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## Molecular characterisation of three species of *Coitocaecum* (Digenea: Opecoelidae) infecting *Clinus superciliosus* (Clinidae) in South Africa, with description of *Coitocaecum brayi* sp. n.

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**Abstract:** The genus *Coitocaecum* Nicoll, 1915 is part of the most speciose digenetic family, the Opecoelidae Ozaki, 1925, which is found globally in both freshwater and marine fishes. Fifteen opecoelid species have been reported from marine fishes in South Africa, yet only one species of *Coitocaecum* has been described from this region: *Coitocaecum capense* Bray, 1987. During an explorative study of the digenetics of the endemic, intertidal fish *Clinus superciliosus* (Linnaeus) from the Saldanha Bay area, Cape Town harbour, Hermanus, the Tsitsikamma section of the Garden Route National Park and Chintsa East in South Africa, a total of three distinct species of *Coitocaecum* were identified based on morphological and molecular (28S rDNA, ITS1-5.8S-ITS2 rDNA and COI mtDNA) data: the previously mentioned *C. capense*, *Coitocaecum brayi* sp. n. and a third, unnamed species. We provide the first molecular characterisation of species of *Coitocaecum* from South Africa, accompanied by detailed morphological descriptions. This study illustrates the importance of an integrated taxonomic approach, especially when studying species with similar morphology. These findings further emphasise the lack of information on the true diversity and molecular 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:** Trematoda, marine fish parasites, genetics, phylogeny, morphology, Afrotropical region

Of all digenetic families, the Opecoelidae Ozaki, 1925 is the most speciose, with more than 900 species from over 90 genera currently known (Bray et al. 2016, Martin et al. 2020a). Members of this family are under continuous re-organisation, mainly due to their homoplastic morphology and the implementation of novel phylogenetic investigative efforts (Bray et al. 2016, Martin et al. 2020a). Opecoelids have a cosmopolitan distribution and utilise marine and freshwater fishes as definitive hosts (Bray et al. 2016).

To date, 15 opecoelids have been reported from marine fishes in South Africa, of which five were described from this region. 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.

Of these 15 species, two were found in the endemic fish *Clinus superciliosus* (Linnaeus) (Blenniiformes: Clinidae): *Coitocaecum capense* Bray, 1987 (localities: Gqeberha, formerly Port Elizabeth, on the south coast; Oudekraal on the west coast) and *Helicometra fasciata* (Rudolphi, 1819), also from Gqeberha (Bray 1987). Clinid species are abun-

dant inter- and subtidal fishes, nearly half of which are endemic to southern Africa (von der Heyden et al. 2011).

The genus *Coitocaecum* Nicoll, 1915 consists of more than 40 known species that occur in both freshwater and marine fishes. Species of *Coitocaecum* are characterised by having caeca that form a cyclocoel, and the absence of papilliform projections on the ventral sucker (Aken’Ova and Cribb 1996). Molecular data on this genus are scarce globally.

During a study exploring the diversity of trematodes of *C. superciliosus* from a large section of its distributional range in South Africa, we found that it is a host to three species of *Coitocaecum*, among others. Thus, this study aimed to contribute distributional, morphological and molecular data on trematodes from this understudied area.

### MATERIALS AND METHODS

#### Specimen collection

Seventy-one specimens of *Clinus superciliosus* were collected in rocky intertidal areas and intertidal rock pools from five localities along the South African coast: Langebaan marina in the Saldanha Bay (henceforth referred to as Saldanha Bay) (-33.045683;

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18.038628) (n=19), Cape Town harbour (-33.908092; 18.418281) (n=16), Hermanus (-34.421072; 19.243767) (n=8), Tsitsikamma National Park (henceforth referred to as TNP) (-34.020892; 23.878675) (n=17) and Chintsa East (-32.836539; 28.116997) (n=11). These localities are situated in an area where the cold, nutrient rich Benguela current and the warmer Agulhas current meet, in varying degrees, thus encompassing a wide variety of environmental conditions and habitats.

Sampling was conducted under the permits MALH-K2016-005a for TNP; RES2018/35 for Hermanus; and RES2019-103 for Saldanha Bay, Cape Town harbour and Chintsa East. Fish were collected with baited traps and hand lines. Following euthanasia, fish were subjected to helminthological examination by inspecting every organ. Digenean trematodes were removed from the organs with fine needles, heat-fixed in hot saline and preserved in 80% ethanol for further analyses. Fish nomenclature follows FishBase (Froese and Pauly 2023).

### Morphological analyses

Trematode specimens were grouped based on their morphology and hologenophores selected for molecular analysis were vouchered following Pleijel et al. (2008). Whole mounts and hologenophores were rehydrated in distilled water, stained with Mayer's haematoxylin, destained with diluted hydrochloric acid (1%), neutralised with diluted ammonia (1%), gradually dehydrated with an ethanol series (70%, 80%, 90%, 96%, 100%), and then permanently mounted on a slide using Dammar gum.

Slides were used to obtain measurements, take photomicrographs and to make detailed drawings for each species. All measurements were obtained using NIS-Elements BR Camera Analysis software and a Nikon Eclipse Ni microscope (Nikon Instruments, Tokyo, Japan), and are given in micrometres ( $\mu\text{m}$ ), unless otherwise specified. The metrical data are presented as a range, followed by the mean in parentheses. Detailed drawings were made using a drawing tube attached to the aforementioned microscope and digitised using Adobe Illustrator v. 26.4.1 and Photoshop v. 23.4.2. Voucher material and type series were deposited at the Parasite Collection of the National Museum (NMB), Bloemfontein, South Africa; the Swedish Museum of Natural History (SMNH), Stockholm, Sweden; and Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic (IPCAS).

### 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 PCR Biosystems Rapid DNA Extraction Kit (PCR Biosystems available from Analytical Solutions, Randburg, South Africa). The manufacturer's protocol for each extraction kit was followed, except for the following alterations made to the latter: only 10  $\mu\text{l}$  lysis buffer and 5  $\mu\text{l}$  protease containing buffer was used; the reaction was finally diluted with 450  $\mu\text{l}$  molecular water instead of 900  $\mu\text{l}$  as recommended, to obtain quality DNA.

Polymerase chain reaction (PCR) was used to amplify the partial D1–D3 fragment of the 28S nuclear ribosomal RNA gene, the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, and the entire internal transcribed spacer region (ITS1-5.8S-ITS2) or 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) were used to amplify the ITS2 gene region, following the protocol of Kudlai et al. (2015).

The COI gene was amplified using the forward primer JB3 (5'- TTT TTT GGG CAT CCT GAG GTT TAT -3') (Bowles et al. 1995) and reverse primer CO1-R trema (5'- CAA CAA ATC ATG ATG CAA AAGG -3') (Koehler et al. 2011); thermocycling conditions were: 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, and final extension at 72°C for 7 min. 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). Newly generated sequences have been deposited in GenBank (see Table 1).

### Phylogenetic analyses

Sequences selected for phylogenetic analyses were based on the analyses of Martin et al. (2018, 2020b). Available sequences for representatives of the subfamily Opecoelinae were retrieved from GenBank as well as sequences for *Holsworthotrema enboulrichthys* Martin, Huston, Cutmore et Cribb, 2018, and *Scorpidotrema longistipes* Aken'ova et Cribb, 2003, which were used as outgroup taxa (Table 1). Three alignments, one of 28S rDNA, one of ITS2 rDNA, and one of COI mtDNA, were built using MUSCLE (Edgar 2004) as implemented in Geneious v. 11.1.4, with sequences retrieved from GenBank together with the novel sequences. As only two novel COI sequences were generated for our taxa and no GenBank data exist for identified opecoeline species, no attempt was made to analyse the COI dataset beyond calculating pairwise differences.

The best nucleotide substitution model for each alignment was determined with jModelTest 2.1 (Posada 2008), 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) was used for the construction of the 28S phylogenetic tree; and the general time reversible model with gamma distribution among site rate variation (GTR + G) was used for the construction of the ITS2 phylogenetic tree.

Phlogenies are based on Bayesian inference (BI) and maximum likelihood (ML) estimate analyses. The analyses for BI were performed with MrBayes software and ML analyses were performed with PhyML v. 3.0 (available at <http://www.atgc-montpellier.fr/phylml/>). For the BI analyses of both alignments, the Markov chain

**Table 1.** Sequences used for phylogenetic analyses of the Opecoelinae.

Species	Host	Locality	GenBank accession numbers			Reference
			28S	ITS2	COI	
<i>Coitocaecum capense</i> Bray, 1987	<i>Clinus superciliatus</i> (Linnaeus)	Tsitsikamma National Park, South Africa	OR129142	OR129261	—	Present study
<i>C. capense</i> Bray, 1987	<i>C. superciliatus</i>	Tsitsikamma National Park, South Africa	OR129143	OR125622	OR125617	Present study
<i>C. capense</i> Bray, 1987	<i>C. superciliatus</i>	Hermanus, South Africa	OR129144	OR129262	—	Present study
<i>C. capense</i> Bray, 1987	<i>C. superciliatus</i>	Hermanus, South Africa	OR129145	OR129263	—	Present study
<i>C. capense</i> Bray, 1987	<i>C. superciliatus</i>	Hermanus, South Africa	—	OR129270	—	Present study
<i>C. capense</i> Bray, 1987	<i>C. superciliatus</i>	Saldanha Bay, South Africa	—	OR129269	—	Present study
<i>Coitocaecum</i> sp.	<i>C. superciliatus</i>	Hermanus, South Africa	OR129276	OR129264	OR125618	Present study
<i>Coitocaecum brayi</i>	<i>C. superciliatus</i>	Saldanha Bay, South Africa	OR129277	OR129265	—	Present study
<i>Coitocaecum brayi</i>	<i>C. superciliatus</i>	Saldanha Bay, South Africa	—	OR129266	—	Present study
<i>Coitocaecum brayi</i>	<i>C. superciliatus</i>	Saldanha Bay, South Africa	—	OR129267	—	Present study
<i>Coitocaecum brayi</i>	<i>C. superciliatus</i>	Saldanha Bay, South Africa	OR129278	OR129268	—	Present study
<i>Anomalotrema koiae</i> Gibson et Bray, 1984	<i>Microankathus fedorovi</i> (Mandrytsa)	Off Simushir Island, North Pacific	MH161429	—	—	Sokolov et al. (2019)
<i>Coitocaecum</i> sp.	<i>Hemigymnus fasciatus</i> (Bloch)	Great Barrier Reef, Australia	—	KJ596418	—	Barnett et al. (2014)
<i>Dimerosaccus oncorhynchi</i> (Eguchi, 1931) Shimazu, 1980	<i>Salvelinus curilis</i> (Pallas)	Kedrovaya River, Russia	FR870262	—	—	Shedko et al. (2015)
<i>Discoverytrema gibsoni</i> Zdziewiecki, 1990	<i>Muraenolepis marmorata</i> Günther	Ross Sea, Antarctica	MH161430	—	—	Sokolov et al. (2019)
<i>Discoverytrema markowskii</i> Gibson, 1976	<i>Muraenolepis marmorata</i> Günther	Ross Sea, Antarctica	MH161431	—	—	Sokolov et al. (2019)
Opecoelidae gen. sp.	<i>Mitrella</i> sp.	Corsica, France	—	AJ241812	—	Jousson et al. (1999)
Opecoelidae gen. sp.	<i>Columbella rustica</i> (Linnaeus)	Corsica, France	—	AJ241813	—	Jousson et al. (1999)
Opecoelidae gen. sp.	<i>Nassarius olivaceus</i> (Bruguière)	Queensland, Australia	—	KJ596417	—	Barnett et al. (2014)
<i>Opecoeloides fimbriatus</i> (Linton, 1934) Sogandares-Bernal et Hutton, 1959	<i>Micropogonias undulatus</i> (Linnaeus)	Gulf of Mexico	KJ001211	—	—	Andres et al. (2014)
<i>Opecoeloides furcatus</i> (Bremser in Rudolphi, 1819) Odhner, 1928	<i>Mullus surmuletus</i> (Linnaeus)	Corsica, France	AF151937	—	—	Tkach et al. (2000)
		Corsica, France	—	AJ241790	—	Jousson et al. (1999)
Opecoelus sp.	<i>Thalassoma jansenii</i> (Bleeker)	Great Barrier Reef, Australia	—	KJ596419	—	Barnett et al. (2014)
<i>Paropecoelus corneliae</i> Rohner et Cribb, 2013	<i>Parupeneus ciliatus</i> (Lacépède)	Off Lizard Island, Australia	—	KC357691	—	Rohner & Cribb (2013)
<i>Paropecoelus elongatus</i> (Ozaki, 1928) Pritchard, 1966	<i>Parupeneus ciliatus</i>	Off Lizard Island, Australia	—	KC357695	—	Rohner & Cribb (2013)
<i>Paropecoelus leonae</i> Rohner et Cribb, 2013	<i>Parupeneus ciliatus</i>	Off Lizard Island, Australia	—	KC357693	—	Rohner & Cribb (2013)
<i>Paropecoelus sogandaresi</i> Pritchard, 1966	<i>Parupeneus ciliatus</i>	Off Lizard Island, Australia	—	KC357697	—	Rohner & Cribb (2013)
<i>Poracanthium furcatum</i> Dollfus, 1948	<i>Mullus surmuletus</i>	Corsica, France	—	AJ241791	—	Jousson et al. (1999)
<i>Pseudopecoeloides engeleri</i> Rohner et Cribb, 2013	<i>Parupeneus ciliatus</i>	Off Lizard Island, Australia	—	KC357702	—	Rohner & Cribb (2013)
Pseudopecoeloides sp.	<i>Parupeneus ciliatus</i>	Off Lizard Island, Australia	—	KC357704	—	Rohner & Cribb (2013)
<i>Pseudopecoeloides tenuis</i> Yamaguti, 1940	<i>Priacanthus hamrur</i> (Forsskål)	New Caledonia	KU320605	—	—	Bray et al. (2016)
<i>Pseudopecoeloides vulgaris</i> (Manter, 1934) von Wicklen, 1946	<i>Sebastes</i> sp.	Off Simushir Island, North Pacific	MH161436	—	—	Sokolov et al. (2019)
Outgroup						
<i>Holsworthotrema enboubalichthys</i> Martin, Huston, Cutmore et Cribb, 2018	<i>Kyphosus cornelii</i> (Whitley)	Off Garden Island, Australia	MK052937	MK052934	—	Martin et al. (2019)
<i>Scorpidotrema longistipes</i> Aken'ova et Cribb, 2003	<i>Scorpis georgiana</i> Valenciennes	Point Peron, Australia	MK052936	MK052933	—	Martin et al. (2019)

**Table 2.** Morphometric data of *Coitocaecum* spp. from the present and previous studies.

Species	<i>Coitocaecum capense</i>	<i>Coitocaecum capense</i>	<i>Coitocaecum brayi</i> sp.n.	<i>Coitocaecum</i> sp.	<i>Coitocaecum tylogenium</i>
Host	<i>Clinus superciliatus</i>	<i>Clinus superciliatus</i> , <i>Clinus cotoides</i> , <i>Clinus rotundifrons</i> , <i>Cirrhibarbis capensis</i> , <i>Xenopoclinus kochi</i> , <i>Xenopoclinus leprosus</i>	<i>Clinus superciliatus</i>	<i>Clinus superciliatus</i>	<i>Centriscops humerosus</i>
Locality	TNP; Hermanus; Chintsa; Saldanha Bay, South Africa	Port Elizabeth and Oudekraal, SA	Saldanha Bay, SA	Hermanus, SA	Portobello, New Zealand
Source	Present study range (n = 13) mean	Bray (1987) range (n = 38) mean	Present study range (n = 12) mean	Present study range (n = 1) mean	Manter (1954) range (n = 1) mean
Body length	1882–3512 2631	960–2560 –	1008–2066 1582	2084 –	3204 –
Body width	352–845 578	280–830 –	309–461 395	510 –	868 –
Forebody length	650–1037 801	– –	346–565 448	429 –	980 –
Hindbody length	1232–2475 1830	– –	889–1501 1163	1655 –	– –
Body width:length ratio	1 : 3.7–5.4 1 : 4.6	– –	1 : 3.7–5.8 1 : 4.3	1 : 4.1 –	– –
Forebody length as % body length	27.5–35.7% 30.6%	28–33%	23.3–45.0% 28.8%	20.6% –	– –
Hindbody length as % body length	64.3–72.5% 68.7%	– –	67.8–88.2% 74.0%	79.4% –	– –
Oral sucker length	148–221 180	110–160 –	95–133 116	141 –	– –
Oral sucker width	133–191 155	100–150 –	94–131 114	125 –	– –
Prepharynx length	Absent –	Absent or very short –	11–32 20	Absent –	– –
Pharynx length	55–81 67	40–70 –	56–98 90	76 –	129 –
Pharynx width	63–107 82	40–80 –	63–90 77	83 –	137 –
Oesophagus length	129–231 168	30–160 –	86–188 131	170 –	– –
Ventral sucker length	184–286 236	150–270 –	181–261 216	282 –	– –
Ventral sucker width	116–289 193	200–280 –	177–292 234	240 –	455 –
Oral and ventral sucker length ratio	1 : 1.2–1.5 1 : 1.3	– –	1 : 1.7–2.3 1 : 1.9	1 : 2 –	– –
Oral and ventral sucker width ratio	1 : 1–1.6 1 : 1.3	1 : 1.6–2.0 –	1 : 1.5–2.4 1 : 2.1	1 : 1.9 –	1 : 1.8 –
Oral sucker and Pharynx length ratio	1 : 0.3–0.5 1 : 0.4	– –	1 : 0.5–0.8 1 : 0.7	1 : 0.5 –	– –
Genital pore to ventral sucker	379–616 468	190–520 –	161–293 228	238 –	– –
Ovary length	69–145 101	50–90 –	57–90 73	116 –	– –
Ovary width	135–300 210	150–260 –	103–209 149	208 –	– –
Egg length	37–52 45	45–51 –	40–62 56	48–49 48	53–57 –
Egg width	– –	– –	27–29 28	– –	30–32 –
Anterior testis length	106–236 161	50–130 –	113–180 142	126 –	– –
Anterior testis width	177–443 297	190–340 –	121–223 191	136 –	– –
Posterior testis length	114–248 185	60–160 –	121–239 183	108 –	– –
Posterior testis width	194–445 308	180–330 –	109–222 192	170 –	– –
Cirrus sac length	41–131 70	– –	62–107 82	78 –	152 –
Cirrus sac width	25–41 32	– –	– –	21 –	95 –
Seminal vesicle length	492–989 666	– –	Anterior portion 79–167 137 Posterior portion 99–188 143	244 –	– –
Seminal vesicle width	59–99 73	80–100 –	Anterior portion 35–67 51 Posterior portion 60–124 78	47 –	– –
Seminal receptacle length	48–100 61	– –	– –	– –	– –
Seminal receptacle width	39–73 71	– –	– –	– –	– –
Post-testicular field	617–1419 1013	– –	126–653 468	806 –	– –
Post-testicular field as % body length	32.8–42.7% 38.2%	– –	12.5–31.6% 29.6%	38.7% –	– –

Abbreviations: SA (South Africa); TNP (Tsitsikamma National Park).

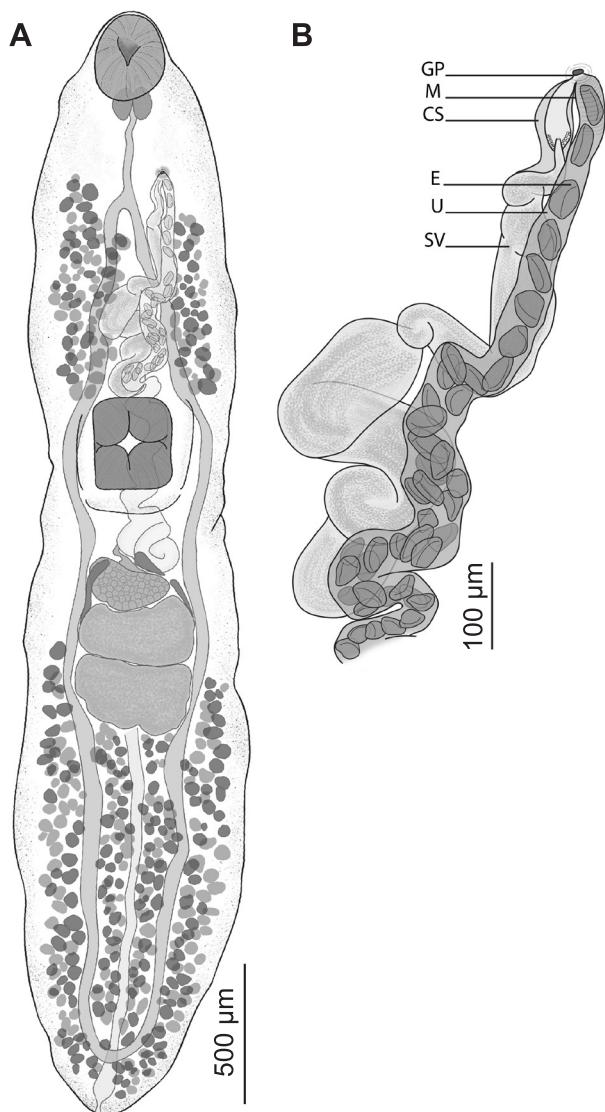
Monte Carlo (MCMC) chains were run for 3,000,000 generations. The ‘burn-in’ parameter was set for the first 25% of the sampled trees. Nodal support for the ML analyses was determined by conducting 100 bootstrap pseudo replicates. FigTree v. 1.4.4 (Rambaut 2012) software was used to visualise all phylogenetic trees and these trees were combined using Adobe Illustrator v. 26.4.1. Pairwise genetic distance matrices (p-distances) were calculated in MEGA v. X.

## RESULTS

### General observations

Of the 71 *Clinus superciliatus* collected from the Saldanha Bay, Hermanus, TNP and Chintsa East, 24 were infected with species of *Coitocaecum*. No trematodes were

present in this fish species collected from Cape Town harbour. Molecular and morphological analyses confirmed the presence of three distinct species of *Coitocaecum* based on the following generic characteristics: in all three species, the caeca form a cyclocoel, the cirrus sac is highly reduced, the eggs are operculate and lack filaments, and the ventral sucker lacks papillae (Cribb 2005). In his paper on the Opecoelidae from marine fishes of South Africa, Bray (1987) divided species of the genus *Coitocaecum* into five groups based on the distribution of the vitellarium and the posterior extent or position of the seminal vesicle. According to this classification, all three species of *Coitocaecum* in the present study belong to group E. Species within this group have interrupted vitelline follicles that reach into the



**Fig. 1.** *Coitocaecum capense* Bray, 1987 ex *Clinus superciliosus* (Linnaeus). **A** – ventral view; **B** – terminal genitalia. Abbreviations: CS – cirrus sac; E – egg; GP – genital pore; M – metraterm; SV – seminal vesicle; U – uterus.

forebody and a seminal vesicle that does not extend posterior to the ventral sucker (Bray 1987). *Coitocaecum tylogenium* Manter, 1954 described from the banded yellowfish *Centrisops humerosus* (Richardson) (Syngnathiformes: Centriscidae) off Portobello, New Zealand (Manter 1954) was the only representative of group E included by Bray (1987). Interestingly, we also note the co-occurrence of species of *Coitocaecum* in *C. superciliosus* from Hermanus and Saldanha Bay.

#### Morphological characterisation

Family Opecoelidae Ozaki, 1925

Subfamily Opecoelinae Ozaki, 1925

Genus *Coitocaecum* Nicoll, 1915

#### *Coitocaecum capense* Bray, 1987

Fig. 1

**Description** (based on 12 whole mounts; Table 2) Body elongate, tapered anteriorly, dorsoventrally flattened, slightly constricted at level of ventral sucker or posterior to ventral sucker, maximum body width in hindbody immediately posterior to testes; width to length ratio 1:3.7–5.4 (1:4.6). Forebody 27.5–35.7% (30.6%) of total body length. Tegument unarmed.

Oral sucker subspherical, subterminal. Prepharynx not observed. Pharynx muscular, transversely oval to subspherical, smaller than oral sucker, with few gland cells laterally. Oesophagus distinct, elongate, sinuous. Intestinal bifurcation in mid-level of forebody, surrounded by few large gland-cells. Intestinal caeca narrow, with distinct epithelial lining, pass ventral sucker and gonads laterally, form cyclocoel near posterior body extremity. Ventral sucker pre-equatorial, transversely oval, almost square, muscular, slightly protuberant, surrounded by folds of body tegument. 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). Oral sucker to pharynx length ratio 1:0.3–0.5 (1:0.4).

Testes two, irregularly lobed, intercaecal, tandem, contiguous with one another, in second third of body; anterior testis transversely-oval or sometimes triangular, contiguous with ovary; posterior testis transversely-oval; post-testicular field 32.8–42.7% (38.2%) of body length. Seminal vesicle elongate, tubular, convoluted, naked, extends to anterior margin of ventral sucker or slightly overlaps ventral sucker dorsally. Cirrus sac encloses anterior portion of seminal vesicle, *pars prostatica* and ejaculatory duct. Genital pore sinistral, close to mid-level of oesophagus.

Ovary transversely-oval, slightly lobed or smooth, intercaecal, median, pretesticular, contiguous with anterior testis. Mehlis' gland dextral, anterior to ovary. Seminal receptacle sinistral, anterior to ovary. Uterus with few loops, restricted to area between ovary and genital pore, dorsal to ventral sucker, contains numerous eggs, provided anteriorly with distinct, short, muscular metraterm. Eggs oval, operculate, yellow, translucent, without filament.

Vitellarium follicular; vitelline follicles numerous, interrupted in midbody, distributed anteriorly from level of genital pore to anterior margin of ventral sucker or sometimes to mid-level of ventral sucker, in two lateral fields, distributed posteriorly from mid-level of anterior testis to near posterior body extremity, in four fields, may overlap caeca and excretory vesicle; vitelline reservoir small, dorsal and anteromedian or anterosinistral to ovary.

Excretory vesicle straight, tubular, extends close to level of ovary. Excretory pore subterminal, opens dorsally, surrounded by small gland cells.

**Type host:** Super klipfish *Clinus superciliosus* (Linnaeus) (Clinidae).

**Type locality:** Gqeberha (as Port Elizabeth), Eastern Cape Province, South Africa.

**Other records:** None.

**New material:** New material: 51 (1–13, 3) specimens from 18 of 54 (33%) *C. superciliosus*; 1 of 19 (5%) from Saldanha Bay (-33.045683; 18.038628), Western Cape Province; 7 of 8 from Hermanus (-34.421072; 19.243767), Western Cape

Province; 9 of 16 (56%) from Tsitsikamma National Park (-34.020892; 23.878675), Eastern Cape Province; 1 of 11 (9%) from Chintsa East (-32.836539; 28.116997), Eastern Cape Province, South Africa.

**Site of infection:** intestine.

**Voucher material:** 16 voucher specimens deposited in NMB P 928–937 – 10 stained and permanently mounted specimens and NMB P 946 – 6 specimens in ethanol; 7 voucher specimens deposited in SMNH 218572–218577 – 6 stained and permanently mounted specimens and 218578 – 1 specimens in ethanol; 6 voucher specimens deposited in IPCAS D 860 – 5 stained and permanently mounted specimens and 1 specimens in ethanol.

**Representative DNA sequences:** OR129142–OR129145 (28S); OR125622, OR129261–OR129263, OR129269–OR129270 (ITS2); OR125617 (COI).

**Remarks.** Our specimens identified as *C. capense* agree well with those described by Bray (1987). However, our specimens are overall larger, and have lower minima for egg length. Specimens of *C. capense* from the present study are also distinct from *C. tylogenium* by being smaller and having a relatively smaller pharynx, ventral sucker, eggs and cirrus sac.

#### *Coitocaecum brayi* sp. n.

Fig. 2

Zoobank number for species:

[urn:lsid:zoobank.org:act:32DE7593-DBE2-49AC-911C-F6A93A8DB85F](https://doi.org/10.15468/zoobank.org:act:32DE7593-DBE2-49AC-911C-F6A93A8DB85F)

**Description** (based on 13 whole mounts; Table 2): Body elongate, dorsoventrally flattened, maximum width posterior to testes or occasionally at level of ventral sucker, posterior to ventral sucker or at level of testes. Forebody long, occupies 23.3–45.0% (28.8%) of total body length. Body width to length ratio 1:3.7–5.8 (1:4.3). Tegument unarmed.

Oral sucker subspherical, subterminal. Prepharynx indistinct, occasionally short. Pharynx large, elongate-oval to subspherical. Oesophagus long, thick-walled, sinuous. Intestinal bifurcation in posterior forebody. Caeca narrow, with distinct epithelial lining, pass ventral sucker dorsolaterally, form cyclocoel near posterior extremity of body. Ventral sucker pre-equatorial, transversely-oval, slightly protuberant, surrounded by folds of the body tegument. Oral sucker to ventral sucker length ratio 1:1.7–2.3 (1:1.9); width ratio 1:1.5–2.4 (1:2.1). Oral sucker to pharynx length ratio 1:0.5–0.8 (1:0.7).

Testes two, irregular, intercaecal, tandem, contiguous; anterior testis transversely oval, contiguous with ovary; posterior testis transversely oval, occasionally subspherical or triangular. Post-testicular field 12.5–31.6% (29.6%) of body length. Seminal vesicle naked, thick-walled, bipartite, both parts saccular to elongate-oval, extends posteriorly to anterior margin of ventral sucker or sometimes to mid-level of ventral sucker; anterior part elongate and tubular or saccular. Posterior part elongate or saccular, usually larger (longer and broader) than anterior part. Cirrus sac small, encloses small portion of anterior seminal vesicle, *pars prostatica* and ejaculatory duct. Genital pore sinistral, just posterior to pharynx or at posterior level of pharynx.

Ovary transversely oval, irregular, pretesticular or slightly overlaps anterior testis, median, intercaecal, contiguous with anterior testis. Mehlis' gland dextral, anterior to ovary, at level of or occasionally anterior to vitelline reservoir. Seminal receptacle observed in two specimens, sinistral, anterior to ovary. Uterus with few coils, restricted to area between ovary and genital pore, dorsal to ventral sucker, contains few eggs; metraterm not observed. Eggs oval, operculate, yellow, translucent, without filament.

Vitellarium follicular; vitelline follicles distributed in two lateral, interrupted fields, distributed from just posterior to pharynx to mid-level of ventral sucker in forebody, and throughout hindbody to posterior extremity, confluent in post-testicular field, overlap caeca and excretory vesicle. Vitelline reservoir small, median to slightly dextral, anterodorsal to ovary.

Excretory vesicle straight, tubular, extends to level of anterior testis, dorsal. Excretory pore subterminal, opens dorsally, surrounded by gland cells.

**Type host:** Super klipfish *Clinus superciliosus* (Clinidae).

**Type locality:** Saldanha Bay (-33.045683; 18.038628), Western Cape Province, South Africa.

**Site of infection:** intestine.

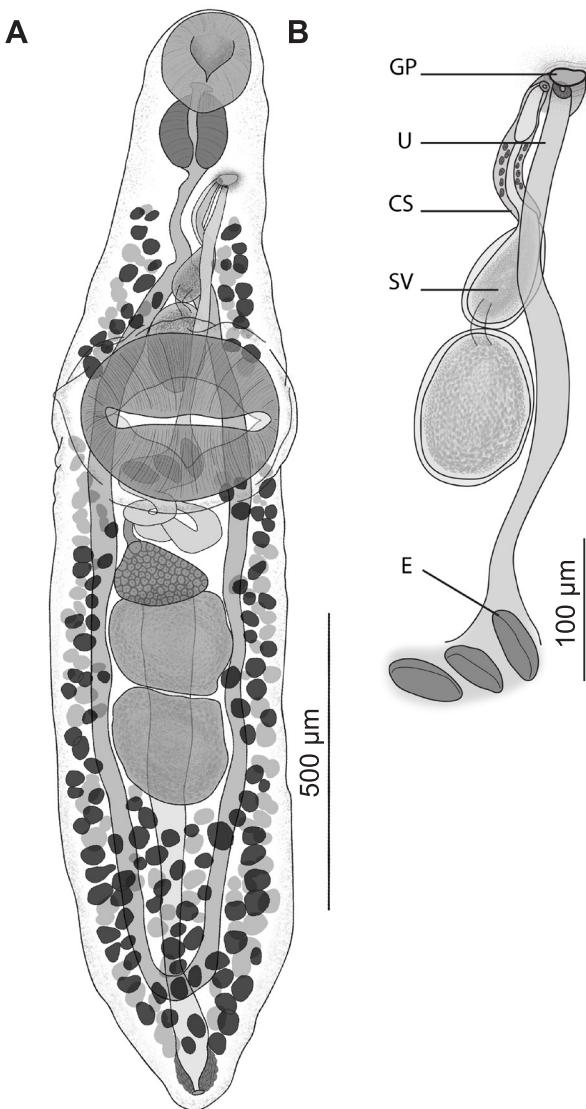
**Prevalence and intensity:** 5 of 19 (26%) fish were infected with 1–22 (7) specimens.

**Type material:** holotype and 17 voucher specimens deposited in NMB P 939–945 – 7 stained and permanently mounted specimens and NMB P 947 – 10 specimens in ethanol; 9 voucher specimens deposited in SMNH TYPE9760–TYPE9763 – 4 stained and permanently mounted specimens and TYPE–9764 – 5 specimens in ethanol; 6 voucher specimens deposited in IPCAS D 861 – 4 stained and permanently mounted specimens and 2 specimens in ethanol.

**Representative DNA sequences:** OR129277–OR129278 (28S), OR129265–OR129268 (ITS2).

**Etymology:** This species is named in honour of Rodney Bray of the Natural History Museum, London, who described the first species of *Coitocaecum* from South Africa and who greatly contributed to the knowledge on digenleans in South African marine environments and elsewhere.

**Remarks.** *Coitocaecum brayi* sp. n. is distinct from all previously described species of *Coitocaecum*, as well as others from the present study by possessing a bipartite seminal vesicle. Additionally, *C. brayi* differs from *C. capense* collected in the present study by having a smaller body, smaller oral and ventral suckers, greater size difference between oral and ventral suckers, distinguishable prepharynx in some specimens, greater difference between oral sucker and pharynx, a shorter and thicker-walled oesophagus, intestinal bifurcation in posterior vs anterior forebody, no discernible metraterm, smaller ovary and anterior testis, narrower posterior testis, larger eggs, genital pore situated closer to the ventral sucker, seminal vesicle occasionally extending to the mid-level of the ventral sucker, vitelline reservoir is median or slightly dextral, post-testicular hindbody is proportionally shorter, vitellarium is not distributed in four fields in hindbody, and the anterior extent of the vitellarium in both the fore- and hindbody is greater (just posterior to the pharynx and posterior to the ventral sucker).



**Fig. 2.** *Coitocaecum brayi* sp. n. ex *Clinus superciliatus* (Linnaeus). A—ventral view; B—terminal genitalia. Abbreviations: CS – cirrus sac; E – egg; GP – genital pore; M – metraterm; SV – seminal vesicle; U – uterus.

The body of *C. brayi* is much smaller than that of *C. tylogenium*, with a proportionally longer forebody, nearly half the body length. Additionally, *C. brayi* differs from *C. tylogenium* by having a smaller ovary, pharynx and suckers, as well as a greater size difference between suckers.

#### *Coitocaecum* sp.

**Description** (based on one whole mount; Table 2): Body elongate, dorsoventrally flattened, distinctly broad at level of ventral sucker, somewhat constricted immediately posterior to ventral sucker, maximum width in hindbody, at level of testes and posterior to them. Forebody short, occupies 20.6% of total body length. Body width to length ratio 1 : 4.1. Tegument unarmed.

Oral sucker subspherical, subterminal. Prepharynx not observed. Pharynx small, transversely oval. Oesophagus distinct, long. Intestinal bifurcation in posterior forebody. Caeca narrow, with distinct epithelial lining, pass ventral

sucker dorsolaterally, form cyclocoel near posterior extremity. Ventral sucker in first third of body, subspherical; surrounded by conspicuous tegumental fold. Oral sucker to ventral sucker length ratio 1 : 2; width ratio 1 : 1.9. Oral sucker to pharynx length ratio 1 : 0.5.

Testes two, intercaecal, obliquely tandem, irregular, not contiguous, in third quarter of body; anterior testis triangular, contiguous with ovary; posterior testis transversely oval. Post-testicular field represents 38.7% of body length. Seminal vesicle short, elongate, tubular, sinuous, naked, extends posteriorly to level of anterior margin of ventral sucker. Cirrus sac small, encloses small portion of seminal vesicle, *pars prostatica* and ejaculatory duct. Genital pore sinistral, at mid-level of forebody.

Ovary irregular, lobed, intercaecal, median, pretesticular, contiguous with anterior testis. Mehlis' gland not observed. Uterus with several loops, restricted to area between ovary and genital pore, dorsal to ventral sucker, contains numerous eggs, provided with distinct, short, muscular metraterm. Eggs oval, operculate, yellow, translucent, without filament.

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

Excretory vesicle straight, tubular, extends close to anterior level of ovary. Excretory pore subterminal, opens dorsally.

**Host:** Super klipfish *Clinus superciliatus* (Clinidae).

**Locality:** Hermanus (-34.421072; 19.243767), Western Cape Province, South Africa.

**Site of infection:** intestine.

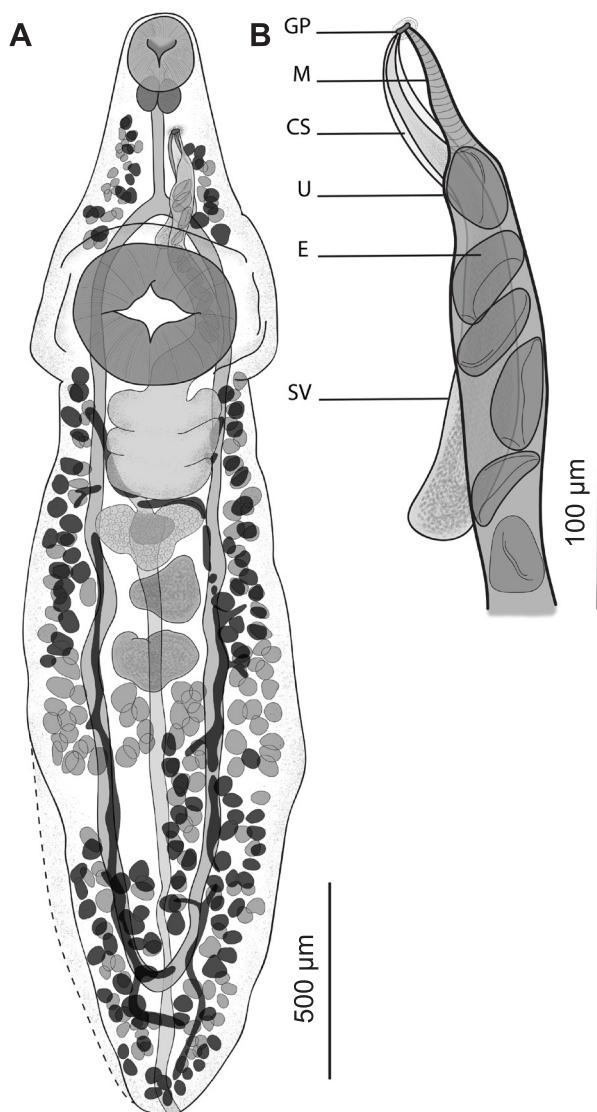
**Prevalence and intensity:** 1 of 8 fish were infected with 1 specimens.

**Voucher material:** 1 voucher specimens deposited in NMB P 938 – 1 stained and permanently mounted specimens.

**Representative DNA sequences:** OR129276 (28S), OR129264 (ITS2) OR125618 (COI).

**Remarks.** *Coitocaecum* sp. differs from *C. capense* in the present study by having a larger body, shorter forebody, genital pore closer to the ventral sucker, a greater sucker width ratio, smaller posterior testis, narrower cirrus sac and smaller seminal vesicle. Additionally, the oesophagus is not sinuous, intestinal bifurcation is in the anterior vs posterior forebody, the ventral sucker is subspherical, testes are irregular and not contiguous, the seminal vesicle is shorter, not convoluted and extends only to the posterior margin of the ventral sucker, ovary is irregular and lobed, and the anterior limit of the vitellarium in the fore- and hindbody extends higher (from the posterior limit of the pharynx and the posterior limit of the ventral sucker). This species also differs from *C. tylogenium* by being much smaller in size, and having a smaller pharynx, ventral sucker, eggs and cirrus sac.

#### Fig. 3



**Fig. 3.** *Coitocaecum* sp. ex *Clinus superciliosus* (Linnaeus). **A** – Ventral view; **B** – terminal genetalia. E – egg; CS – cirrus sac; GP – genital pore; M – metraterm; SV – seminal vesicle; U – uterus.

#### Molecular characterisation

The new 28S and ITS2 phylogenetic analyses produced phylogenograms with similar topology (Fig. 4). Novel sequences generated for the three species of *Coitocaecum* found in this study formed a strongly supported subclade within the subfamily Opecoelinae for the 28S dataset (Fig 4A). Interestingly, the sequences of *Coitocaecum* sp. clustered among isolates of *C. capense*. *Coitocaecum brayi* sp. n. formed a highly supported basal branch in this subclade. The overall interspecific divergence between species of *Coitocaecum* for the 28S dataset of the present study is 0.3–5.8% (4–70 nt). The intraspecific divergence between isolates of *C. capense* is 0–0.1% (0–1 nt). Sequences of *C. capense* differed from *Coitocaecum* sp. by 0.3–0.4% (4–5 nt). Sequences of *C. brayi* are identical but differed from *C. capense* by 5.5–5.6% (66–67 nt) and *Coitocaecum* sp. by 5.8% (70 nt).

Novel ITS2 sequences for species of *Coitocaecum* formed a highly supported subclade with representatives of the Opecoelinae, along with an unidentified/unde-

scribed species of *Coitocaecum* from *Hemigymnus fasciatus* (Bloch) (Labridae) and a species identified to the genus *Opecoelus* Ozaki, 1925 from *Thalassoma jansenii* (Bleeker) (Labridae) from Australia (Barnett et al. 2014) (Fig. 4B). These two species clustered close to isolates of *C. brayi*. The isolate of *Coitocaecum* sp. again clustered among isolates of *C. capense*. Interspecific divergence between isolates of *Coitocaecum* in this dataset is 0.7–13.7% (2–41 nt). Isolates of *C. capense* have a low intraspecific variability of 0–0.3% (0–1 nt), whereas isolates of *C. brayi* were identical. *Coitocaecum capense* differed from *Coitocaecum* sp. by 0.7–1.1% (2–3 nt), whereas *C. brayi* differed from *C. capense* by 12.8–13.7% (38–41 nt) and from *Coitocaecum* sp. by 12.8–13.4% (38–40 nt).

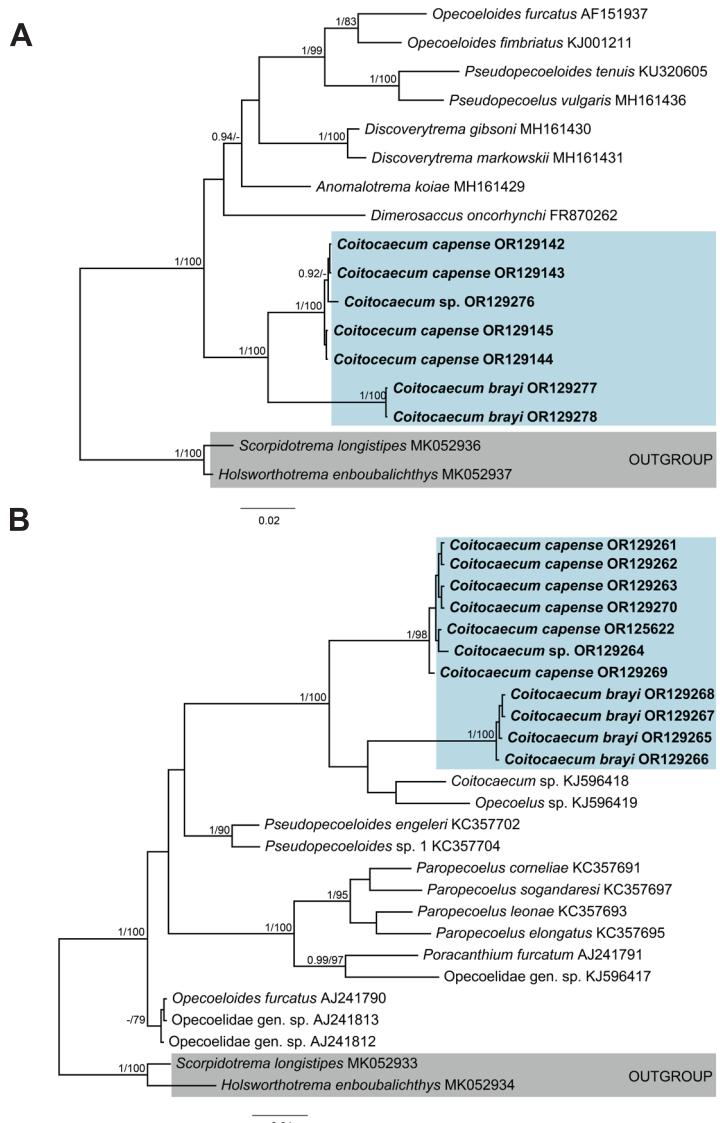
Analysis of the COI dataset showed that the single sequence of *C. capense* differs from that of *Coitocaecum* sp. by 14.9% (80 nt).

#### DISCUSSION

This study reports on the high diversity of trematodes belonging to the genus *Coitocaecum* infecting the clinid fish *Clinus superciliosus*. The known species *Coitocaecum capense*, previously described from this fish host, was found to co-occur with a novel species, *Coitocaecum brayi*, and an unnamed third species of *Coitocaecum*. Additional specimens are required for a detailed species description of the third species. Prior to this study, *C. capense* was the only species of this genus reported from South Africa (Bray 1987). According to Aken’Ova and Cribb (1996), many marine species of *Coitocaecum* normally have a narrow host specificity or infect ecologically similar hosts, emphasising the need for more explorative studies on intertidal digenleans from this region to expand our understanding of the zoogeography of this genus.

Although being morphologically distinct on account of numerous features, *C. capense* and *Coitocaecum* sp. are nested together in both the 28S and ITS2 phylogenies. However, they differ considerably (14.9% / 80 nt) based on the more variable mitochondrial COI gene. Martin et al. (2020a) observed similar results with two morphologically distinct species of the genus *Pseudoplagioporus* Yamaguti, 1938, where more conservative markers (18S, 28S and ITS) did not prove sufficient for species delineation, compared to the results of the COI gene. They considered the 63 nucleotide difference between the two species as interspecific (Martin et al. 2020a). The mitochondrial COI gene is generally a good marker to use, in combination with less variable ribosomal genes such as ITS2 and 28S, when characterising trematode species (Bray et al. 2022). The morphology of *C. capense* and *Coitocaecum* sp. is much too distinct to be considered as intraspecific variation. The high divergence between the COI genes of these isolates further strengthen the notion that these are two distinct species. This study once again highlights the importance of an integrated taxonomic approach and making use of various genetic markers for species distinction.

Our study provides the first molecular characterisation based on 28S rDNA, ITS2 rDNA and COI mtDNA sequences for species of *Coitocaecum* and the family Opecoelidae from South Africa. Furthermore, these new



**Fig. 4.** Bayesian inference (BI) tree based on the 28S rDNA (A) and ITS2 (B) datasets of the Opecoelinae. 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 a blue rectangle

discoveries from a previously examined fish species highlight the potential for biodiversity exploration of marine trematodes from this highly diverse coastal region.

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