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 digenean 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 exploratory study of the digeneans 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 digenean 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 reorganisation, 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). Clidid species are abundant 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;
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 ammonium (1%), gradually dehydrated with an ethanol series (70%, 80%, 90%, 96%, 100%), and then permanently mounted on a slide using Dammann 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 (µm), 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 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, 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 1 (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 D1g2 (5′-AAG CAT ACT ATC AAG CCG-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 GGG CCG TGT TCC AAG ACG GG-3′) (Tkach et al. 2003) and 300F (5′-CAA GTA CCG TGA GGG AAA GGT 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 GAG GAT CCT GGT-3′) (Galasso et al. 2002). The protocol of Galasso et al. (2002) was followed. The forward primer 3S (5′-GTT 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 CTC TTC 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 AAA ATC ATG ATG CAA AAGG-3′) (Koehler et al. 2011); thermostabilising 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 enbuctive and Scorpiodromes longistipes (Aken’Ova et al. 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.

Phylogenies 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/phyml/). For the BI analyses of both alignments, the Markov chain
Table 1. Sequences used for phylogenetic analyses of the Opecoelinae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Locality</th>
<th>GenBank accession numbers</th>
<th>Reference</th>
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<tr>
<td>Coitoecum capense Bray, 1987</td>
<td><em>Climus superciliosus</em> (Linnaeus)</td>
<td>Tsetsikamanna National Park, South Africa</td>
<td>OR129142 OR129261 –</td>
<td>Present study</td>
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<td>C. capense Bray, 1987</td>
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<td>Present study</td>
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<td>C. capense Bray, 1987</td>
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<td>Hermanus, South Africa</td>
<td>– OR129270 –</td>
<td>Present study</td>
</tr>
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<td>C. capense Bray, 1987</td>
<td><em>C. superciliosus</em></td>
<td>Saldanha Bay, South Africa</td>
<td>– OR129269 –</td>
<td>Present study</td>
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<td>Coitoecum sp.</td>
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<td>Hermanus, South Africa</td>
<td>OR129276 OR129264 OR125618</td>
<td>Present study</td>
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<td>Saldanha Bay, South Africa</td>
<td>OR129277 OR129265 –</td>
<td>Present study</td>
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<td>Saldanha Bay, South Africa</td>
<td>– OR129266 –</td>
<td>Present study</td>
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<td><em>C. superciliosus</em></td>
<td>Saldanha Bay, South Africa</td>
<td>– OR129267 –</td>
<td>Present study</td>
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<td>Coitoecum brayi</td>
<td><em>C. superciliosus</em></td>
<td>Saldanha Bay, South Africa</td>
<td>OR129278 OR129268 –</td>
<td>Present study</td>
</tr>
<tr>
<td>Anomalotrema koiae Gibson et Bray, 1984</td>
<td><em>Microankerushus fedorovi</em> (Mandrytsya)</td>
<td>Off Simushir Island, North Pacific</td>
<td>MH161429 – –</td>
<td>Sokolov et al. (2019)</td>
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<td>Coitoecum sp.</td>
<td><em>Hemigennus fasciatus</em> (Bloch)</td>
<td>–</td>
<td>–</td>
<td>Barnett et al. (2014)</td>
</tr>
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<td>Discoverytrema gibsoni Zdzislowicki, 1996</td>
<td><em>Muraenoleptis marmorata</em> Günther</td>
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<td>Opecoelidae gen. sp.</td>
<td><em>NASSARIUS OLIVACEUS</em> (BRUGUIERE)</td>
<td>Queensland, Australia</td>
<td>– KJ596417 –</td>
<td>Barnett et al. (2014)</td>
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<td>Opecoeloides fimbriatus (Linton, 1934)</td>
<td>Sogandares-Bernal et Hutton, 1959</td>
<td>Gulf of Mexico</td>
<td>KJ001211 – –</td>
<td>Andres et al. (2014)</td>
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<td>Opecoeloides furcatus (Bremer in Rudolph, 1819)</td>
<td><em>Mullus surmuletus</em> (Linnaeus)</td>
<td>Corsica, France</td>
<td>AF151937 – –</td>
<td>Tkach et al. (2000)</td>
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<td>Pseudopocoeoloides tenuis Yamauchi, 1940</td>
<td><em>Priacanthus hamrur</em> (Forsskål)</td>
<td>New Caledonia</td>
<td>KU320605 – –</td>
<td>Bray et al. (2016)</td>
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<td>Pseudopocoeoloides vulgaris (Manter, 1934)</td>
<td><em>Sebastes sp.</em></td>
<td>Simushir Island, North Pacific</td>
<td>MH161436 – –</td>
<td>Sokolov et al. (2019)</td>
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<td>Scorpidotrema longistipes Ak-en’Ova et Cribb, 2003</td>
<td><em>Scorpius georgiana</em> Valenciennes</td>
<td>Point Peron, Australia</td>
<td>MK052936 MK052933 –</td>
<td>Martin et al. (2019)</td>
</tr>
</tbody>
</table>
 presente 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

### RESULTS

#### General observations

Of the 71 *Clinus superciliosus* collected from the Saldanha Bay, Hermanus, TNP and Chintsa East, 24 were infected with species of *Coitocaecum*. No nematodes were
forebody and a seminal vesicle that does not extend posterior to the ventral sucker (Bray 1987). *Coitocaecum tylogonium* Manter, 1954 described from the banded yellowfish *Centriscops 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

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**Coitocaecum capense** Bray, 1987

![Fig. 1](image-url)

**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 subterminal. Prepharynx not observed. Pharynx muscular, transversely oval to subcircular, 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 Chintsha 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 specimen 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 *Capense* agree well with those described by Bray (1987). However, our specimens are overall larger, and have lower minima for egg length. Specimens of *Capense* from the present study are also distinct from *Cylogonomium* 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-D8E2-49AC-911C-F6A93A8DB85E

**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 subspheri- cal or triangular. Post-testicular field 12.5–31.6% (29.6%) of body length. Seminal vesicle naked, thick-walled, bipartite, both parts saclike 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 sacular. Posterior part elongated or sacular, usually 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.

Eggs prostrated from sternum of the bivalve host. In the 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, anterior to ovary.

Excretory vesicle straight, tubular, extends to level of posterior 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 TYPEP9760–TYPEP9763 – 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 digeneans 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).

**Voucher material:** 16 voucher specimens deposited in both the fore- and hindbody is greater (just posterior to the pharynx and posterior to the ventral sucker).
The body of *C. brayi* is much smaller than that of *C. tylogonium*, with a proportionally longer forebody, nearly half the body length. Additionally, *C. brayi* differs from *C. tylogonium* 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. Segument 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, protesticular, 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.

Vitellium 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, 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 superciliosus* (Linnaeus).

**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. tylogonium* by being much smaller in size, and having a smaller pharynx, ventral sucker, eggs and cirrus sac.

![Fig. 2. Coitocaecum brayi sp. n. 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.](image-url)
The known species 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 janseni (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 clind 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, empha-
sising the need for more explorative studies on intertidal digeneans 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 Pseudoplagiogiporus 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

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**Molecular characterisation**

The new 28S and ITS2 phylogenetic analyses produced phylogenograms with similar topology (Fig. 4). Novel sequenc-
es 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 n. formed a highly supported basal branch in this clade. 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-
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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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 bold and can be seen in a blue rectangle.

REFERENCES


