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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* Cuvier, 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 eye vitreous humour and brain of *C. superciliosus* and in the eye vitreous humour 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, 1926) 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), *Haemogregarina 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), *Ceratomyxa dehoopi* Reed, Basson, Van As et Dyková, 2007 (Reed et al. 2007), *Ceratomyxa obovalis*

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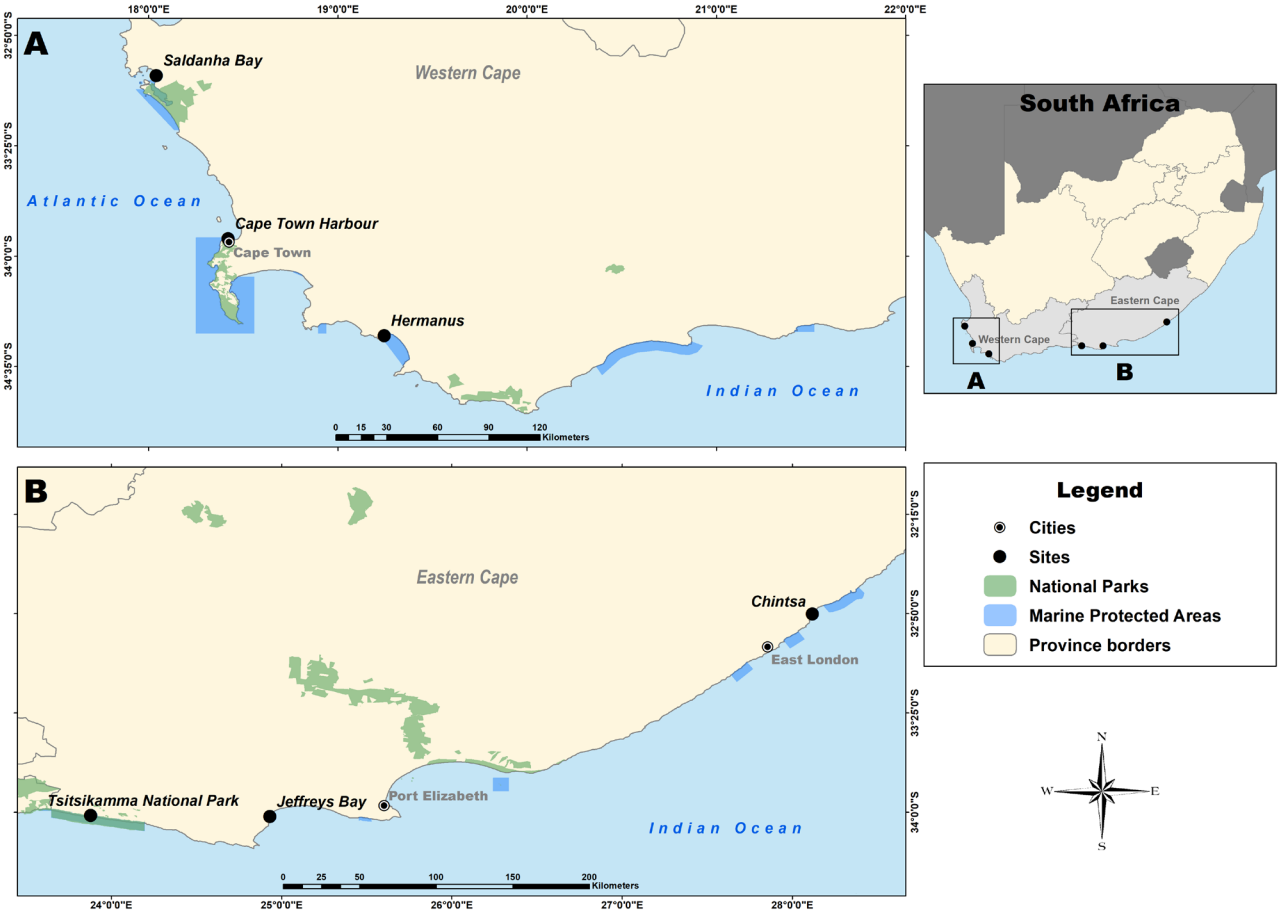

Fig. 1. Map illustrating the sampling localities along the South African coast.

(Fantham, 1930) (Ceratomyxidae) (Fantham 1919, 1930), *Sphaeromyxa clini* Bartošová-Sojková, Kodádková, Pecková, Kuchta et Reed, 2015 (Sphaeromyxidae) (Bartošová-Sojková et al. 2015), *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

Table 1. Primers used for 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. (2015)
	DICE14R	5’-TAA TAC GAC TCA CTA TAC CHA CMR TAA ACA TAT GAT G-3′	Van Steenkiste et al. (2015)

of South Africa. We report, for the first time, clinid fishes as intermediate hosts for trematodes in South Africa, including species of *Cardiocephaloides*. These novel data contribute 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 six localities along the west and south coasts of South Africa: Saldanha Bay (33.045683S, 18.038628E), Cape Town (33.908092S, 18.418281E), Hermanus (34.421071S, 19.243766E), Tsitsikamma National Park (34.020892S, 23.878674E), Jeffreys Bay (34.026389S, 24.930833E) and Chintsa (32.836538S, 28.116997E) (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 metacercariae were removed from the eye vitreous humour and brain of the host, they were excysted with fine needles and preserved in 96% molecular grade ethanol. Metacercariae were sorted and selected for further molecular and morphological analyses. Additionally, adult worms of *Cardiocephaloides physalis* collected by Horne et al. (2011) from *Spheniscus demersus* and donated to us, were used to generate DNA 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 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 are presented as the range followed by the mean, while the 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 sequence 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’ protocols. 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. 2015, 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

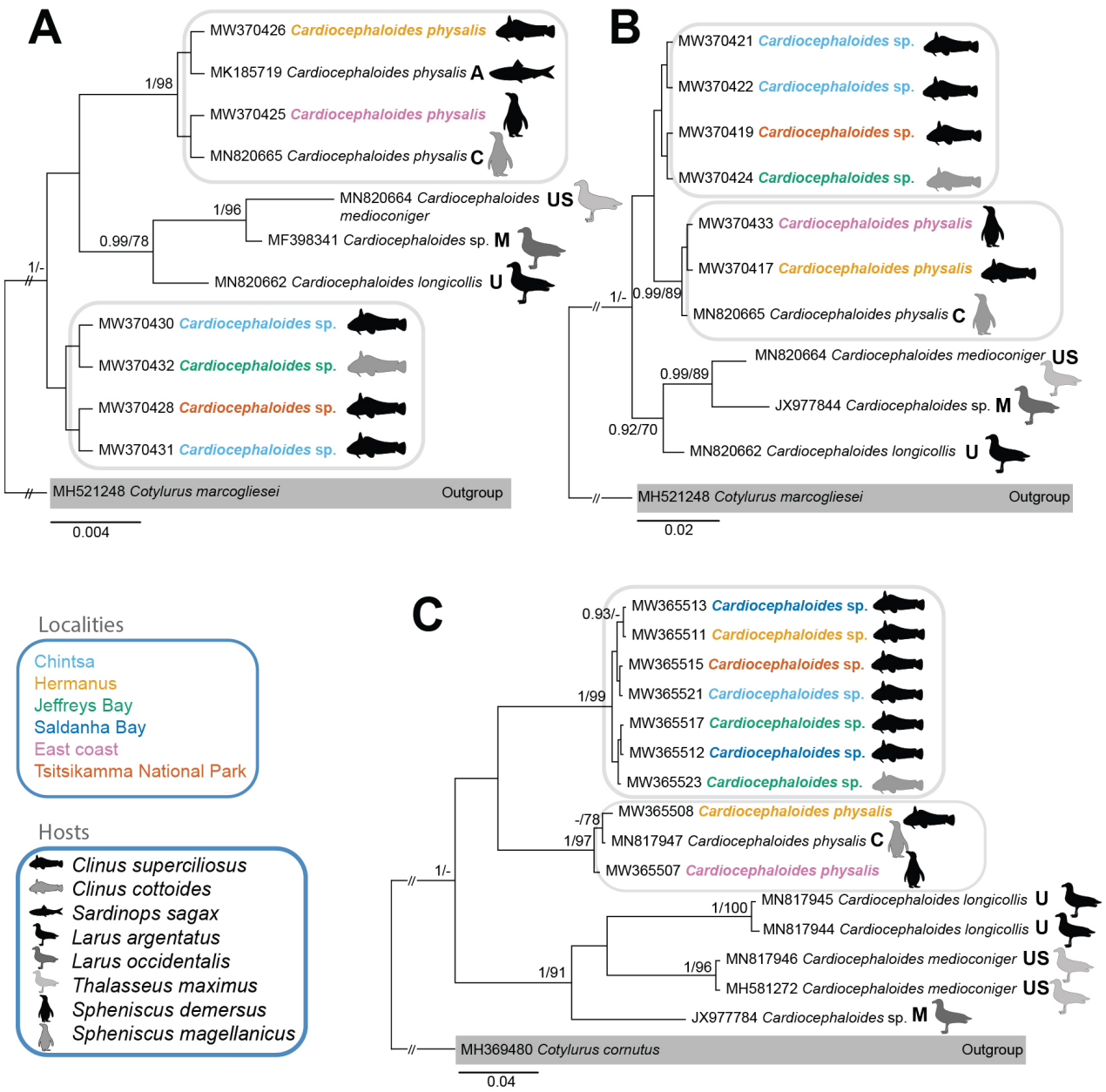

Fig. 2. Bayesian inference (BI) trees based on the 28S rDNA (A), ITS2 (B) and COI (C) sequences. Nodal support values are given as BI/ML (maximum likelihood). Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. Newly generated 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.

(P 723). Accession numbers for the sequences of metacercarial isolates are provided in the relevant taxonomic summaries.

Phylogenetic analysis

In total, 37 novel sequences were generated for 21 metacercarial isolates (seven for 28S, one for ITS1-5.8S-ITS2, eight 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 six sequences obtained from GenBank, including an unpublished sequence for *C. physalis* (MK185719) obtained from *Sardinops 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).

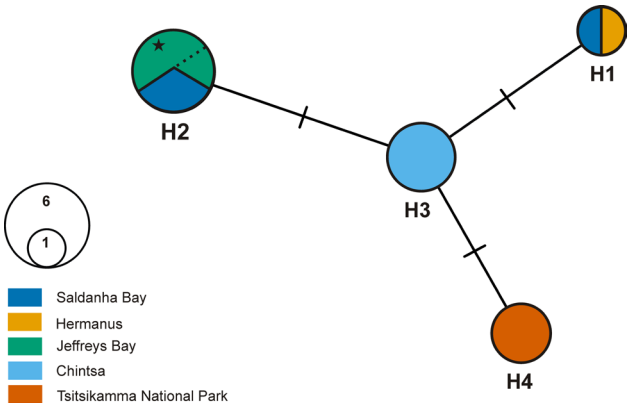

Fig. 3. Haplotype network for *Cardiocephaloides* sp. based on novel COI sequences from metacercarial isolates collected at five sampling localities along the coast of South Africa from *Clinus superciliosus* (Linnaeus) and *Clinus cottoides* Valenciennes. Unsampled intermediate haplotype is represented 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 *C. cottoides* are indicated by a star. Abbreviation: H, haplotype. Numbers indicate the haplotype code number (see Table 2 for details).

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).

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 eye vitreous humour 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 *Clinus cottoides* examined from Jeffreys Bay, the eyes of four were infected with 1–6 metacercariae of *Cardiocephaloides*.

Molecular genetic 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 *Cardiocephaloides physalis* were identical with sequences of three metacercarial specimens obtained from the eyes of *C. superciliosus* from Hermanus and with the sequence 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 genetic 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 *Spheniscus 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 *Cardiocephaloides medioconiger* (Dubois et Vigueras, 1949) 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

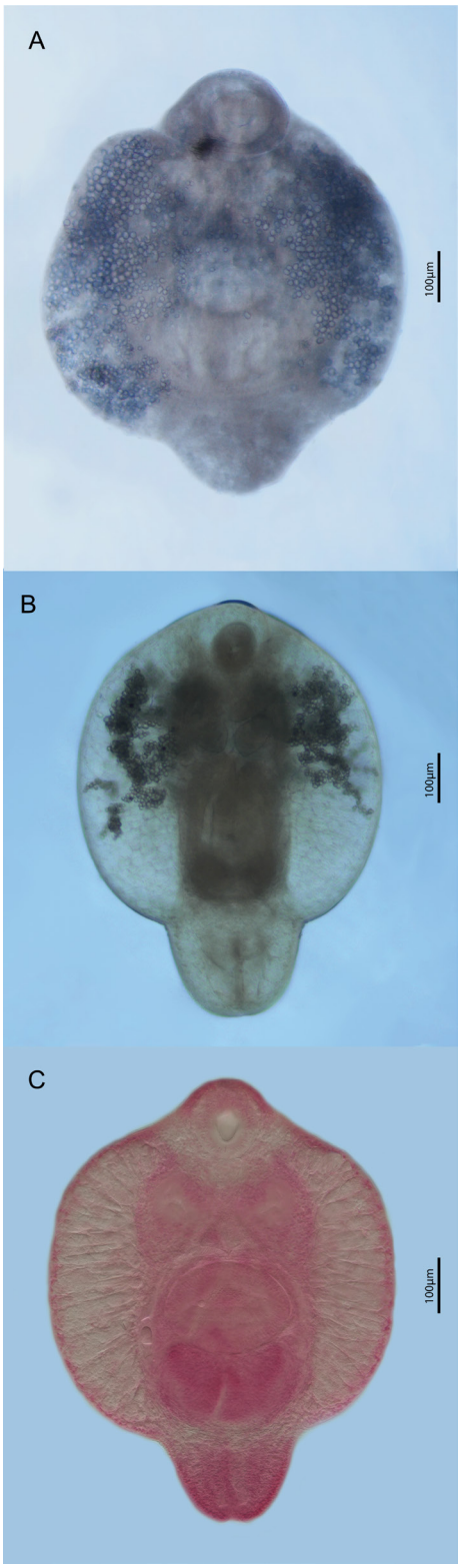

Fig. 4. Photomicrographs of metacercariae of *Cardiocephaloides* spp. **A** – *Cardiocephaloides physalis* (Lutz, 1926) ex *Clinus superciliosus* (Linnaeus) from Hermanus, excysted, fixed specimen, ventral view (voucher, GenBank MW370427 (28S), MW370418 (ITS2), MW365510 (COI)); **B** – *Cardiocephaloides* sp. ex *C. superciliosus* from Jeffreys Bay, excysted, fixed specimen, ventral view (voucher, GenBank MW365517 (COI)); **C** – *Cardiocephaloides* sp. ex *Clinus cottoides* Valenciennes from Jeffreys Bay, excysted, stained specimen, ventral view (voucher, NMB P 708).

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 *Cardiocephaloides 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).

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).

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 '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,

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

Species	Host	Locality	GenBank accession numbers					Reference
			28S	ITS1-5.8S-ITS2	ITS2	COI	Haplotype	
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, 1926)	*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
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 gen 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

Abbreviation: SA, South Africa, [a]Sequence only of ITS1 + 5.8S

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

Species	*Cardiocephaloides physalis* (Lutz, 1926)		*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, digital vouchers)		Ukomadu (2017)		Timi et al. (1999)		(present study, 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/ body length	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/ body length as %	40–41	40	–		–	–	33–51	40	33–42	39
Oral sucker/ 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/ 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

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 (34.421071S, 19.243766E), South Africa.

Site of infection: Vitreous humour of eye.

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

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

Description (based on 24 whole excysted specimens – digital vouchers and stained specimens; metrical data in Table 3). Metacercariae of '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 (33.045683S, 18.038628E), Hermanus (34.421071S, 19.243766E), Tsitsikamma National

Park (34.020892S, 23.878674E), Jeffreys Bay (34.026389S, 24.930833E) and Chintsa (32.836538S, 28.116997E), 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. This author found encysted metacercariae of *Cardiocephaloides* sp. (reported as "Tetracotyle sp. larvae") in the vitreous humour of the eyes of 26 *Sardinops 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 *Clinus superciliosus* and the second species of the genus, *Cardiocephaloides* sp. parasitising both *C. superciliosus* and *Clinus 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) 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), *Cominella glandiformis* (Reeve) and *Cominella 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 level.

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), *Cardiocephaloides musculosus* (Johnston, 1904) from *Chlidonias hybrida* (Pallas) and *Hydroprogne caspia* (Pallas) (Australia), and *Cardiocephaloides ovicorpus* Dubois et Angel, 1972 from *Phalacrocorax varius* (Gmelin) (Australia) and *Microcarbo 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 maxi-

ma for length than those 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* has been 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 Cli-

nidae were recently reported as a part of the diet of a larid, the great crested tern *Thalasseus bergii* (Lichtenstein) 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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