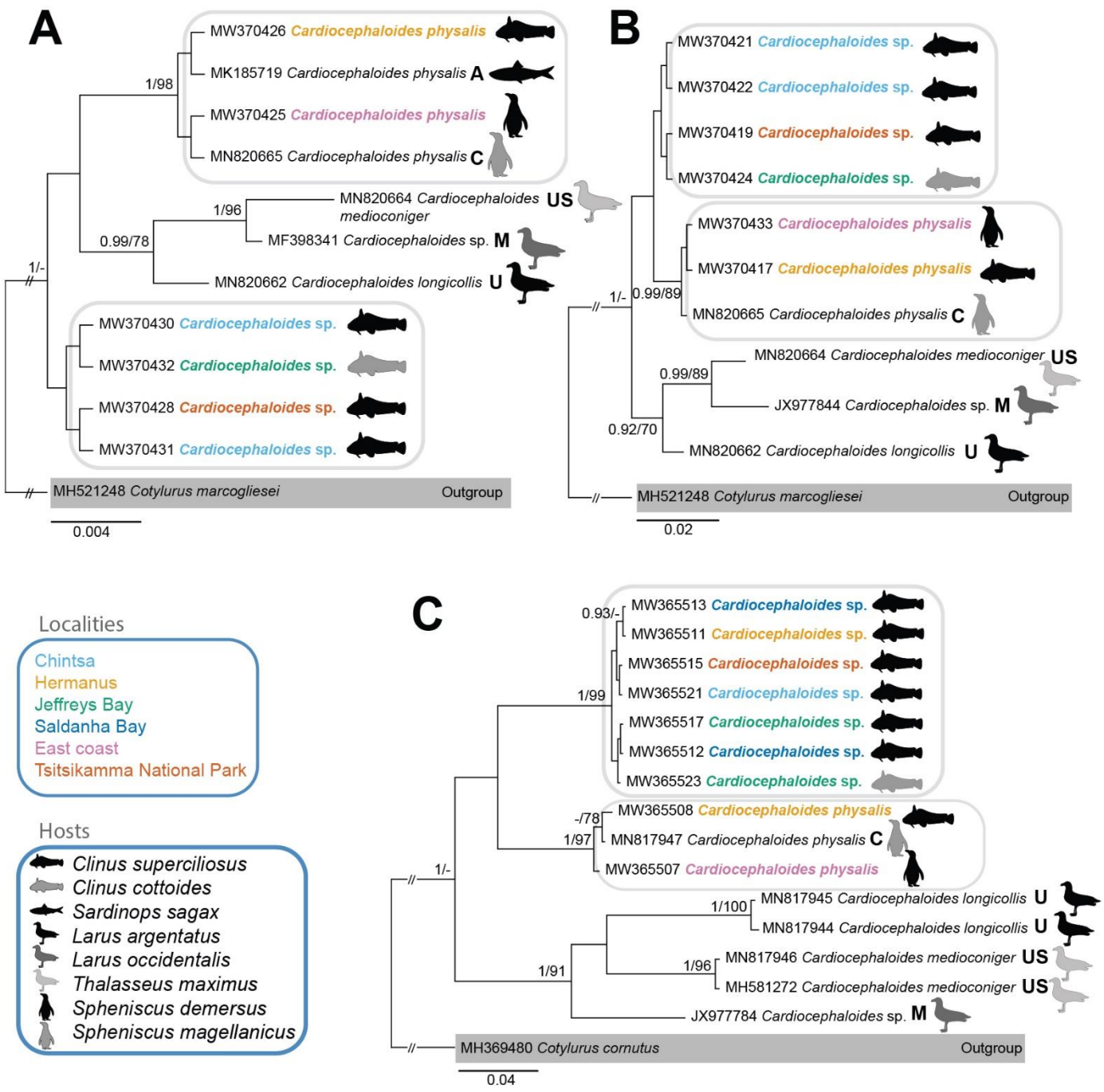

Figure 2. Bayesian inference (BI) trees based on the 28S rDNA (A), ITS2 (B) and COI (C) sequences. Nodal support values are given as BI/ML (maximum likelihood). Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. Newly generated sequences are highlighted in bold. Colours of the species names refer to sampling localities as indicated in the key. Abbreviations: A, Atlantic Ocean, South Africa; C, Chile; M, Mexico; U, Ukraine; US, United States of America

The 15 COI sequences (625 nt) generated in the present study for isolates of *Cardiocephaloides* sp. collected at five localities along the coast of South Africa, were collapsed into four haplotypes (Fig. 3). Two of the haplotypes (H1 and H2) found in *C. superciliosus* in Saldanha Bay (west coast) were detected in two distant localities: H1 in *C. superciliosus* in Hermanus (south coast) and H2 (shared with six isolates) in *C. superciliosus* and *C. cottoides* in Jeffreys Bay (south coast). Two other haplotypes were recovered in *C. superciliosus* from Tsitsikamma National Park (H4 shared with three isolates) and in *C. superciliosus* from Chintsa (H3 shared with four isolates).

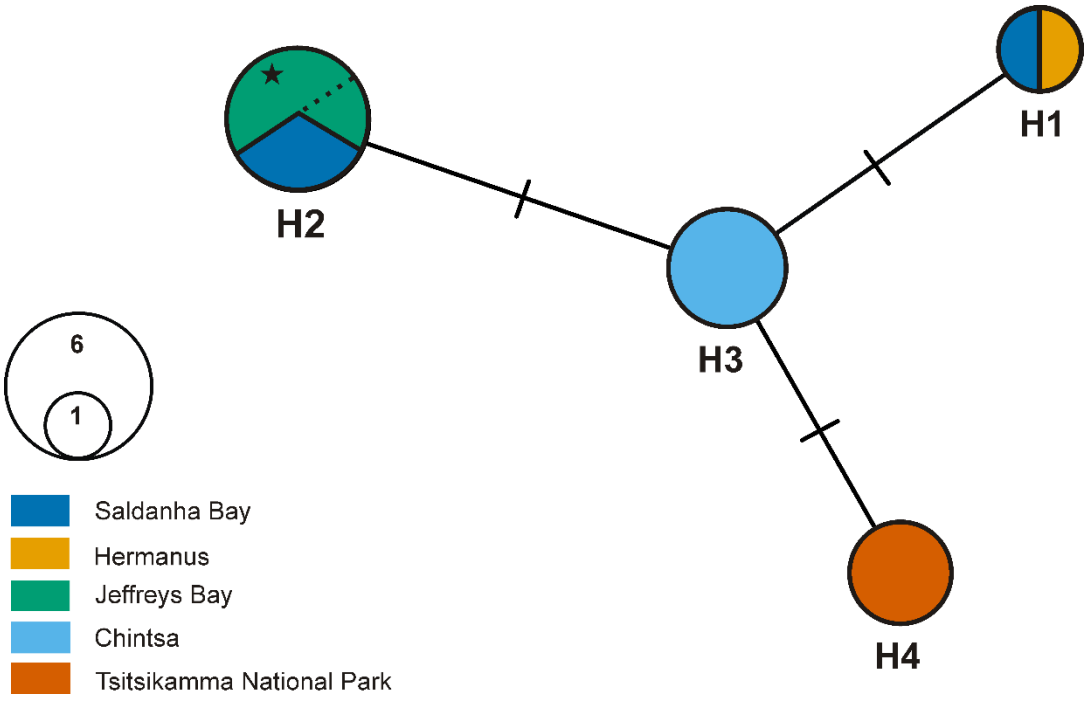

Figure 3. Haplotype network for *Cardiocephaloides* sp. based on novel COI sequences from metacercarial isolates collected at five sampling localities along the coast of South Africa from *Clinus superciliosus* and *Clinus cottoides*. Unsampled intermediate haplotype is represented by short intersecting line; each branch corresponds to a single mutational difference and connective lines represent one mutational step. Circle size is proportional to the number of isolates sharing a haplotype; haplotype frequency is indicated by colourless circles. Isolates obtained from *Clinus cottoides* are indicated by a star. Abbreviation: H, haplotype. Numbers indicate the haplotype code number (see Table 2 for details)

Morphological characterisation of metacercariae

***Cardiocephaloides physalis* (Lutz, 1926)** Fig. 4A

Description (based on three whole excysted specimens – digital vouchers; metrical data in Table 3). Metacercariae of the 'tetracotyle' type, encysted in thin-walled subspherical, colourless, transparent cysts. Body pyriform, indistinctly bipartite, with maximum width just anterior to ventral sucker, body longer than wide. Forebody short, representing 40–41% (40%) of body length, larger than hindbody, with ventral concavity. Hindbody more flattened ventrally. Tegument thick, unarmed. Oral sucker transversely oval, muscular, subterminal. Oral opening ventro-subterminal. Pseudosuckers large, elongate-oval, lateral to oesophagus, between oral and ventral suckers. Prepharynx, pharynx, oesophagus and caeca not observed. Ventral sucker transversely oval, larger than oral sucker; oral/ventral sucker length ratio 1 : 1.2−1.4 (1 : 1.3), oral/ventral sucker width ratio 1 : 1.3−1.5 (1 : 1.4), at mid-body length or slightly anterior. Distance from anterior extremity of body to ventral sucker 360−363 (362). Holdfast organ large, transversely oval, bipartite, with longitudinal slit-like aperture, contiguous with and partially overlapping ventral sucker ventrally. Excretory system of 'tetracotyle' type, composed of network filling body with free excretory granules in canals. Numerous medium-sized excretory granules distributed in two lateral fields between posterior margin of oral sucker and posterior margin of holdfast organ; fields confluent at the level of ventral sucker. Excretory vesicle not observed. Excretory pore terminal.

Second intermediate host: Super klipfish *Clinus superciliosus* (Linnaeus) (Perciformes: Clinidae).

Locality: Hermanus (34°25'15.86"S; 19°14'37.56"E), South Africa.

Site of infection: Vitreous humour of eye.

Molecular data: MW370426–MW370427 (28S), MW370417–MW370418 (ITS2), MW365508–MW365510 (COI).

Table 3. Comparative metrical data (in µm) for *Cardiocephaloides* spp.

Species	*Cardiocephaloides physalis* (Lutz, 1927)		*Cardiocephaloides physalis*		*Cardiocephaloides* sp.		*Cardiocephaloides* sp.		*Cardiocephaloides* sp.	
Host	*Clinus superciliosus* (Linnaeus)		*Sardinops sagax* (Jenyns)		*Engraulis anchoita* Hubbs et Marini		*Clinus superciliosus*, *Clinus cottoides* Valenciennes		*Clinus superciliosus*, *Clinus cottoides*	
Locality	Hermanus, South Africa		Gansbay, South Africa		Argentina, Uruguay		Tsitsikamma, Jeffreys Bay, South Africa		Tsitsikamma, Jeffreys Bay, South Africa	
Source	(present study)		Ukomadu (2017)		Timi et al. (1999)		(present study, not stained digital vouchers)		(present study, stained vouchers)	
	range (n = 3)	mean	range (n = 77)	mean	range (n = 25)	mean	range (n = 14)	mean	range (n = 10)	mean
Body length	791–907	861	762–967	875	550–860	656	661–977	793	502–1407	866
Body width	568–787	675	512–677	605	360–620	468	500–740	605	272–946	670
Forebody length	360–363	362	249–386	336	210–420	194	231–474	320	208–485	336
Hindbody length	525–544	535	370–440*	405*	185–310*	244*	371–648	473	291–922	531
Oral sucker length	116–121	119	87–112	104	70–110	93	76–125	100	89–211	124
Oral sucker width	113–130	124	92–116	107	80–120	97	62–136	96	66–233	115
Ventral sucker length	142–160	151	122–146	135	100–150	132	90–186	144	122–226	169
Ventral sucker width	164–196	180	121–147	135	110–148	133	113–205	155	124–268	195
Pseudosuckers length	–	–	165–233	–	109–160	142	101–138	120	64–234	132
Pseudosuckers width	–	–	79–116	–	–	–	67–105	87	44–197	105
Holdfast organ length	292 (n = 1)	–	127–200	172	100–200	140	191–433	298	212–416	300
Holdfast organ width	319 (n = 1)	–	216–321	281	240–310	276	202–298	246	148–363	234
Body width:length ratio	1:1.2–1.4	1:1.3	–	–	–	–	1:1.1–1.6	1:1.3	1:1.3–1.9	1:1.5
Forebody length as % body length	40.0–40.7	40.4	–		–	–	32.8–51	40	33–42	39
Oral sucker length:Ventral sucker length	1:1.2–1.4	1:1.3	–	–	–	–	1:1–2	1:1.5	1:1–1.6	1:1.4
Oral sucker width:Ventral sucker width	1:1.3–1.5	1:1.4	–	–	–	–	1:1.2–2.5	1:1.6	1:1.2–2	1:1.8

*values represent length of post acetabular region, not hindbody.

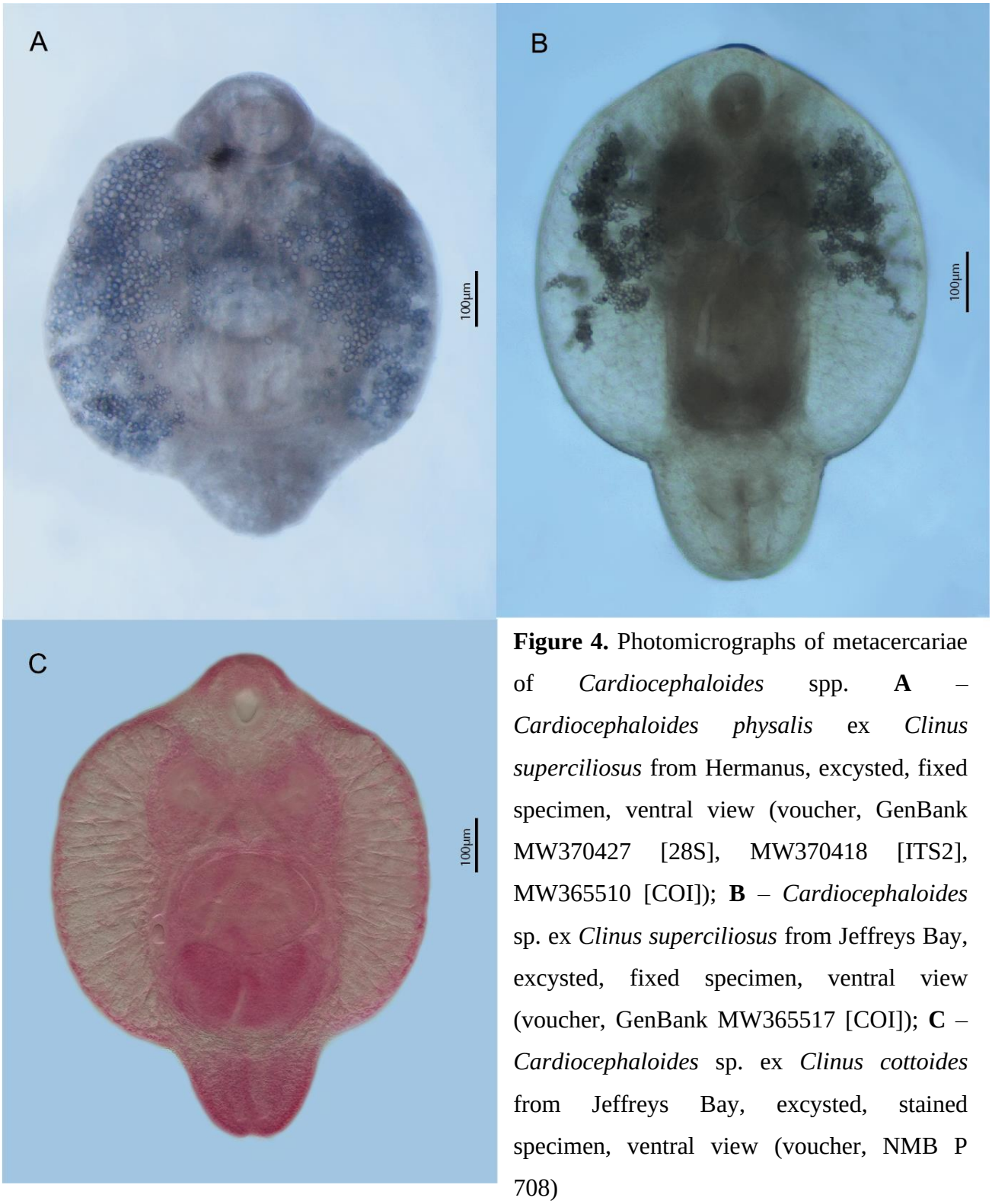

Figure 4. Photomicrographs of metacercariae of *Cardiocephaloides* spp. **A** – *Cardiocephaloides physalis* ex *Clinus superciliosus* from Hermanus, excysted, fixed specimen, ventral view (voucher, GenBank MW370427 [28S], MW370418 [ITS2], MW365510 [COI]); **B** – *Cardiocephaloides* sp. ex *Clinus superciliosus* from Jeffreys Bay, excysted, fixed specimen, ventral view (voucher, GenBank MW365517 [COI]); **C** – *Cardiocephaloides* sp. ex *Clinus cottoides* from Jeffreys Bay, excysted, stained specimen, ventral view (voucher, NMB P 708)

***Cardiocephaloides* sp.** Fig. 4B, C

Description (based on 32 whole excysted specimens – digital vouchers and stained specimens; metrical data in Table 3). Metacercariae of the 'tetracotyle' type, encysted in thin-walled, subspherical, fluid-filled, colourless, transparent cysts. Body pyriform, indistinctly bipartite, with maximum width anterior to ventral sucker, body longer than wide. Forebody short, representing 33–51% (40%) of body length of digital voucher specimens and 33–42% (39%) of stained specimens, with ventral concavity. Hindbody more flattened ventrally, shorter than forebody. Tegument thick, unarmed. Oral sucker elongate-oval, muscular, subterminal. Oral opening ventro-subterminal. Pseudosuckers large, elongate-oval, lateral to oesophagus, between oral and ventral suckers. Prepharynx absent, pharynx small, subspherical, muscular; oesophagus short, bifurcates at mid-level between pharynx and ventral sucker; intestinal caeca short, narrow, reach posterior to ventral sucker. Ventral sucker transversely oval, distinctly larger than oral sucker; oral/ventral sucker length ratio 1 : 1–2 (1 : 1.5) of digital voucher specimens and 1 : 1–1.6 (1 : 1.4) of stained specimens, oral/ventral sucker width ratio 1 : 1.2–2.5 (1 : 1.6) of digital voucher and 1 : 1.2–2 (1 : 1.8) of stained specimens, at mid-body length or slightly anterior. Distance from anterior extremity of body to ventral sucker 231–474 (320) of digital voucher specimens and 208–485 (336) of stained specimens. Holdfast organ large, transversely oval, bipartite, with longitudinal slit-like aperture between two large lobes, overlapping ventral sucker ventrally, extends beyond anterior and posterior margins of ventral sucker. Excretory system of 'tetracotyle' type, composed of network filling body with free excretory granules in canals. Numerous small excretory granules occupy most of body between posterior margin of oral sucker and level of mid-length of holdfast organ. Excretory vesicle V-shaped. Excretory pore terminal.

Second intermediate hosts: Super klipfish *Clinus superciliosus* (Linnaeus), Bluntnose klipfish *Clinus cottoides* Valenciennes (Perciformes: Clinidae).

Locality: Saldanha Bay (33°2'44.46"S; 18°2'19.06"E), Hermanus (34°25'15.86"S; 19°14'37.56"E), Tsitsikamma National Park (34°1'15.21"S; 23°52'43.23"E), Jeffreys Bay (34°1'35"S; 24°55'51"E) and Chintsa (32°50'11.54"S; 28°7'1.19"E), South Africa.

Site of infection: Vitreous humour of eye (*C. superciliosus*, *C. cottoides*), brain (*C. superciliosus*).

Voucher material: 96 voucher specimens deposited in NMB P 706–722: NMB P 706–716 – 11 stained and permanently mounted specimens and NMB P 717–722 – 85 specimens in ethanol.

Molecular data: MW370428–MW370432 (28S), MW370434 (ITS1-5.8S-ITS2), MW370419–MW370424 (ITS2), MW365511–MW365525 (COI).

DISCUSSION

Cardiocephaloides physalis was known to be the only representative of the genus *Cardiocephaloides* in South Africa for decades since it was first recorded in *Spheniscus demersus* (Randall and Bray 1983, Horne et al. 2011, Espinaze et al. 2019). Interestingly, metacercariae of this genus were previously reported by Parukhin (1968, 1975), prior to the discovery of adults. Parukhin found encysted metacercariae of *Cardiocephaloides* sp. (reported as "Tetracotyle sp. larvae") in the vitreous humour of the eyes of 26 *S. sagax* (P = 10.6%, 1–7 specimens in the eyes) collected from the waters around South Africa. Later, metacercariae of *Cardiocephaloides* were found in the same species of pilchards collected along the South African coast by Reed et al. (2012), Weston et al. (2015) and Ukomadu (2017), and recently a 28S rDNA sequence of these metacercariae confirming their identity as *C. physalis*, was deposited in GenBank (Ukomadu 2017; as Uzonnah et al. unpublished in GenBank).

Our study has revealed that the species diversity within *Cardiocephaloides* in South Africa is higher than previously known and that the spectrum of their intermediate hosts is not limited to pilchards. With morphological and molecular evidence based on three genetic markers (28S, ITS2 and COI) we report metacercariae of *C. physalis* from *C. superciliosus* and the second species of the genus, *Cardiocephaloides* sp. parasitising both *C. superciliosus* and *C. cottoides* along the west and south coasts of South Africa.

Morphologically, specimens of *C. physalis* in our study were consistent with specimens of the same species found in *S. sagax* by Ukomadu (2017) and specimens described by Timi et al. (1999) as *Cardiocephaloides* sp. from *Engraulis anchoita* Hubbs et Marini, suggested to belong to *C. physalis*. Despite similarities in body size of metacercariae from our material and metacercariae described by Ukomadu (2017) (Table 3), our specimens differed in possessing a more elongate holdfast organ (292 µm *vs* 127–200 µm), larger oral (length 116–121 µm *vs* 87–112 µm; width 113–130 µm *vs* 92–116 µm) and ventral (length 142–160 µm *vs* 122–146 µm; width 164–196 µm *vs* 121–147 µm) suckers, and in exhibiting higher minima and maxima for body width (568–787 µm *vs* 512–677 µm). In comparison to the metacercariae of *Cardiocephaloides* sp. of Timi et al. (1999), our specimens of *C. physalis* had higher minima and maxima for all dimensions (see Table 3 for details). These differences can be ascribed to the limited sample size of metacercariae in the present study and variations in maturation of the metacercarial stages from the different studies.

It is interesting that metacercariae of *C. physalis* were found in both an intertidal fish host as well as an offshore fish host from different families. This suggests that the cercariae of this species do not exhibit strict host specificity, infecting a broad spectrum of fish intermediate

hosts. However, further studies are required to confirm transmission patterns of this trematode species.

Achatz et al. (2020) cautiously confirmed, based on molecular evidence, the broad distribution of *C. physalis* in both Africa and South America. However, the authors suggested that further comparison of data from faster mutating genes such as the mitochondrial COI gene, might provide a better substantiated conclusion. From our COI analyses, it is evident that the species does not exhibit high genetic intraspecific variability (0.4–0.9%), even in isolates from distant geographical localities. Thus, *C. physalis* can indeed be regarded as a single, widely distributed trematode species.

The presence of the second species of *Cardiocephaloides*, *Cardiocephaloides* sp. in our material was confirmed by the results of the phylogenetic analyses. Interestingly, based on comparative sequence analysis of the ITS1 region, this species appeared to be conspecific to the species of sporocyst isolates reported from the whelks *Cominella adspersa* (Bruguière), *C. glandiformis* (Reeve) and *C. virgata* Adams et Adams in New Zealand (Donald and Spencer 2016). This suggests that the distribution of *Cardiocephaloides* sp. is broader and not restricted to the coast of southern Africa. However, the characterisation of adult specimens is required to identify these larval trematodes to a lower taxonomic position. To date, three species of *Cardiocephaloides* were described and reported from Australia and New Zealand (Dubois and Angel 1972): *Cardiocephaloides hilli* (Johnston, 1904) from *Chroicocephalus novaehollandiae* (Stephens) (Australia), *C. musculosus* (Johnston, 1904) from *Chlidonias hybrida* (Pallas) and *Hydroprogne caspia* (Pallas) (Australia), and *C. ovicorpus* Dubois et Angel, 1972 from *Phalacrocorax varius* (Gmelin) (Australia) and *P. melanoleucos brevirostris* (Gould) (New Zealand). Therefore, *Cardiocephaloides* sp. may either be one of these known species for which adult specimens have not yet been molecularly characterised or represents a new species within the genus. While it is difficult to predict the distribution pathway of this species between the continents at the present stage, our study once again demonstrates that DNA sequencing is an extremely efficient and precise tool of advancing our knowledge not only in species identification, but in geographical distribution of parasites, even if based on their larval stages.

Specimens of *Cardiocephaloides* sp. from the two clinid species were morphologically relatively similar to, but overall larger than *Cardiocephaloides* sp. found by Timi et al. (1999). Yet, the pseudosuckers of our specimens from digital vouchers showed slightly lower minima and maxima for length than that noted by Timi et al. (1999) (101–138 µm *vs* 109−160 µm). Additionally, it is evident that stained specimens of *Cardiocephaloides* sp. in our material were overall slightly larger than specimens from digital vouchers, with the exception of the holdfast

organ width (Table 3). When compared to our specimens of *C. physalis*, *Cardiocephaloides* sp. (digital vouchers) was morphometrically similar, but smaller in all body dimensions with the exceptions of the forebody as a proportion of body length (higher maxima, 33–51% *vs* 40–41%) and sucker ratio (higher maxima for length 1 : 1–2 *vs* 1 : 1.2–1.4 and width 1 : 1.2–2.5 *vs* 1 : 1.3–1.5).

Detailed morphological and morphometric analysis of metacercariae of the two species collected in South Africa during the present study, demonstrated remarkably little variation that can be used for species differentiation or identification. However, we found a high degree in variability of the number, size and distribution of excretory granules. Excretory granules in metacercariae of *C. physalis* were medium-sized, dense and distributed in two lateral fields between the posterior margin of the oral sucker and the posterior margin of the holdfast organ, with fields confluent at the level of the ventral sucker (Fig. 4A). In contrast, excretory granules in metacercariae of *Cardiocephaloides* sp. were small, less dense and distributed between the posterior margin of the oral sucker and the mid-level of the holdfast organ length (Fig. 4B). We could not compare our data to those of Timi et al. (1999) and Ukomadu (2017) as the authors did not describe or illustrate excretory granules in detail, but we suggest that this characteristic can be potentially useful for species delineation prior to molecular identification. Previously, differences in the number, size and distribution of excretory granules were considered in several studies when differentiating between metacercariae of the genus *Diplostomum* von Nordmann, 1832 (see Shigin 1986, Pérez-del-Olmo et al. 2014, Kudlai et al. 2017).

Due to the simple morphology, distinction between metacercariae of *C. physalis* and *Cardiocephaloides* sp. is not obvious. Therefore, molecular characterisation is essential for the accurate identification of these species, pointing out the importance of an integrative approach to the study of trematodes (Blasco-Costa et al. 2016), especially their larval stages that do not yet possess various characteristic features (Hoogendoorn et al. 2020).

Four haplotypes of *Cardiocephaloides* sp. were shared among isolates collected from *C. superciliosus* and *C. cottoides* in five localities. According to the haplotype analyses, haplotype H1 was found in *C. superciliosus* from Saldanha Bay and Hermanus, whereas haplotype H2 was present in Saldanha Bay and Jeffreys Bay (Figs. 1, 3) in both *C. superciliosus* and *C. cottoides*. The haplotype mixture in the geographically distant localities suggests that there is gene flow between populations from the west coast and south-east coast study sites. The low host specificity at the level of host species was illustrated by the presence of haplotype H2 in both species of *Clinus*, which agrees with the general rule for specificity of trematodes to their second intermediate hosts (Galaktionov and Dobrovolskij 2003). As H2 is more abundant in Jeffreys

Bay, it can be assumed that this is where the haplotype originated and has subsequently spread to Saldanha Bay. This may be due to direct transmission of digenean descendants between these sites via final host migration. Haplotypes H3 and H4 were found in Chintsa and Tsitsikamma National Park, respectively. Interesting to note is the complete absence of trematodes from *C. superciliosus* collected in Cape Town harbour, a locality with high commercial fishing activity and anthropogenic influences. Further investigation into the presence of suitable intermediate hosts from this locality or the effects of anthropogenic activity on these parasites, might give more insight into this anomaly.

Metacercariae of *Cardiocephaloides* sp. occurred more frequently and widespread in *C. superciliosus*, than *C. physalis* did, which may suggest that infection with *C. physalis* observed in our study was accidental or rather rare. Due to the much higher prevalence and widespread occurrence of metacercariae of *C. physalis* in *S. sagax* along the coast of South Africa (Reed et al. 2012, Weston et al. 2015, Ukomadu 2017), it is most likely that this is their primary host. Intertidal clinid species are likely the preferred second intermediate host for *Cardiocephaloides* sp. since infection with this trematode species is widespread along the South African coast. However, further research will be needed to confirm this assumption and to elicit the life cycles and host range of these species of *Cardiocephaloides*. As nassarid and buccinid molluscs have been reported as first intermediate hosts for species of *Cardiocephaloides* in the Mediterranean (Osset et al. 2005) and in the waters around New Zealand (Donald and Spencer 2016) respectively, they might be involved in the life cycles of these parasites in southern Africa. There are at least 12 species of the Nassariidae (Mollusca: Gastropoda), namely, *Bullia annulata* (Lamarck), *B. callosa* (Wood), *B. digitalis* (Dillwyn), *B. diluta* (Krauss), *B. laevissima* (Gmelin), *B. melanoides* (Deshayes), *B. natalensis* (Krauss), *B. pura* Melvill, *B. rhodostoma* Reeve, *B. tenuis* Reeve, *B. vittata* (Linnaeus) (see Brown 1982), *Nassarius kraussianus* (Dunker) (see Perissinotto et al. 2014), *N. capensis* (Dunker), *N. arcularia plicatus* (Röding), *N. niveus* (Adams) and *N. speciosus* (Adams) (see Branch et al. 2007), and six species of the Buccinidae, namely *Afrocominella capensis simoniana* (Petit de la Saussaye), *Burnupena cincta* (Röding), *B. lagenaria* (Lamarck), *B. catarrhacta* (Gmelin), *B. papyracea* (Bruguière) and *B. pubescens* (Küster) (Branch et al. 2007), occurring along the coast of South Africa that can potentially serve as the first intermediate hosts for *C. physalis* and *Cardiocephaloides* sp.

Metacercariae of *Cardiocephaloides* spp. are trophically transmitted to definitive hosts when infected fish are consumed by seabirds (Osset et al. 2005). To date, only *C. physalis* was reported from penguins, whereas the rest of the species within the genus are known to parasitise birds from the family Laridae that includes gulls, terns and skimmers (Niewiadomska 2002,

Hernández-Mena et al. 2014, Achatz et al. 2020). Due to their widespread occurrence along the South African coast, these seabirds along with others such as cormorants, may act as definitive hosts for *Cardiocephaloides* spp. Moreover, members of the Clinidae were recently reported as a part of the diet of a larid, the great crested tern *Thalasseus bergii* (Lichtenstein, MHC) in the Western Cape, South Africa (Gaglio et al. 2018).

Clinus spp. commonly occupy intertidal habitats, especially rock pools that support a wide diversity of organisms that may act as first or second intermediate hosts for digenean trematodes. Therefore, these fishes are likely potential intermediate and definitive hosts for a variety of trematodes, as they are easily targeted by cercariae and feed on a wide variety of organisms. Thus, it is not surprising that *C. superciliosus* and *C. cottoides* were reported as intermediate hosts for trematodes in the present study. Further dedicated research focusing on the role of klipfish in the life cycles of parasites along the coast of southern Africa and their natural range of distribution, could potentially lead to numerous and valuable discoveries.

The present study is not only reporting on the diversity of trematodes in marine fishes from South Africa, but also highlights the potential utility of easily accessible species of *Clinus* infected with eye and brain parasites (metacercariae of *Cardiocephaloides* spp.), for studying natural host-parasite relationships, in particular parasite manipulation of host behaviour in the marine environment.

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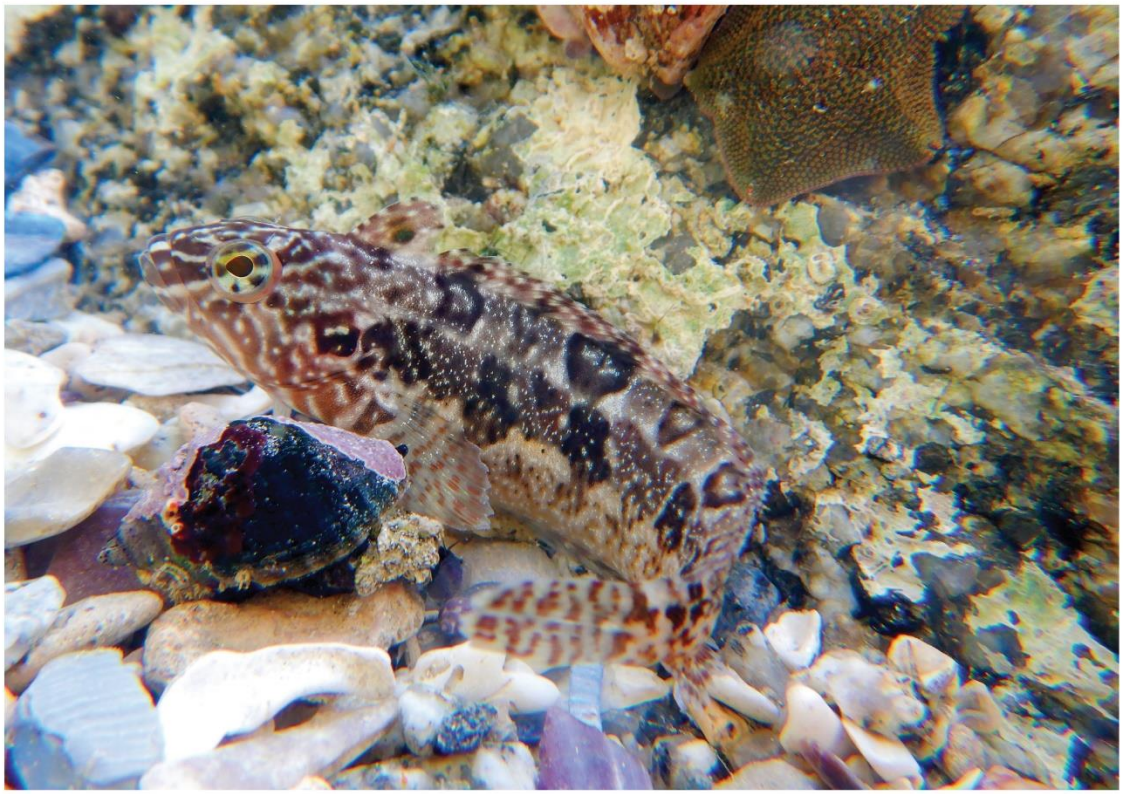
Chapter 4

Six species of the Opecoelidae (Trematoda: Digenea) infecting *Clinus superciliosus* (Linnaeus) (Perciformes, Clinidae) from South Africa



Chapter 4. Six species of the Opecoelidae (Trematoda: Digenea) infecting *Clinus superciliosus* (Linnaeus) (Perciformes, Clinidae) from South Africa

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Abstract

The Opecoelidae is the largest digenean family, thus it is no surprise to discover six opecoelid species, of which five may be new to science, from a single marine fish species along the coast of South Africa. By means of molecular (28S rDNA and ITS/ITS2) data combined with morphological characterisation, *Coitocaecum capense* Bray, 1987, three unidentified species of *Coitocaecum* and two unidentified species of *Helicometra* have been detected from *Clinus superciliosus* (Linnaeus) collected in Saldanha Bay, Hermanus, Tsitsikamma National park and Chintsa along the coast of South Africa. A previous study reported *Helicometra fasciata* (Rudolphi, 1819) and described *C. capense* from this fish host. Contrary to what was expected, no digeneans were found from fish collected in Cape Town harbour. This study thus provides the first molecular characterisation of species of *Coitocaecum* based on 28S rDNA and ITS2 sequences, which aided in the confirmation of the taxonomic position of this genus within the subfamily Opecoelinae. Furthermore, this study provides the first DNA sequence data of *Helicometra* spp. from South Africa and found adults of a species of *Helicometra*, of which

larval stages were previously reported from the Mediterranean, thus proving that the distribution of these species might be much broader than previously believed. These findings further emphasise the lack of diversity data for trematodes of marine fishes in South Africa, creating a great capacity for future explorative taxonomic studies and highlighting the use of intertidal areas for conducting such research.

Keywords: digenean, klipfish, marine fish parasite, DNA, morphology

Introduction

Of all digenean families, the Opecoelidae Ozaki, 1925 is the most speciose, with more than 900 species from over 90 genera (Bray et al. 2016; Martin 2019). Members of this family are under continuous reorganisation, greatly due to their homoplastic morphology and the implementation of novel phylogenetic investigative efforts (Bray et al. 2016; Martin et al. 2019a). Opecoelids have a cosmopolitan distribution and utilise marine and freshwater fishes as definitive hosts (Bray et al. 2016). Various groups of animals are involved in the life cycles of this group of trematodes. While molluscs play the role of first intermediate hosts, the range of second intermediate hosts is much broader and includes a variety of invertebrates (crustaceans, aquatic insects, oligochaetes, leeches, gastropods, cephalopods, echinoids, turbellarians and scleractinian corals) and even fish (Martin et al. 2019a).

To date, merely 15 opecoelids have been reported from marine fishes in South Africa, of which five were described from this region: *Allopodocotyle recifensis* Bray, 1987 ex *Pterogymnus laniarius* (Valenciennes) from Cape Recife; *Coitocaecum capense* Bray, 1987 ex *Clinus superciliosus* (Linnaeus), *Clinus cottoides* Valenciennes and *Cirrhibarbis capensis* Valenciennes from Port Elizabeth, as well as *Cl. superciliosus*, *C. cottoides*, *Clinus rotundifrons* Barnard, *Xenopoclinus kochi* Smit and *Xenopoclinus leprosus* Smit from Oudekraal; *Dactylostomum griffithsi* Bray, 1987 ex *Cheilodactylus fasciatus* Lacepède from Oudekraal, *Margolisia vidalensis* Bray, 1987 ex *Trachinocephalus myops* (Forster) from Cape Vidal; and *Pseudopecoelus ablennesi* Bray, 1987 ex *Ablennes hians* (Valenciennes) from Durban and St. Lucia (Bray 1987). Given the high number of endemic fish species, it can be assumed that this is not an accurate representation of the Opecoelidae from the unique, biodiversity-rich habitats along the South African coast.

Clinid species (Perciformes: Clinidae) are abundant inter- and subtidal fishes, nearly half of which are endemic to southern Africa (von der Heyden et al. 2011). One of the most abundant

and endemic species, *Cl. superciliosus*, has been reported as definitive host for two opecoelid trematodes: *Helicometra fasciata* (Rudolphi, 1819) and *C. capense* (Bray, 1987).

During a study exploring the diversity of digenean trematodes of *Cl. superciliosus* from a large portion of its distributional range in South Africa, we found that among other, it is a host to four species of *Coitocaecum* and two species of *Helicometra* – this high diversity of opecoelid trematodes from a single fish species, again emphasises the expanse of this species-rich digenean family. Overall, there is a deficiency not only of trematode descriptions and reports from marine fishes in South Africa, but especially of molecular data of such trematodes. This is the first study to molecularly characterise species of the Opecoelidae from South Africa and to provide data of good quality for the ongoing reorganisation of this trematode family, especially considering their homoplastic morphology. Additionally, these newfound discoveries from a previously studied fish species, further highlight the prospect for explorative biodiversity studies of marine trematodes from this richly abundant coastline.

Materials and Methods

In total, 71 specimens of *Cl. superciliosus* were collected in rocky intertial areas and intertidal rock pools from five localities along the South African coast: Saldanha Bay (33°2'44.46"S; 18°2'19.06"E) (n=19), Cape Town harbour (33°54'29.13"S; 18°25'5.81"E) (n=16), Hermanus (34°25'15.86"S; 19°14'37.56"E) (n=8), Tsitsikamma National Park (henceforth referred to as TNP) (34°1'15.21"S; 23°52'43.23"E) (n=17) and Chintsa (32°50'11.54"S; 28°7'1.19"E) (n=11) (Fig. 1). As this fish species is found from the Skeleton Coast in Namibia to the Kei River in South Africa (von der Heyden et al. 2011), these dispersed sampling sites cover a large portion of the host's distribution. Additionally, the sites are situated within both the cold, nutrient-rich Benguela current as well as the warmer Agulhas current, thus the sampling sites encompass a wide variety of environmental conditions and unique habitats. Sampling was carried out under the permit MALH–K2016–005a for TNP; RES2018/35 for Hermanus; RES2019–103 for Saldanha Bay, Cape Town harbour and Chintsa. Fish were collected with baited traps and hand lines. After euthanisation, fish were subjected to helminthological examination, by inspecting every organ. Digenean trematodes were removed with fine needles, after which they were relaxed in hot saline and fixed in 80% ethanol for further analyses.

Morphological analysis

Trematode specimens were grouped based on their morphology and hologenophores chosen for molecular analysis were vouchered following the concept of Pleijel et al. (2008). The hologenophore along with other representatives of the same species, were stained with Mayer's

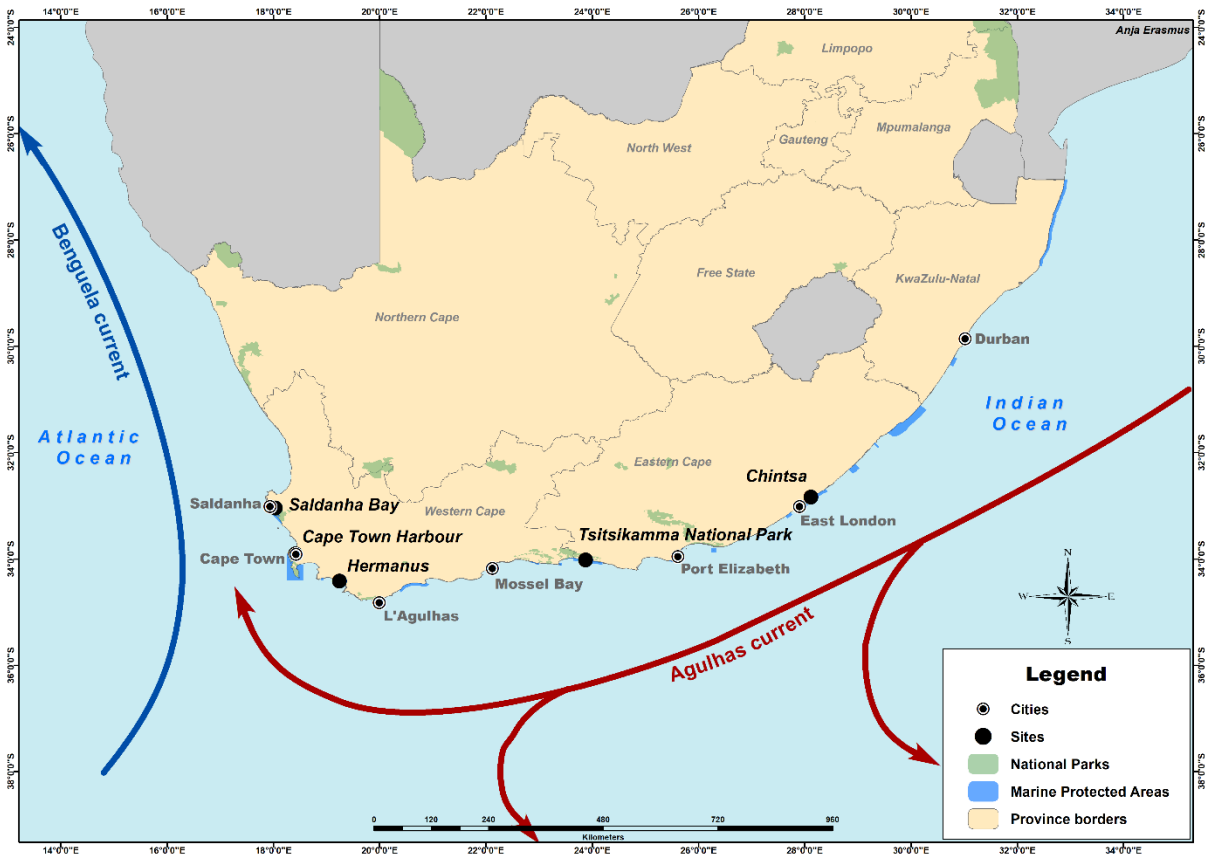

Figure 1. Map illustrating the study sites along the South African coast, included in the present study

haematoxylin and mounted on a permanent slide with Dammar gum. Slides were used to obtain morphometric measurements, to take photomicrographs and to make detailed morphological drawings for each species. All measurements were taken with NIS–Elements BR Camera Analysis software and are given in micrometres (µm), unless otherwise stated. The metrical data is presented as the range followed by the mean, while the number of specimens measured (n) is in parentheses. Digital illustrations were created using Adobe Illustrator CC v. 22.0 and Photoshop CC v. 25.0. Voucher material has been submitted in the Parasite Collection of the National Museum (NMB), Bloemfontein, South Africa.

Generation of molecular data

DNA was extracted from the excised sections of voucher specimens by using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) or PCRBiosystems Rapid DNA Extraction Kit (PCRBiosystems available from Analytical Solutions, Randburg, South Africa). The manufacturer's protocol for each extraction kit was followed, except the following alterations to the latter: only 10 µl lysis buffer and 5 µl protease containing buffer was used; the

reaction was finally diluted with 450 µl molecular water instead of 900 µl as recommended. Polymerase chain reaction (PCR) was used to amplify the partial D1-D3 fragment of the 28S nuclear ribosomal RNA gene, the entire internal transcribed spacer region (ITS1-5.8S-ITS2) and the complete internal transcribed spacer 2 (ITS2) of the ribosomal gene cluster.

For amplification of the 28S rRNA gene, the forward primer Digl2 (5′-AAG CAT ATC ACT AAG CGG-3′) (Tkach et al. 2001) and reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Snyder and Tkach 2001) were used, following the protocol of Tkach et al. (2003). Additionally, two internal primers were used for sequencing of 28S rDNA: ECD2 (5'-CTT GGT CCG TGT TTC AAG ACG GG-3') (Tkach et al. 2003) and 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') (Littlewood et al. 2000). The ITS region was amplified with the forward primer D1 (5'-AGG AAT TCC TGG TAA GTG CAA G-3') and the reverse primer D2 (5'-CGT TAC TGA GGG AAT CCT GGT-3') (Galazzo et al. 2002). The protocol of Galazzo et al. (2002) was followed. The forward primer 3S (5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′) (Morgan and Blair 1995) and the reverse primer ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) (Cribb et al. 1998) was used to amplify the ITS2 gene region, following the protocol of Kudlai et al. (2015). Resultant PCR amplicons were visualised with 1% agarose gel electrophoresis and sent to Inqaba Biotechnical Industries (Pty). Ltd. in Pretoria, South Africa, for purification and sequencing. Obtained sequences were assembled and edited with Geneious v. 11.1.4 bioinformatics software (Biomatters, Auckland, New Zealand). Sequences have been deposited in GenBank.

Phylogenetic analyses

Available sequences for representatives of the family Opecoelidae were retrieved from GenBank as well as sequences for the outgroups (Table A-A2). Two alignments incorporating 28S rDNA and ITS2 sequences retrieved from GenBank and the novel sequences generated during our study, were built using MUSCLE (Edgar 2004) as implemented in Geneious v. 11.1.4. Outgroups were selected based on the analyses of Martin et al. (2018a). The jModelTest 2.1 (Posada 2008) software was used to determine the best nucleotide substitution model for each alignment, based on the Akaike information criterion (AIC). The general time reversible model with estimates of invariable sites and gamma distribution among site rate variation (GTR + I + G) was used for the construction of both the 28S and ITS2 phylogenetic trees. Both phylogenies are based on Bayesian inference (BI) and maximum likelihood (ML) estimate analyses. The analyses for BI were performed with MrBayes software as run on CIPRES Science gateway v. 3.3 (available at https://www.phylo.org/) and ML analyses were performed with PhyML v. 3.0

(available at http://www.atgc-montpellier.fr/phyml/). For the BI analyses of both alignments, the Markov chain Monte Carlo (MCMC) chains were run for 10,000,000 generations. The 'burn-in' parameter was set for the first 25% of the sampled trees. The results were visualised in Tracer ver. 1.6 (Rambaut et al. 2014) to assess proper sampling and to identify the 'burn-in' period. Nodal support for the ML analyses was determined by conducting 100 bootstrap pseudoreplicates. FigTree v. 1.4.3 (Rambaut 2012) software was used to visualise all phylogenetic trees. Pairwise genetic distance matrices (p-distances) were calculated in MEGA v. 7.

Results

General observations

Of the 71 specimens of *Cl. superciliosus* collected, 23 fish from Saldanha Bay, Hermanus, TNP and Chintsa were found to be infected with species of *Coitocaecum*; and 32 fish from Saldanha Bay, Hermanus, TNP and Chintsa were infected with species of *Helicometra*. No trematodes were present in the fish collected from Cape Town harbour. Molecular and morphological analyses confirmed the presence of four species of *Coitocaecum* and two species of *Helicometra* (Table 1). In total, 18 novel sequences have been generated for *Coitocaecum* spp.: eight sequences for 28S, one sequences for ITS1-5.8S-ITS2, and nine sequences for ITS2; and a total of 26 novel sequences have been generated for *Helicometra* spp.: nine sequences for 28S, three sequences for ITS1-5.8S-ITS2, and 14 sequences for ITS2.

Table 1. Summary data for trematodes detected during the present study. Prevalence and intensity of infection are given for each species per sampling locality

Taxa	Locality	Prevalence	Intensity of infection (individuals per host)
Coitocaecum capense	Saldanha Bay	5%	1-5
	Hermanus	7 of 8	1-5
	TNP	53%	1-13
	Chintsa	9%	7
Coitocaecum sp. 1	Hermanus	1 of 8	1
Coitocaecum sp. 2	Chintsa	9%	1
Coitocaecum sp. 3	Saldanha Bay	26%	1-22
Helicometra sp. 1	Saldanha Bay	21%	1-15
	Hermanus	8 of 8	1-21
	TNP	65%	1-6
	Chintsa	82%	1-11
Helicometra sp. 2	Hermanus	1 of 8	1

Morphological characterisation

Family Opecoelidae Ozaki, 1925
Subfamily Opecoelinae Ozaki, 1925
Genus *Coitocaecum* Nicoll, 1915

***Coitocaecum capense* Bray, 1987**

Host: Super klipfish *Clinus superciliosus* (Linnaeus) (Clinidae).
Locality: Saldanha Bay (33°2'44.46"S; 18°2'19.06"E), Hermanus (34°25'15.86"S; 19°14'37.56"E), Tsitsikamma National Park (34°1'15.21"S; 23°52'43.23"E) and Chintsa (32°50'11.54"S; 28°7'1.19"E), South Africa.
Site of infection: intestine.
Representative DNA sequences: four sequences for 28S; one sequence for ITS1-5.8S-ITS2; two sequences for ITS2.
Voucher material: 12 slides and 41 ethanol-fixed specimens deposited in NMB.

Description (based on 12 whole mounts; Fig. 2; Table 2) Body elongate, tapered anteriorly, dorso-ventrally flattened, waist posterior to ventral sucker, maximum body width in hindbody, just posterior to testes, 1882–3512 × 1232–2475 (2631 × 1830). Forebody 650–1037 (801) long, making up 27–36% (31%) of total body length. Body width to length ratio 1:3.7–5.4 (1:4.6). Tegument unarmed.

Oral sucker subspherical, subterminal, 148–221 × 113–191 (180 × 155). Prepharynx short, 18–51 (35). Pharynx muscular, elongate-oval to transversely oval, 55–81 × 63–107 (67 × 82), smaller than oral sucker. Oesophagus distinct, long, sinuous, 129–231 (168). Intestinal bifurcation in mid-level of forebody. Few large gland-cells situated in area of intestinal bifurcation. Caeca narrow, with distinct epithelial lining, pass ventral sucker and gonads dorso-laterally, form cyclocoel near posterior body extremity. Ventral sucker preequitorial, transversely oval, muscular, 184–286 × 116–289 (236 × 193). Oral sucker to ventral sucker length ratio 1:1.2–1.5 (1:1.3); width ratio 1:1.0–1.6 (1:1.3).

Testes two, irregularly lobed, intercaecal, tandem, contiguous, in third quarter of body; anterior testis transversely oval, contiguous with ovary, 106–236 × 177–443 (161 × 297); posterior testis transversely oval, 114–248 × 194–445 (185 × 308). Cirrus sac small, encloses seminal vesicle anteriorly, 51–131 × 18–41 (70 × 32). Seminal vesicle long, tubular, sinuous,

492–989 × 59–99 (666 × 73), extending to anterior margin of ventral sucker or slightly overlapping ventral sucker dorsally. Genital pore sinistral, at mid-level of oesophagus.

Ovary transversely oval, slightly lobed, intercaecal, median, pretesticular, contiguous with anterior testis, 69–145 × 135–300 (101 × 210). Mehlis' gland median, anterior to ovary. Uterus with few loops, restricted to area between ovary and genital pore, dorsal to ventral sucker, contains numerous eggs. Metraterm muscular, very short, in anterior portion of uterus. Eggs elongate-oval, operculate, yellow, translucent, length 37–50 (45).

Vitellarium follicular, vitelline follicles numerous, small, distributed in two lateral interrupted, non-confluent fields, extending anteriorly from level of intestinal bifurcation to anterior margin of ventral sucker in forebody, and in hindbody, extending from posterior level of posterior testis to posterior body extremity, may overlap caeca and excretory vesicle. Vitelline reservoir small, dorsal, anterior and slightly sinistral to ovary.

Excretory vesicle tubular, dorsal, extending close to level of ovary. Excretory pore terminal.

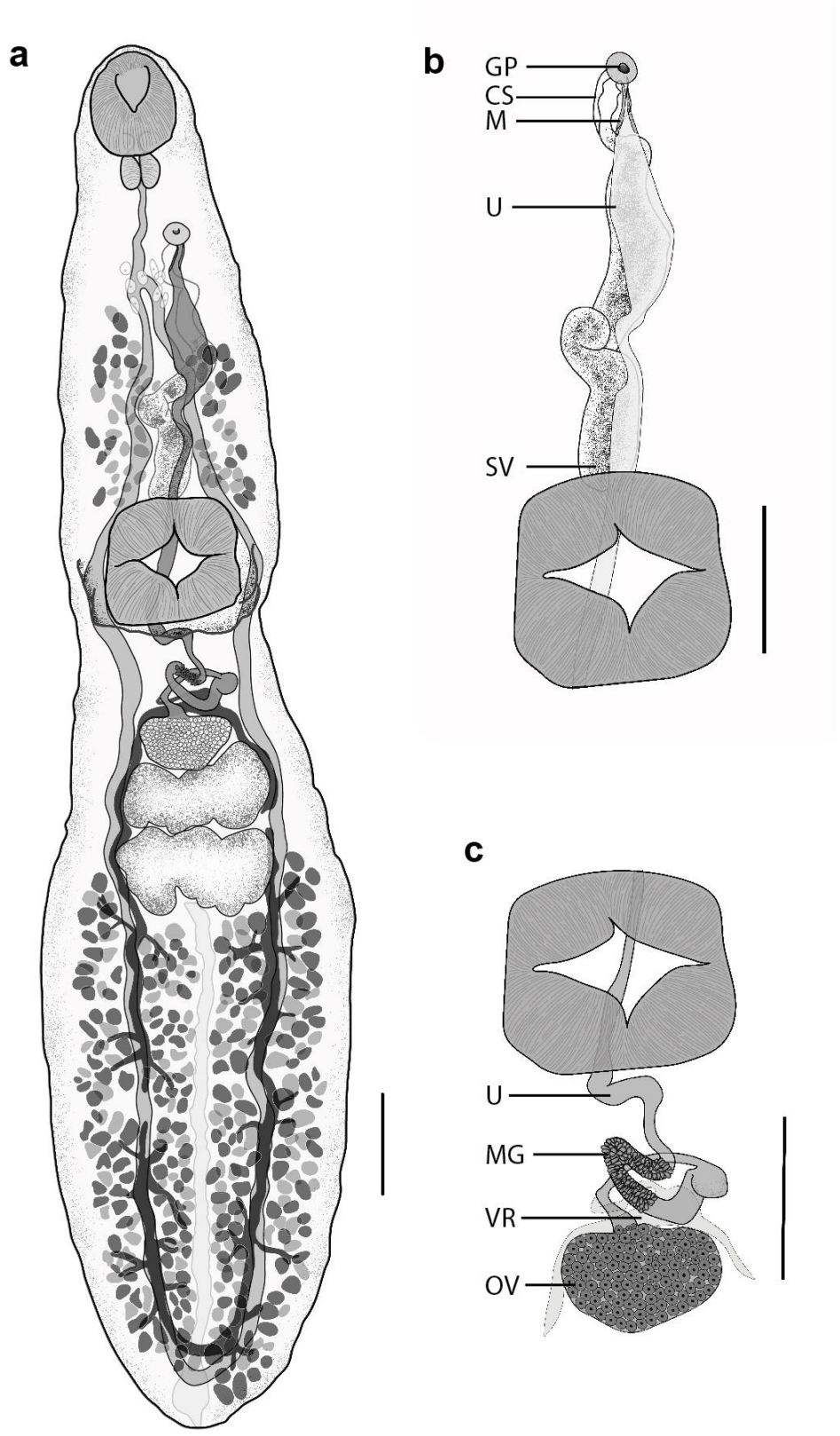

Figure 2. *Coitocaecum capense* ex *Clinus superciliosus*. Ventral view (A), terminal genitalia (B), ovarian complex (C). *Abbreviations*: CS, cirrus sac; GP, genital pore; M, metraterm; MG, Mehlis' gland; OV, ovary; SV, seminal vesicle; U, uterus; VR, vitelline reservoir. Scale bars: 200 µm

***Coitocaecum* sp. 1**

Host: Super klipfish *Clinus superciliosus* (Linnaeus) (Clinidae).
Locality: Hermanus (34°25'15.86"S; 19°14'37.56"E), South Africa.
Site of infection: intestine.
Representative DNA sequences: one sequence for 28S; one sequence for ITS2.
Voucher material: One slide deposited in NMB.

Description (based on one whole mount; Fig. 3; Table 2) Body elongate, dorso-ventrally flattened, distinctly wide at the level of ventral sucker, waist posterior to ventral sucker, maximum width in hindbody, at level of testes and posterior to them, 2084 × 510. Forebody short, 429, making up 21% of total body length. Body width to length ratio 1:4.1. Tegument unarmed.

Oral sucker subspherical, subterminal, 141 × 125. Prepharynx absent. Pharynx small, subspherical, 61 × 82. Oesophagus distinct, long, 170. Intestinal bifurcation in posterior forebody. Caeca narrow, with distinct epithelial lining, pass ventral sucker dorso-laterally, form cyclocoel near posterior extremity. Ventral sucker in first third of body, subspherical, 278 × 253; surrounded by conspicuous tegumental fold. Oral sucker to ventral sucker length ratio 1:2; width ratio 1:2.

Testes two, intercaecal, tandem, in third quarter of body; anterior testis irregular, smooth, 126 × 136, contiguous with ovary; posterior testis transversely oval, slightly irregularly lobed, 114 × 167. Cirrus sac small, encloses seminal vesicle anteriorly, 70 × 20. Seminal vesicle elongate, tubular, sinuous, 244 × 44, extending to level of anterior margin of ventral sucker. Genital pore sinistral, at mid-level of forebody.

Ovary transversely oval, trilobed, intercaecal, median, pretesticular, 114 × 211. Mehlis' gland was not observed. Uterus with several loops, restricted to area between ovary and genital pore, dorsal to ventral sucker, contains numerous eggs. Metraterm not observed. Eggs elongate-oval, operculate, yellow, translucent, length 48–49 (48).

Vitellarium follicular, vitelline follicles small, numerous, distributed in two lateral interrupted non-confluent fields extending from posterior level of pharynx to anterior margin of ventral sucker in forebody, and in hindbody, extending from level of posterior margin of ventral sucker to posterior extremity, may overlap caeca and excretory vesicle. Vitelline reservoir anterior to ovary.

Excretory vesicle tubular, dorsal, extends close to anterior level of ovary. Excretory pore terminal.

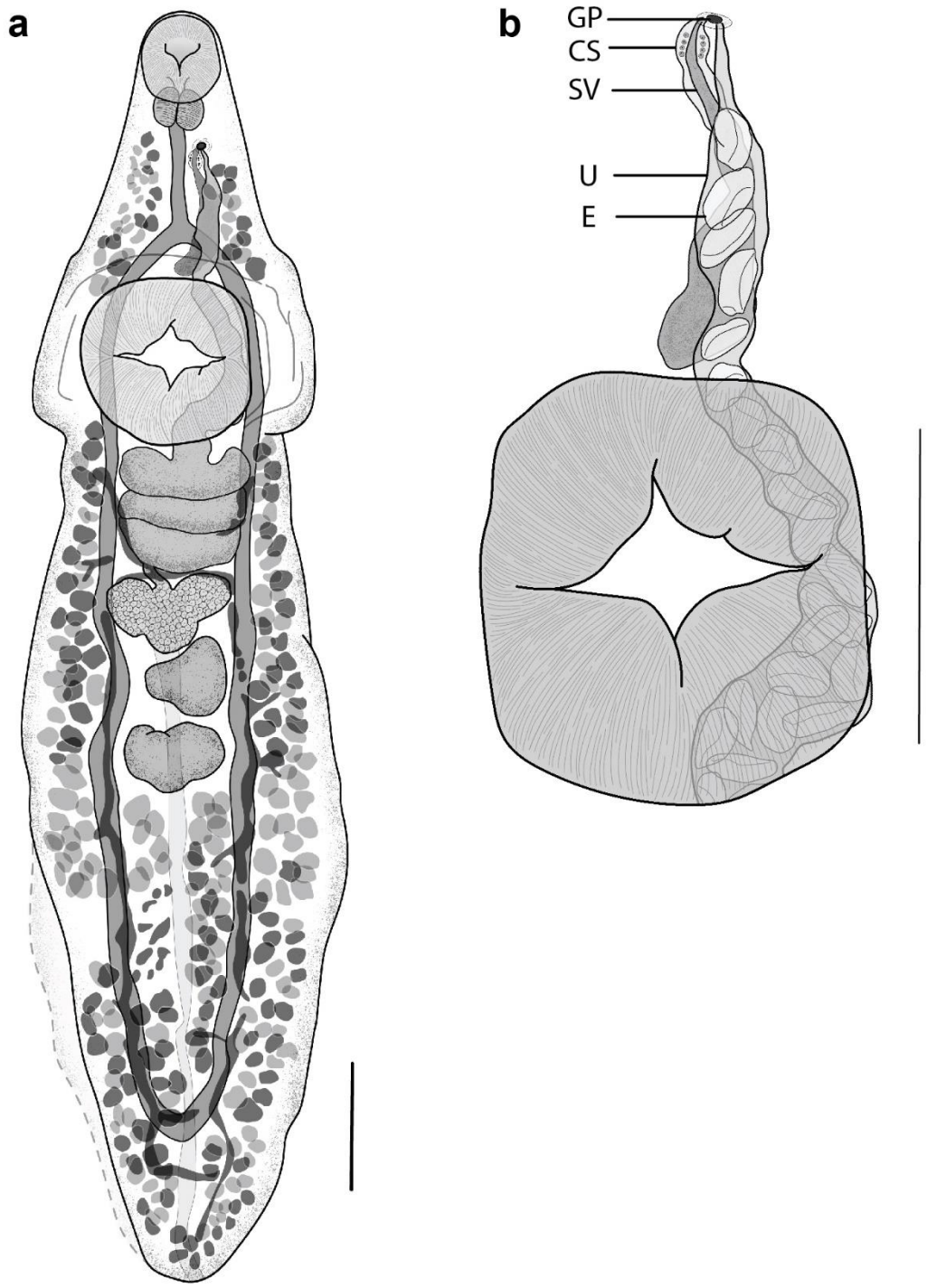

Figure 3. *Coitocaecum* sp. 1 ex *Clinus superciliosus*. Ventral view (A), terminal genitalia (B). *Abbreviations*: CS, cirrus sac; E, egg; GP, genital pore; SV, seminal vesicle; U, uterus. Scale bars: 200 µm

***Coitocaecum* sp. 2**

Host: Super klipfish *Clinus superciliosus* (Linnaeus) (Clinidae).
Locality: Chintsa (32°50'11.54"S; 28°7'1.19"E), South Africa.
Site of infection: intestine.
Representative DNA sequences: one sequence for 28S; one sequence for ITS2.
Voucher material: One slide deposited in NMB.

Description (based on one whole mount; Fig. 4; Table 2) Body elongate, slender, dorso-ventrally flattened, maximum width at level of ventral sucker, 1762 × 278. Forebody 458 long, making up 26% of total body length. Body width to length ratio 1:6.3. Tegument unarmed.

Oral sucker subspherical, subterminal, 150 × 130. Prepharynx distinct, 35. Pharynx large, elongate, 98 × 76. Oesophagus very short, 67. Intestinal bifurcation at mid-level between pharynx and ventral sucker. Caeca narrow, with distinct epithelial lining, pass ventral sucker dorso-laterally, form cyclocoel near posterior extremity. Ventral sucker preequitorial, subspherical, with ridges on luminal surface, 256 × 261, larger than oral sucker. Oral sucker to ventral sucker length ratio 1:1.7; width ratio 1:2.

Testes two, smooth, intercaecal, tandem, contiguous, in third quarter of body; anterior testis elongate-oval, contiguous with ovary, 143 × 95; posterior testis elongate-oval, 140 × 110. Cirrus sac small, encloses seminal vesicle anteriorly, 49 × 24. Seminal vesicle long, tubular, elongate, sinuous, 182 × 29, extending to anterior margin of ventral sucker. Few medium-sized prostatic gland cells situated near genital pore. Genital pore sinistral, at mid-level of pharynx.

Ovary transversely oval, irregular, intercaecal, median, pretesticular, 72 × 90. Mehlis' gland not observed. Uterus with few loops, restricted to area between ovary and genital pore, dorsal to ventral sucker. Metraterm not observed.

Vitellarium follicular, vitelline follicles small, numerous, distributed in two lateral interrupted fields, extending from mid-level of pharynx to anterior margin of ventral sucker in forebody, and in hindbody, extending from level of posterior margin of ventral sucker to posterior extremity, confluent at level just posterior to intestinal bifurcation and in last quarter of the post-testicular region, may overlap caeca and excretory vesicle. Vitelline reservoir small, anterior to ovary, median.

Excretory vesicle tubular, dorsal, extends close to level of posterior testis. Excretory pore terminal.

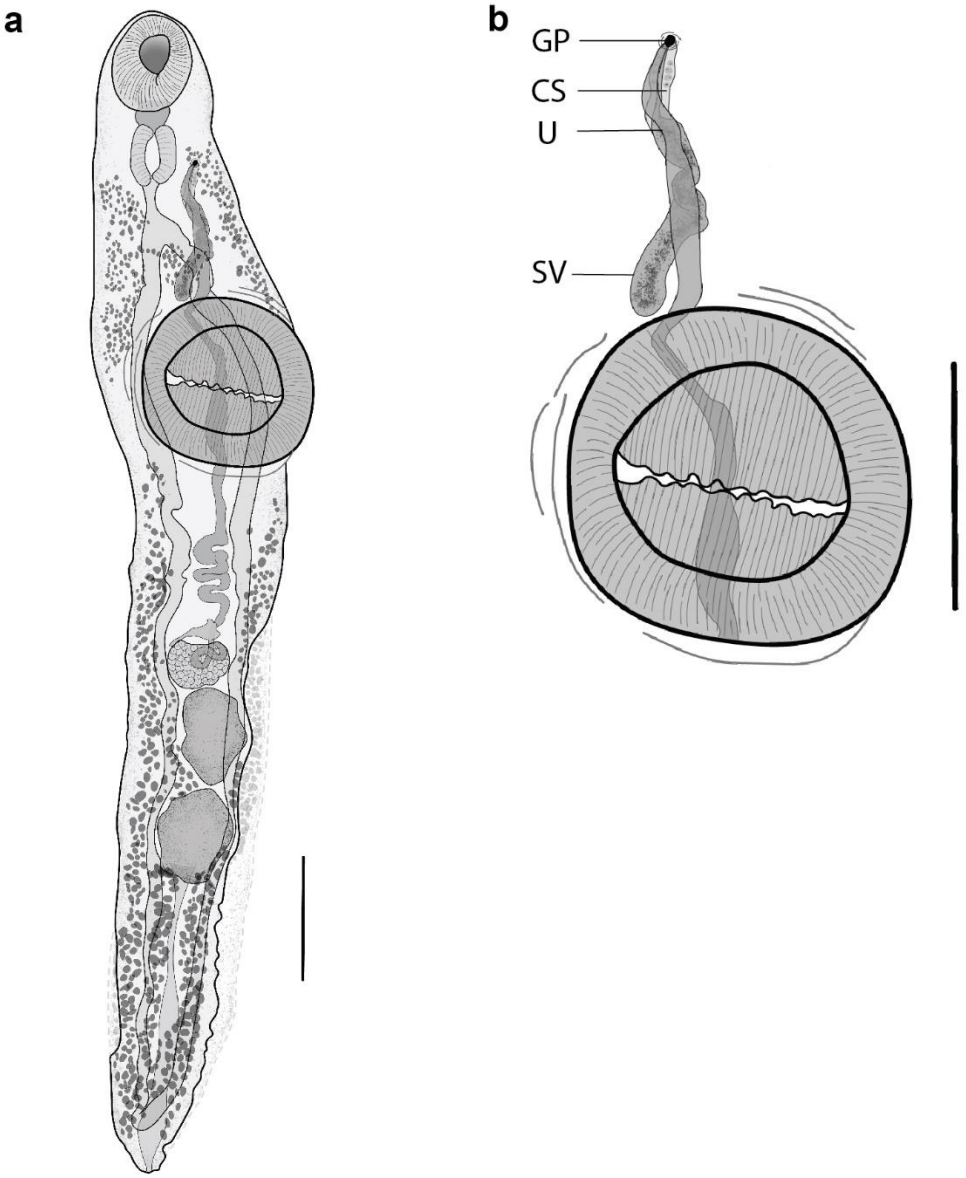

Figure 4. *Coitocaecum* sp. 2 ex *Clinus superciliosus*. Ventral view (A), terminal genitalia (B). *Abbreviations*: CS, cirrus sac; GP, genital pore; SV, seminal vesicle; U, uterus. Scale bars: 200 µm

***Coitocaecum* sp. 3**

Host: Super klipfish *Clinus superciliosus* (Linnaeus) (Clinidae).
Locality: Saldanha Bay (33°2'44.46"S; 18°2'19.06"E), South Africa.
Site of infection: intestine.
Representative DNA sequences: two sequence for 28S; four sequences for ITS2.
Voucher material: Three slides and 30 ethanol-fixed specimens deposited in NMB.

Description (based on three whole mounts; Fig. 5; Table 2) Body elongate, dorso-ventrally flattened, maximum width just posterior to ventral sucker, 1352–1732 × 257–438 (1521 × 333). Forebody 346–557 (460) long, making up 26–32% (30%) of total body length. Body width to length ratio 1:4–5.8 (1:4.7). Tegument unarmed.

Oral sucker subspherical, subterminal, 103–126 × 100–118 (113 × 112). Prepharynx short, 28–32 (30). Pharynx large, subspherical, 56–87 × 69–90 (75 × 80). Oesophagus sinuous, 96–163 (131). Intestinal bifurcation in mid forebody. Caeca narrow, with distinct epithelial lining, pass ventral sucker dorso-laterally, form cyclocoel near posterior extremity. Ventral sucker preequitorial, transversely oval, 181–232 × 177–262 (199 × 215). Oral sucker to ventral sucker length ratio 1:1.7–1.8 (1:1.8); width ratio 1:1.5–2.2 (1:1.9).

Testes two, smooth, tandem, contiguous, intercaecal; anterior testis transversely oval, contiguous with ovary, 113–180 × 121–223 (142 × 177); posterior testis elongate-oval, 121–239 × 109–219 (172 × 173). Cirrus sac sinuous, small, encloses seminal vesicle anteriorly, 49 × 32. Seminal vesicle saccular, elongate, bipartite, 246–456 × 62–113 (351 × 88), dorsal to ventral sucker, extending slightly posterior to its anterior margin. Genital pore sinistral, at level of posterior margin of pharynx.

Ovary transversely oval, irregular, pretesticular, median, contiguous with anterior testis, intercaecal, 57–90 × 103–160 (75 × 131). Mehlis' gland not observed. Seminal receptacle anterior to ovary, sinistral, dorsal to uterus. Uterus with few loops, restricted to area between ovary and genital pore, dorsal to ventral sucker, contains few eggs. Metraterm not observed. Eggs elongate-oval, operculate, yellow, translucent, 49–60 × 27–29 (55 × 28).

Vitellarium follicular, vitelline follicles distributed in two lateral interrupted fields, extending from mid-level of oesophagus to mid-level of ventral sucker in forebody, and in hindbody, posterior to ventral sucker and reaching to posterior extremity, confluent in post-testicular region, overlap caeca and excretory vesicle. Vitelline reservoir small, anterior to ovary, slightly dextral, overlaps ovary ventrally.

Excretory vesicle tubular, sinuous, extends to level of anterior testis, dorsal. Excretory pore terminal.

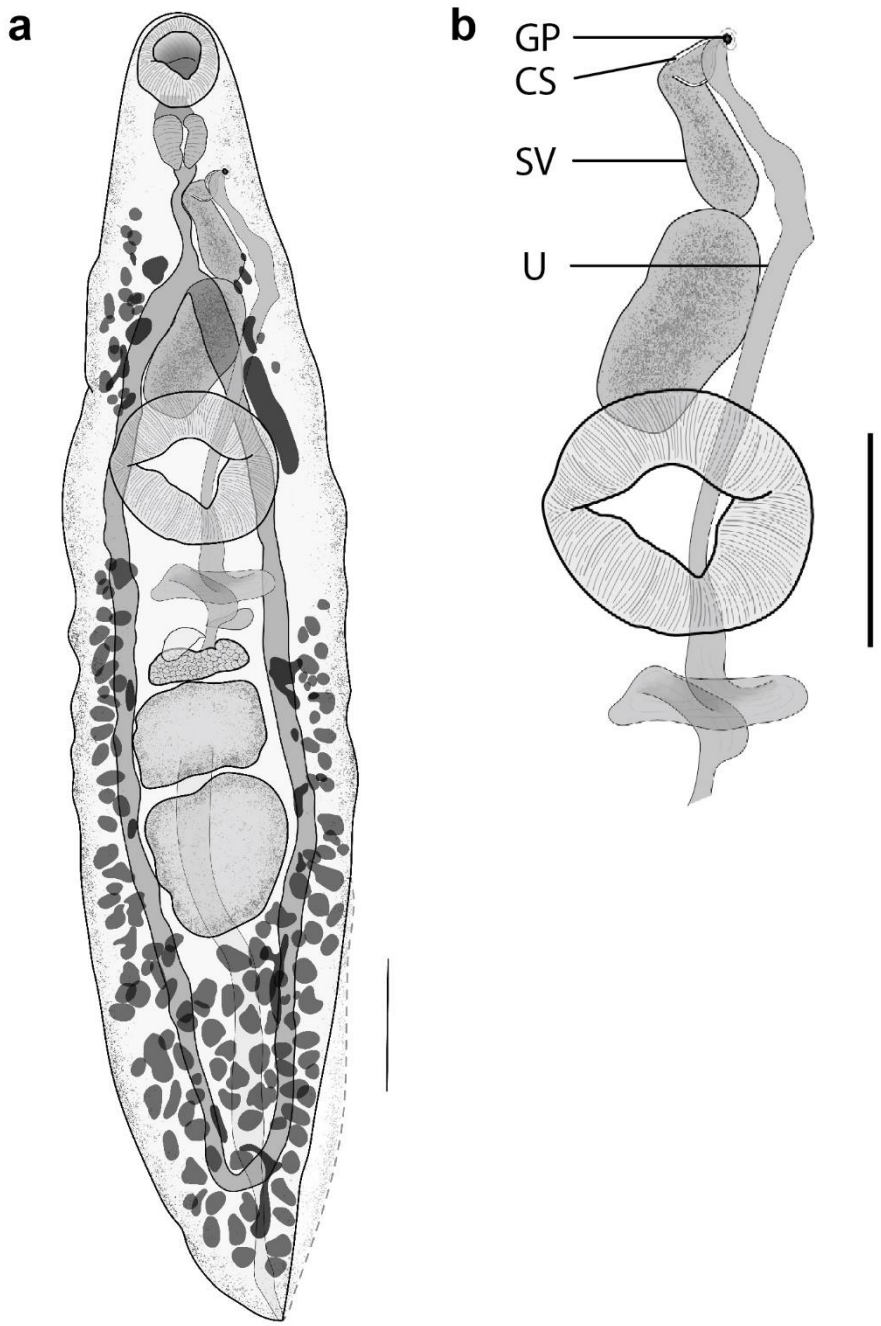

Figure 5. *Coitocaecum* sp. 3 ex *Clinus superciliosus*. Ventral view (A), terminal genitalia (B). *Abbreviations*: CS, cirrus sac; GP, genital pore; SV, seminal vesicle; U, uterus. Scale bars: 200 µm

Table 2. Morphometric data of *Coitocaecum* spp.

Species	*Coitocaecum capense* Bray, 1987		*Coitocaecum capense*		*Coitocaecum* sp. 1		*Coitocaecum* sp. 2		*Coitocaecum* sp. 3		*Coitocaecum tylogonium* Manter, 1954	
Host	*Clinus superciliosus* L.		*Clinus superciliosus*, *Clinus cottoides* Valenciennes, *Clinus rotundifrons* Barnard, *Cirrhibarbis capensis* Valenciennes, *Xenopoclinus kochi* Smith, *Xenopoclinus leprosus* Smith		*Clinus superciliosus*		*Clinus superciliosus*		*Clinus superciliosus*		*Centriscops humerosus* (Richardson)	
Locality	TNP; Hermanus; Chintsa; Saldanha Bay, SA		Port Elizabeth and Oudekraal, SA		Hermanus, SA		Chintsa, SA		Saldanha Bay, SA		Portobello, NZ	
Source	Present study		Bray (1987)		Present study		Present study		Present study		Manter (1954)	
	range (n = 12)	mean	range (n = 38)	mean	range (n = 1)	mean	range (n = 1)	mean	range (n = 3)	mean	range (n = 1)	mean
Body length	1882–3512	2631	960–2560	–	2084	–	1762	–	1352–1732	1521	3204	–
Body width	352–845	578	280–830	–	510	–	278	–	257–438	333	868	–
Forebody length	650–1037	801	–	–	429	–	458	–	346–557	460	980	–
Hindbody length	1232–2475	1830	–	–	1655	–	1304	–	1003–1175	1061	–	–
Oral sucker length	148–221	180	110–160	–	141	–	150	–	103–126	113	–	–
Oral sucker width	133–191	155	100–150	–	125	–	130	–	100–118	112	–	–
Prepharynx length	18–51	35	Absent or very short	–	30	–	35	–	28–32	30	–	–
Pharynx length	55–81	67	40–70	–	61	–	98	–	56–87	75	129	–
Pharynx width	63–107	82	40–80	–	82	–	76	–	69–90	80	137	–
Oesophagus length	129–231	168	30 –160	–	170	–	67	–	96–163	131	–	–
Ventral sucker length	184–286	236	150–270	–	278	–	256	–	181–232	199	–	–
Ventral sucker width	116–289	193	200–280	–	253	–	261	–	177–262	215	455	–
Genital pore to ventral sucker	379–616	468	–	–	229	–	212	–	209–315	252	–	–

Table 2. Continued

Ovary length	69–145	101	50–90	–	114	–	72	–	57–90	75	–	–
Ovary width	135–300	210	150–260	–	211	–	90	–	103–160	131	–	–
Egg length	37–50	45	45–51	–	48–49	48	–	–	49–60	55	53–57	–
Egg width	–	–	–	–	–	–	–	–	27–29	28	30–32	–
Anterior testis length	106–236	161	50–130	–	126	–	143	–	113–180	142	–	–
Anterior testis width	177–443	297	190–340	–	136	–	95	–	121–223	177	–	–
Posterior testis length	114–248	185	60–160	–	114	–	140	–	121–239	172	–	–
Posterior testis width	194–445	308	180–330	–	167	–	110	–	109–219	173	–	–
Cirrus sac length	51–131	70	–	–	70	–	49	–	49	–	152	–
Cirrus sac width	18–41	32	–	–	20	–	24	–	32	–	95	–
Seminal vesicle length	492–989	666	–	–	244	–	182	–	246–456	351	–	–
Seminal vesicle width	59–99	73	80–100	–	44	–	29	–	62–113	88	–	–
Seminal receptacle length	48–100	71	–	–	–	–	–	–	–	–	–	–
Seminal receptacle width	39–73	61	–	–	–	–	–	–	–	–	–	–
Post-testicular region	617–1419	1013	–	–	808	–	426	–	422–564	493	–	–
Body width:length ratio	1:3.7–5.4	1:4.6	–	–	1:4.1	–	1:6.3	–	1:4–5.8	1:4.7	–	–
Forebody length as % body length	27–36%	31%	28–33%	–	21%	–	26%	–	26–32%	30%	–	–
Oral sucker length:Ventral sucker length	1:1.2–1.5	1:1.3	–	–	1:2	–	1:1.7	–	1:1.7–1.8	1:1.8	–	–
Oral sucker width:Ventral sucker width	1:1–1.6	1:1.3	1:1.62–2	–	1:2	–	1:2	–	1:1.5–2.2	1:1.9	1:1.8	–

Remarks

Specimens of the four species described above possess features that are fully consistent with species of the genus *Coitocaecum*, in particular the presence of a caecum that forms a cyclocoel, a highly reduced cirrus-sac, operculate eggs and the absence of papillae of any sort on the aperture of the ventral sucker (Cribb 2005). Species identified in this study as *C. capense* agrees well with the original description of Bray (1987). However, it differs in having a larger body (1882–3512 × 352–845 *vs* 960–2560 × 280–830), oral sucker (148–221 × 133–191 *vs* 110–160 × 100–150), pharynx (55–81 × 63–107 *vs* 40–70 × 40–80), a longer oesophagus (129–231 *vs* 30–160), larger ovary (69–145 × 135–300 *vs* 50–90 × 150–260), lower minima for egg length (37 *vs* 45), as well as larger testes (anterior testis 106–236 × 177–443 *vs* 50–130 × 190–340; posterior testis 114–248 × 194–445 *vs* 60–160 × 180–330).

The remaining three species of *Coitocaecum* were morphologically identified to genus level. All four species of *Coitocaecum* possess caeca that form a cyclocoel. One of the noticeable differences between the four species of *Coitocaecum* is the shape and position of the gonads: the testes of *C. capense* are more indented than in the other three species and are contiguous, whereas all the other species have entire or slightly indented testes that are not contiguous; the anterior testis of *Coitocaecum* sp. 1 is irregular, whereas that of *Coitocaecum* sp. 2 and sp. 3 are elongate-oval and *C. capense* has transversely oval testes; The ovary of *Coitocaecum* sp. 1 has three lobes, where the other species have an entire or a slightly lobed ovary. The three unnamed species of *Coitocaecum* have noticeably shorter forebodies than *C. capense* from the present study (mean: 429 *vs* 458 *vs* 460 *vs* 801); *Coitocaecum* sp. 2 has a more elongate body than the other species, a longer pharynx that is much more elongate than that of the others (mean length: 98 *vs* 61 *vs* 75 *vs* 67), and has ridges on the luminal surface of the ventral sucker; and *Coitocaecum* sp. 3 has a much smaller ventral sucker in relation to body size when compared to *Coitocaecum* sp. 1 and sp. 2 (mean: 199 × 125 *vs* 256 × 261 *vs* 278 × 253 *vs* 236 × 193). *Coitocaecum capense* can be further distinguished from the three other species by the limit of the anterior level of vitelline follicles in the hindbody (level of posterior testis *vs* level of ventral sucker) and *Coitocaecum* sp. 3 by the vitellarium being confluent in the post-testicular region.

In his paper on the Opecoelidae from marine fishes of South Africa, Bray (1987) divided species of the genus *Coitocaecum* into five distinct groups based on the distribution of their vitellarium and posterior limit of position of the seminal vesicle. According to this classification, all four species of *Coitocaecum* in the present study belong to group E. Species within this group have interrupted vitelline follicles that reach into the forebody and a seminal vesicle that does not extend posterior to the ventral sucker (Bray 1987). To date, *Coitocaecum tylogonium* Manter

1954 (Bray 1987) described from the Banded yellowfish *Centriscops humerosus* (Richardson) in Portobello, New Zealand (Manter 1954) was the only representative of group E. Species in the present study differ from *C. tylogonium* in a combination of morphological features. *C. tylogonium* is much larger; nearly double the size of *Coitocaecum* sp. 2 and *Coitocaecum* sp. 3. Thus, this species also has a larger oral sucker, ventral sucker and pharynx. Manter (1954) reports the pharynx and oesophagus to be similar in length, however this is not observed in the species examined in the present study, in which the oesophagus can reach more than double the length of the pharynx.

Even though the eggs of *C. tylogonium* are quite shorter than that of *Coitocaecum* sp. 3, they are almost similar in width (27 × 29 *vs* 30 × 32). Except for the aforementioned species, the eggs of *C. tylogonium* exhibit lower minima and higher maxima for egg length. Of the *Coitocaecum* species observed in the present study, only the ovary of *Coitocaecum* sp. 1 is distinctly lobed, but not as deeply lobed as that of *C. tylogonium*. The genital pore of *C. tylogonium* was found to be median and slightly posterior to intestinal bifurcation, whereas the genital pores of all four species from the present study are sinistral and pre-bifurcal. However, the vitelline follicles exhibit similar dispersal patterns in all these species, the major difference is that the vitelline follicles of *C. capense* is distributed posterior to the testes in the hindbody, whereas in all other species, the follicles are distributed posterior to the ventral sucker in the hindbody. The vitelline follicles for *Coitocaecum* sp. 2 appear much smaller than that for the other species, but this may be due to the specimen not being fully developed yet. *Coitocaecum* sp. 3 has the fewest and more widely dispersed vitelline follicles of all four species from the present study.

This is the first study to report species of *Coitocaecum* from the fish host *Cl. superciliosus,* other than *C. capense,* which was described from this fish host. To our knowledge, *Coitocaecum* sp. 1, *Coitocaecum* sp. 2 and *Coitocaecum* sp. 3 have not been reported from any other fish host along the coast of South Africa or globally.

Genus *Helicometra* Odhner, 1902

***Helicometra* sp. 1**

Host: Super klipfish *Clinus superciliosus* (Linnaeus) (Clinidae).
Locality: Saldanha Bay (33°2'44.46"S; 18°2'19.06"E), Hermanus (34°25'15.86"S; 19°14'37.56"E), Tsitsikamma National Park (34°1'15.21"S; 23°52'43.23"E) and Chintsa (32°50'11.54"S; 28°7'1.19"E), South Africa.
Site of infection: intestine.
Representative DNA sequences: seven sequences for 28S; three sequences for ITS1-5.8S-ITS2; 13 sequences for ITS2.
Voucher material: 15 slides and 126 ethanol-fixed voucher specimens deposited in NMB.

Description (based on 15 whole mounts; Fig. 6; Table 3) Body dorso-ventrally flattened, elongate, maximum width in post-testicular hindbody, 1211–2163 × 362–605 (1744 × 473). Forebody 362–648 (473) long, making up 29–33% (30%) of total body length. Body width to length ratio 1:2.9–4.5 (1:3.7). Tegument unarmed.

Oral sucker transversely oval subterminal, 87–135 × 106–143 (110 × 119). Prepharynx relatively short 18–33 (24) or absent. Pharynx transversely oval, 18–33 × 43–64 (24 × 57). Oesophagus distinct, sinuous 81–251 (166). Intestinal bifurcation between oral and ventral suckers. Caeca narrow, with distinct epithelial lining, pass ventral sucker dorso-laterally, terminate blindly close to posterior extremity. Ventral sucker subspherical, 190–242 × 128–323 (215 × 205). Oral to ventral sucker length ratio 1:1.5–2 (1:2); width ratio 1:1.2–2.7 (1:1.7).

Testes two, tandem, contiguous, intercaecal, at mid-level of hindbody; anterior testis smooth, transversely oval, 88–171 × 130–219 (120 × 171); posterior testis slightly irregular, transversely oval, 120–199 × 128–243 (137 × 173). Cirrus sac elongate, sinuous, 59–505 × 45–441 (350 × 96), antero-dorsal to ventral sucker, extending slightly posterior to its mid-level. Internal seminal vesicle tubular, 50–531 × 31–429 (346 × 106). Pars prostatica long, tubular; prostatic cells medium-sized, not numerous; cirrus with spines. Genital pore median, at mid-level of oesophagus.

Ovary transversely oval, lobed, intercaecal, pretesticular, contiguous or slightly overlaps anterior testis ventrally, 55–121 × 113–180 (84 × 150). Mehlis' gland subspherical, anterior to ovary, dextral, overlapping caeca dorsally. Seminal receptacle anterior to ovary, sinistral, overlaps ovary dorsally. Uterus long, helical, with main bulk between region of ovary and

ventral sucker, with numerous eggs, passes ventral sucker dorsally. Metraterm not observed. Eggs elongate-oval, with one long terminal filament, yellow, translucent, length 52–75 (63).

Vitellarium follicular, vitelline follicles small, distributed in two lateral fields, between oesophagus and posterior extremety of body, overlap caeca and excretory vesicle, non-confluent in post-testicular field. Vitelline reservoir large, anterior to ovary, slightly dextral.

Excretory vesicle tubular, extends to level of ovary, dorsal. Excretory pore terminal.

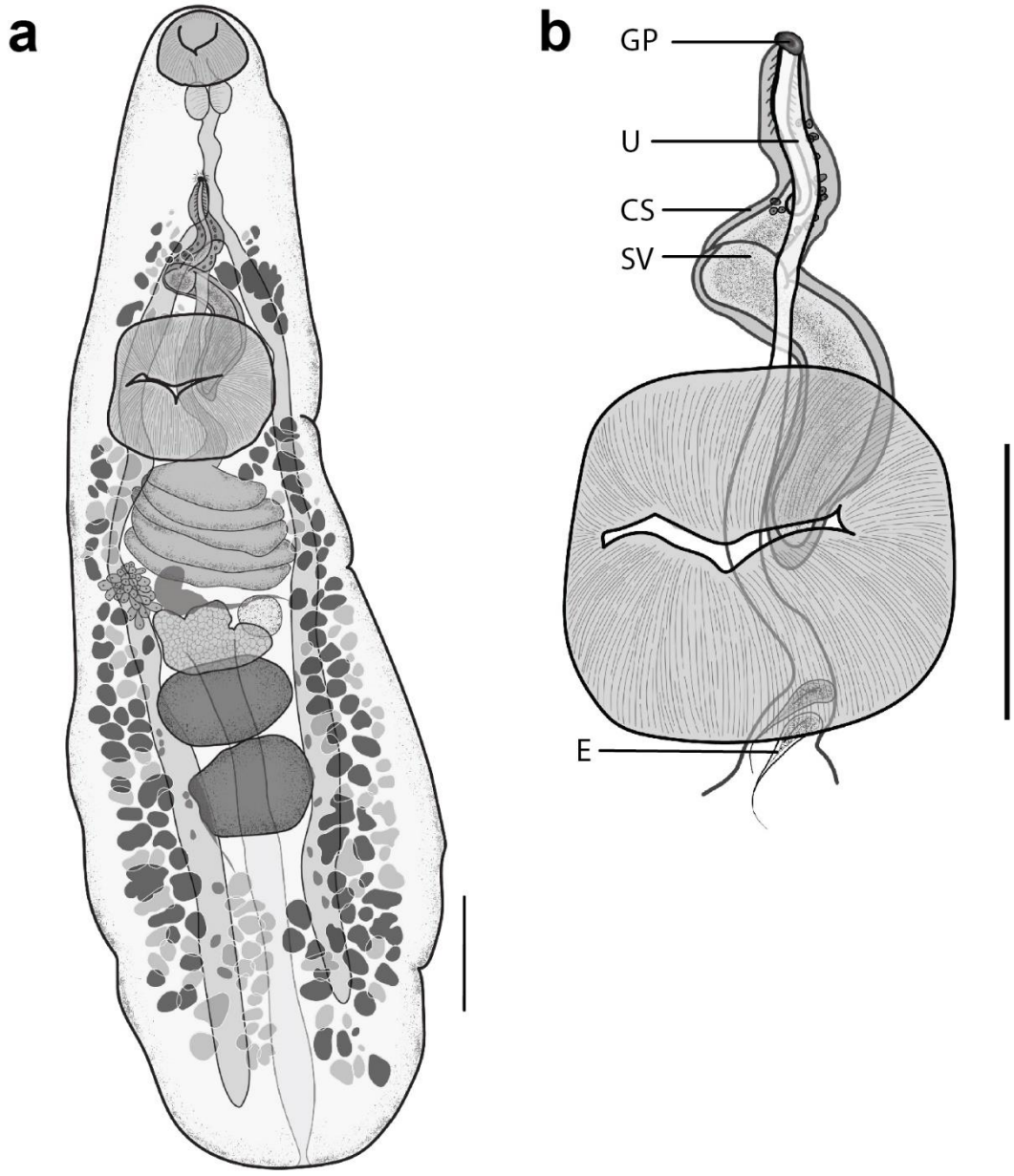

Figure 6. *Helicometra* sp. 1 ex *Clinus superciliosus*. Ventral view (A), terminal genitalia (B). *Abbreviations*: CS, cirrus sac; E, egg; GP, genital pore; SV, seminal vesicle; U, uterus. Scale bars: 200 µm

***Helicometra* sp. 2**

Host: Super klipfish *Clinus superciliosus* (Linnaeus) (Clinidae).
Locality: Chintsa (32°50'11.54"S; 28°7'1.19"E), South Africa.
Site of infection: intestine.
Representative DNA sequences: one sequence for 28S; one sequence for ITS2.
Voucher material: One slide deposited in NMB.

Description (based on one whole mount; Fig. 7; Table 3) Body elongate, dorso-ventrally flattened, maximum width at level of seminal receptacle, 1078 × 303. Forebody 312 long, making up 29% of total body length. Body width to length ratio 1:3.6. Tegument unarmed.

Oral sucker subspherical, subterminal, 100 × 124. Prepharynx absent. Pharynx large, transversely oval, 56 × 70. Oesophagus short, sinuous, 96. Intestinal bifurcation in posterior forebody. Caeca narrow, with distinct epithelial lining, pass ventral sucker dorso-laterally, end blindly close to posterior extremity. Ventral sucker subspherical, 186 × 194. Oral to ventral sucker length ratio 1:1.9; width ratio 1:1.6.

Testes two, irregular, tandem, contiguous, intercaecal; anterior testis transversely oval, 79 × 93; posterior testis elongate-oval 90 × 69. Cirrus sac short, sinuous, 140 × 29, extending to level of anterior margin of ventral sucker. Internal seminal vesicle elongate, tubular, sinuous, 133 × 26. Genital pore median, at mid-level of oesophagus.

Ovary transversely oval, lobed, intercaecal, pretesticular, slightly overlaps anterior testis ventrally, 96 × 153. Seminal receptacle anterior to ovary, sinistral, overlaps ovary ventrally. Uterus coiled, with main bulk between region of ovary and ventral sucker, with numerous eggs, passes ventral sucker dorsally. Metraterm not observed. Eggs elongate-oval, with one long terminal filament, yellow, translucent, length 63–67 (65).

Vitellarium follicular, vitelline follicles small, in two lateral, uninterrupted fields, between pharynx and posterior extremety of body, confluent in forebody and in post-testiculat region. Vitelline reservoir large, anterior to ovary, slightly dextral.

Excretory vesicle tubular, extends to level of ovary, dorsal. Excretory pore not observed.

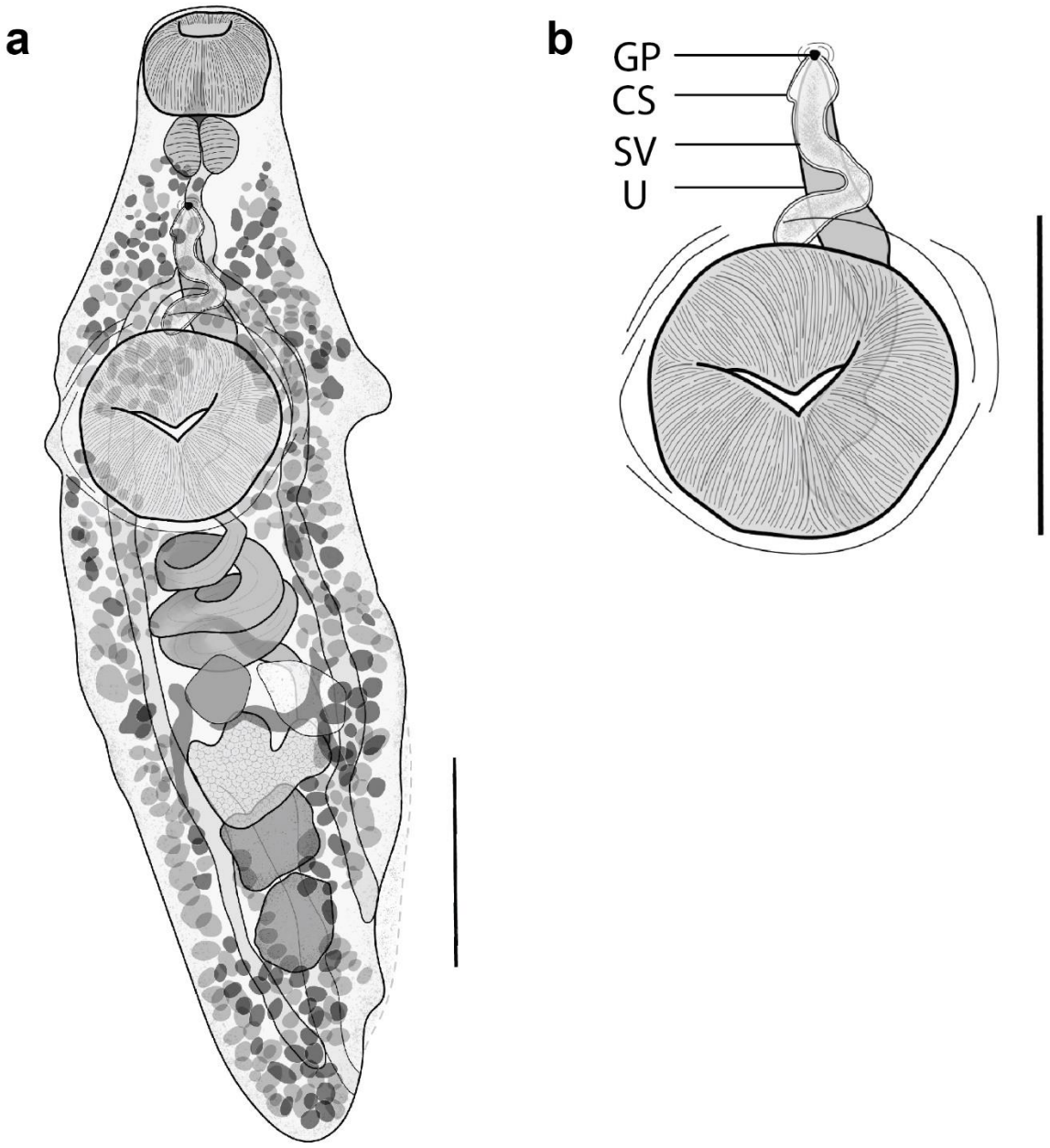

Figure 7. *Helicometra* sp. 2 ex *Clinus superciliosus*. Ventral view (A), terminal genitalia (B). *Abbreviations*: CS, cirrus sac; GP, genital pore; SV, seminal vesicle; U, uterus. Scale bars: 200 µm

Table 3. Morphometric data of *Helicometra* spp.

Species	*Helicometra* sp. 1		*Helicometra* sp. 2		*Helicometra fasciata* (Rudolphi, 1819)		*Helicometra fasciata*		*Helicometra scorpaenae* Prudhoe & Bray, 973	
Host	*Clinus superciliosus*		*Clinus superciliosus*		12 host species (for full host list see reference below)		nine host species (for full host list see reference below)		*Scorpaena jacksoniensis* Steindachner	
Locality	TNP; Hermanus; Chintsa; Saldanha Bay, SA		Hermanus, SA		Port Elizabeth, Oudekral and Durban, SA		Queensland and New South Wales, Australia		Tasmania	
Source	Present study		Present study		Bray (1987)		Aken'Ova et al. (2006)		Prudhoe & Bray (1973)	
	range (n = 15)	mean	range (n = 1)	mean	range (unspecified)	mean	range (unspecified)	mean	range (n=2 contracted specimens)	mean
Body length	1211–2163	1744	1078	–	1020–1820	–	1247–2336	–	1000–1140	–
Body width	362–605	473	303	–	350–780	–	389–696	–	550–650	–
Forebody length	362–648	524	312	–	–	–	412–646	–	–	–
Hindbody length	849–1531	1189	766	–	–	–	–	–	–	–
Oral sucker length	87–135	110	100	–	130–240	–	115–173	–	–	–
Oral sucker width	106–143	119	124	–	130–220	–	130–186	–	130–140	–
Prepharynx length	18–33	24	–	–	≤30	–	–	–	–	–
Pharynx length	43–64	57	56	–	70–110	–	58–83	–	69–77	–
Pharynx width	61–91	76	70	–	70–110	–	71–91	–	82–84	–
Oesophagus length	81–251	166	96	–	30–130	–	–	–	100	–
Ventral sucker length	190–242	215	186	–	190–320	–	169–288	–	–	–
Ventral sucker width	128–323	205	194	–	200–350	–	175–294	–	150	–
Genital pore to ventral sucker	162–348	266	98	–	–	–	–	–	–	–
Ovary length	55–121	84	96	–	80–210	–	88–194	–	–	–
Ovary width	113–180	150	153	–	130–430	–	156–282	–	–	–
Egg length	52–75	63	63–67	65	51–84	–	38–72	–	72–80	–
Anterior testis length	88–171	120	79	–	80–190	–	81–181	–	–	–
Anterior testis width	130–219	171	93	–	110–500	–	117–259	–	–	–
Posterior testis length	102–199	137	90	–	110–230	–	81–214	–	–	–
Posterior testis width	128–243	173	69	–	150–450	–	117–233	–	–	–
Seminal receptacle length	135–164	151	81	–	–	–	–	–	–	–
Seminal receptacle width	60–77	70	45	–	–	–	–	–	–	–
Cirrus sac length	59–505	350	140	–	250–500	–	292–486	–	210–260	–
Cirrus sac width	45–441	96	29	–	50–110	–	58–84	–	–	–

Table 3. Continued

Seminal vesicle length	50–531	346	133	–	–	–	–	–	–	–
Seminal vesicle width	31–429	106	26	–	–	–	–	–	–	–
Post-testicular region	218–641	445	155	–	–	–	220–582	–	–	–
Body width:length ratio	1:2.9–4.5	1:3.7	1:3.6	–	–	–	1:2.9–3.8	–	–	–
Forebody length as % body length	29–33%	30%	29%	–	23–34%	–	27.3–34.4%	–	–	–
Oral sucker length:Ventral sucker length	1:1.5–2	1:2	1:1.9	–	–	–	–	–	–	–
Oral sucker width:Ventral sucker width	1:1.2–2.7	1:1.7	1:1.6	–	1:1.36–2.08	–	–	–	–	–

Remarks

Specimens of the two species described above possess features that are fully consistent with species of the genus *Helicometra*, in particular a tightly coiled, helical uterus, eggs with a single, polar filament, seminal vesicle enclosed in a well-developed cirrus sac, caeca ending blindly and two testes (Cribb 2005). According to the most recent revision of *Helicometra* by Blend and Dronen (2015) this genus contains 34 nominal species that are divided into four groups, based on the shape and position of their cirrus sac and oral sucker, as well as the distribution of vitelline follicles in the body. According to these characters, *Helicometra* sp. 1 from the present study belong to Group I and is morphologically similar to *H. fasciata*, except for possessing a smaller oral sucker (87–135 × 106–143 *vs* 130–240 × 130–220 (Bray 1987) and 115–173 x 130–186 (Aken'Ova et al. 2006)), a shorter pharynx than that described by Bray (1987) (43–64 *vs* 70–110), a longer oesophagus (81–251 *vs* 30–130) (Bray 1987), a smaller ovary (55–121 × 113–180 *vs* 80–210 × 130–430 (Bray, 1987) and 88–194 × 156–282 (Aken'Ova et al. 2006)), and lower maxima for testis width than that observed by Bray (1987) (anterior testis 130–219 *vs* 110–500; posterior testis 128–243 *vs* 150–450). The specimens of *Helicometra* sp. 1 have a clear interruption between the vitelline follicles of the fore- and hindbody, whereas those illustrated by Bray (1985) and Blend and Dronen (2015) are less distinct. The specimen of *H. fasciata* illustrated by Bray (1985) also has testes with deeper indentations than those of *Helicometra* sp. 1.

According to the key of Blend and Dronen (2015) *Helicometra* sp. 2 is morphologically similar to *Helicometra scorpaenae* Prudhoe and Bray, 1973 and also belongs to Group I. Unfortunately the description provided by Prudhoe and Bray (1973) is based on two contracted and one distorted specimen, thus comparison with certainty is not possible at this stage. Although *Helicometra* sp. 2 is morphologically similar to *H. scorpaenae*, it differs by having testes without strongly indented margins, non-confluent vitelline follicles in the forebody or in the post-testicular region, and in having a narrower body (303 *vs* 550–650), a smaller pharynx (56 × 70 *vs* 69–77 × 82–84), a wider ventral sucker (194 *vs* 150) and shorter eggs (63–67 *vs* 72–80).

Specimens of the two species of *Helicometra* reported on during the present study, are similar by having a coiled uterus, eggs with a single polar filament, two testes with smooth edges, an ovary that is slightly tri-lobed, caeca that end in two blind ends, a seminal vesicle that is enclosed in a cirrus sac, and a seminal receptacle. However, *Helicometra* sp. 1 has a clear vitelline interruption between the fore- and hindbody, whereas *Helicometra* sp. 2 has no vitelline interruptions in this area. Also, the seminal vesicle of *Helicometra* sp. 2 does not overlap the

ventral sucker as far as that of *Helicometra* sp. 1 does. Additionally, specimens of *Helicometra* sp. 1 have a larger body (1121–2163 × 362–605 *vs* 1078 × 303), a longer forebody (362–648 *vs* 312), a longer hindbody (849–1531 vs 766), a longer ventral sucker (190–242 *vs* 186), larger anterior (88–171 × 130–219 *vs* 120 × 171) and posterior (102–199 × 128–243 *vs* 137 × 173) testes, a seminal receptacle nearly double in size (135–164 × 60–77 *vs* 151 × 70), overall larger cirrus sac (59–505 × 45–441 *vs* 350 × 96) and seminal vesicle (50–531 × 31–429 *vs* 346 × 106), and the genital pore is situated much further from the ventral sucker (162–348 *vs* 98) than *Helicometra* sp. 2.

Molecular characterisation

Altogether, this study has generated sequences for four unique species of *Coitocaecum* and two species of *Helicometra*. Novel sequences were compared to those of the Opecoelidae available in GenBank (Table 1). Comparative 28S rDNA sequence analysis demonstrated that *Helicometra* sp. 2 from the present study was identical to the metacercarial isolate, Opecoelidae gen. sp. (AJ241817), obtained from the crustacean *Hippolyte inermis* Leach in Corsica, France (Jousson et al. 1999). All other sequences belong to species which have not yet been molecularly characterised or are possibly new to science.

Further phylogenetic analyses based on Bayesian inference and maximum likelihood methods, produced phylogenetic trees for the 28S and ITS2 datasets with similar topology (Figs. 8, 9). All the species of *Helicometra* from the present and previous studies, formed a highly supported clade at the basal position to the rest of the Opecoelidae included in the analyses of the 28S dataset (Fig. 8). Isolates of *Helicometra* sp. 2 clustered with *Helicometra manteri* (KJ701238) collected from the Spiny searobin *Prionotus alatus* Goode and Bean in the Gulf of Mexico (Andres et al. 2014b) with strong support. Sequences of *Coitocaecum* spp. formed a strongly supported subclade within a clade with other representatives of the subfamily Opecoelinae. This is in agreement with morphology and molecular based systematics of the Opecoelidae (Cribb 2005; Bray et al. 2016; Martin et al. 2019a). Interestingly, the novel sequence of *Coitocaecum* sp. 1 did not form a separate branch within the "*Coitocaecum* clade", but appeared among sequences of *C. capense*.

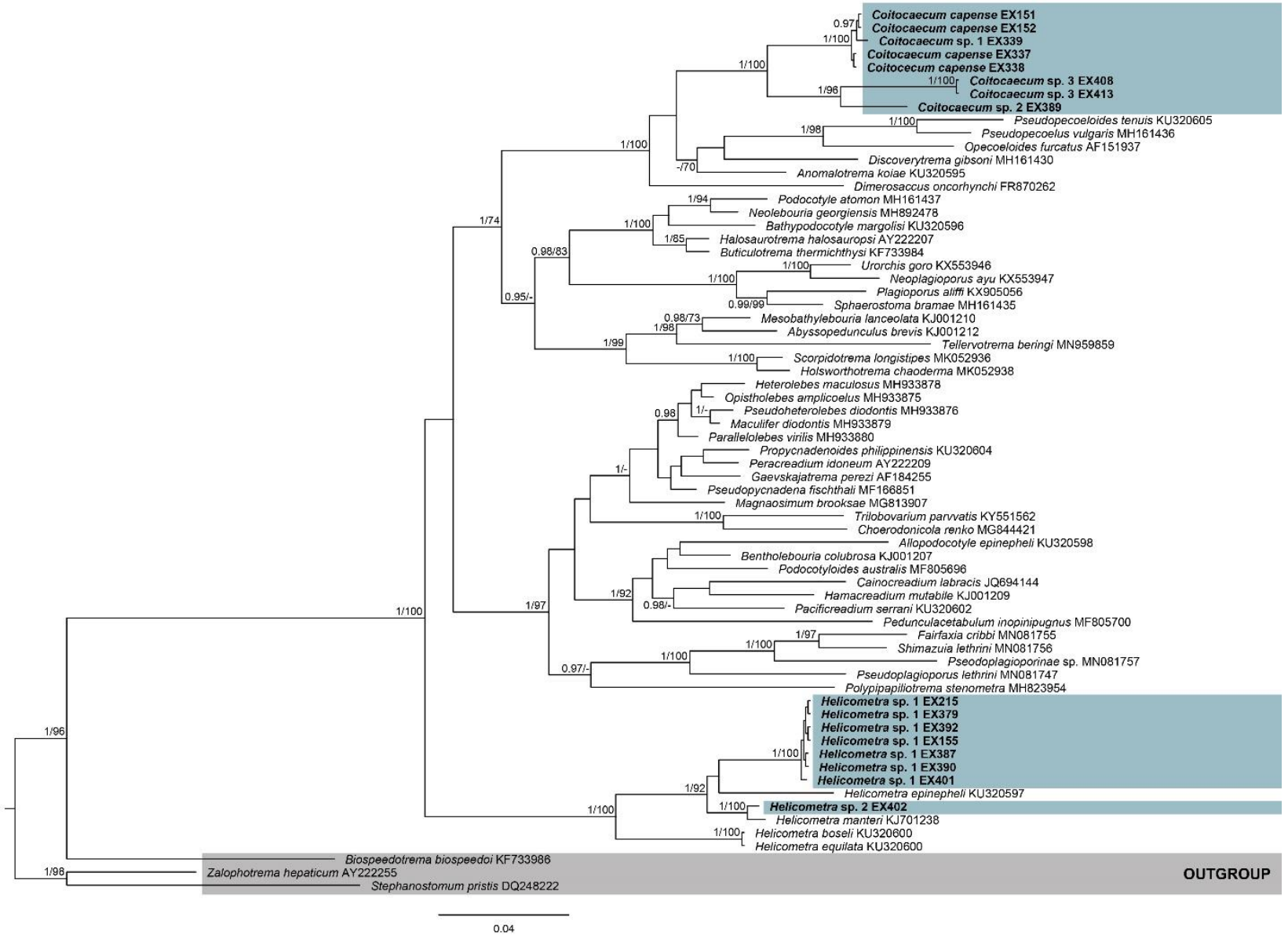

Figure 8. Bayesian inference (BI) tree based on the 28S rDNA dataset for the Opecoelidae. Nodal support given as BI/ML (maximum likelihood). Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. Newly generated sequences are highlighted in bold and can be seen in a blue rectangle

The overall interspecific divergence between species of *Helicometra* in the 28S dataset was 0.7−7.3% (8−85 nucleotides (nt) with *Helicometra epinepheli* being the most divergent and *Helicometra* sp. 1 being the least divergent. The intraspecific divergence between isolates of *Helicometra* sp. 1 was 0−0.1% (0−1 nt). Between the two species of *Helicometra* from the present study, the overall interspecific divergence was 3.2−3.3% (37−38 nt). The overall interspecific divergence between the species of *Coitocaecum* from the present study was 0.3−5.9% (3−69 nt) with *Coitocaecum* sp. 1 being the most divergent. The intraspecific divergence between isolates of *C. capense* was 0−0.1% (0−1 nt), sequences of *Coitocaecum* sp. 3 were identical. Sequences of *C. capense* differed from *Coitocaecum* sp. 1 by 0.3% (3−4 nt).

The phylogenetic tree resulting from the ITS2 dataset (Fig. 9), grouped isolates of *Helicometra* sp. 1 together in a clade with high support. Isolate of *Helicometra* sp. 2 clustered in a highly supported clade with an isolate of Opecoelidae gen. sp. (AJ241817), metacercariae collected from the crustacean host *H. inermis* from Corsica, France (Jousson et al. 1999). Similarly to results of the 28S rDNA data analysis, isolates of *Coitocaecum* spp. formed a subclade within the clade with members of the Opecoelinae. The isolate of *Coitocaecum* sp. 1 clustered in a strongly supported clade with isolates of *C. capense* but at the basal position. The isolates of *Coitocaecum* sp. 2 and *Coitocaecum* sp. 3 were grouped together in a separate branch within the subclade.

The overall interspecific divergence between species of *Helicometra* in the ITS2 dataset was 2.3−7.9% (8−27 nt). The interspecific divergence between the two species of *Helicometra* from the present study was 6.1−7.9% (17−27 nt), whereas the intraspecific variation between isolates of *Helicometra* sp. 1 was 0−0.6% (0−2 nt). Interspecific divergence between isolates of *Coitocaecum* was 0.3−14.1% (1−42 nt); isolates of *Coitocaecum* sp. 3 had low intraspecific divergence 0−0.3% (0−1 nt), while no intraspecific divergence was noted for isolates of *C. capense*. Isolates of *C. capense* differed by 0.7% (2 nt) from *Coitocaecum* sp. 1.

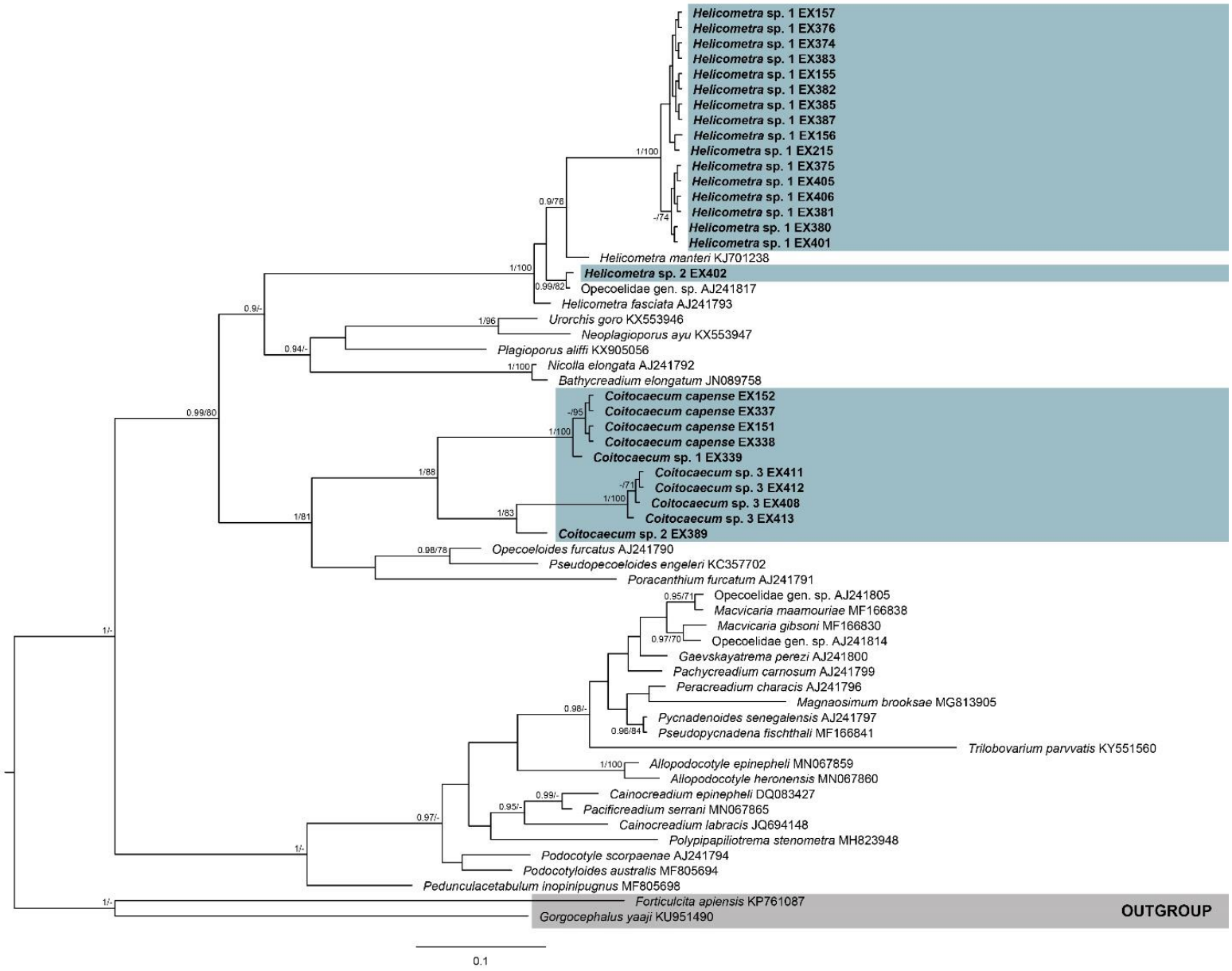

Figure 9. Bayesian inference (BI) tree based on the ITS2 dataset for the Opecoelidae. Nodal support given as BI/ML (maximum likelihood). Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. Newly generated sequences are highlighted in bold and can be seen in a blue rectangle

Discussion

Despite the lack of recent digenean descriptions from South African marine life, the abundance of the Opecoelidae found from a single marine fish species in this study, remains astonishing. This is especially surprising when taking into account that one of the most recent studies on trematodes of marine fishes from South Africa (Bray 1987) reported on merely one species (*H. fasciata*) and described another from *Cl. superciliosus* (*C. capense*). Discovering six opecoelid trematodes from this fish host emphasises the importance of incorporating combined molecular and morphological identification techniques, as well as including various localities along the host's distributional range when studying parasite communities, especially from varying habitats or environmental conditions.

This study found a great diversity of trematodes within the genus *Coitocaecum*: *C. capense*, which was previously described from this fish host, and three species that are possibly new to science. Prior to this study, *C. capense* was the only species of this genus to be described or reported from the marine biotope in South Africa (Bray 1987). According to Aken'Ova and Cribb (1996), many marine species of *Coitocaecum* normally have a narrow host specificity or infect hosts that are ecologically similar. *Coitocaecum capense* has been reported from six fish hosts of the same family (Clinidae) (Bray 1987), this might indicate that *C. capense* represents a species complex, rather than a single species with low host specificity at the level of definitive host. However, broader explorative studies on intertidal digeneans from this region are needed to further elicit information regarding the zoogeography of this genus.

Although the interspecific variation between *Coitocaecum capense* and *Coitocaecum* sp. 1 is not very high (interspecific divergence for 28S: 0.3% (3−4 nt); and ITS2: 0.7% (2 nt)), on the basis of numerous morphological variances we can confirm that these are indeed two distinct species and that the interspecific variation for species of *Coitocaecum* might be much lower than initially thought.

Our study provides the first molecular characterisation based on 28S rDNA and ITS2 sequences for species of *Coitocaecum* from South Africa and confirms the taxonomic position of this genus within the subfamily Opecoelinae, based on molecular evidence. Furthermore, we added DNA sequence data of *Helicometra* spp. from South Africa to the molecular library of the Opecoelidae.

An excellent example of the homoplasticity exhibited by opecoelids and familiar participants of taxonomic reorganisation, are species of the genus *Helicometra* (Blend and Dronen 2015). One species from this genus, *H. fasciata*, has been reported from 12 species of marine fishes belonging to six families, in South Africa (Bray 1987). This species is known to

have a cosmopolitan distribution, yet surprisingly little molecular data is available for this, most likely, species complex. As noted by previous studies, the combination of molecular and morphological identification techniques is not to be underestimated (Blasco-Costa et al. 2016; Bray et al. 2018), especially when concerning homoplastic species, such as *H. fasciata*. Morphologically, the specimens of *Helicometra* sp. 1 are similar to *H. fasciata*, but are genetically unique (interspecific divergence for 28S 3.2% (37−38 nt); and for ITS2 6.1−7.9% (6.1−7.9 nt)). It is possible that *Helicometra* sp. 1 is conspecific to the trematodes also found in this fish host and others from South Africa by Bray (1987), identified as *H. fasciata*. However, comparison to voucher specimens is needed to confirm this.

The isolate of *Helicometra* sp. 2. is genetically identical to a metacercarial isolate (AJ241817) identified as Opecoelidae gen. sp. collected from *H. inermis* in the Mediterranean (Corsica, France), indicating that the distribution of this species is much higher than we initially believed. The discovery of these adult trematodes can now lead to the further identification of these metacercariae, currently identified as *Helicometra* sp. 2, to species level. Only one other opecoelid species has been reported from both South Africa and the Mediterranean, *Macvicaria obovata* (Molin, 1859) Bartoli, Bray and Gibson, 1989 (Bartoli et al. 1989; Jousson et al. 1999; Born-Torrijos et al. 2012). Thus, we provide the partially elucidated life cycle of this species.

Intertidal areas are inhabited by a plethora of organisms, especially on the biologically rich south coast of South Africa. The biodiversity of such areas is yet to be fully explored, with many parasitic species possibly being overlooked, as has been demonstrated by the present study. As a result of the high biodiversity, in other words the large number of possible hosts, as well as the close proximity within which these organisms reside, these intertidal pools and intertidal areas are ideal for a parasitic lifestyle. Thus, serving as ideal study areas for parasitological surveys or studies that involve the ecology or biology of these organisms.

However, this research could be improved by conducting seasonal sampling from each locality. As the addition of new sampling localities has led to the reports of five more opecoelid trematodes from *C. superciliosus*, further sampling in additional localities along the hosts distributional range, would benefit our knowledge on the true diversity of trematodes for which it can act as an intermediate or definitive host. The incorporation of molecular identification techniques has also proven extremely helpful for the identification of trematodes, and further molecular data from this geographic region can greatly aid future research endeavours.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Code availability

Not applicable

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Chapter 5

A fluke discovery – unexpected diversity of digeneans from *Clinus superciliosus* (L. 1758) (Perciformes, Clinidae) in South Africa

Chapter 5. A fluke discovery – unexpected diversity of digeneans from *Clinus superciliosus* (L. 1758) (Perciformes, Clinidae) in South Africa

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Abstract

Often biodiversity studies only include macroorganisms, while the array of parasites which they host are sometimes overlooked or greatly underestimated. It is estimated that the diversity of marine parasites in South Africa remains vastly unexplored, and the results of this study indicate that this estimation is accurate, at least for the fish host *Clinus superciliosus* and its digenean parasites. We collected specimens of this host species from various localities along the South African coast and were found to be infected with a total of 14 digenean species of the families Acanthocolpidae, Bucephalidae, Fellodistomidae, Hemiuridae, Opecoelidae, Strigeidae and Zoogonidae. This was confirmed by analyses of DNA sequence data (28S rDNA, ITS1-5.8S-ITS2, ITS2, *cox*1 mtDNA) for all species as well as morphological analyses for representatives of the families Opecoelidae and Strigeidae. This study provides the first report of *C. superciliosus* as second intermediate host to metacercariae of four digenean species (*Cardiocephaloides physalis*, *Cardiocephaloides* sp., *Dollfustrema* sp. and *Stephanostomum* sp.) and definitive host to 10 diegenan species (*Coitocaecum capense*, *Coitocaecum* sp. 1, *Coitocaecum* sp. 2, *Coitocaecum* sp. 3, *Helicometra* sp. 1, *Helicometra* sp. 2, Hemiuridae gen. sp. 1, Hemiuridae gen. sp. 2, *Proctoeces* sp. and Zoogonidae gen. sp.). Not only does the present

study highlight the importance of incorporating multiple study sites along the host's distributional range when examining the parasite community that it harbours, but also demonstrates the multitude of opportunities for further explorative parasitological studies from this biodiversity-rich area.

Keywords: Trematoda, klipfish, DNA sequences, community composition

Introduction

Rohde (2005) states that the majority of living organisms can be seen as a diversity of hosts for parasites. With that in mind, Smit and Hadfield (2015) noted that if each marine fish species in South Africa hosted merely one parasite species, the majority of marine parasites from this area is yet to be discovered. Astonishing as it may be, this is very likely to be true as the blending of the cold, nutrient-rich Benguela current and warm Agulhas current (Griffiths et al. 2010) gives rise to unique habitats, accommodating the numerous species in this biodiversity-rich area.

Helminths are among the most important marine parasites (Rohde 2005), yet the last description of a digenean trematode (Platyhelminthes) from marine fishes in South Africa are from 1991 (Bray 1991) and the latest reports of trematodes from marine fishes in this region are from 2013 and 2017 (Le Roux et al. 2013; Nunkoo et al. 2017; Ukomadu 2017). In an attempt to further explore the digenean diversity of marine fishes in South Africa, the Super klipfish, *Clinus superciliosus* (Linnaeus, 1758), was the focus of the present study. Members of the Clinidae are abundant in intertidal and nearshore marine areas along the coast of southern Africa, with many species being endemic (Day 1969; Stepien 1992; von der Heyden et al. 2011). As a result of this fish species' close proximity to various organisms which may act as first or second intermediate hosts to trematodes (such as molluscs, crustaceans, sponges, urchins, polychaetes and fishes), it is easily accessible to cercariae that infect by penetrating the outer surface of the host. Also, as an omnivore, *C. superciliosus* consumes a wide variety of organisms through which trematode infection can also be facilitated. Thus, this fish has the potential to be both a second intermediate and/or definitive host to digenean trematodes.

Previous studies have found *C. superciliosus* from Port Elizabeth, South Africa to be a definitive host for two digenean species: *Helicometra fasciata* (Rudolphi, 1819) and *Coitocaecum capense* Bray, 1987, whilst being the type-host to the latter (Bray 1987a). The present study found *C. superciliosus* to host a total of 14 digenean species: second intermediate host to four species and definitive host to ten species – a much higher diversity than anticipated.

The aim of this paper is therefore to provide a detailed account of the diversity of digenean species infecting *C. superciliosus* from five localities throughout its distributional range.

Materials and Methods

Sample collection

In total, 71 specimens of *C. superciliosus* were collected from five localities along the South African coast: Saldanha Bay (33°2'44.46"S; 18°2'19.06"E) (n=19), Cape Town harbour (33°54'29.13"S; 18°25'5.81"E) (n=16), Hermanus (34°25'15.86"S; 19°14'37.56"E) (n=8), Tsitsikamma National Park (henceforth referred to as TNP) (34°1'15.21"S; 23°52'43.23"E) (n=17) and Chintsa (32°50'11.54"S; 28°7'1.19"E) (n=11). Sampling was carried out under the permit MALH–K2016–005a for TNP; RES2018/35 for Hermanus; RES2019–103 for Saldanha Bay, Cape Town harbour and Chintsa. Fish were collected using both baited traps and hand lines. Fish were subjected to helminthological examination following euthanisation. All metacercariae were excysted with fine needles. Adult digeneans were relaxed in hot saline and fixed in ethanol; ethanol concentration for the fixation of adults was 80%, while 96% ethanol was used for the fixation of metacercariae.

Morphological analysis

Trematodes from each fish host were sorted according to their morphology and specimens were selected for further genetic analyses. See Chapters 3 and 4 of this dissertation for the materials and methods used during the staining, fixation, making of permanent slides and microscopic examination of both metacercarial and adult trematodes respectively. The prevalence of a species at each sampling site was determined by calculating the percentage of fish hosts infected with that species, of the total number of fishes sampled from that site (Bush et al. 1997). The intensity of infection is given as a range of the lowest and highest number of trematodes that infect a single fish host from a specific locality (Bush et al. 1997).

Generation of molecular data

DNA was extracted from the selected specimens by using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) or PCRBiosystems Rapid DNA Extraction Kit (PCRBiosystems available from Analytical Solutions, Randburg, South Africa). The manufacturer's protocol for each extraction kit was followed, with the modification for PCRBiosystems Rapid DNA Extraction Kit described in Chapter 3. Amplification by means of polymerase chain reaction (PCR), was done for: the partial D1-D3 fragment of the 28S nuclear

ribosomal RNA gene, the entire internal transcribed spacer region (ITS1-5.8S-ITS2), the complete internal transcribed spacer 2 (ITS2) of the ribosomal gene cluster and the cytochrome c oxidase subunit I (*cox*1) mitochondrial gene. See Chapter 2 for information on primers used during amplification and sequencing. The PCR amplicons were visualised with agarose gel electrophoresis and sent to Inqaba Biotechnical Industries (Pty). Ltd. in Pretoria, South Africa, for purification and sequencing. Geneious v. 11.1.4 bioinformatics analyses software (Biomatters, Auckland, New Zealand) was used to assemble and edit obtained sequences.

Phylogenetic analyses

Novel sequences were compared to those available on GenBank by using the basic local alignment search tool (BLAST). Sequences were obtained from GenBank according to the trematode group and gene/region fragment amplified in our study (Table A-A3). Alignments including these sequences and those generated during this study, were built using MUSCLE (Edgar 2004) as implemented in Geneious v. 11.1.4. Outgroup selection was based on the phylogenetic analyses of Bray et al. (2005); Sokolov et al. (2016); Wee et al. (2017); Sokolov et al. (2018) and Hammond et al. (2020). The best nucleotide substitution model for each alignment, based on the Akaike information criterion (AIC), was determined with the jModelTest 2.1 software (Posada 2008). The construction of all phylogenetic trees was based on the general time reversible model with estimates of invariable sites and gamma distribution among site rate variation. The trees contain both Bayesian inference (BI) and maximum likelihood (ML) estimate analyses data. Analyses for BI were performed with MrBayes software, run on CIPRES Science gateway v. 3.3 (available at https://www.phylo.org/); while ML analyses were performed with PhyML v. 3 (available at http://www.atgc-montpellier.fr/phyml/). Markov chian Monte Carlo (MCMC) chains were run for 10 000 000 generations for all alignments. The 'burn-in' parameter was set for the first 25% of the sampled trees. Bootstrap pseudoreplicates (100) were used to determine nodal support for the ML analyses. Phylogenetic trees were visualised using FigTree v. 1.4.3 (Rambaut 2012). Only BI values greater than or equal to 0.9 and ML values greater than or equal to 70 are shown. Pairwise genetic distance matrices (p-distances) were calculated in MEGA v. 7.

Results

General observations

A total of 14 distinct digenean species from seven families, have been found in *C. superciliosus* from five localities along the South African coast (Table 1). This fish species is utilised as a definitive host for ten digenean species and as a second intermediate host for four digenean species. Detailed information on species belonging to the Strigeidae Railliet, 1919 and Opecoelidae Ozaki, 1925 (*Cardiocephaloides* spp., *Coitocaecum* spp. and *Helicometra* spp.) is presented in Chapters 3 and 4 of this dissertation).

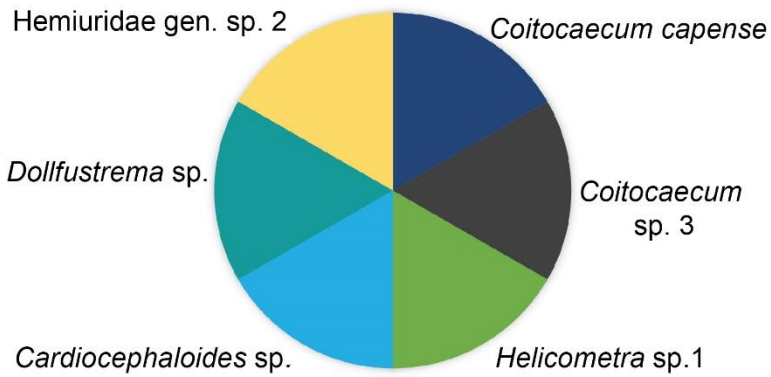
This study found *C. superciliosus* from Saldanha Bay to harbour the highest number of digeneans per host individual. This is due to the very high intensity of infection by metacercariae of the family Bucephalidae, with some hosts harbouring more than 1000 individuals of this species. Fish from Chintsa along the eastern coast, had the highest diversity of digenean species, followed by Hermanus, Saldanha Bay and TNP (Fig. 1). No trematodes were found parasitising the fish from Cape Town harbour. The three most common species are *Co. capense*, *Helicometra* sp. 1 and *Cardiocephaloides* sp., which occurred at all sampling localities, except Cape Town harbour. Other species of *Coitocaecum* and *Helicometra* were each only found in a single locality and in lower numbers, mostly only a single individual.

At each sampling locality, there appeared to be one species that was more dominant to the others, except for Tsitsikamma National Park, where the species diversity and intensity of infection were more balanced. The metacercariae of the family Bucephalidae were dominant in *C. superciliosus* from Saldanha Bay due to the extremely high intensity of infection and presence in all fish examined. Individuals of *Helicometra* sp. 1 dominantly occurred in Hermanus. Due to the high intensity of infection of a single *C. superciliosus* specimen with 87 individuals of *Cardiocephaloides* sp. in the eyes and brain, this was the dominant species at Chintsa. A summary of the prevalence and intensity of infection of *C. superciliosus* with different digenean species per sampling locality is provided in Table 2.

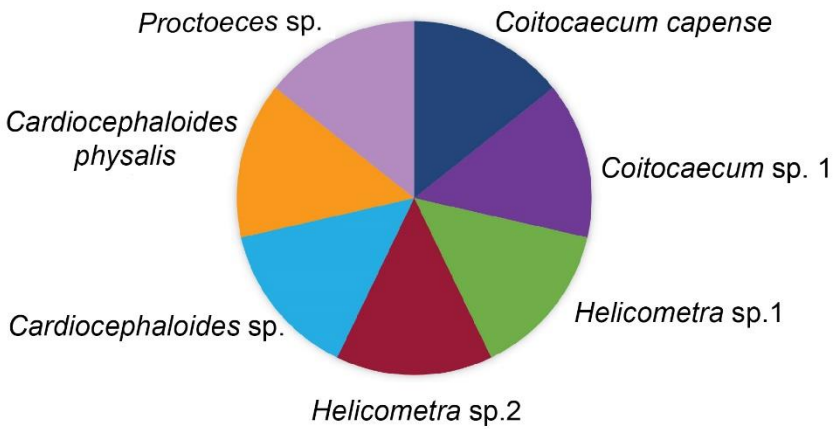
Table 1. Summary of digenean trematodes from *C. superciliosus*

Family	Species	Stage	Site in host	Locality	DNA sequences			
					28S	ITS1-5.8S-ITS2	ITS2	*cox*1
Acanthocolpidae								
	Stephanostomum sp.	M	Visceral organs, eyes, brain	Chintsa	+	–	+	+
Bucephalidae								
	Dollfustrema sp.	M	Visceral organs, muscles, eyes, brain	Saldanha Bay	+	–	+	–
Fellodistomidae								
	Proctoeces sp.	A	Intestine	Hermanus	–	–	–	–
				TNP	+	–	–	–
				Chintsa	+	–	–	–
Hemiuridae								
	Hemiuridae gen. sp. 1	A	Male and female gonads; intestine	TNP	+	–	+	–
				Chintsa	+	–	+	–
	Hemiuridae gen. sp. 2	A	Intestine	Saldanha Bay	+	–	+	–
Opecoelidae								
	Coitocaecum capense	A	Intestine	Saldanha Bay	–	–	–	–
				Hermanus	+	–	+	–
				TNP	+	–	+	–
				Chintsa	–	–	–	–
	Coitocaecum sp. 1	A	Intestine	Hermanus	+	–	+	–
	Coitocaecum sp. 2	A	Intestine	Chintsa	+	–	+	–
	Coitocaecum sp. 3	A	Intestine	Saldanha Bay	+	–	+	–
	Helicometra sp. 1	A	Intestine	Saldanha Bay	–	–	+	–
				Hermanus	+	–	+	–
				TNP	+	–	+	–
				Chintsa	+	–	+	–
	Helicometra sp. 2	A	Intestine	Hermanus	+	–	+	–
Strigeidae								
	Cardiocephaloides physalis	M	Eye	Hermanus	+	–	+	+
	Cardiocephaloides sp.	M	Eye	Saldanha Bay	–	–	–	+
				Hermanus	–	–	–	+
				TNP	+	+	+	+
				Jeffreys Bay	+	–	+	+
				Chintsa	+	–	+	+
Zoogonidae								
	Zoogonidae gen. sp.	A	Intestine	Chintsa	+	–	+	–

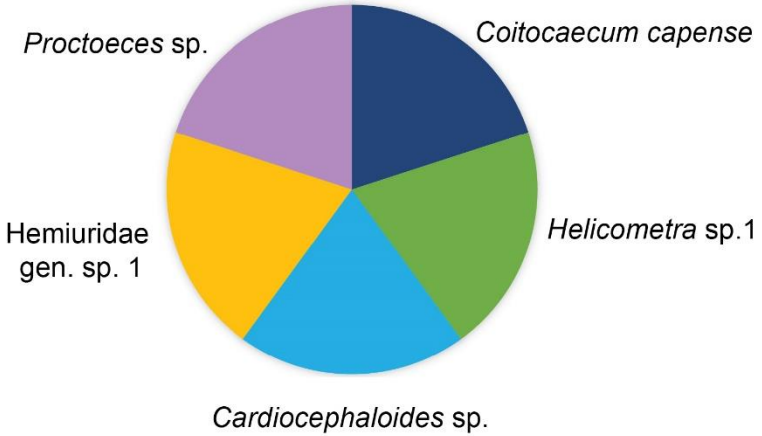
Abbreviations: A, adult; M, metacercariae; +, sequences generated for this gene/region; –, no sequences generated for this gene/region



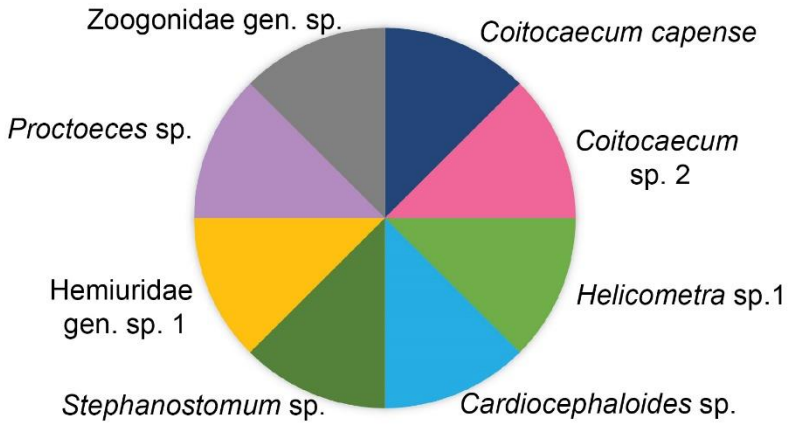
Saldanha Bay



Hermanus



Tsitsikamma National Park



Chintsa

Figure 1. Graphical presentations of the digenean species found at each sampling locality

Table 2. Summary on the prevalence and mean intensity of infection of *C. superciliosus* with digenean trematodes

Taxa	Locality (site in host)	Prevalence	Intensity of infection
Acanthocoplidae			
Stephanostomum sp.	Chintsa (brain, eyes, gills, body cavity)	36%	1–5
Bucephalidae			
Dollfustrema sp.	Saldanha Bay (eyes, brain, muscle, visceral organs)	100%	≥200
Fellodistomidae			
Proctoeces sp.	Chintsa (intestine)	27%	3–4
	Hermanus (intestine)	1 of 8	1
	TNP (intestine)	30%	1
Hemiuridae			
Hemiuridae gen. sp. 1	Chintsa (ovaries)	18%	2
	Chintsa (testes)	18%	1–2
	TNP (ovaries)	10%	6
	TNP (testes)	70%	1–5
	TNP (intestine)	10%	3
Hemiuridae gen. sp. 2	Saldanha Bay (intestine)	5%	4
Opecoelidae			
Coitocaecum capense	Chintsa (intestine)	9%	7
	Hermanus (intestine)	7 of 8	1–5
	Saldanha Bay (intestine)	5%	1
	Tsitsikamma National Park (intestine)	53%	1–13
Coitocaecum sp. 1	Hermanus (intestine)	1 of 8	1
Coitocaecum sp. 2	Chintsa (intestine)	9%	1
Coitocaecum sp. 3	Saldanha Bay (intestine)	26%	1–22
Helicometra sp. 1	Chintsa (intestine)	82%	1–11
	Hermanus (intestine)	8 of 8	1–21
	Saldanha Bay (intestine)	21%	1–15
	Tsitsikamma National Park (intestine)	65%	1–6
Helicometra sp. 2	Hermanus (intestine)	1 of 8	1
Strigeidae			
Cardiocephaloides physalis	Hermanus (eyes)	1 of 8	3
Cardiocephaloides sp.	Chintsa (eyes, brain)	27%	1–87
	Hermanus (eyes)	2 of 8	3
	Saldanha Bay (eyes, brain)	42%	1–16
	Tsitsikamma National Park (eyes)	29%	1–6
Zoogonidae			
Zoogonidae gen. sp.	Chintsa (intestine)	27%	1–6

Family Acanthocolpidae Lühe, 1906

Previous studies have reported five species of the Acanthocolpidae from marine fishes in South Africa: ***Pleorchis sciaenae*** Yamaguti, 1938 ex *Argyrosomus hololepidotus* (Lacepède, 1801) from Port Elizabeth (Bray 1986); ***Stephanostomum ditrematis*** (Yamaguti, 1939) Manter, 1947 ex *Megalaspis cordyla* (Linnaeus, 1758) from Durban (Bray 1985); ***Stephanostomum imparispine*** (Linton, 1905) Manter, 1940 ex *Engraulis japonicus* Temminck and Schlegel, 1846, *Sardinops sagax* (Jenyns, 1842) and *Sardina pilchardus* (Walbaum, 1792) from the south Atlantic (Parukhin 1976); ***Stephanostomum solontschenkae*** Parukhin, 1968 ex *Merluccius capensis* Castelnau, 1861 from Cape Recife; ***Stephanostomum* spp.** ex *M. capensis* from Cape Town and *Chaetodon marleyi* Regan, 1921 from Port Elizabeth (Bray 1985). Four specimens, of which three belong to the genus *Stephanostomum*, were reported as adults, and the latter was found as metacercariae. This study reports on the presence of metacercariae of *Stephanostomum* sp. found in *C. superciliosus* from Chintsa, South Africa (Fig. 2).

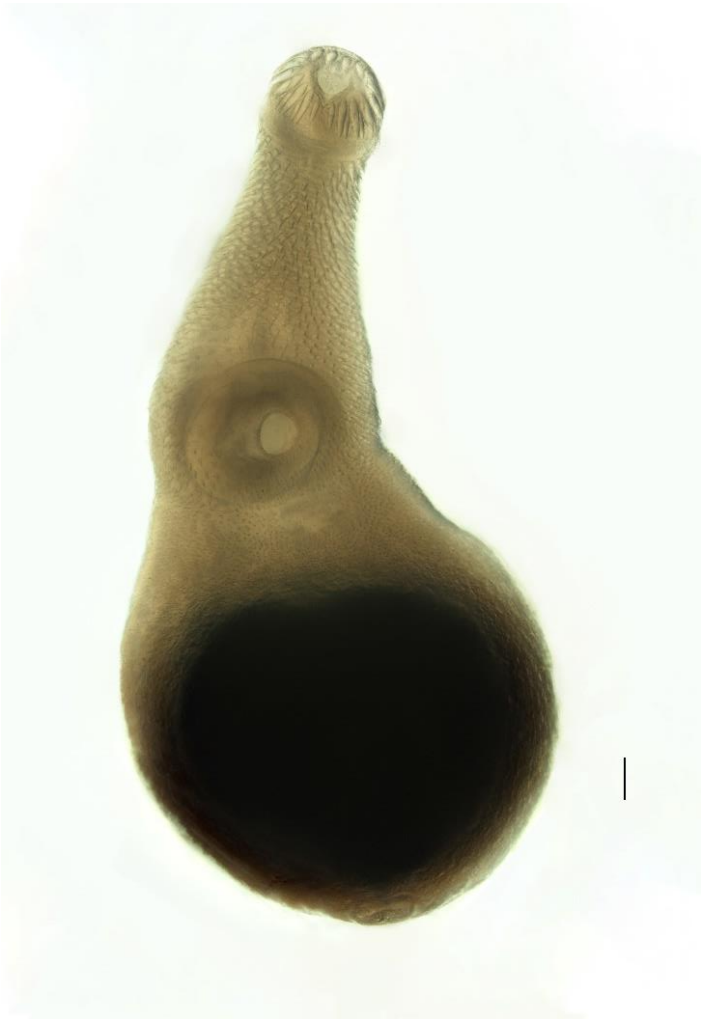


Figure 2. *Stephanostomum* sp. Ventral view of unstained, fixed specimen. Scale bar: 50 µm

Genus *Stephanostomum* Looss, 1899

***Stephanostomum* sp.**

Host: Super klipfish *Clinus superciliosus* (L. 1758) (Clinidae).
Locality: Chintsa, South Africa (32°50'11.54"S; 28°7'1.19"E)
Site of infection: brain, eyes, gills, body cavity.
Representative DNA sequences: one sequence for 28S; two sequences for ITS2; and one sequence for *cox*1.
Voucher material: three slides and eight ethanol-fixed specimens kept in the collection of the Water Research Group (WRG) at North-West University (NWU) in Potchefstroom.

BI and ML phylogenetic analyses of the 28S dataset, including a sequence of the metacercarial isolate collected in our study and representatives of the Acanthocolpidae available in GenBank, produced trees with consensus topology (Fig. 3). The alignment was 874 nucleotides (nt) long and contains 22 sequences (Table A–A3). Novel sequence clustered within the clade that consists of isolates of the genus *Stephanostomum* together with *Stephanostomum bicoronatum* (Stossich, 1883) Fuhrmann, 1928 collected from the fish host *Sciaena umbra* L., 1758 as well as *Stephanostomum cesticillum* (Molin, 1858) collected from angler fish *Lophius piscatorius* L., 1758, both collected in Corsica, France (Bray et al. 2005). The genetic divergence between the novel sequence of *Stephanostomum* sp. and *S. cestillium* was 0.3% (3 nt), whereas it differed from *S. bicoronatum* by 0.6% (5 nt). The overall interspecific divergence observed in this dataset is 0–17.9% (0–156 nt), with the interspecific variation between isolates of the genus *Stephanostomum* being 0.3– 10.6% (0–92 nt).

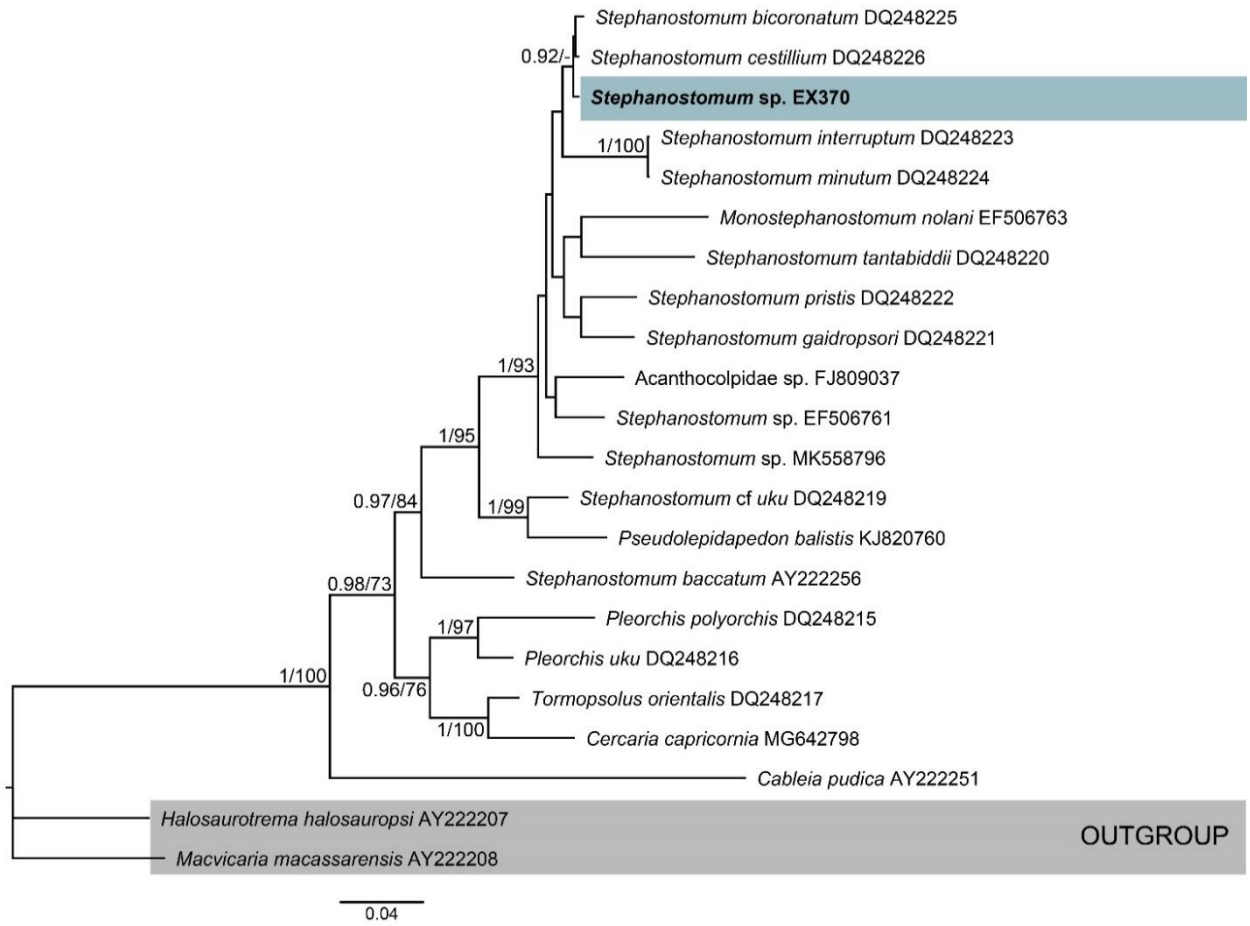


Figure 3. Bayesian inference (BI) phylogenetic tree for representatives of the Acanthocolpidae based on the partial 28S rDNA sequences. Newly generated sequences are highlighted in bold and can be seen in a blue rectangle. Nodal support given as BI/ML (maximum likelihood). The scale bar indicates the expected number of substitutions per site

Family Bucephalidae Poche, 1907

To date, five species of the Bucephalidae have been reported from marine fishes along the South African coast: ***Bucephalus margaritae*** Ozaki and Ishibashi, 1934 ex *Carangoides hedlandensis* (Whitley, 1934) from Durban, *Caranx heberi* (Bennett, 1830) from Sodwana (Bray 1984), and *Pomatomus saltatrix* (Linnaeus, 1766) from the south Atlantic (Parukhin 1976); ***Prosorhynchoides arcuatus*** (Linton, 1900) Love and Moser, 1983 ex *P. saltatrix* from Sodwana; ***Prosorhynchus caudovatus*** Manter, 1940 ex *Epinephelus andersoni* Boulenger, 1903 from Umvoti (Bray 1984); ***Rhipidocotyle paruchini*** Gavrilyuk-Tkachuk, 1979 ex *Otolithes ruber* (Bloch and Schneider, 1801) from the Agulhas bank in the Indian Ocean (Gavriljuk-Tkachuk 1979); and ***Rhipidocotyle* sp.** ex *Ruvettus pretiosus* Cocco, 1833 from the west coast of South Africa (Nunkoo et al. 2017). The present study reports on metacercariae belonging to the same

family and identified as *Dollfustrema* sp. collected from *C. superciliosus* in Saldanha Bay (Fig. 4). Fishes from this site had a high intensity of infection with meatacercariae of *Dollfustrema* sp. (Table 2), infecting nearly all organs and tissues.

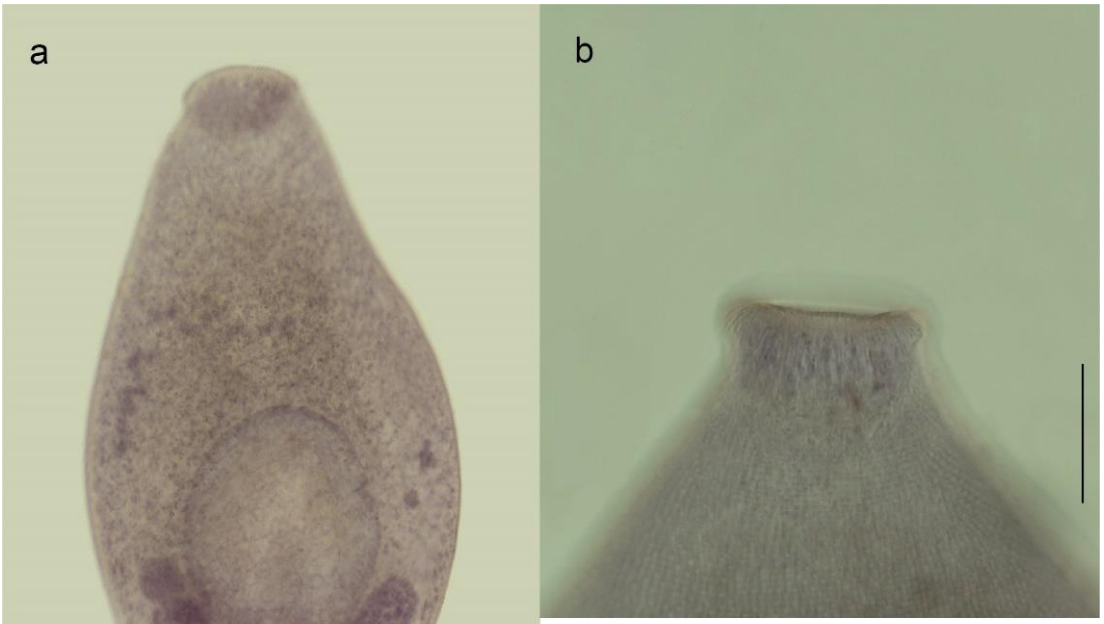


Figure 4. *Dollfustrema* sp. a, ventral view of whole specimen. b, ventral view of armed tegument behind the oral sucker. Specimen stained with Mayers haematoxylin. Scale bars: 100 µm

Genus *Dollfustrema* Eckmann, 1934

***Dollfustrema* sp.**

Host: Super klipfish *Clinus superciliosus* (L. 1758) (Clinidae).
Locality: Saldanha Bay, South Africa (33°2'44.46"S; 18°2'19.06"E).
Site of infection: brain, eyes, muscle, on visceral organs.
Representative DNA sequences: one sequence for 28S and two sequences for ITS2.
Voucher material: two slides and 15 ethanol-fixed specimens kept in the collection of the WRG at NWU in Potchefstroom.

The 28S sequence of the metacercarial isolate of *Dollfustrema* sp. was analysed along with other species of the same family. BI and ML analyses produced a tree with resolved topologies (Fig. 5). The alignment contained 28 sequences and was 1034 nt long. The newly generated sequence formed a highly supported basal clade with other isolates of the genus *Dollfustrema*: *Dollfustrema durum* Nolan, Curran, Miller, Cutmore, Cantacessi & Cribb, 2015 from the Giant Moray *Gymnothorax javanicus* (Bleeker, 1859) in Australia and *Dollfustrema hefeiensis* Liu, 1999 from the fish host *Rhinogobius giurinus* (Rutter, 1897) in China (Nolan et al. 2015). Genetic divergence between *Dollfustrema* sp. and *D. durum* was 2.6% (27 nt), whereas 4.3% (44 nt) differences were observed between this species and *D. hefeiensis*. The overall interspecific divergence between bucephalid isolates in this analysis was 0.7–14% (7–142 nt). This is the first report of a species of the genus *Dollfustrema* from marine fishes in South Africa.

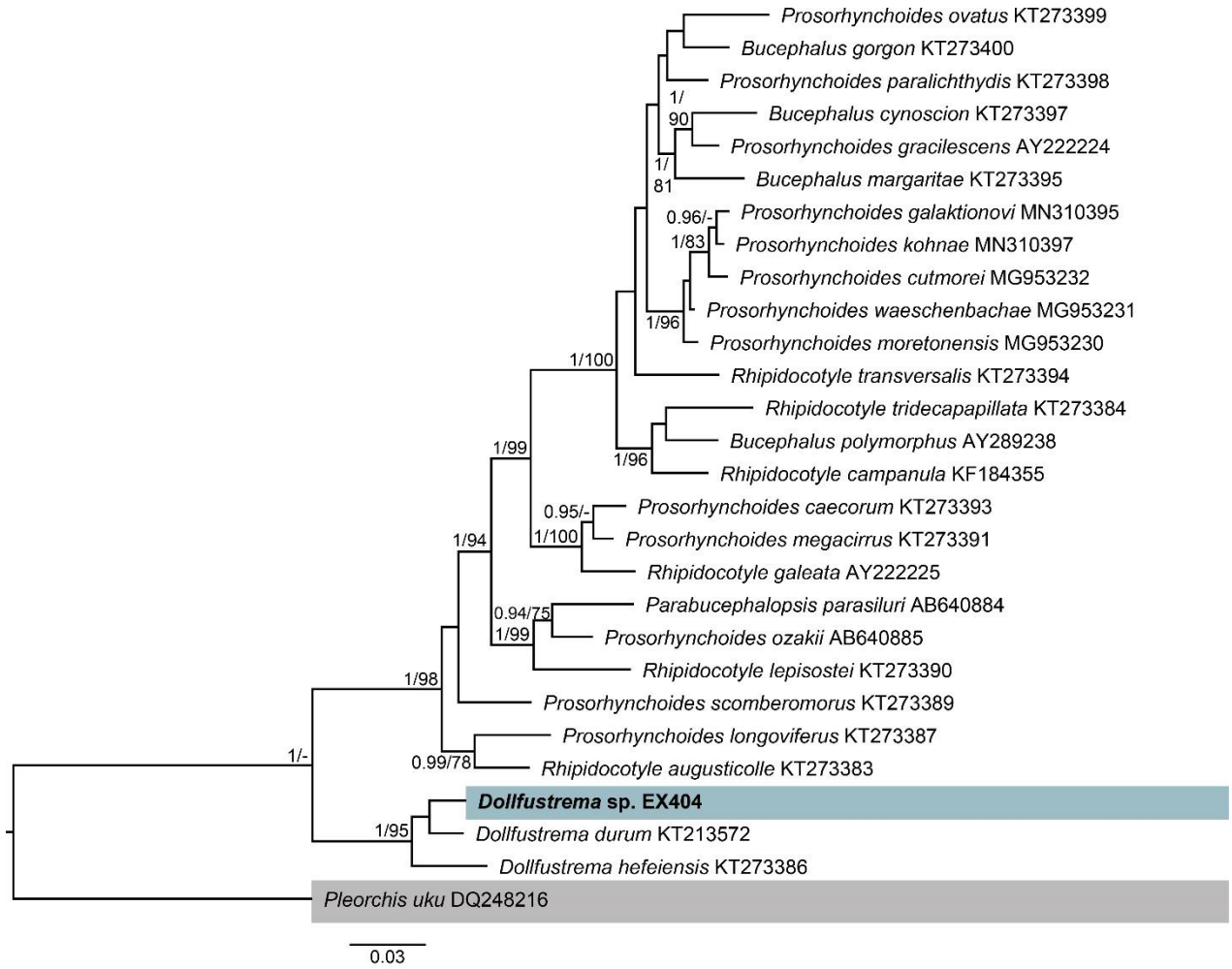


Figure 5. Bayesian inference (BI) phylogenetic tree for representatives of the Bucephalidae based on the partial 28S rDNA sequences. Newly generated sequences are highlighted in bold and can be seen in a blue rectangle. Nodal support given as BI/ML (maximum likelihood). The scale bar indicates the expected number of substitutions per site

Family Fellodistomidae Nicoll, 1909

Prior to our study, five species of the Fellodistomidae have been reported from marine fishes in South Africa: ***Fellodistomum* sp. 1** ex *Lophius piscatorius* Linnaeus, 1758 from the south Atlantic (Parukhin 1989); ***Steringotrema pagelli*** (van Beneden, 1871) Odhner, 1911 ex *Spondyliosoma emarginatum* Valenciennes, 1830 from Algoa Bay; ***Tergestia pauca*** Texeira de Freitas and Kohn, 1965 ex *P. saltatrix* from Sodwana (Bray 1984); ***Theledera pectinata*** (Linton, 1905) Linton, 1910 ex *P. saltatrix* from the south Atlantic (Parukhin 1976); and ***Tergestia laticollis*** (Rudolphi, 1819) Stossich, 1899 ex *Trachurus trachurus* L., 1758 and *Decapterus punctatus* (Cuvier, 1829) from Durban (Parukhin 1976) as well as *Trachurus capensis* Castelnau, 1861 from Port Elizabeth (Le Roux 2013). Bray (1984) also reported metacercarial specimens of ***Proctoeces maculatus*** (Looss, 1901) Odhner, 1911 from the common octopus *Octopus vulgaris*

Cuvier, 1797 from Durban. This study reports on the presence of a species of *Proctoeces* collected from *C. superciliosus* in both TNP, Hermanus and Chintsa (Fig. 6).

Figure 6. *Proctoeces* sp. Ventral view of stained specimen. Scale bar: 100 µm

Genus *Proctoeces* Odhner, 1911

***Proctoeces* sp.**

Host: Super klipfish *Clinus superciliosus* (L. 1758) (Clinidae).
Locality: Hermanus (34°25'15.86"S; 19°14'37.56"E), Tsitsikamma National Park (34°1'15.21"S; 23°52'43.23"E), Chintsa (32°50'11.54"S; 28°7'1.19"E), South Africa.
Site of infection: intestine.
Representative DNA sequences: three sequences for 28S.
Voucher material: one slide and 12 ethanol-fixed specimens kept in the collection of the WRG at NWU in Potchefstroom.

Phylogenetic analyses (BI and ML) based on the 28S dataset included sequences of *Proctoeces* sp. and available sequences of other representatives of the genus *Proctoeces* (alignment consisted of 10 sequences, 842 nt long), produced trees with similar topologies (Fig. 7). The three novel identical sequences formed a highly supported clade together with an isolate of *Proctoeces maculatus* (Looss, 1901) Odhner, 1911 retrieved from the bream *Sparus aurata* L., 1758 in Tunisia (Wee et al. 2017). Genetic divergence of 0.1% (1 nt) was noted between sequences of *Proctoeces* sp. and sequences of *P. maculatus* (KX671302). The overall genetic divergence within the genus *Proctoeces* was 0.1–8.9% (1–75 nt).

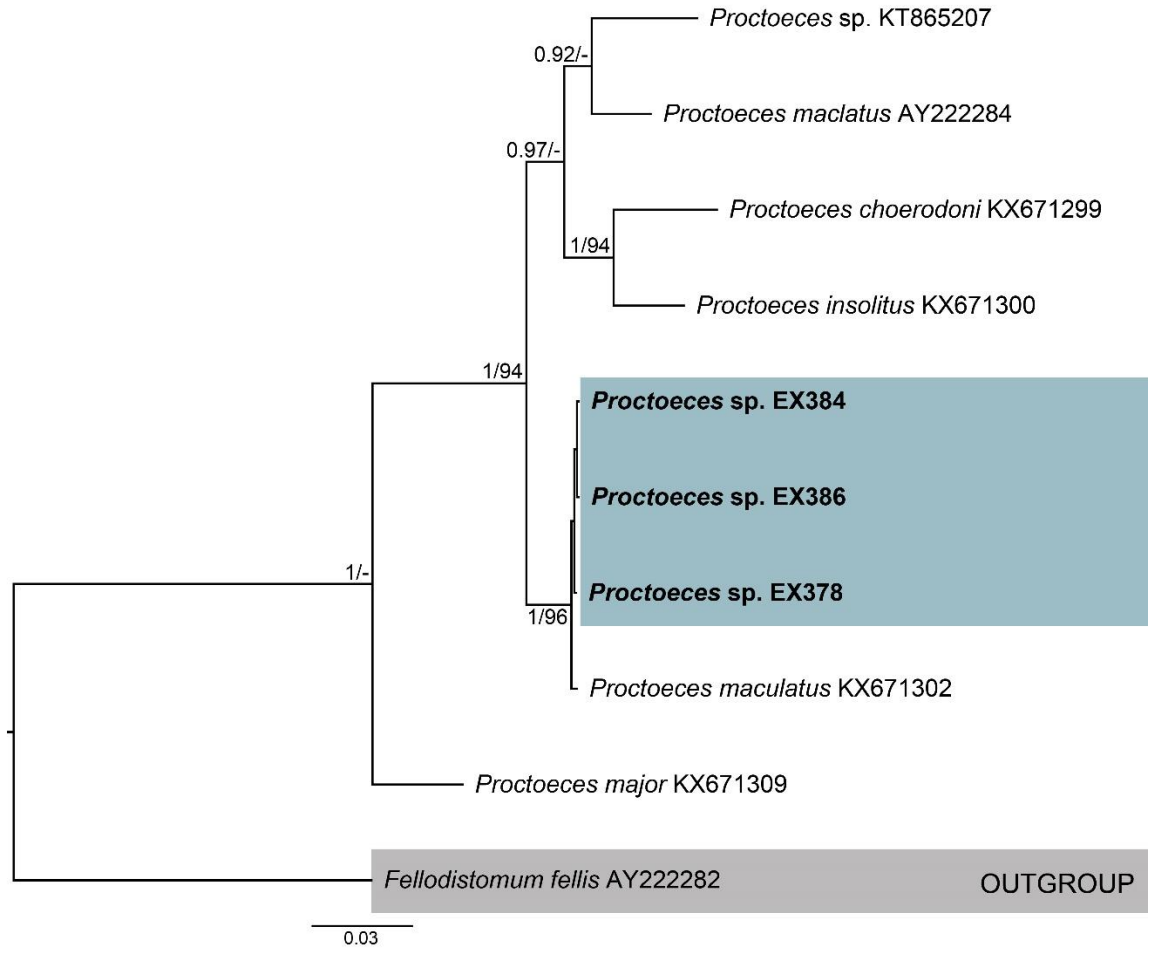


Figure 7. Bayesian inference (BI) phylogenetic tree for representatives of the genus *Proctoeces* based on the partial 28S rDNA sequences. Newly generated sequences are highlighted in bold and can be seen in a blue rectangle. Nodal support given as BI/ML (maximum likelihood). The scale bar indicates the expected number of substitutions per site

Family Hemiuridae Looss, 1899

The Hemiuridae is the digenean family with the most reports (18) from marine fishes in South Africa (Table 3). The present study adds to these reports with two unidentified species of the Hemiuridae, one of which was found in TNP and Chintsa (Hemiuridae gen. sp. 1) (Fig. 8), whereas the other was present only in Saldanha Bay (Hemiuridae gen. sp. 2) (Fig. 9). Interestingly, Hemiuridae gen. sp. 1 was found to infect different organs within *C. superciliosus*. Specimens of this species were found in the ovaries, testes and scarcely in the intestine.

Table 3. Summary of Hemiuridae reported or described from marine fishes in South Africa

Hemiuridae species	Host species	Locality	Reference
Dinurus longisinus Looss, 1907	*Coryphaena hippurus* Linnaeus, 1758	Eastern Cape	Bray 1990
Dissosaccus laevis (Linton, 1898) Manter, 1947	*Helicolenus dactylopterus* (Delaroche, 1809)	Durban	Parukhin 1989
Ectenurus lepidus Looss, 1907	*T. capensis*	Port Elizabeth	Le Roux 2013
Ectenurus virgula Linton, 1910	*L. piscatorius*	south Atlantic	Parukhin 1989
	Unspecified	Durban	Parukhin 1976
Elytrophalloides humerus Bray, 1990	*Trachinotus botla* (Shaw, 1803)	Sodwana	Bray 1990
Lecithochirium floridense (Manter, 1934) Crowcroft, 1946	*Hoplobrotula gnathopus* (Regan, 1921)	Cape Town	Parukhin 1989
Lecithochirium genypteri Manter, 1954	*Genypterus capensis* (Smith, 1847)	Algoa Bay	Bray 1991
		Cape Town	Bray 1991
		van Stadens River mouth	Bray 1991
Lecithochirium jaffense Fischthal, 1982	*Blennioclinus brachycephalus* (Valenciennes, 1836)	Port Elizabeth	Bray 1991
Lecithochirium kawakawa Yamaguti, 1970	*Euthynnus affinis* (Cantor, 1849)	Durban	Bray 1991
		Sodwana	Bray 1991
	Chrysoblephus anglicus (Gilchrist and Thompson, 1908)	St. Lucia	Bray 1991
Lecithochirium parafusiforme Bray, 1991	*Gymnothorax flavimarginatus* (Rüppell, 1830)	La Mercy	Bray 1991
Lecithochirium sp.	*Saurida undosquamis* (Richardson, 1848)	Richards Bay	Bray 1991
Lecithochirium sp.	*Alectis ciliaris* (Bloch, 1787)	Durban	Bray 1991
Lecithocladium angustiovum Yamaguti, 1953	*Rastrelliger kanagurta* (Cuvier, 1816)	Durban	Bray 1990
Lecithocladium excisum (Rudolphi, 1819) Lühe, 1901	*Scomber japonicus* Houttuyn, 1782	Durban	Parukhin 1976
Lecithocladium magnacetabulum Yamaguti, 1934	*Ariomma indicum* (Day, 1871)	Durban	Parukhin 1976
	Cubiceps whiteleggii (Waite, 1894)	Durban	Parukhin 1976

Table 3. Continued

Hemiuridae species	Host species	Locality	Reference
	Pseudocaranx dentex (Bloch and Schneider, 1801)	Durban	Parukhin 1976
	Parupeneus cyclostomus (Lacepède, 1801)	south Atlantic	Parukhin 1976
Ectenurus selari (Parukhin, 1966) Yamaguti, 1971	*T. trachurus*	Durban	Parukhin 1976
Parahemiurus merus (Linton, 1910) Manter, 1940	*Chlorophthalmus agassizi* Bonaparte, 1840	Durban	Parukhin 1976
Plerurus digitatus (Looss, 1899) Looss, 1907	*E. affinis*	Durban	Bray 1990
		Sodwana	Bray 1990
	Scomberomorus commerson (Lacepède, 1800)	Durban	Bray 1990
		Cape Vidal	Bray 1990
	Thunnus albacares (Bonnaterre, 1788)	Umhlanga Rocks	Bray 1990
	Lichia amia (Linnaeus, 1758)	Kerridene	Bray 1990
		Port Elizabeth	Bray 1990
	Scomberoides commersonnianus Lacepède, 1801	Umhlanga Rocks	Bray 1990
	A. ciliaris	Durban	Bray 1990
	P. saltatrix	Eastern Cape	Bray 1990
		Sodwana	Bray 1990
	S. undosquamis	Richards Bay	Bray 1990
	Rachycentron canadum (Linnaeus, 1766)	Umhlanga Rocks	Bray 1990

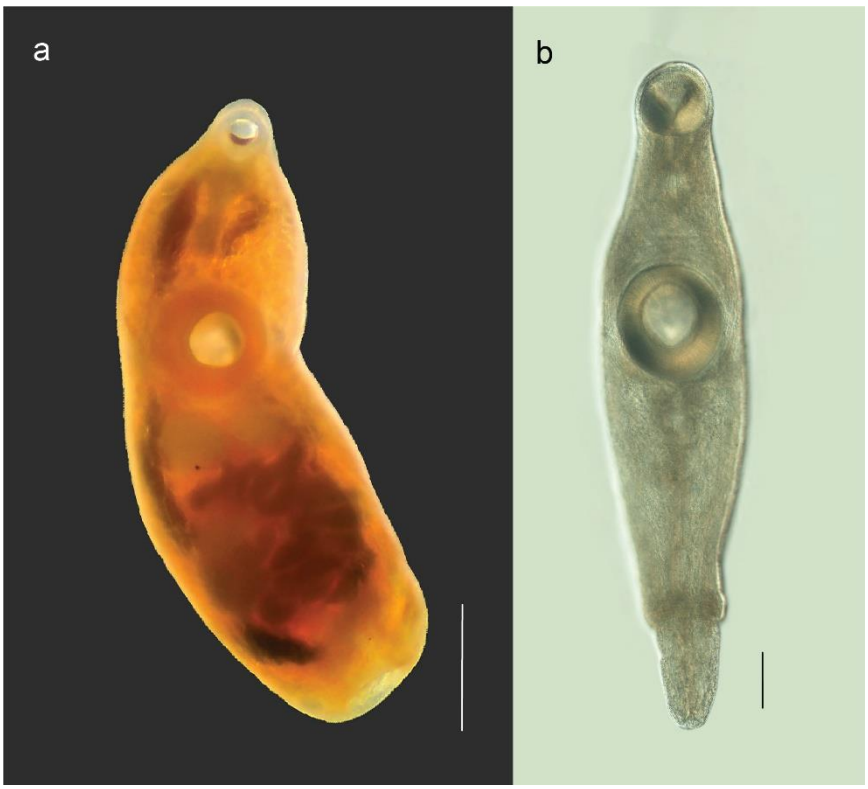


Figure 8. Hemiuridae gen. sp. 1. a, Ventral view of a live specimen found in the ovaries. b, Ventral view of fixed, unstained pregravid adult specimen found in the testes. Scale bars: a, 1000 µm; b, 100 µm

Hemiuridae gen. sp. 1

Host: Super klipfish *Clinus superciliosus* (L. 1758) (Clinidae).
Locality: Tsitsikamma National Park (34°1'15.21"S; 23°52'43.23"E) and Chintsa (32°50'11.54"S; 28°7'1.19"E), South Africa.
Site of infection: intestine, ovaries and testes
Representative DNA sequences: two sequences for 28S and six sequences for ITS2.
Voucher material: two slides and 32 ethanol-fixed specimens kept in the collection of the WRG at NWU in Potchefstroom.

Figure 9. Hemiuridae gen. sp. 2. Ventral view of stained voucher specimen. Scale bar: 100 µm

Hemiuridae gen. sp. 2

Host: Super klipfish *Clinus superciliosus* (L. 1758) (Clinidae).
Locality: Saldanha Bay, South Africa (33°2'44.46"S; 18°2'19.06"E).
Site of infection: intestine.
Representative DNA sequences: one sequence for 28S and one sequence for ITS2.
Voucher material: one slide and three ethanol-fixed specimens kept in the collection of the WRG at NWU in Potchefstroom.

The alignment consisting of 24 28S rDNA sequences (952 nt) of members of the Hemiuridae collected in the present and previous studies, was analysed using BI and ML methods. The consensus tree had high nodal support for most of the nodes (Fig. 10). Sequences generated during the present study clustered within a strongly supported clade with representatives of the subfamilies Hemiurinae, Lecithochiriinae and Pulmoverminae. Within this clade Hemiuridae gen. sp. 2 clustered with *Pulmovermis cyanovitellosus* Coil & Kuntz, 1960

from the Chinese sea snake *Laticauda semifasciata* (Reinwardt, 1837) from Japan (Sokolov et al. 2018), albeit without high support. Sequences of isolates of Hemiuridae gen. sp. 1 formed a separate branch basal to the 'Hemiuridae gen. sp. + *P. cyanovitellosus*' subclade. These species formed a sister clade to isolates from the genera *Lecithochirium* and *Hemiurus*. The interspecific divergence between the two hemiurid species reported during this study was 3.3–3.5% (31–33 nt). Intraspecific divergence between the two isolates of Hemiuridae gen. sp. 1 was 0.4% (4 nt). Hemiuridae gen. sp. 2 was most closely related to *P. cyanovitellosus*, with genetic divergence of 3.2% (30 nt), and Hemiuridae gen. sp. 1 differing by 2.7–3% (26–28 nt) from *P. cyanovitellosus*. The overall genetic divergence within this dataset of the Hemiuridae was 1.2–21.6% (8–196 nt).

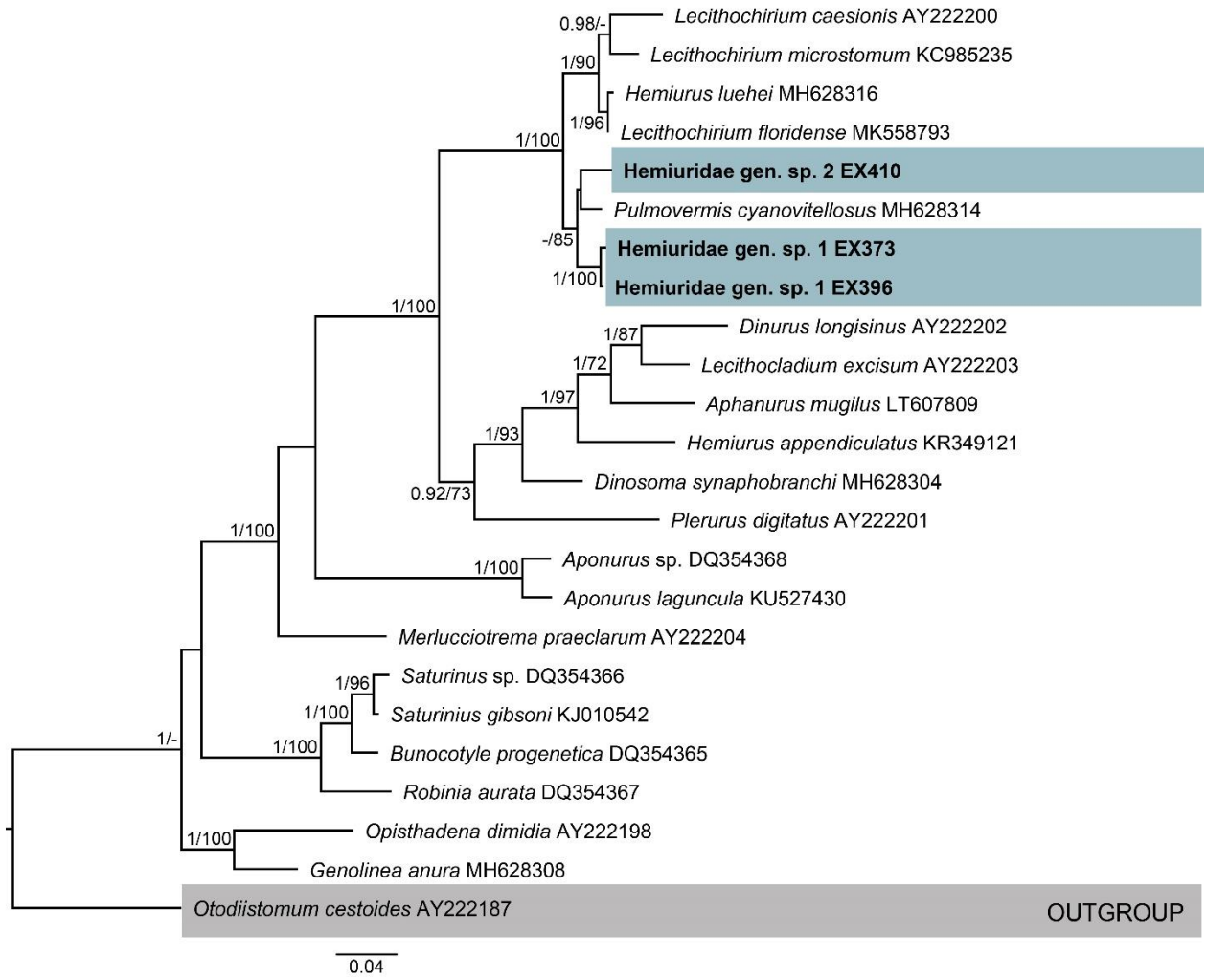


Figure 10. Bayesian inference (BI) phylogenetic tree for representatives of the Hemiuridae based on the partial 28S rDNA sequences. Newly generated sequences are highlighted in bold and can be seen in a blue rectangle. Nodal support given as BI/ML (maximum likelihood). The scale bar indicates the expected number of substitutions per site

Family Zoogonidae Odhner, 1902

In total, five species of Zoogonidae have been found in marine fishes in South Africa: ***Cephaloporus bakeri*** Bray, 1985 ex *Pervagor melanocephalus* (Bleeker, 1853) from Sodwana (Bray 1985); ***Lecithostaphylus retroflexus*** (Molin, 1859) Odhner, 1911 ex *Pachymetopon blochii* (Valenciennes, 1830) from Cape Town (Fantham 1938; Bray 1987b); ***Overstreetia sodwanaensis*** Bray, 1985 ex *Atherinomorus pinguis* (Lacepède, 1803) from Sodwana (Bray 1985) and the Natal region (Bray 1987b); ***Steganoderma* sp.** ex *Setarches guentheri* Johnson, 1862 from Durban (Parukhin 1989); and ***Steganodermatoides allocytti*** (Tkachuk, 1979) Bray, 1985 ex *Neocyttus rhomboidalis* Gilchrist, 1906, *Allocyttus verrucosus* (Gilchrist, 1906) and *Oreosoma atlanticum* Cuvier, 1829 from Cape Town (Bray 1985, 1987b) and the Agulhas Bank (Bray 1987b). A single species of the Zoogonidae has been found from *C. superciliosus* in Chintsa, during the present study (Fig. 11).

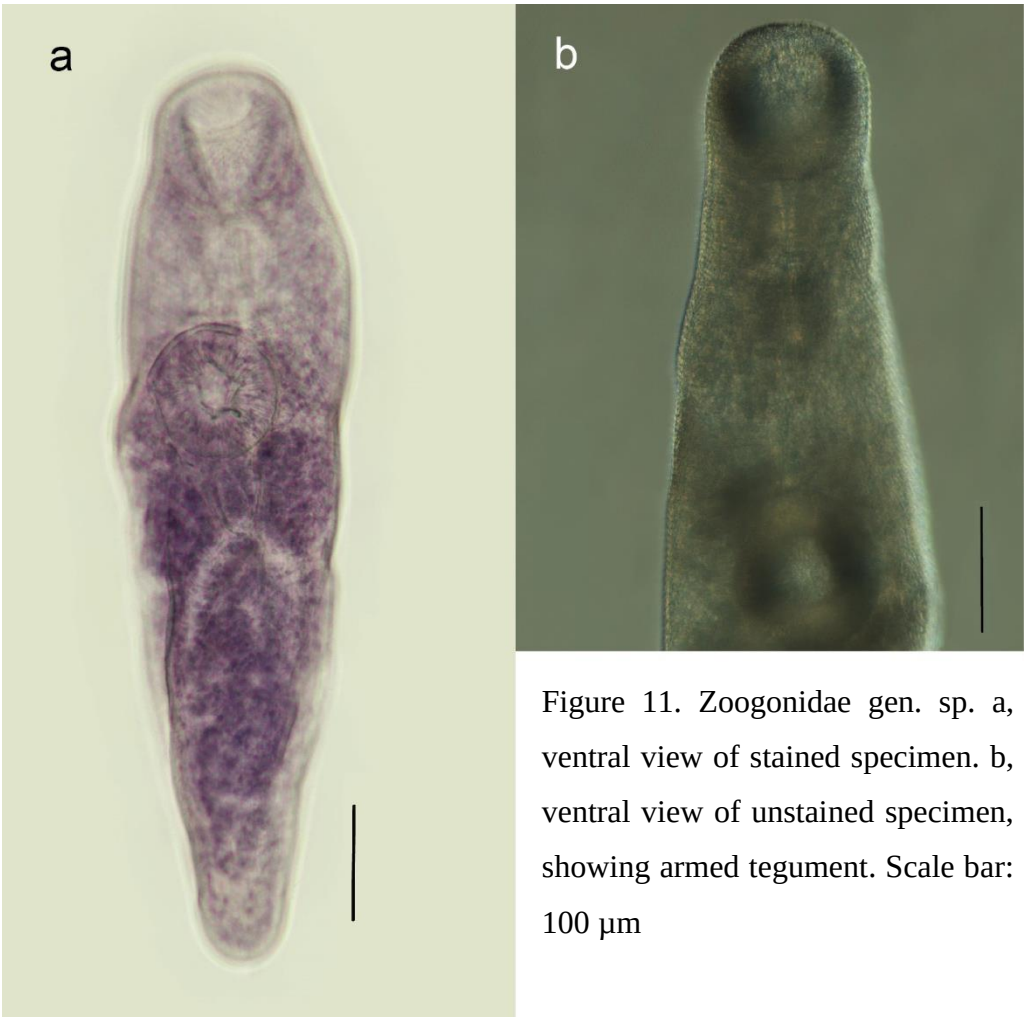


Figure 11. Zoogonidae gen. sp. a, ventral view of stained specimen. b, ventral view of unstained specimen, showing armed tegument. Scale bar: 100 µm

Zoogonidae gen. sp.

Host: Super klipfish *Clinus superciliosus* (L. 1758) (Clinidae).
Locality: Chintsa, South Africa (32°50'11.54"S; 28°7'1.19"E).
Site of infection: intestine.
Representative DNA sequences: one sequence for 28S and two sequences for ITS2.
Voucher material: two slides and eight ethanol-fixed specimens kept in the collection of the WRG at NWU in Potchefstroom.

A dataset containing novel sequences for the 28S gene and available sequences of the representatives of the family Zoogonidae (alignment consisted of 7 sequences and is 1236 nt long) was used to conduct phylogenetic analyses using BI and ML methods. Both analyses produced trees with topology consistent with those of previous studies (Sokolov et al. 2016) (Fig. 12). The novel sequence grouped together with a sequence of *Zoogonoides viviparus* (Olsson, 1868) Odhner, 1902 from the fish host *Callionymus lyra* L. 1758 found near the United Kingdom (Olson et al. 2003). Genetic divergence between Zoogonidae gen. sp. and *Z. viviparus* is 14.2% (172 nt), whereas the genetic divergence between Zoogonidae gen. sp. and *Diptherostomum* sp. was 17.2% (206 nt). Overall, the interspecific divergence noted between representatives of this family was 14.2–24% (172–296 nt).

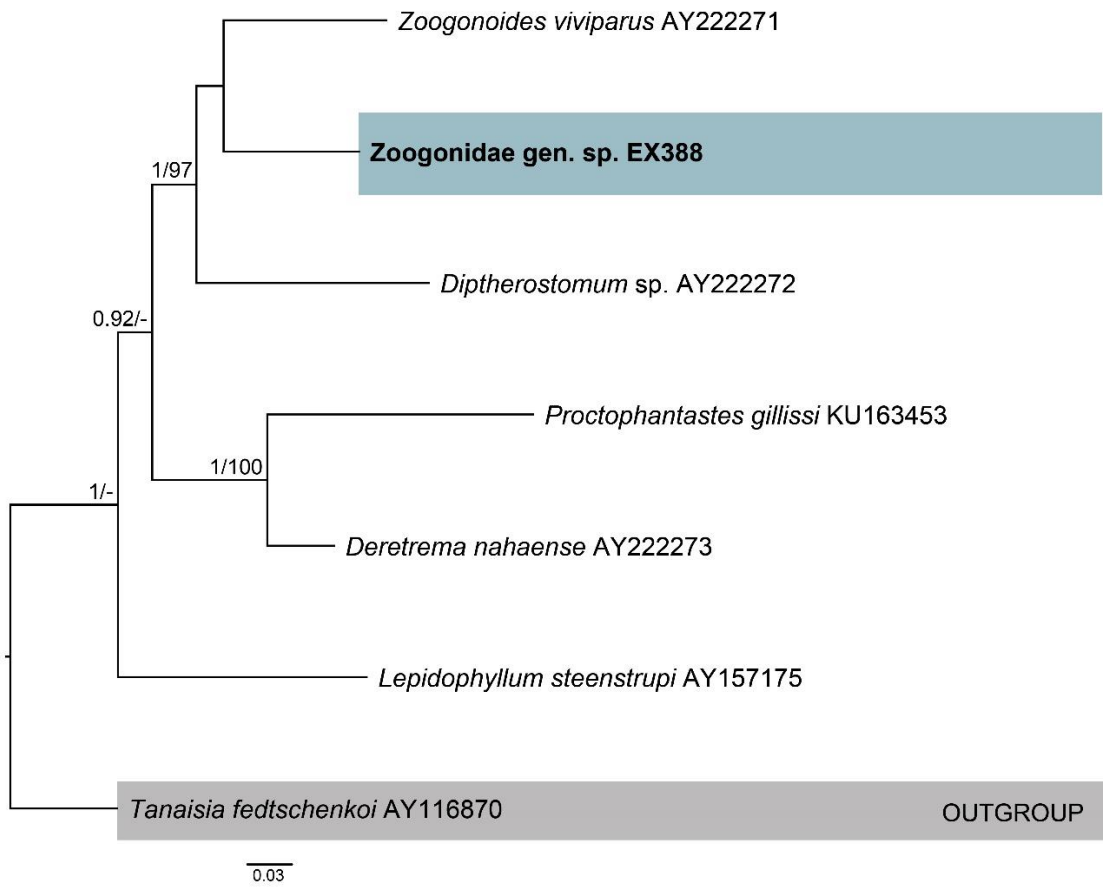


Figure 12. Bayesian inference (BI) phylogenetic tree for representatives of the Zoogonidae based on the partial 28S rDNA sequences. Newly generated sequences are highlighted in bold and can be seen in a blue rectangle. Nodal support given as BI/ML (maximum likelihood). The scale bar indicates the expected number of substitutions per site

Discussion

Prior to this study, merely two trematode species have been reported from *C. superciliosus* (*Co. capense* and *H. fasciata* (Bray 1987a)), both utilising this fish as definitive host. The present study reports *C. superciliosus* as a host to 14 digenean species in South Africa: second intermediate host for four digenean species and definitive host for ten species. This confirms that the suggestion of Smit and Hadfield (2015), stating that the parasites of marine fishes from South Africa are vastly understudied, is accurate.

The unidentified species of *Stephanostomum* found during this study is not genetically identical to any of the representatives for which genetic information is available. However, considering the relative close proximity of our the sampling locality to Port Elizabeth and Cape Recife, where Bray (1985) found an unidentified species of *Stephanostomum* from the fish host *Chaetodon marleyi* Regan, 1921; and *Stephanostomum solontschenkae* Parukhin, 1968 from the

fish host *Merluccius capensis* Castelnau, 1861 respectively, our specimens might represent one of these species.

According to the 28S phylogenetic analyses of the Bucephalidae, it is evident that the unidentified bucephalid species found during the present study is a member of the genus *Dollfustrema*. This is the first report of metacercariae of the Bucephalidae from a marine fish in South Africa. However, as with all digenean metacercariae, the sequences of adult specimens will be needed to identify these specimens to a lower taxonomic level. The extremely high intensity of infection with this species can be attributed to the geography of the sampling locality, which was a rocky area in a marina – as there is very little turbulence or currents in this type of area, it is theoretically easier for the cercariae to find and penetrate the fish host. *Clinus superciliosus* were found in high numbers at this site and are often very docile fishes, thereby making the infection with parasites even more likely. Further investigation into the presence and abundance of first intermediate (molluscs) and definitive (seabirds or larger fishes) hosts, may provide interesting data on the composition of parasitic communities in man-made marine habitats.

The 28S rDNA analysis has revealed that the sequences for the unidentified species of *Proctoeces* are identical and might in fact represent the species *P. maculatus*. Antar and Gargouri (2015) noted that the intraspecific variation for the 28S gene of *P. maculatus* is 0–0.42% (0–5 nt), thus the 0.1% (1 nt) difference between novel sequences of *Proctoeces* sp. and those of P. *maculatus* is likely intraspecific. Further comparative morphological and genetic analyses with faster mutating genes is necessary to confirm this. As Bray (1984) reported *P. maculatus* from the common octopus *O. vulgaris* in Durban, it is very likely that the specimens found during the present study may represent this species, especially when considering that these two host species share a habitat and are thereby both exposed to cercariae of the same species.

Members of the family Hemiuridae are commonly found in the stomach of their marine fish hosts (Pankov et al. 2006), which makes the findings of this study even more interesting and serves as a valuable report of hemiurids infecting different organs of a single fish species. According to the morphological analyses, two distinct, unidentified species of the Hemiuridae infect *C. superciliosus*. As mentioned, adult Hemiuridae gen. sp. 1 infect the intestine and ovaries of *C. superciliosus*, whereas pregravid adults infect the male gonads. As these two unidentified hemiurid species did not match molecularly with other species, they likely belong to species which have not yet been molecularly characterised or may even be new to science. From the phylogenetic analyses based on the 28S rRNA gene, it seems that both species likely belong

to the genus *Pulmovermis*. Further morphological characterisation and comparison to previously described species will be needed to identify these specimens to a lower taxonomic level.

Not many representative sequences are available for members of the Zoogonidae, thus the phylogenetic analyses did not aid substantially in the identification of this unidentified zoogonid species from *C. superciliosus*. Although it did cluster with *Z. viviparus* with low support, the genetic divergence between these isolates can be regarded as interspecific 14.2% (172 nt). However, this unidentified species may be from the same genus, but will require further morphological characterisation and comparison to previously described species to identify it to a lower taxonomic level.

Due to the overall lack of molecular data for species that were described long ago and more recently, we would strongly encourage the use of genetic analyses alongside morphological analyses when identifying trematodes, to ensure easier, faster and more accurate identification in future studies. This study also highlights the importance of collecting samples from various localities along the hosts' distributional ranges, when examining the parasitic community composition. It is evident that digeneans from marine fishes in South Africa are understudied and require further research focused on species discoveries, elucidation of their life cycles and identification of host ranges.

The present study thus provides the first molecular characterisation of digeneans from the families Acanthocolpidae, Bucephalidae, Fellodistomidae, Hemiuridae and Zoogonidae found in marine fishes in South Africa. Also, this study reports on 13 digenean species more than previously known from the fish host *C. superciliosus*.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Code availability

Not applicable

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Chapter 6

Summative Discussion

Chapter 6. Summative Discussion

Prior to this study, 87 digenean species from 63 genera of 24 families have either been described or reported from marine fishes along the coast of South Africa (see Table A-A1). As mentioned, the amount of marine parasite descriptions from South Africa is estimated to be much lower than reality, thus the aim of the current study was to further explore the diversity of digeneans from the fish host *Clinus superciliosus* in South Africa. This was achieved by collecting specimens of *C. superciliosus* from six dispersed localities along the South African coast: Saldanha Bay, Cape Town Harbour, Hermanus, Tsitsikamma National Park, Jeffreys Bay and Chintsa. Each fish was then examined for the presence of digenean trematodes, which were then removed and fixed appropriately. Through a combination of genetic and morphological analyses, a combination which has proven to be essential for the identification of some species, the trematodes were identified to the lowest possible taxonomic position. Although, some species still require further in-depth morphological analyses to be either identified as a known species that has yet to be molecularly characterised, or as a species that is new to science.

A previous study has found two digenean species that parasitise this fish host, *Coitocaecum capense* and *Helicometra fasciata*, from a single locality (Port Elizabeth). However, the inclusion of five additional sampling localities along this host's distributional range has led to an unexpected discovery by the present study – a further 13 digenean species and *Co. capense*. To our surprise, no digeneans were found from *C. superciliosus* in Cape Town harbour, as well as no specimens of *H. fasciata* from any of the sampling localities. Of the newfound species, two have been identified to species level (*Co. capense* and *Cardiocephaloides physalis*), whereas the others have been identified only to genus or family level (*Cardiocephaloides* sp., *Coitocaecum* sp. 1, *Coitocaecum* sp. 2, *Coitocaecum* sp. 3, *Dollfustrema* sp., *Helicometra* sp. 1, *Helicometra* sp. 2, Hemiuridae gen. sp. 1, Hemiuridae gen. sp. 2, *Proctoeces* sp., *Stephanostomum* sp., and Zoogonidae gen. sp.).

This study provides the largest contribution of molecular information on marine digeneans from South Africa, whilst also being the first to molecularly characterise adult digeneans from this area as well as trematodes from *C. superciliosus*. We also provide the first report of *C. superciliosus* as second intermediate host for digeneans. The fieldwork conducted for this study has also revealed that the use of baited traps (we utilised traps designed to collect aquatic amphibians) is an excellent means to collect these curious fish, as it is not only a speedy and effective method, but the release of by-catch or excess fish is easy and injury-free for the animals.

It is clear that the hidden biodiversity of marine areas in South Africa is understudied and still requires further research to determine the true biodiversity of this area. As the South African coast is one of the richest in biodiversity in the world, due to the unique coastal conditions created by the meeting of two varying currents, we can expect that there are many more microorganisms such as parasites, that await discovery.

Future Research

This study has given some insight into the estimated amount of undescribed or unreported species from marine fishes in South Africa. Thus, as mentioned, there is much room for future explorative taxonomic research to be done from this area. Firstly, further morphological analyses will be needed to identify the newfound species, to the lowest possible taxonomic position. This research can also be improved by incorporating more sampling sites along the host's distributional range or by conducting seasonal sampling to explore the changes in parasite community composition and abundance as environmental conditions fluctuate. As *C. superciliosus* has proven to be easily accessible and to host various digenean species of different life stages, it will be a good candidate to study host-parasite interactions such as changes in behaviour, histological effects on the host and so forth. Comparison between the parasite communities of these fish and others with whom they share the intertidal areas, might also yield interesting results and give more insight into the transmission mechanisms and host selection of these parasites. Thus, these areas will also be ideal for studies to elucidate the life cycles of digeneans.

Conclusion

The identification of trematodes and provision of molecular data from the present study will hopefully facilitate the easy and accurate identification of digeneans in future studies. Furthermore, the need for future explorative taxonomic and biodiversity studies has once again been highlighted by the astonishing diversity of trematodes found from a single fish species along the biodiversity-rich coast of South Africa. It is important to know the true diversity of an area, so as to better incorporate all species into future conservation efforts and planning. It may well be that some of the species awaiting discovery might have biological, ecological or economic value in these systems as well, so it is advantageous to know what organisms we are dealing with. When considering the loss of global biodiversity that we are experiencing, research projects such as this become increasingly essential in order to study the overall diversity of species present in this world, before they vanish. It would truly be devastating if a species went extinct, before ever being discovered.

APPENDIX

APPENDIX A – TABLES

Table A-A1. Summary on digenean trematodes reported and described from marine fishes from South Africa

Taxon name	Stage	Host name	Host family	Locality	Source
Acanthocolpidae Lühe, 1906					
Pleorchis sciaenae Yamaguti, 1938	A	*Argyrosomus hololepidotus*	Sciaenidae	Port Elizabeth, EC	Bray, 1986a
Stephanostomum ditrematis (Yamaguti, 1939)	A	*Megalaspis cordyla*	Carangidae	Durban, N	Bray, 1985
Stephanostomum imparaspine (Linton, 1905)	A	*Engraulis japonicus*	Engraulidae	South Atlantic	Parukhin, 1976
		Sardinella pilchardus	Clupeidae	South Atlantic	Parukhin, 1976
		Sardinops sagax	Clupeidae	South Atlantic	Parukhin, 1976
Stephanostomum solontschenkae Parukhin, 1968	A	*Merluccius capensis*	Merlucciidae	Cape Recife, EC	Bray, 1985
Stephanostomum spp.	M	*Merluccius capensis*	Merlucciidae	Cape Town, WC	Bray, 1985
		Chaetodon marleyi	Chaetodontidae	Port Elizabeth, EC	Bray, 1985
Aephnidiogenidae Yamaguti, 1934					
Holorchis pycnoporus Stossich, 1901	A	*Sparodon durbanensis*	Sapridae	Port Elizabeth, EC	Bray, 1985
		Diplodus sargus	Sparidae	Port Elizabeth, EC	Bray, 1985
Pseudaephnidiogenes rhabdosargi (Prudhoe, 1956)	A	*Rhabdosargus sarba*	Sparidae	Durban, N	Bray, 1985
		Rhabdosarugs sarba	Sparidae	Durban, N	Prudhoe, 1956
		Rhabdosargus holubi	Sparidae	Algoa Bay, EC	Bray, 1985
Pseudaephnidiogenes rossi Bray, 1985	A	*Caffrogobius nudiceps*	Gobiidae	Algoa Bay, EC	Bray, 1985
Aspidogastridae Poche, 1907					
Cotylogaster basiri Siddiqi & Cable, 1960	A	*Rhabdosargus sarba*	Sparidae	Durban, N	Bray, 1984
Azygiidae Lühe, 1909					
Otodistomum sp.	A	*Coelorhynchus fasciatus*	Macrouridae	South Atlantic	Parukhin, 1989
		Macrourus berglax	Macrouridae	South Atlantic	Parukhin, 1989
Bucephalidae Poche, 1907					
Bucephalus margaritae Ozaki & Ishibashi, 1934	A	*Carangoides hedlandensis*	Carangidae	Durban, N	Bray, 1984
		Caranx heberi	Carangidae	Sodwana, N	Bray, 1984
		Pomatomus saltatrix	Pomatiomidae	South Atlantic	Parukhin, 1976
Prosorhynchoides arcuatus (Linton, 1900)	A	*Pomatomus saltatrix*	Pomatomidae	Sodwana, N	Bray, 1984
Prosorhynchus caudovatus Manter 1940	A	*Epinephelus andersoni*	Serranidae	Umvoti, N	Bray, 1984
Rhipidocotyle paruchini Gavrilyuk-Tkachuk, 1979	A	*Otolithes ruber*	Sciaenidae	Agulas Bank	Gavrilyuk-Tkahuk, 1979
Rhipidocotyle sp.	A	*Ruvettus pretiosus*	Gempylidae	West coast, SA	Nunkoo *et al*., 2017
Cryptogonimidae Ward, 1917					
Aphallus rubalo (Bray, 1986) Bartoli & Bray, 1987	A	*Cheimerius nufar*	Sparidae	St. Lucia, N Umvoti, N Port Elizabeth, EC Durban, N	Bray, 1986b

Table A–A1. Continued

Taxon name	Stage	Host name	Host family	Locality	Source
Didymozoidae Monticelli, 1888					
Skrjabinozoum vodjanitskii Nikolaeva & Parukhin, 1974	A	*Ariomma indicum*	Ariommatidae	Durban, N	Parukhin, 1976
Derogenidae Nicoll, 1910					
Derogenes varicus (Müller, 1784)	A	*Cheilodonichthys capensis*	Triglidae	South Atlantic	Parukhin, 1989
		Coelorhynchus fasciatus	Macrouridae	South Atlantic	Parukhin, 1989
		Chlorophthalmus agassizi	Chlorophthalmidae	Durban, N	Parukhin, 1976
Enenteridae Yamaguti, 1958					
Enenterum elsti Bray, 1978	A	*Neoscorpis lithophilus*	Kyphosidae	Mapelane, N	Bray, 1978
				Tongaat, N	Bray, 1986a
				Uvongo, N	Bray, 1986a
				Mapelane, N	Bray, 1978
Enenterum kyphosi Yamaguti, 1970	A	*Kyphosus vaigiensis*	Kyphosidae	Sodwana, N	Bray, 1986a
Enenterum mannarense Hafeezullah, 1980	A	*Kyphosus vaigiensis*	Kyphosidae	Sodwana, N	Bray, 1986a
Enenterum prudhoei Bray, 1978	A	*Neoscorpis lithophilus*	Kyphosidae	Mapelane, N	Bray, 1978
				Tongaat, N	Bray, 1986a
Enenterum stinkvis Bray, 1986	A	*Neoscorpis lithophilus*	Kyphosidae	Uvongo, N	Bray, 1986a
				Tongaat, N	
Enenterum tongaatense Bray, 1986	A	*Neoscorpis lithophilus*	Kyphosidae	Tongaat, N	Bray, 1986a
Pseudozakia hatampo Machida & Araki, 1977	A	*Pempheris oualensis*	Pempheridae	Mbibi, N	Bray, 1986a
Faustulidae Poche, 1926					
Paradiscogaster farooqii Hafeezullah & Siddiqi, 1970	A	*Monocactylus argenteus*	Monodactylidae	Sodwana, N	Bray, 1984
Fellodistomidae Nicoll, 1909					
Fellodistomum sp.	A	*Lophius piscatorius*	Lophiidae	South Atlantic	Parukhin, 1989
Steringotrema pagelli (Van Beneden, 1871)	A	*Spondyliosoma emarginatum*	Sparidae	Algoa Bay, EC	Bray, 1984
Tergestia laticollis (Rudolphi, 1819)	A	*Decapterus punctatus*	Carangidae	Durban, N	Parukhin, 1976
		Trachurus trachurus	Carangidae	Durban, N	Parukhin, 1976
		Trachurus capensis	Carangidae	Port Elizabeth, EC	Le Roux, 2013
Tergestia pauca Texeira de Freitas & Kohn, 1965	A	*Pomatomus saltatrix*	Pomatomidae	Sodwana, N	Bray, 1984
Theledera pectinata (Linton, 1905)	A	*Pomatomus saltatrix*	Pomatomidae	South Atlantic	Parukhin, 1976
Gonocercidae Skrjabin & Guschanskaja, 1955					
Gonocerca crassa Manter, 1934	A	Not specified	–	Cape Town, WC	Parukhin, 1989
Gonocerca phycidis Manter, 1925	A	*Coelorhynchus fasciatus*	Macrouridae	Cape Town, WC	Parukhin, 1989

Table A–A1. Continued

Taxon name	Stage	Host name	Host family	Locality	Source
Gorgoderidae Looss, 1899					
Phyllodistomum tongaatense Bray, 1985	A	*Dichistius multifasciatus*	Dichistiidae	Tongaat, N	Bray, 1985
Probolitrema richiardii (López, 1888)	A	*Haploblepharus pictus*	Scyliorhinidae	West coast, WC	Yeld, 2009
		Scyllium sp.	Scyliorhinidae	Cape Town, WC	Looss, 1902
Haplosplanchnidae Poche, 1926					
Haplosplanchnus caudatus (Srivastava, 1937)	A	*Mugil cephalus*	Mugilidae	Sodwana, N	Bray, 1984
Haplosplanchnus purii Srivastava, 1939	A	*Chelon tricuspidens*	Mugilidae	Algoa Bay, EC	Bray, 1984
Hemiuridae Looss, 1899					
Dinurus longisinus Looss, 1907	A	*Coryphaena hippurus*	Coryphaenidae	EC	Bray, 1990
Dissosaccus laevis (Linton, 1898)	A	*Helicolenus dactylopterus*	Sebastidae	Durban, N	Parukhin, 1989
Ectenurus Lepidus	A	*Trachurus capensis*	Carangidae	Port Elizabeth, EC	Le Roux, 2013
Ectenurus selari (Parukhin, 1966)	A	*Trachurus trachurus*	Carangidae	Durban, N	Parukhin, 1976
Ectenurus virgula Looss, 1910	A	Not specified	–	Durban, N	Parukhin, 1976
		Lophius piscatorius	Lophiidae	South Atlantic	Parukhin, 1989
Elytrophalloides humerus Bray, 1990	A	*Trachinotus botla*	Carangidae	Sodwana, N	Bray, 1990
Lecithochirium floridense	A	Hoplobrotula gnathopus	Ophidiidae	Cape Town, WC	Parukhin 1989
Lecithochirium genypteri Manter, 1954	A	*Genypterus capensis*	Ophidiidae	Algoa Bay, EC	Bray, 1991
				Cape Town, WC	Bray, 1991
				Van Stadens River Mouth, EC	Bray, 1991
Lecithochirium jaffense Fischthal, 1982	A	*Blennioclinus brachycephalus*	Clinidae	Port Elizabeth, EC	Bray, 1991
Lecithochirium kawakawa Yamaguti, 1970	A	*Euthynnus affinis*	Scombridae	Durban, N	Bray, 1991
				Sodwana, N	Bray, 1991
		Chrysoblephus anglicus	Sparidae	St. Lucia, N	Bray, 1991
Lecithochirium parafusiforme Bray, 1991	A	*Gymnothorax flavimarginatus*	Muraenidae	La Mercy, N	Bray, 1991
Lecithochirium sp.	A	*Saurida undosquamis*	Synodontidae	Richard's Bay, N	Bray, 1991
Lecithochirium sp.	A	*Alectis ciliaris*	Carangidae	Durban, N	Bray, 1991
Lecithocladium angustiovum Yamaguti, 1953	A	*Rastrelliger kanagurta*	Scombridae	Durban, N	Bray, 1990
Lecithocladium excisum (Rudolphi, 1819)	A	*Scomber japonicus*	Scombridae	Durban, N	Parukhin, 1976
Lecithocladium magnacetabulum Yamaguti, 1934	A	*Ariomma indicum*	Ariommatidae	Durban, N	Parukhin, 1976
		Cubiceps whiteleggii	Nomeidae	Durban, N	Parukhin, 1976
		Parupenaeus cyclostomus	Mullidae	South Atlantic	Parukhin, 1976
		Pseudocaranx dentex	Carangidae	Durban, N	Parukhin, 1976
Parahemiurus merus (Linton, 1910)	A	*Chlorophthalmus agassizi*	Chlorophthalmidae	Durban, N	Parukhin, 1976

Table A–A1. Continued

Taxon name	Stage	Host name	Host family	Locality	Source
Plerurus digitatus (Looss, 1899)	A	*Alectis ciliaris*	Carangidae	Durban, N	Bray, 1990
		Euthynnus affinis	Scombridae	Durban, N	Bray, 1990
				Sodwana, N	Bray, 1990
		Lichia amia	Carangidae	Kerridene, N	Bray, 1990
				Port Elizabeth, EC	Bray, 1990
		Pomatomus saltatrix	Pomatomidae	EC	Bray, 1990
				Sodwana, N	Bray, 1990
		Rachycentron canadum	Rachycentridae	Unhlanga Rocks, N	Bray, 1990
		Saurida undosquamis	Synodontidae	Richard's Bay, N	Bray, 1990
		Scomberoides commersonnianus	Carangidae	Unhlanga Rocks, N	Bray, 1990
		Scomberoides commersonnianus	Scombridae	Cape Vidal, N	Bray, 1990
				Durban, N	Bray, 1990
		Thunnus albacares	Scombridae	Umhlanga Rocks, N	Bray, 1990
Lecithasteridae Odhner, 1905					
Aponurus laguncula Looss, 1907	A	*Parupenaeus cyclostomus*	Mullidae	South Atlantic	Parukhin, 1976
Lepidapedidae Yamaguti, 1958					
Lepidapedon alvigae Tkachuk, 2002	A	*Coelorinchus fasciatus*	Macrouridae	Agulas Bank	Gavrilyuk-Tkachuk, 1979
Lepidapedoides nicolli (Manter, 1934)	A	*Epinephelus albomarginatus*	Serranidae	Richard's Bay, N	Bray, 1985
Lepocreadiidae Odhner, 1905					
Clavogalea gaevskayae Bray, 1985	A	*Trachinotus botla*	Carangidae	Sodwana, N	Bray, 1985
Prodistomum orientale (Layman, 1930)	A	*Macrourus berglax*	Macrouridae	South Atlantic	Parukhin, 1989
		Scomber japonicus	Scombridae	Durban, N	Parukhin, 1976
		Macrourus berglax	Macrouridae	South Atlantic	Parukhin, 1989
Mesometridae Poche, 1926					
Elstia stossichianum (Monticelli, 1892)	A	*Sarpa salpa*	Sparidae	Durban, N	Bray, 1984
Multicalycidae Gibson & Chinabut, 1984					
Multicalyx cristata Faust & Tang, 1936	A	*Sphyrna lewini*	Sphyrnidae	Uvongo, N	Bray, 1984
		Carcharias taurus	Odontaspididae	EC	Bray, 1984
Opecoelidae Ozaki, 1925					
Allopodocotyle recifensis Bray, 1987	A	*Pterogymnus laniarius*	Sparidae	Cape Recife, EC	Bray, 1987a
Coitocaecum capense Bray, 1987	A	*Clinus superciliosus*	Clinidae	Port Elizabeth, EC	Bray, 1987a
				Oudekraal, WC	Bray, 1987a

Table A–A1. Continued

Taxon name	Stage	Host name	Host family	Locality	Source
		Cirrhibarbis capensis	Clinidae	Port Elizabeth, EC	Bray, 1987a
		Clinus cottoides	Clinidae	Oudekraal, WC	Bray, 1987a
		Clinus rotundifons	Clinidae	Port Elizabeth, EC	Bray, 1987a
		Xenopoclinus kochi	Clinidae	Oudekraal, WC	Bray, 1987a
		Xenopoclinus leprosus	Clinidae	Oudekraal, WC	Bray, 1987a
Dactylostomum griffithsi Bray, 1987	A	*Cheilodactylus fasciatus*	Cheilodactylidae	Oudekraal, WC	Bray, 1987a
Helicometra fasciata (Rudolphi, 1819)	A	*Galeichthys feliceps*	Ariidae	Port Elizabeth, EC	Bray, 1987a
		Chirodactylus brachydactylus	Cheilodactylidae	Port Elizabeth, EC	Bray, 1987a
		Cirrhibarbis capensis	Clinidae	Port Elizabeth, EC	Bray, 1987a
		Clinus cottoides	Clinidae	Oudekraal, WC	Bray, 1987a
		Clinus superciliosus	Clinidae	Port Elizabeth, EC	Bray, 1987a
		Clinus rotundifons	Clinidae	Oudekraal, WC	Bray, 1987a
		Pachymetopon blochii	Sparidae	Oudekraal, WC	Bray, 1987a
		Pomadasys olivaceus	Haemulidae	Durban, N	Bray, 1987a
		Pomatomus saltatrix	Pomatomidae	Durban, N	Bray, 1987a
		Xenopoclinus kochi	Clinidae	Oudekraal, WC	Bray, 1987a
		Xenopoclinus leprosus	Clinidae	Oudekraal, WC	Bray, 1987a
		Chelidonichthys capensis	Triglidae	South Atlantic	Parukhin, 1989
Helicometrina nimia Linton, 1910	A	*Pomadasys furctatus*	Haemulidae	Mbibi, N	Bray, 1987a
Macvicaria obovata (Molin, 1859)	A	*Sparodon durbanensis*	Sparidae	Port Elizabeth, EC	Bray, 1987a
		Diplodus cervinus	Sparidae	Port Elizabeth, EC	Bray, 1987a
		Cheilodactylus fasciatus	Cheilodactylidae	Port Elizabeth, EC	Bray, 1987a
Margolisia vidalensis Bray, 1987	A	*Synodus myops*	Synodontidae	Cape Vidal, N	Bray, 1987a
Neonotoporus decapteri Parukhin, 1966	A	Not specified	–	Durban, N	Parukhin, 1976
Opecoelus gonorhynchi Gavrilyuk-Tkachuk, 1979	M	*Gonorynchus gonorynchus*	Gonorynchidae	Agulas Bank	Gavrilyuk-Tkachuk, 1979
Opecoelina helicoleni Manter, 1934	A	*Helicolenus dactylopterus*	Sebastidae	Durban, N	Parukhin, 1989
Opecoelina scorpaenae Manter, 1934	A	*Setarches guentheri*	Setarchidae	Durban, N	Parukhin, 1989
Pseudoheterolebes cotylophorus Ozaki, 1935	A	*Amblyrhynchotes honckenii*	Tetraodontidae	Algoa Bay, EC	Bray, 1986a
Pseudopecoelus ablennesi Bray, 1987	A	*Ablennes hians*	Belonidae	Durban, St Lucia, N	Bray, 1987 Bray, 1987a
Pseudopecoelus japonicus (Yamaguti, 1938)	A	*Chlorophthalmus punctatus*	Chlorophthalmidae	Durban, N	Parukhin, 1976
Pseudopecoelus vulgaris (Manter, 1934)	A	*Setarches guentheri*	Setarchidae	Durban, N	Parukhin, 1989
Sclerodistomidae Odhner, 1927					
Kenmackenzia gigas (Nardo, 1827)	A	*Luvarus imperialis*	Luvaridae	Cape Town, WC	Gibson, 1983
Prosorchis palinurichthi Kurochkin, Parukhin & Korotaeva, 1971	A	*Hyperoglyphe pringlei*	Centrolophidae	South Atlantic	Parukhin, 1976

Table A–A1. Continued

Taxon name	Stage	Host name	Host family	Locality	Source
Strigeidae Railliet, 1919					
Cardiocephaloides physalis (Lutz, 1927)	M	*Engraulis capensis*	Engraulidae	South Atlantic	Parukhin, 1976
		Sardinops sagax	Clupeidae	WC, EC	Uzonnah, 2017 (unpublished)
"Tetracotyle" sp.	M	*Sardinops sagax*	Clupeidae	WC, EC, N	Reed *et a*l., 2012
Zoogonidae Odhner, 1902					
Cephaloporus bakeri Bray, 1985	A	*Pervagor melanocephalus*	Monocanthidae	Sodwana, N	Bray, 1985
Lecithostaphylus retroflexus (Molin, 1859) Odhner, 1911	A	*Pachymetopon blochii*	Sparidae	Cape Town, WC	Fantham, 1938
				Cape Town, WC	Bray, 1987b
Overstreetia sodwanaensis Bray, 1985	A	*Atherinomorus pinguis*	Atherinidae	Sodwana, N	Bray, 1985
				IO	Bray, 1987b
Steganoderma sp.	A	*Setarches guentheri*	Setarchidae	Durban, N	Parukhin, 1989
Steganodermatoides allocytti (Tkachuk, 1979)	A	*Neocyttus rhomboidalis*	Oreosomatidae	Cape Town, WC	Bray, 1985
				Cape Town, WC	Bray, 1987b
				Agulhas Bank	Bray, 1987b
		Allocyttus verrucosus	Oreosomatidae	Cape Town, WC	Bray, 1987b
				Agulhas Bank	Bray, 1987 b
		Oreosoma atlanticum	Oreosomatidae	Cape Town, WC	Bray, 1987b
				Agulhas Bank	Bray, 1987b

Table A-A2. Sequences used for phylogenetic analyses of the Opecoelidae

Species	Host	Locality	GenBank accession numbers		Reference
			28S	ITS2/ ITS1-5.8S-ITS2	
Coitocaecum capense Bray, 1987	*Clinus superciliosus* Linnaeus	TNP, SA	Ex151	Ex151	Present study
C. capense Bray, 1987	*Cl. superciliosus*	TNP, SA	Ex152	–/ Ex152	Present study
C. capense Bray, 1987	*Cl. superciliosus*	Hermanus, SA	Ex337	Ex337	Present study
C. capense Bray, 1987	*Cl. superciliosus*	Hermanus, SA	Ex338	Ex338	Present study
Coitocaecum sp. 1	*Cl. superciliosus*	Hermanus, SA	Ex339	Ex339	Present study
Coitocaecum sp. 2	*Cl. superciliosus*	Chintsa, SA	Ex389	Ex389	Present study
Coitocaecum sp. 3	*Cl. superciliosus*	Saldanha Bay, SA	–	Ex408	Present study
Coitocaecum sp. 3	*Cl. superciliosus*	Saldanha Bay, SA	–	Ex411	Present study
Coitocaecum sp. 3	*Cl. superciliosus*	Saldanha Bay, SA	–	Ex412	Present study
Coitocaecum sp. 3	*Cl. superciliosus*	Saldanha Bay, SA	–	Ex413	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	Ex155	Ex155	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	–/Ex156	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	–/Ex157	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	Ex215	–/Ex215	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex374	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex375	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex376	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	Ex377	–	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	Ex379	–	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex380	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex381	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex382	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex383	Present study
Helicometra sp. 1	*Cl. superciliosus*	TNP, SA	–	Ex385	Present study
Helicometra sp. 1	*Cl. superciliosus*	Chintsa, SA	Ex387	Ex387	Present study
Helicometra sp. 1	*Cl. superciliosus*	Chintsa, SA	Ex390	–	Present study
Helicometra sp. 1	*Cl. superciliosus*	Chintsa, SA	Ex392	–	Present study
Helicometra sp. 1	*Cl. superciliosus*	Hermanus, SA	Ex401	Ex401	Present study
Helicometra sp. 1	*Cl. superciliosus*	Saldanha Bay, SA	–	Ex405	Present study
Helicometra sp. 1	*Cl. superciliosus*	Saldanha Bay, SA	–	Ex406	Present study
Helicometra sp. 2	*Cl. superciliosus*	Hermanus, SA	Ex402	Ex402	Present study
Abyssopedunculus brevis (Andres et Overstreet, 2013) Martin, Huston, Cutmore et Cribb, 2018	*Conger esculentus* Poey	Caribbean Sea	KJ001212	–	Andres et al. (2014a)

Table A–A2. Continued

Species	Host	Locality	GenBank accession numbers		Reference
			28S	ITS2/ ITS1-5.8S-ITS2	
Allopodocotyle epinepheli (Yamaguti, 1942) Pritchard, 1966	*Epinephelus cyanopodus* (Richardson)	New Caledonia	KU320598	MN067859	Bray et al. (2016); Martin et al. (2019a)
Anomalotrema koiae Gibson et Bray, 1984	*Sebastes viviparus* Krøyer	Shetland Islands	KU320595	–	Bray et al. (2016)
Bathycreadium elongatum (Maillard, 1970) Bray, 1973	*Trachyrincus scabrous* (Rafinesque)	Spain	–	JN089758	Constenla et al. (2011)
Bathypodocotyle margolisi (Gibson, 1995) Martin, Huston, Cutmore et Cribb, 2018	*Coryphaenoides mediterraneus* (Giglioli)	Rockall Trough	KU320596	–	Bray et al. (2016)
Bentholebouria colubrosa Pulis et Overstreet, 2014	*Pristipomoides aquilonaris* (Goode et Bean)	Gulf of Mexico	KJ001207	–	Andres et al. (2014a)
Buticulotrema thermichthysi Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014	*Thermichthys hollisi* (Cohen, Rosenblatt et Moser)	South East Pacific Rise	KF733984	–	Bray et al. (2014)
Cainocreadium labracis (Dujardin, 1845) Nicoll, 1909	*Steromphala adansonii*[a] (Payraudeau)	Spain	JQ694144	JQ694148	Born-Torrijos et al. (2012)
Choerodonicola renko Machida, 2014	*Dentex abei* Akazaki et Taniguchi	Japan	MG844421	–	Martin et al. (2018a)
Dimerosaccus oncorhynchi (Eguchi, 1931) Shimazu, 1980	*Salvelinus curilus* (Pallas)	Russia	FR870262	–	Shedko et al. (2015)
Discoverytrema gibsoni Zdzitowiecki, 1990	*Muraenolepis marmorata* Günther	The Ross Sea	MH161430	–	Sokolov et al. (2019)
Fairfaxia cribbi Hassanine et Gibson, 2005	*Lethrinus atkinsoni* Seale	Australia	MN081755	–	Martin et al. (2020)
Gaevskajatrema perezi (Mathias, 1926) Gibson et Bray, 1982	?Fish; *Symphodus roissali* (Risso)	Corsica, France	AF184255	AJ241800	Tkach et al. (2001); Jousson et al. (1999)
Halosaurotrema halosauropsi (Bray et Campbell, 1996) Martin, Huston, Cutmore et Cribb, 2018	*Halosauropsis macrochir* (Günther)	Atlantic Ocean	AY222207	–	Olson et al. (2003)
Hamacreadium mutabile Linton, 1910	*Lutjanus griseus* (Linnaeus)	Gulf of Mexico	KJ001209	–	Andres et al. (2014a)
Helicometra boseli Nagaty, 1956	*Sargocentron spiniferum* (Forsskål)	New Caledonia	KU320600	–	Bray et al. (2016)
Helicometra epinepheli Yamaguti, 1934	*Epinephelus fasciatus* (Forsskål)	New Caledonia	KU320597	–	Bray et al. (2016)
Helicometra equilata (Manter, 1933) Siddiqi et Cable, 1960	*S. spiniferum* (Forsskål)	New Caledonia	KU320600	–	Bray et al. (2016)
Helicometra fasciata (Rudolphi, 1819) Odhner, 1902	*Symphodus rostratus* (Bloch)	France	–	AJ241793	Jousson et al. (1999)

Table A–A2. Continued

Species	Host	Locality	GenBank accession numbers		Reference
			28S	ITS2/ ITS1-5.8S-ITS2	
Helicometra manteri Andres, Ray, Pulis, Curran et Overstreet, 2014	*Prionotus alatus* Goode et Bean	Gulf of Mexico	KJ701238	KJ701238	Andres et al. (2014b)
Heterolebes maculosus Ozaki, 1935	*Diodon hystrix* (Linnaeus)	Australia	MH933878	–	Martin et al. (2018d)
Holsworthotrema chaoderma Martin, Huston, Cutmore et Cribb, 2018	*Kyphosus gladius* Knudsen et Clements	Australia	MK052938	–	Martin et al. (2019b)
Maculifer diodontis Martin, Ribu, Cutmore et Cribb, 2018	*Diodon hystrix* (Linnaeus)	Australia	MH933879	–	Martin et al. (2018d)
Macvicaria gibsoni Rima, Marzoug, Pérez-del-Olmo, Kostadinova, Bouderbala et Georgieva, 2017	*Diplodus vulgaris* (Saint-Hilaire)	Algeria	–	MF166830	Rima et al. (2017)
Magnaosimum brooksae Martin, Crouch, Cutmore et Cribb, 2018	*Tripodichthys angustifrons* (Hollard)	Australia	MG813907	MG813905	Martin et al. (2018b)
Mesobathylebouria lanceolata (Price, 1934) Martin, Huston, Cutmore et Cribb, 2018	*Polymixia lowei* Günther	Gulf of Mexico	KJ001210	–	Andres et al. (2014a)
Neolebouria georgiensis Gibson, 1976	*Trematomus pennellii* Regan	Southern Ocean	MH892478	–	Faltýnková et al. (2017)
Neoplagioporus ayu (Takahashi, 1929) Shimazu, 1990	*Plecoglossus altivelis altivelis* (Temminck et Schlegel)	Japan	KX553947	KX553947	Fayton and Andres (2016)
Nicolla elongate Maillard, 1970	*Phycis phycis* (Linnaeus)	France	–	AJ241792	Jousson et al. (1999)
Opecoelidae gen. sp.	*Hippolyte inermis* Leach	France	–	AJ241817	Jousson et al. (1999)
Opecoelidae gen. sp.	*Paracentrotus lividus* (Lamarck)	France	–	AJ241814	Jousson et al. (1999)
Opecoelidae gen. sp.[b]	*Sparus aurata* (Linnaeus)	France	–	AJ241805	Jousson et al. (1999)
Opecoeloides furcatus (Bremser in Rudolphi, 1819) Odhner, 1928	*Mullus surmuletus* (Linnaeus)	France	AF151937	AJ241790	Tkach et al. (2000); Jousson et al. (1999)
Opistholebes amplicoelus Nicoll, 1915	*Tetractenos hamiltoni* (Richardson)	Australia	MH933875		Martin et al. (2018b)
Pachycreadium carnosum (Rudolphi, 1819) Cortini et Ferretti, 1959	*Dentex dentex* (Linnaeus)	France	–	AJ241799	Jousson et al. (1999)
Pacificreadium serrani (Nagaty et Abdel Aal, 1962) Durio et Manter, 1968	*Plectropomus leopardus* (Lacepède)	New Caledonia	KU320602	–	Bray et al. (2016)
Parallelolebes virilis Martin, Ribu, Cutmore et Cribb, 2018	*Meuschenia hippocrepis* (Quoy et Gaimard)	Australia	MH933880	–	Martin et al. (2018d)

Table A–A2. Continued

Species	Host	Locality	GenBank accession numbers		Reference
			28S	ITS2 / ITS1-5.8S-ITS2	
Pedunculacetabulum inopinipugnus Martin, Curmore et Cribb, 2017	*Plectorhinchus chrysotaenia* (Bleeker)	Australia	MF805700	MF805698	Martin et al. (2018b)
Peracreadium characis (Stossich, 1886) Bartoli, Gibson et Bray, 1989	*Diplodus puntazzo* (Walbaum)	Spain	–	AJ241796	Sánchez-Garcia et al. (2015)
Peracreadium idoneum (Nicoll, 1909) Gibson et Bray, 1982	*Anarhichas lupus* Linnaeus	United Kingdom	AY222209	–	Olson et al. (2003)
Plagioporus aliffi Fayton, Choudhury, McAllister et Robinson, 2017	*Etheostoma blennioides newmanni* Miller	USA	KX905056	KX905056	Fayton et al. (2017)
Podocotyle atomon (Rudolphi, 1802) Odhner, 1905	*Littorina saxatilis* (Olivi)	Russia	MH161437	–	Sokolov et al. (2019)
Podocotyle scorpaenae (Rudolphi, 1919) Bartoli et Gibson, 1991	*Scorpaena scrofa* Linnaeus	France	–	AJ241794	Jousson et al. (1999)
Podocotyloides australis Martin, Cutmore et Cribb, 2017	*Diagramma pictum labiosum* Macleay	Australia	MF805696	MF805694	Martin et al. (2018c)
Polypipapiliotrema stenometra (Pritcherd, 1966) Martin, Cutmore et Cribb in Martin, Sasal, Cutmore, Ward, Aeby et Cribb, 2018	*Chaetodon quadrimaculatus* Gray	Hawai'i	MH823954	MH823948	Martin et al. (2018e)
Poracanthium furcatum Dollfus, 1948	*M. surmuletus* (Linnaeus)	France	–	AJ241791	Jousson et al. (1999)
Propycnadenoides philippinensis Fischthal et Kuntz, 1964	*Gymnocranius grandoculis* (Valenciennes)	New Caledonia	KU320604	–	Bray et al. (2016)
Pseudoheterolebes diodontis (Cable, 1956) Martin, Ribu, Cutmore et Cribb, 2018	*D. hystrix* (Linnaeus)	Australia	MH933876	–	Martin et al. (2018d)
Pseudopecoeloides engeleri Tohner et Cribb, 2013	*Parupeneus ciliatus* (Lacepède)	Australia	–	KC357702	Rohner and Cribb (2013)
Pseudopecoeloides tenuis Yamaguti, 1940	*Priacanthus hamrur* (Forsskål)	New Caledonia	KU320605	–	Bray et al. (2016)
Pseudopecoelus vulgaris (Manter, 1934) von Wicklen, 1946	*Sebastes* sp.	North Pacific	MH161436	–	Sokolov et al. (2019)
Pseudoplagioporinae sp.	*Lethrinus nebulosus* (Forsskål)	Australia	MN081757	–	Martin et al. (2020)
Pseudoplagioporus lethrini Yamaguti, 1938	*Lathrinus nebulosus* (Forsskål)	Australia	MN081747	–	Martin et al. (2020)
Pseudopycnadena fischthali Saad-Fares et Maillar, 1986	*D. vulgaris* (Saint-Hilaire)	Algeria	MF166851	MF166841	Rima et al. (2017)

Table A–A2. Continued

Species	Host	Locality	GenBank accession numbers		Reference
			28S	ITS2 / ITS1-5.8S-ITS2	
Pycnadenoides senegalensis Fischthal et Thomas, 1972	*S. aurata* (Linnaeus)	France	–	AJ241797	Jousson et al. (1999)
Scorpidotrema longistipes Aken'Ova et Cribb, 2003	*Scorpis georgiana* Valenciennes	Australia	MK052936	–	Martin et al. (2019b)
Shimazuia lethrini (Yamaguti, 1938) Cribb, 2005	*L. atkinsoni* Seale	Australia	MN081756	–	Martin et al. (2020)
Sphaerostoma bramae (Müller, 1776)	*Abramis brama* (Linnaeus)	Russia	MH161435	–	Sokolov et al. (2018)
Tellervotrema beringi (Mamaev, 1965) Gibson et Bray, 1982	*Antimora microlepis* Bean	Kuril Islands	MN959859	–	Sokolov et al. (2020)
Trilobovarium parvvatis Martin, Cutmore et Cribb, 2017	*L. nebulosus* (Forsskål)	Australia	KY551562	KY551560	Martin et al. (2017)
Urorchis goro Ozaki, 1927	*Rhinogobius* sp.	Japan	KX553946	KX553946	Fayton and Andres (2016)
Outgroup					
Forticulcita apiensis Andres, Curran, Fayton, Pulis et Overstreet, 2015	*Mugil cephalus* Linnaeus	USA	–	KP761087	Andres et al. (2015)
Gorgocephalus yaaji Bray et Cribb, 2005	*Echinolittorina austrotrochoides* Reid	Australia	–	KU951490	Huston et al. (2016)
Stephanostomum pristis (Deslongchamps, 1824) Looss, 1899	*P. phycis* (Linnaeus)	Corsica, France	DQ248222	–	Bray et al. (2005b)
Zalophotrema hepaticum Stunkard et Alvey, 1929	*Zalophus californianus* (Lesson)	USA	AY222255	–	Olson et al. (2003)

Abbreviations: TNP, Tsitsikamma National Park; SA, South Africa. [a]Name in paper: *Gibbula adansonii*; [b]Name in paper: *Allopodocotyle pedicellate*.

Table A-A3. Sequences used for phylogenetic analyses of the Acanthocolpidae, Bucephalidae, Fellodistomidae, Hemiuridae and Zoogonidae

Species	Host	Locality	GenBank ID (28S sequences)	Reference
Hemiuridae gen. sp. 1	*Clinus superciliosus*	TNP, SA	EX373	Present study
Hemiuridae gen. sp. 1	*Clinus superciliosus*	Chintsa, SA	EX396	Present study
Hemiuridae gen. sp. 2	*Clinus superciliosus*	Saldanha Bay, SA	EX410	Present study
Proctoeces sp.	*Clinus superciliosus*	TNP, SA	EX378; EX384	Present study
Proctoeces sp.	*Clinus superciliosus*	Chintsa, SA	EX386	Present study
Stephanostomum sp.	*Clinus superciliosus*	Chintsa, SA	EX370	Present study
Zoogonidae gen. sp.	*Clinus superciliosus*	Chintsa, SA	EX388	Present study
Acanthocolpidae sp.	*Nassarius dorsatus*	Australia	FJ809037	Barnett et al. (2010)
Aphanurus mugilus Tang, 1981	–	Vietnam	LT607809	Atopkin et al. (2016) (unpublished)
Aponurus sp.	*Mullus surmuletus*	–	DQ354368	Pankov et al. (2006)
Aponurus laguncula Looss, 1907	*Rhomboplites aurorubens*	Western Atlantic Ocean	KU527430	Claxton et al. (2017)
Bucephalus cynoscion Hopkins, 1956	*Bairdiella chrysoura*	USA	KT273397	Nolan et al. (2015)
Bucephalus gorgon (Linton, 1905) Eckmann, 1932	*Seriola dumerili*	Gulf of Mexico	KT273400	Nolan et al. (2015)
Bucephalus margaritae Ozaki et Ishibashi, 1934	*Caranx crysos*	Gulf of Mexico	KT273395	Nolan et al. (2015)
Bucephalus polymorphus von Baer, 1827	*Dreissena polymorpha*	Belarus	AY289238	Stunžėnas et al. (2004)
Bunocotyle progenetica (Markowski, 1936) Chabaud et Buttner, 1959	*Hybrobia ulvae*	White Sea	DQ354365	Pankov et al. (2006)
Cableia pudica Bray, Cribb et Barker, 1996	*Cantherines pardalis*	Australia	AY222251	Olson et al. (2003)
Cercaria Capricornia Barnett, Smales et Cribb, 2008	*Nassarius dorsatus*	Australia	MG642798	Barnett & Miller (2018)
Deretrema nahaense Yamaguti, 1942	*Thalassoma lunare*	Australia	AY222273	Olson et al. (2003)
Dinosoma synaphobranchi Yamaguti, 1938	*Antimora microlepis*	Sea of Okhotsk, Russia	MH628304	Sokolov et al. (2018)
Dinurus longisinus Looss, 1907	*Coryphaena hippurus*	Jamaica	AY222202	Olson et al. (2003)
Diptherostomum sp.	*Scolopsis monogramma*	Australia	AY222272	Olson et al. (2003)
Dollfustrema durum Nolan, Curran, Miller, Cutmore, Cantacessi et Cribb, 2015	*Gymnothorax javanicus*	Australia	KT213572	Nolan et al. (2015)
Dollfustrema hefeiensis Liu, 1999	*Rhinogobius giurinus*	China	KT273386	Nolan et al. (2015)
Genolinea anura (Layman, 1930) Manter, 1954	*Pleurogrammus monopterugius*	Simushir Island	MH628308	Sokolov et al. (2018)
Hemiurus appendiculatus (Rudolphi, 1802) Looss, 1899	*Hemiurus appendiculatus*	Spain	KR349121	Bao et al. (2015)
Hemiurus luehei Odhner, 1905	*Ophidion rochei*	Russia	MH628316	Sokolov et al. (2018)
Lecithochirium caesionis Yamaguti, 1942	*Caesio cuning*	Australia	AY222200	Olson et al. (2003)
Lecithochirium floridense (Manter, 1934) Crowcroft, 1946	*Syacium papillosum*	Gulf of Mexico	MK558793	Vidal-Martínez et al. (2019)

Table A–A3. Continued

Species	Host	Locality	GenBank ID (28S sequences)	Reference
Lecithochirium microstomum Chandler, 1935	*Trichiurus lepturus*	Gulf of Mexico	KC985235	Calhoun et al. (2013)
Lecithocladium excisum (Rudolphi, 1819) Lühe, 1901	*Scomber scombrus*	UK	AY222203	Olson et al. (2003)
Lepidophyllum steenstrupi Odhner, 1902	*Anarhichas lupus*	UK	AY157175	Lockyer et al. (2003)
Merlucciotrema praeclarum (Manter, 1934) Yamaguti, 1971	*Caraetyx laticeps*	North-east Atlantic	AY222204	Olson et al. (2003)
Monostephanostomum nolani Bray et Cribb, 2007	*Carangoides plagiotaenia*	Australia	EF506763	Bray et al. (2007)
Opisthadena dimidia Linton, 1910	*Kyphosus cinerascens*	Australia	AY222198	Olson et al. (2003)
Parabucephalopsis parasiluri Wang, 1985	*Limnoperna fortunei*	Japan	AB640884	Baba et al. (2012)
Pleorchis polyorchis (Stossich, 1889) Stiles, 1896	*Sciaena umbra*	Corsica	DQ248215	Bray et al. (2005)
Pleorchis uku Yamaguti, 1970	*Aprion virescens*	Australia	DQ248216	Bray et al. (2005)
Plerurus digitatus (Looss, 1899) Looss, 1907	*Scomberomorus commerson*	Australia	AY222201	Olson et al. (2003)
Proctoeces sicyases Oliva, Valdivia, Cárdenas, Muñoz, Escribano et George-Nascimento, 2018	*Sicyases sanguineus*	Chile	KT865207	Oliva et al. (2018)
Proctoeces choerodoni Wee, Cribb, Bray et Cutmore, 2017	*Choerodon cyanodus*	Australia	KX671299	Wee et al. (2017)
Proctoeces insolitus (Nicoll, 1915) Yamaguti, 1954	*Acanthopagrus australis*	Australia	KX671300	Wee et al. (2017)
Proctoeces maculatus (Looss, 1901) Odhner, 1911	*Archosargus probatocephalus*	Gulf of Mexico	AY222284	Olson et al. (2003)
Proctoeces maculatus (Looss, 1901) Odhner, 1911	*Sparus aurata*	Tunisia	KX671302	Wee et al. (2017)
Proctoeces major Yamaguti, 1934	*Monodactylus argenteus*	Australia	KX671309	Wee et al. (2017)
Proctophantastes gillissi (Overstreet et Pritchard, 1977) Bray et Gibson, 1986	*Muraenolepis marmorata*	Ross Sea	KU163453	Sokolov et al. (2016)
Prosorhynchoides caecorum (Hopkins, 1956) Bott et Cribb, 2005	*Monodactylus argenteus*	Australia	KT273393	Wee et al. (2017)
Prosorhynchoides cutmorei Hammond, Cribb et Bott, 2018	*Tylosurus gavialoides*	Australia	MG953232	Hammond et al. (2018)
Prosorhynchoides galaktionovi Hammond, Cribb, Nolan et Bott, 2020	*Tylosurus crocodilus*	Australia	MN310395	Hammond et al. (2020)
Prosorhynchoides gracilescens (Rudolphi, 1819) Stunkard, 1976	*Lophius piscatorius*	UK	AY222224	Olson et al. (2003)
Prosorhyncoides kohnae Hammond, Cribb, Nolan et Bott, 2020	*Tylosurus crocodilus*	Australia	MN310397	Hammond et al. (2020)
Prosorhynchoides longoviferus (Manter, 1940) Curran et Overstreet, 2009	*Sphyraena barracuda*	USA	KT273387	Nolan et al. (2015)
Prosorhynchoides megacirrus (Riggin et Sparks, 1962) Curran et Overstreet, 2009	*Sciaenops ocellatus*	USA	KT273391	Nolan et al. (2015)
Prosorhynchoides moretonensis Hammond, Cribb et Bott, 2018	*Tylosurus gavialoides*	Australia	MG953230	Hammond et al. (2018)
Prosorhynchoides ovatus (Linton, 1900) Dollfus, 1929	*Lobotes surinamensis*	USA	KT273399	Nolan et al. (2015)
Prosorhynchoides ozakii (Nagaty, 1937)	*Lomnoperna fortune*	Japan	AB640885	Baba et al. (2012)
Prosorhynchoides paralichthydis (Corkum, 1961) Curran et Overstreet, 2009	*Paralichthys lethostigma*	USA	KT273398	Nolan et al. (2015)

Table A–A3. Continued

Species	Host	Locality	GenBank ID (28S sequences)	Reference
Prosorhynchoides scomberomorus (Corkum, 1968) Curran et Overstreet, 2009	*Scomberomorus cavalla*	Gulf of Mexico	KT273389	Nolan et al. (2015)
Prosorhynchoides waeschenbacha Hammond, Cribb et Bott, 2018	*Tylosurus gavialoides*	Australia	MG953231	Hammond et al. (2018)
Pseudolepidapedon balistis Manter, 1940	*Balistis capriscus*	Gulf of Mexico	KJ820760	Curran & Pulis (2014)
Pulmovermis cyanovitellosus Coil & Kuntz, 1960	*Laticauda semifasciata*	Japan	MH628314	Sokolov et al. (2018)
*Rhipidocotyle angusticollis** Chandler, 1941	*Euthynnus alletteratus*	USA	KT273383	Nolan et al. (2015)
Rhipidocotyle campanula (Dujardin, 1845) Dollfus, 1968	*Anodonta anatine*	Lithuania	KF184355	Petkevičiūtė et al. (2014)
Rhipidocotyle galeata (Rudolphi, 1819) Eckmann, 1932	*Eurtigla gurnardus*	UK	AY222225	Olson et al. (2003)
Rhipidocotyle lepisostei Hopkins, 1954	*Lepisosteus occeus*	USA	KT273390	Nolan et al. (2015)
Rhipidocotyle transversale Chandler, 1935	*Strongylura marina*	USA	KT273394	Nolan et al. (2015)
Rhipidocotyle tridecapapillata Curran et Overstreet, 2009	*Micropterus salmoides*	USA	KT273384	Nolan et al. (2015)
Robinia aurata Pankov, Webster, Blasco-Costa, Gibson, Littlewood, Balbuena et Kostadinova, 2006	*Liza aurata*	Spain	DQ354367	Pankov et al. (2006)
Saturnius sp.	*Mugil cephalus*	Spain	DQ354366	Pankov et al. (2006)
Saturnius gibsoni Marzoug, Rima, Boutiba, Georgieva, Kostadinova et Pérez-del-Olmo, 2014	*Mugil cephalus*	Algeria	KJ010542	Marzoug et al. (2014)
Stepahanostomum sp.	*Syacium papillosum*	Mexico	MK558796	Vidal-Martínez et al. (2019)
Stephanostomum adlardi Bray, Cribb, Waeschenbach et Littlewood, 2007	*Plectropomus leopardus*	Australia	EF506761	Bray et al. (2007)
Stephanostomum baccatum (Nicoll, 1907) Manter, 1934	*Eutrigla gurnardus*	UK	AY222256	Bray et al. (2005)
Stephanostomum bicoronatum (Stossich, 1883) Fuhrmann, 1928	*Sciaena umbra*	Corsica	DQ248225	Bray et al. (2005)
Stephanostomum cesticillum (Molin, 1858)	*Lophius piscatorius*	Corsica	DQ248226	Bray et al. (2005)
Stephanostomum gaidropsari Bartoli et Bray, 2001	*Gaidropsarus mediterraneus*	France	DQ248221	Bray et al. (2005)
Stephanostomum interruptum Sparks et Thatcher, 1958	*Menticirrhus americanus*	USA	DQ248223	Bray et al. (2005)
Stephanostomum minutum (Looss, 1901) Manter, 1940	*Uranoscopus scaber*	Corsica	DQ248224	Bray et al. (2005)
Stephanostomum pristis (Deslongchamps, 1824) Looss, 1899	*Phycis phycis*	Corsica	DQ248222	Bray et al. (2005)
Stephanostomum tantabiddii Bray et Cribb, 2004	*Carangoides fulvoguttatus*	Australia	DQ248220	Bray et al. (2005)
Stephanostomum cf. *uku* Yamaguti, 1970	*Aprion virescens*	Australia	DQ248219	Bray et al. (2005)
Tormopsolus orientalis Yamaguti, 1934	*Seriola dumerili*	Corsica	DQ248217	Bray et al. (2005)
Zoogonoides viviparus (Olsson, 1868) Odhner, 1902	*Callionymus lyra*	UK	AY222271	Olson et al. (2003)
Outgroup				
Azygia longa (Leidy, 1851) Manter, 1926	*Esox niger*	USA	KT808319	Womble et al. (2016)
Fellodistomum fellis (Olsson, 1868) Nicoll, 1909	*Anarhichas lupus*	UK	AY222282	Olson et al. (2003)

Table A–A3. Continued

Species	Host	Locality	GenBank ID (28S sequences)	Reference
*Halosaurotrema halosauropsi*** (Bray et Campbell, 1996) Martin, Huston, Cutmore et Cribb, 2018	*Halosauropsis macrochir*	UK	AY222207	Olson et al. (2003)
Macvicaria macassarensis (Yamaguti, 1952) Bray et Cribb, 1989	*Lethrinus miniatus*	Australia	AY222208	Olson et al. (2003)
Otodistomum cestoides (Van Beneden, 1870) Odhner, 1911	*Raja montagui*	UK	AY222187	Olson et al. (2003)
Pleorchis uku Yamaguti, 1970	*Aprion virescens*	Australia	DQ248216	Bray et al. (2005)
Tanaisia fedtschenkoi Skrjabin, 1924	*Anas plathrhynchos*	Ukraine	AY116870	Olson et al. (2003)

Abbreviations: SA, South Africa; TNP, Tsitsikamma National Park; UK, United Kingdom; USA, United States of America. *Registered in GenBank as *Rhipidocotyle angusticolle*. **Registered in GenBank as *Gaevskajatrema halosauropsi*.

APPENDIX B – PERMITS

agriculture,
forestry & fisheries
Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Enquiries: Dr Kim Prochazka
Tel: 021-402 3546
Fax : 021-402 3639
E-mail : researchpermits@daff.gov.za

Dr. Rachel Welicky
North-West University
Potchefstroom Campus
11 Hoffman Street
Building E6, Office 111
Potchefstroom
2520

Attention: Dear Dr. Welicky

PERMIT FOR THE PURPOSES OF A SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT NO. 18 OF 1998).

I, the undersigned, Chief Director: Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agriculture, Forestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby permit, in terms of Section 83 of the Act, the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below:

PERMIT REFERENCE NUMBER: RES2018/35
PERSON(S)/ INSTITUTION: Dr. Rachel Welicky, North-West University,
SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT: Collection and possession of marine organisms for the purposes of research,

subject to the following conditions:

1. GENERAL CONDITIONS

1.1. This permit is issued subject to the provisions and regulations of the following laws:
(a) The Marine Living Resources Act, 1998 (Act No. 18 of 1998) ("the Act"), and all regulations published in terms thereof;
(b) The National Environmental Management Act, 1998 (Act No. 107 of 1998) ("NEMA"), and in particular, the regulations that control vehicle use in the coastal zone (as amended);
(c) The National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973); and
(f) The Prevention of Pollution from Ships Act (Act No. 2 of 1986).

1.2. This permit is intended to facilitate *bona fide* research, and should not be used for the purposes of financial gain by the Permit Holder.

1.3. If, in the opinion of the Chief Director there are sound reasons for doing so, the Chief Director may amend the conditions of the permit.

1.4. A breach of the provisions of the Act, regulations or these permit conditions by the Permit Holder may result in the initiation of legal proceedings (civil or criminal). A breach includes:

(a) furnishing information to which the Department of Agriculture, Forestry and Fisheries (the Department) is entitled, which is not true or complete;
(b) contravening or failing to comply with a permit condition or with the provisions of the Act;
(c) being convicted of an offence in terms of the Act; or

1.5. An application for a permit may be refused if the conditions of a previously issued permit had not been adhered to.

1.6. In terms of the Act, the permit holder is obliged to report to the Minister any contravention of the provisions of the Act by any other person.

1.7. This permit does not in any way absolve the holder from the obligations of and adhering to the remainder of the provisions and conditions of the Act.

1.8. Any individual utilizing this permit shall in addition to the above conditions have a certified copy of any other permit/exemption required in terms of other legislation including any permit or exemption in terms of the Marine Living Resources Act, 1998 (Act No. 18 of 1998).

1.9. Any reference to the Permit Holder in these permit **conditions includes the entity or person, his/her or its employees (whether permanent, full-time or part-time**), registered students, contractors, agents or advisers, being cognisant of the course and scope of their contractual relationship.

1.10. If the permit is in the name of an institution/company/close corporation, the individual utilizing the permit shall in addition to a certified copy of this permit, be in possession of identification and/or a letter which identifies the individual as an authorized person or employee of the permit holder.

1.11. The Permit Holder shall store at their registered-place of business/residence the original permit issued. The Permit Holder shall at all times, have available a true certified copy of this permit which should be produced on demand by any Fishery Control Officer or any other law enforcement official.

1.12. The Permit Holder must safely store all inorganic waste material, garbage and pollutants on board the vessel or at the site of research activities. Should the Permit Holder discard any inorganic waste material, garbage or pollutants into the sea or coastal environment, this permit will be suspended for a period determined by the Department and the Permit Holder shall take those steps considered necessary in terms of NEMA to remedy any pollution caused.

1.13. Specimens collected in terms of this permit shall not be sold or offered for sale.

1.14. No vehicle may be used in the coastal zone in terms of this permit, and an application for an exemption to use a vehicle in the coastal zone shall be made to the Minister in terms of regulation 6(1)(a) of GN Regulation 1399 of 21 December 2001: Control of Vehicles in the Coastal Zone, as amended.

1.15. Report(s), as stipulated in the Specific Conditions must be submitted to the Chief Director: Fisheries Research and Development, Department of Agriculture, Forestry and Fisheries, Branch: Fisheries Management (Attention: Dr Kim Prochazka), Private Bag X2, Vlaeberg, 8018. This should be submitted within one month of the expiry date of this permit, or with the application for renewal of the permit, as required.

2. SPECIFIC CONDITIONS

2.1. This permit allows for the collection and possession of marine species for *bona fide* research projects of the North-West University, as authorised by the Head of the Department.

2.2. A certified copy of this permit shall be carried by staff during collections and must be shown to a Fishery Control Officer or any other authorized person on demand. Staff undertaking collections shall identify themselves, if requested to do so, by means of an identification document issued by the North-West University.

2.3. A maximum of forty (40) specimens of the following list of species may be collected by the Permit Holder during the validity period of this permit.

Hyporhamphus affinis
Strongylura leuira
Diplodus capensis
Diplodus hottentotus
Monodactylus falciformis
Monodactylus argenteus
Trachinotus botla
Trachinotus africanus
Scarus cyanescens
Scarus rubroviolaceus
Amblyrhynchotes honckenii
Acanthurus dussumieri
Acanthurus triostegus
Acanthurus lineatus
Suttiamen bursa
Balistoides conspiciullum
Odonus niger
Apolemichthyes trimaculatus
Chaetodon guttatissimus
Pomacanthus imperator
Pygolites diacanthus
Rhabdosargus sarba
Rhabdosrgus holubi
Pachymetopon blochii
Clinus superciliosus

Chirodactylus brachydactylus
Pempheris adusta
Sparadon durbanensis
Atennablennius bifulum
Scartella emarginata
Helcogramma obtuirostre

2.4. No harmful chemicals are to be used when collecting marine species. Limited use of fish anaesthetics (including rotenone) is permitted if no other suitable technique is available to collect fishes, and should be kept to a minimum. Local authorities should be advised when rotenone is to be used to collect fish.

2.5. Any installations must be removed on termination of the project(s).

2.6. The report, as required under Condition 1.15, should provide details of the dates, locations, species and quantities collected.

3. PERMIT VALIDITY PERIOD

This permit is valid from **1 January 2018** until **31 December 2018.**

MR BELEMANI SEMOLI
ACTING CHIEF DIRECTOR: FISHERIES RESEARCH AND DEVELOPMENT
DATE: 27/11/2017

To acquire and manage a system of national parks which represents the indigenous wildlife, vegetation, landscapes and significant cultural assets of South Africa for the pride and benefits of the nation.

South African NATIONAL PARKS

Permit Number: MALH-K/2016-005a
Issue Date: 2018/12/07
Research Permit: Tsitsikamma Section - Garden Route National Park

addo elephant
agulhas
augrabies
bontebok
camdeboo
garden route
- *tsitsikamma*
- *knysna*
- *wilderness*
golden gate highlands
karoo
kgalagadi transfrontier
kruger
mapungubwe
marakele
mokala
mountain zebra
namaqua
table mountain
tankwa
|ai-|ais/richtersveld
west coast

Project title: Biodiversity and systematics of marine isopods from southern Africa.
Senior Researcher: Dr K Malherbe
Co Workers: Prof NJ Smit, Dr W Malherbe, Dr R Gerber, Dr NL Bruce

Herewith the extension permit for your research project valid from 07 December 2018 until 31 December 2019. The approval is subject to the following conditions:

The Park Management staff must be contacted prior to entry into the park (see list of staff members below). This approval grants you and your co-workers free entrance to the Park *for bona fida* research.

Standard Conditions:

- The use of non-demarcated areas will lead to the disturbance of animals and eco-systems, trampling of vegetation and soil erosion and only the use of accepted pathways and areas is therefore permitted, UNLESS BY SPECIAL ARRANGEMENTS. PLEASE CONTACT THE PARK MANAGEMENT STAFF IF RESTRICTED AREAS NEED TO BE ACCESSED.
- No damage shall be permitted to any natural vegetation, environment or property.
- Animals may not be disturbed in any way.
- Uncontrolled vehicle access and parking could cause damage to vegetation and soil erosion and therefore only the use of approved vehicles routes and parking areas is allowed.
- Fires can cause loss of vegetation, soil erosion and life and therefore fires, and braai's are not permitted unless in dedicated braai areas.
- Remove all rubbish and waste as it has an effect on the health of visitors, animals and plants.
- Other visitors to the area and or neighbours may not be hindered in any way.
- Pollution affects the health and safety of animals, plants, visitors and neighbours and is not permitted.
- Excessive noise affects animals (e.g. birds nesting in the areas), visitors and or neighbours and is not permitted.
- Your permit must be retained and kept on your person at all times, and produced on request.
- The areas under the control of SANParks are used entirely at your own risk. South African National Parks, its Board, directors, employees and agents are not liable for any loss or damage to the property or possession of any guest or participant (or accompanying minor) whether such damage is caused by the negligent act or omission of South African National Parks; arising from death or any bodily injuries of whatsoever nature sustained by a guest or participant (or accompanying minor) whether such injuries are caused by the negligent act or omission by South African National Parks, and/or by the defective functioning of any apparatus. The guest or participant and/or his/her/their estate hereby indemnifies South African National Parks against any claim, action, judgment, costs and/or expenses which may be made against South African National Parks and as may in any way be related to the above. The onus lies with the company or applicant to ensure that they are adequately insured.
- Please note that you (your delegates, staff etc) are subject to the conditions set in terms of Section 86(1) of the National Environmental Management Act (107 of 1998) and the National Environmental Act: Protected Areas Act (Act 57 of 2003) for the duration of your stay in the

PO Box 176
Sedgefield
6573

Garden Route National Park
Scientific Services
Office of General Manager

Tel: (044) 343 1302
Fax: (044) 343 2331
www.sanparks.org

To acquire and manage a system of national parks which represents the indigenous wildlife, vegetation, landscapes and significant cultural assets of South Africa for the pride and benefits of the nation.

South African NATIONAL PARKS

National Park. Your attention is specifically drawn to sections 64(1) (a), (b) & (c) which refers to penalties in terms of the Act.

- SANParks staff's instructions, notices, regulations and signs must be complied with.
- The activity shall be restricted to the area applied for.
- Gate and operating times to be complied with.
- NO PETS ALLOWED

Special Conditions:

New study site to be identified in collaboration with Garden Route Scientific Services Staff members.

All conditions contained within the research agreement linked to this permit must be complied with. It is the Researcher's responsibility to familiarize themselves (including co-workers and research assistants) with these conditions prior to commencement of work within the Park.

Any contraventions of the above will result in your permit being revoked.

Park Management Contact Details:

Tsitsikamma: Area Manager	Bulelwa Msengi
Telephone:	042 281 1607
Email:	Bulelwa.msengi@sanparks.org
Tsitsikamma: Senior Section Ranger	Euginia Mkhatshwa
Telephone:	042 281 1557
Email:	euginia.mkhatshwa@sanparks.org

Kind Regards

Nerina Kruger, South African National Parks, Tel: 044 343 1302, Fax: 086 621 5340
E-mail: nerina.kruger@sanparks.org

addo elephant

agulhas

augrabies

bontebok

camdeboo

garden route
- *tsitsikamma*
- *knysna*
- *wilderness*

golden gate highlands

kgalagadi transfrontier

mapungubwe

marakele

mokala

mountain zebra

namaqua

table mountain

tankwa

|ai-|ais/richtersveld

west coast

PO Box 176
Sedgefield
6573

Garden Route National Park
Scientific Services
Office of General Manager

Tel: (044) 343 1302
Fax: (044) 343 2331
www.sanparks.org

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Enquiries: Dr Kim Prochazka
Tel: 021-402 3546
Fax : 021-402 3639
E-mail : researchpermits@daff.gov.za

Ms Catharina Greyling
Unit for Environmental Sciences
and Management
Potchefstroom Campus
North-West University
Private Bag X6001
Potchefstroom
252

Attention: Dear Ms Greyling

PERMIT FOR THE PURPOSES OF A SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT NO. 18 OF 1998).

I, the undersigned, Chief Director: Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agriculture, Forestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby permit, in terms of Section 83 of the Act, the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below:

PERMIT REFERENCE NUMBER: RES2019/103
PERSON(S)/ INSTITUTION: Ms Catharina Greyling, North-West University,
SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT: Collection and possession of marine organisms for the purposes of research,

subject to the following conditions:

1. GENERAL CONDITIONS

1.1. This permit is issued subject to the provisions and regulations of the following laws:
(a) The Marine Living Resources Act, 1998 (Act No. 18 of 1998) ("the Act"), and all regulations published in terms thereof;
(b) The National Environmental Management Act, 1998 (Act No. 107 of 1998) ("NEMA"), and in particular, the regulations that control vehicle use in the coastal zone (as amended);
(c) The National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973); and
(f) The Prevention of Pollution from Ships Act (Act No. 2 of 1986).

1.2. This permit is intended to facilitate *bona fide* research, and should not be used for the purposes of financial gain by the Permit Holder.

1.3. If, in the opinion of the Chief Director there are sound reasons for doing so, the Chief Director may amend the conditions of the permit.

1.4. A breach of the provisions of the Act, regulations or these permit conditions by the Permit Holder may result in the initiation of legal proceedings (civil or criminal). A breach includes:

(a) furnishing information to which the Department of Agriculture, Forestry and Fisheries (the Department) is entitled, which is not true or complete;
(b) contravening or failing to comply with a permit condition or with the provisions of the Act;
(c) being convicted of an offence in terms of the Act; or

1.5. An application for a permit may be refused if the conditions of a previously issued permit had not been adhered to.

1.6. In terms of the Act, the permit holder is obliged to report to the Minister any contravention of the provisions of the Act by any other person.

1.7. This permit does not in any way absolve the holder from the obligations of and adhering to the remainder of the provisions and conditions of the Act.

1.8. Any individual utilizing this permit shall in addition to the above conditions have a certified copy of any other permit/exemption required in terms of other legislation including any permit or exemption in terms of the Marine Living Resources Act, 1998 (Act No. 18 of 1998).

1.9. Any reference to the Permit Holder in these permit **conditions includes the entity or person, his/her or its employees (whether permanent, full-time or part-time**), registered students, contractors, agents or advisers, being cognisant of the course and scope of their contractual relationship.

1.10. If the permit is in the name of an institution/company/close corporation, the individual utilizing the permit shall in addition to a certified copy of this permit, be in possession of identification and/or a letter which identifies the individual as an authorized person or employee of the permit holder.

1.11. The Permit Holder shall store at their registered-place of business/residence the original permit issued. The Permit Holder shall at all times, have available a true certified copy of this permit which should be produced on demand by any Fishery Control Officer or any other law enforcement official.

1.12. The Permit Holder must safely store all inorganic waste material, garbage and pollutants on board the vessel or at the site of research activities. Should the Permit Holder discard any inorganic waste material, garbage or pollutants into the sea or coastal environment, this permit will be suspended for a period determined by the Department and the Permit Holder shall take those steps considered necessary in terms of NEMA to remedy any pollution caused.

1.13. Specimens collected in terms of this permit shall not be sold or offered for sale.

1.14. This permit may not be used in marketing materials of the Permit Holders, or in any way to contract business to the Permit Holder.

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1.15. No vehicle may be used in the coastal zone in terms of this permit, and an application for an exemption to use a vehicle in the coastal zone shall be made to the Minister in terms of regulation 6(1)(a) of GN Regulation 1399 of 21 December 2001: Control of Vehicles in the Coastal Zone, as amended.

1.16. Report(s), as stipulated in the Specific Conditions must be submitted to the Chief Director: Fisheries Research and Development, Department of Agriculture, Forestry and Fisheries, Branch: Fisheries Management (Attention: Dr Kim Prochazka), Private Bag X2, Vlaeberg, 8018. This should be submitted within one month of the expiry date of this permit, or with the application for renewal of the permit, as required.

2. SPECIFIC CONDITIONS

2.1. This permit allows for the collection and possession of marine species for *bona fide* research projects of the Unit for Environmental Sciences and Management, North-West University, as authorised by the Head of the Department.

2.2. A certified copy of this permit shall be carried by staff during collections and must be shown to a Fishery Control Officer or any other authorized person on demand. Staff undertaking collections shall identify themselves, if requested to do so, by means of an identification document issued by the Unit for Environmental Sciences and Management, North-West University.

2.3. The following amounts of specimens per species may be collected, as detailed below:

Species/ Group	Quantity
Clinus spp. (klipfish)	20
Monodactylus falciformis (Cape moony)	15
Chirodactylus brachydactylus (two-tone fingerfin)	15
Diplodus capensis (black tail)	20
Diplodus sargus (white sea bream)	15
Diplodus hottentotus (zebra fish)	15
Galeichthys feliceps (white sea-catfish)	15
Amblyrhynchotes honckenii (evil-eye pufferfish)	15
Rhabdosargus holubi (Cape stumpnose)	20
Liza richardsonii (South African mullet)	15
Liza dumerili (grooved mullet)	15
Mugil cephalus (flathead mullet)	15
Pachymetopon blochii (hottentot sea bream)	15
Sparodon durbanensis (musselcracker)	15
Epinephelus marginatus (Yellowbelly Rockcod)	15
Chrysoblephus laticeps (red roman)	15

2.4. No harmful chemicals are to be used when collecting marine species. Limited use of fish anaesthetics (including rotenone) is permitted if no other suitable technique is available to collect fishes, and should be kept to a minimum. Local authorities should be advised when rotenone is to be used to collect fish.

2.5. Any installations must be removed on termination of the project(s).

2.6. The report, as required under Condition 1.16, should provide details of the dates, locations, species and quantities collected.

3. PERMIT VALIDITY PERIOD

This permit is valid from **date of issue** until **31 December 2019.**

MR SAASA PHEEHA
ACTING CHIEF DIRECTOR: FISHERIES RESEARCH AND DEVELOPMENT
DATE: 25/7/19

APPENDIX C – ETHICAL APPROVAL

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

20 September 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 20/09/2019, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Diversity and systematics of digeneans from residential and transient intertidal fishes from the coast of South Africa
Principal Investigator/Study Supervisor/Researcher: Prof NJ Smit
Student: A Vermaak - 25476076

Ethics number:

N	W	U	-	0	0	5	6	5	-	1	9	-	A	5
Institution				Study Number						Year			Status	

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

Application Type: Single
Commencement date: 20/09/2019
Expiry date: 30/09/2020

Risk: Category 2

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of September annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal*

without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.

- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC 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 NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual 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.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Prof Tiaan Brink
Chairperson NWU-AnimCareREC

Prof Minrie Greeff
Head of the Faculty of Health Sciences Ethics Office

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
20 August 2019

File Reference: 9.1.5.4.2

APPENDIX D – PAPERS RESULTING FROM THIS DISSERTATION

Folia Parasitologica

OPEN ACCESS JOURNAL
founded 1954

Folia Parasitologica - Article detail

Return back

Status	Text
ver. no. 3	**Article in print** Olena Kudlai **722. 2020/I :: Molecular and morphological characterisation of the metacercariae of two species of *Cardiocephaloides* (Digenea: Strigeidae) infecting endemic South African klipfish (Perciformes: Clinidae)**

South African clinids are a major component of the temperate intertidal regions that are also known to participate in life cycles and transmission of several groups of parasites. However, the knowledge of trematode diversity of these fishes is incomplete. In this study, two species of *Clinus*, the super klipfish *Clinus superciliosus* (Linnaeus) and the bluntnose klipfish *Clinus cottoides* Valenciennes were collected from six localities along the South African coast and examined for the presence of trematodes. Metacercariae of *Cardiocephaloides* were found in the eyes and brain of *C. superciliosus* and in the eyes of *C. cottoides*. Detailed analyses integrating morphological and molecular sequence data (28S rDNA, ITS2 rDNA-region, and *cox*1 mtDNA) revealed that these belong to two species, *Cardiocephaloides physalis* (Lutz, 1927) and an unknown species of *Cardiocephaloides*. This study provides the first report of clinid fishes serving as intermediate hosts for trematodes, reveals that the diversity of *Cardiocephaloides* in South Africa is higher than previously recorded, and highlights the need for further research to elucidate the life cycles of these trematode species. The broad geographical distribution of *Cardiocephaloides* spp. was confirmed in the present study based on molecular sequence data. The host-parasite interactions between clinid fishes and metacercariae of *Cardiocephaloides* are yet to be explored.

View history

Author's comments:

...not specified

Return back

APPENDIX E – EDITING GUIDELINES OF JOURNALS

A-E1: Folia Parasitologica

Preparation of manuscripts

1. PREPARATION OF MANUSCRIPTS – GENERAL INFORMATION

1.1. Language. Manuscripts should be written in simple and concise scientific English using British spelling. Authors whose mother tongue is not English are strongly urged to have their manuscripts checked and improved linguistically by a native speaker (preferably, a life scientist) before submission. In fact, editorial office does not take responsibility of the quality of the English. Professional provider of pre-submission editing services, such as Enago (www.enago.com) or Edanz (www.edanzediting.com) can be consulted. Submissions written in poor English will be returned immediately.

1.2. Names. Scientific names of taxa up to the generic level should be italicised throughout. Current, valid names of hosts should be used (with synonyms, such as used in cited papers, given in parentheses). Scientific names of parasites and hosts studied should be given with authorities (and, for parasites, year of description) in the Abstract and, again, when first mentioned in the main body of text, and also in tables and figures legends. Otherwise, citing these authorities is to be avoided or used sparingly. Authority and year of description should also be given for parasites mentioned in taxonomic comparison. References to authors of names should not be included in the References section. The following style is required: *Fasciola hepatica* Linnaeus, 1758, *Allocreadium patagonicum* Shimazu, Urawa et Coria, 2000. (Note that names of all authors are given; 'et' connects two last authors' names; and last author's name is separated from year by comma.).

1.3. Numerals. All numerals from one to ten are spelled out in the text except for morphological descriptions.

1.4. Taxonomic descriptions. Authors should follow all requirements of the current International Code of Zoological Nomenclature (1999). This journal requires that at least holotype be deposited in an institution that provides long-term care of collections and access for study of deposited materials. One of such institutions is the Institute of Parasitology, Biology Centre ASCR (publisher of this journal). Whenever possible, manuscripts containing description of new species of helminths or arthropods are expected to be accompanied with at least one paratype of each new taxon for scrutiny by reviewers and for subsequent deposition in the collection of the Institute (postal address: Tomáš Scholz, Curator, Institute of Parasitology, Biology Centre CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic).
Within taxonomic treatment the following order of data is preferred: species name, author with year of description (if not new species), reference to figures; synonymy; description in telegraphic style (i.e. omitting articles and as many verbs as can be elided without loss of meaning); after description: data for the material studied or taxonomic summary (type host, other hosts, type locality, other localities, site, prevalence, specimens deposited and their collection numbers); etymology; differential diagnosis (best headed as 'Remarks'); other comments if appropriate.

1.5. Citations. For papers by two authors, names should be connected with 'and', e.g. Koskivaara and Valtonen (1992). For papers by more than two authors, the first author should be cited with 'et al.', e.g. Iglesias et al. (1997). Names and years should not be separated by commas, e.g. (Koskivaara and Valtonen 1992); commas, not semicolons, should separate citations in parentheses, e.g. (Koskivaara and Valtonen 1992, Iglesias et al. 1997a,b). In the text, references should be listed in chronological order; papers published in the same year are ordered alphabetically.

1.6. References to illustrations and tables. Examples: Fig. 1. Figs. 1, 2. Figs. 2A,B. Figs. 1–3. Table 1. Use fig. 1, i.e. do not capitalise, for figures in other papers.

2. PREPARATION OF MANUSCRIPTS – ORGANISATION

The standard order of constituent parts for original full papers is: Title page (with Title, Author name[s], Running header, Abstract, Keywords, Address for correspondence); Introduction (which, however, does not bear this heading); Materials and Methods; Results; Discussion; Acknowledgements; References; Tables; Figure legends. The layout of research notes is similar to that of full papers but section headings

(except for Abstract, Keywords and References) are omitted. For your convenience Folia Parasitologica has developed template word file to help you prepare your manuscript.

2.1. Title. Title should not mention authors of taxa and years of description unless in special cases (e.g. taxonomic revisions and redescriptions). Title is expected to contain names of higher taxa (such as order and family) accommodating the parasites and hosts under study; the names of higher taxa may not be appropriate for multiple parasites/hosts or widely used models in papers of experimental nature.

2.2. Authorship. Author names should be given in bold with full first names and with institutional addresses. Start each different address (not postal, i.e. without details such as street and ZIP code) on a separate line and separate them with semicolon.

2.3. Running header. Suggest a short version of title for use as running header (max. 5 words).

2.4. Abstract. It should be a factual summarisation of the main results and conclusions so that it can be published in abstracting journals without change. Abstracts, written without paragraphs, should not exceed 300 words (reviews and full papers) or 150 words (research notes). Abstracts of taxonomic papers should mention all nomenclatural acts and newly proposed nominal taxa with their brief differential diagnoses.

2.5. Keywords. Suggest a set of keywords (index terms). The set should be complete but keep it as short as possible (typically, under 10 words/phrases). Words from the title should not be repeated.

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2.9. Results. Results should be clear and concise, and only new, original data should be presented here.

2.10. Discussion. It should discussed new data in relation to existing information. It should be straightforward, not wordy, and perspectives of future research should be outlined.

2.11. Acknowledgements.

2.12. References. Entries should be listed alphabetically by names of all authors and, subordinately, chronologically. Author names should **not** be typed in capital letters. Title of journal article should be cited in full, followed by abbreviated name of journal as listed in Serial Sources for the BIOSIS Data Base (BioSciences Information Service, Philadelphia), volume and pagination (example 1). Issue number should be given (after volume, in parentheses) only if volume is not continuously paginated. Title and chapter pagination are required for chapters in monographs (2). Publisher, place where published and number of pages are required for monographs (3). Titles in languages other than English, German, French, Spanish, Portuguese and Italian should be translated into English and their original language stated in parentheses. Titles whose English version is not that given in the original publication should be in square brackets (4). Electronic publication should be cited with the most recent version (5). Examples:

- (1) Moravec F., Bilal S.J., Abdullah S.M.A. 2012: Two species of *Rhabdochona* (Nematoda: Rhabdochonidae) from the cyprinid fish *Luciobarbus kersin* (Heckel) in northern Iraq, including *R.* (*Globochona*) *kurdistanensis* sp. n. Folia Parasitol. 59: 139–147. [Note that dash (–), not hyphen (-), should be used.]
- (2) Scholz T., Kuchta R., Williams C. 2012: *Bothriocephalus acheilognathi* Yamaguti, 1934. In: P.T.K. Woo and K. Buchmann (Eds.), Fish Parasites: Pathobiology and Protection. CABI, Wallingford, pp. 292–307.
- (3) Růžek D. (Ed.) 2011: Flavivirus Encephalitis. InTech, Rijeka, 478 pp.

- (4) Lykova K.A., Gulyaev V.D., Melnikova Yu.A., Karpenko S.V. 2006: [On the species independence of *Mathevolepis larbi* Karpenko, 1982 (Cyclophyllidea: Hymenolepididae: Ditestolepidini).] Parazitologiya 40: 299–305. (In Russian.)
- (5) Froese R., Pauly D. (Eds.) 2016: FishBase. World Wide Web electronic publication, www.fishbase.org, 1/2016.
- (6) Beveridge I. 2014: A review of the genus *Paramoniezia* Maplestone et Southwell, 1923 (Cestoda: Anoplocephalidae), with a new genus, *Phascolocestus*, from wombats (Marsupialia) and redescriptions of *Moniezia mettami* Baylis, 1934 and *Moniezia phacochoeri*. Folia Parasitol. 61: (in press).

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2.13. Legends. All abbreviations and symbols appearing in figures should be explained in legends or (if too numerous and frequently repeated) collected in a list (preferably in Materials and Methods). For composite plates, a summary statement, if possible, should precede specific explanations to separate figures. Parasite (with authority and year) and host names (with authorities but not year) should be spelled out completely in each legend.

2.14. Tables. Tables, typed on separate files and numbered consecutively with Arabic numerals, should be self-explanatory, i.e. their headings and explanations should make them fully understandable without reference to text.

3. PREPARATION OF MANUSCRIPTS – FIGURES

3.1. Illustrations. Illustrations should be numbered consecutively with Arabic numerals in left upper corner. Whenever possible, individual figures should be grouped together in a single block (composite figure) of rectangular shape, using space efficiently. Without change or after reduction, illustrations should neatly fit into one or two columns. The maximum printing area is 165 × 235 mm, but an appropriate space below figures should be left for legends. The elements of composite figure can be identified with consecutive Arabic numerals or with letters in Arial (in upper left corner). A scale bar is required for each figure (on right side), with units directly in figure.

3.2. Graphs. Two-dimensional black-and-white graphics are preferred. Lettering (in Arial) should be of such size that the height of characters, after possible reduction (often to one-column width, 80 mm), will be at least 2.5 and 1.25 mm (upper and lower case, respectively). The same font style and lettering size should be used for all graphs.

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4. POLICY ON THE ACCESSIBILITY OF MOLECULAR DATA

Molecular data supporting publication results should be made available for scrutiny during the review process and for future use. Specifically, alignments of DNA sequences or RNA datasets are required to be made available either as Supporting information on the Folia Parasitologica web site or at publicly accessible repositories (e.g. http://datadryad.org/). Supporting information should be provided with sufficient details so that the data can be interpreted correctly by a third party. Supporting data will be made publicly available at the time of publication. Upon request, embargos may be exceptionally granted by the editorial team for data containing sensitive information (e.g. human medical data).

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Some specific remarks are listed below (in the case of any queries or doubts, please do not hesitate to request editorial assistant at folia@paru.cas.cz):

- Mean ± standard deviation (or standard error) should be provided only if n > 30; otherwise, mean with range should be presented.
- Symbols for protein-coding genes should be italicised (e.g. *cox1*), whereas symbols for proteins should not be italicised (e.g. cox1). The formatting of symbols for RNA-coding genes should not be italicised (e.g. 18S rDNA, 12S rRNA gene, ITS or SSU rDNA).
- Primers are written by regular font.
- Descriptions should not include units; statement such as “All measurements are in micrometres unless otherwise indicated” should appear in the Materials and Methods section.
- Terms in Latin, e.g. *in vitro*, *in vivo*, *pars prostatica*, *receptaculum seminis* (but not seminal receptacle!) should be italicised.
- For coordinates please use these symbols: 47°25'04"S; 22°49'14"W.
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A-E2: Systematic Parasitology

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For life science journals only (when applicable)
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Manuscripts should be submitted in Word.

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Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

IMPORTANT NOTES ON STYLE AND NOMENCLATURE

The authorities and date of all parasite taxa should be given when the names (full binomen) are first cited in the abstract and the text. Subsequently, the generic component is abbreviated except at the beginning of a sentence.
The authorities, but not the date, should be given for the hosts of described parasites.
Publications cited only as authorities for taxa should not be included in the References.
The International Code of Zoological Nomenclature will be strictly adhered to:
Online First for the articles accepted for publication in Systematic Parasitology prior to print publication is available. In order to comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012) concerning the electronic Online First publication of new scientific names and nomenclatural acts, registration of the publication on the Official Register of Zoological Nomenclature (ZooBank.org) is required. Although the Code amendment does not require registration of new names, Systematic Parasitology will also require that new names are registered in ZooBank. Authors are responsible for registration of the article and all new acts at the time of manuscript acceptance.

Museum accession numbers for all deposited type and voucher specimens are required at submission. The museum should be a recognised national or international museum which is protected by law and not a university collection.
New molecular sequences reported in manuscripts should be deposited in the GenBank database and the accession numbers included in the manuscript. For taxonomic studies based on molecular sequences, the source specimen of molecular information should be deposited in a recognised national or international museum.

REFERENCES

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Ideally, the names of six authors should be given before et al. (assuming there are six or more), but names will not be deleted if more than six have been provided.

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The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
Reference list entries should be alphabetized by the last names of the first author of each work.
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- Journal article Harris, M., Karper, E., Stacks, G., Hoffman, D., DeNiro, R., Cruz, P., et al. (2001). Writing labs and the Hollywood connection. *Journal of Film Writing, 44*(3), 213–245.
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- Book Calfee, R. C., & Valencia, R. R. (1991). *APA guide to preparing manuscripts for journal publication.* Washington, DC: American Psychological Association.
- Book chapter O'Neil, J. M., & Egan, J. (1992). Men's and women's gender role journeys: Metaphor for healing, transition, and transformation. In B. R. Wainrib (Ed.), *Gender issues across the life cycle* (pp. 107–123). New York: Springer.
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TABLES

- All tables are to be numbered using Arabic numerals.
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- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.
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- Indicate what graphics program was used to create the artwork.
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- Vector graphics containing fonts must have the fonts embedded in the files.
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- All lines should be at least 0.1 mm (0.3 pt) wide.
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A-E3: Parasitology Research

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- Use the equation editor or MathType for equations.
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Manuscripts with mathematical content can also be submitted in LaTeX.
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The International Code of Zoological Nomenclature (ICZN) must be observed. Genus and species names should be in italics. Authors of scientific names of the genus and species group should not be italicized. At first mention, a specific name should be cited with nomenclatural author and year, e.g. Catenula lemnae (in italics) Dugès, 1832. When three or more joint authors have been responsible for a name, then the citation of the name of the authors may be expressed by use of the term "et al." following the name of the first author, provided that all authors of the name are cited in full elsewhere in the same work, either in the text or in a bibliographic reference. Authors unfamiliar with the taxonomy of the group to which a species belongs should consult an expert to ensure that it is properly identified and that the correct name is used.

REFERENCES

Citation
Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list
The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
Reference list entries should be alphabetized by the last names of the first author of each work. Order multi-author publications of the same first author alphabetically with respect to second, third, etc. author. Publications of exactly the same author(s) must be ordered chronologically.
Journal article
Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8
Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:
Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
Article by DOI
Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086
Book
South J, Blass B (2001) The future of modern genomics. Blackwell, London
Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

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- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

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