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Echinostomatids from South African freshwater limpets: phylogenetic analyses and diagnostic morphological features for cercariae of *Petasiger*

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Research Paper

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Abstract

Species of the family Echinostomatidae use diverse gastropod taxa as first intermediate hosts. However, identification of echinostomatid larvae often proves difficult because of incomplete information on their life cycles and lack of molecular data that can link larvae to the corresponding known adults. Here, echinostomatids that were isolated from freshwater limpets in South Africa were described using light and scanning electron microscopy, and ribosomal (28S, ITS, and 18S) and mitochondrial (*cox1*) DNA sequences. The analyses revealed three species: *Petasiger radiatus*, *Petasiger* sp., and Echinostomatidae gen. sp. Considering the close morphological resemblance between cercariae of *Petasiger* spp., the current species were compared with data from literature. The results showed that cercarial size is generally unsuitable for species discrimination. The numbers of flame cells and refractile granules in the excretory system, and penetration gland cell patterns, may indicate, but do not prove species identity. Although papillary patterns were distinct between species, papillae were clearly discernible only using scanning electron microscopy and are known for only a few species. Phylogenetic reconstruction indicated that 28S rDNA sequences of *Petasiger* on GenBank are for *P. exaeretus*, *P. phalacrocoracis*, *P. radiatus*, and six unnamed species. Furthermore, the results revealed that multiple ITS rDNA and *cox1* sequences labelled as *Stephanoprora amurensis* and *P. phalacrocoracis* on GenBank, are from isolates whose identities are questionable. Echinostomatidae gen. sp. could not be assigned to any currently known genus. Expansion of the genetic database of the family Echinostomatidae is necessary for the delineation of putative species and elucidation of intergeneric relationships.

Introduction

The family Echinostomatidae Looss, 1899 is composed of diverse digeneans that are globally distributed (Kostadinova, 2005; Laidemitt *et al.*, 2019; Pantoja *et al.*, 2021). Echinostomatids typically use molluscs as the first intermediate host, and the second intermediate hosts can be crustaceans, molluscs, amphibians, or fish, depending on the species (Tkach *et al.*, 2016; Toledo & Esteban, 2016). Adults of echinostomes have been reported from various vertebrates, with the highest diversity occurring in birds (Kostadinova & Jones, 2005; Tkach et al., 2016). Some echinostomatids belonging to *Artyfechinostomum*, *Echinostoma*, *Echinoparyphium*, *Hypoderaeum*, and *Isthmiophora* are intestinal parasites of humans who become infected by consuming raw or undercooked second intermediate hosts (Toledo & Fried, 2014; Toledo & Esteban, 2016). Echinostomatid infections in humans have been reported from several countries in Asia and Europe (Toledo & Fried, 2014). Although species of *Echinostoma*, *Echinoparyphium* and *Isthmiophora* occur in Africa (Bisseru, 1967; Appleton *et al.*, 1983; Toledo & Fried, 2014; Laidemitt *et al.*, 2019), reports of human echinostomiasis are very few from the continent. Indeed, data on the infections in humans are available only from Kenya, Tanzania, and Egypt. According to Poland *et al.* (1985), a group of American tourists who had visited Kenya and Tanzania were diagnosed with echinostomiasis. However, the species that caused the infections were not identified (Poland *et al.*, 1985). In Egypt, human echinostomiasis is attributed to *Echinostoma revolutum* (Fröhlich, 1802) and *Echinoparyphium recurvatum* (von Linstow, 1873) (Toledo & Fried, 2014). Considering the ecological importance and zoonotic potential of echinostomatids, they have been the subject of numerous investigations (Pinheiro *et al.*, 2004; Toledo & Esteban, 2016).

For many years, taxonomic knowledge of the family Echinostomatidae was based mainly on morphological characterisation of their adults (Kostadinova, 2005; Kostadinova & Jones, 2005). However, there has been considerable discussion on the morphological criteria used for species delimitation within Echinostomatidae, leading to revisions within the family (Pinheiro *et al.*, 2004; Kostadinova, 2005; Faltýnková *et al.*, 2008a; Tkach *et al.*, 2016). For instance, systematic relationships within the cosmopolitan genus *Petasiger* have been the subject of various studies.

Although Faltýnková *et al.* (2008a) recognised 18 *Petasiger* spp. following a comprehensive morphological study, phylogenetic analyses later inferred that *Petasiger* was polyphyletic. Thus, only 11 species: *Petasiger azerbaydjanicus* (Sailov, 1963); *Petasiger carbonis* (Mendheim, 1940); *Petasiger exaeretus* Dietz, 1909; *Petasiger lobulatus* Odhner, 1910; *Petasiger mexicanus* (Lamothe-Argumedo & Pérez-Ponce de León, 1989); *Petasiger parvicephalus* (Rietschel & Werding, 1978); *Petasiger phalacrocoracis* (Yamaguti, 1939); *Petasiger radiatus* (Dujardin, 1845); *Petasiger segregatus* (Dietz, 1909); *Petasiger testitrifolius* (Gogate, 1934); and *Petasiger variospinosus* (Odhner, 1910) were retained within the genus (Tkach *et al*., 2016). Unfortunately, molecular data are available only for three of the known species: *P. exaeretus*, *P. phalacrocoracis*, and *P. radiatus* (Tkach *et al*., 2016). In Africa, adult stages of *Petasiger* have been reported only for *P. phalacrocoracis*, *P. variospinosus*, and *P. radiatus*, from Tanzania, South Africa, and Zambia (Bisseru, 1957; King & Van As, 2000; Chibwana & Katandukila, 2021). Because of the paucity of studies on adult specimens and absence of molecular data for most *Petasiger* spp., knowledge on the actual diversity and phylogenetic relationships within the genus remain incomplete (Tkach *et al*., 2016; Laidemitt *et al*., 2019).

Similar to the adults, descriptions and identification of larvae of echinostomatids have largely been based on morphological characterisation. Unfortunately, identification of digeneans based on morphological descriptions of larvae alone often prove difficult or unreliable (Frandsen & Christensen, 1984; Laidemitt *et al*., 2019). For instance, the taxonomic positions of many echinostomes from Africa remain uncertain because they were described using cercarial morphology and given provisional names without the assignment of generic names (Cawston, 1923; Faust, 1926; Porter, 1938; Fain, 1953). In recent years, the incorporation of genetic data in studying intramolluscan stages of African echinostomes has proved beneficial for discriminating between morphotypes and providing information on their phylogenetic relationships (Laidemitt *et al*., 2019; Outa *et al*., 2020; Schols *et al*., 2020; Hammoud *et al*., 2022; Outa *et al*., 2024). On the other hand, comprehensive morphological data are lacking for most of those echinostomatids for which genetic data are available (Laidemitt *et al*., 2019; Schols *et al*., 2020; Hammoud *et al*., 2022). Therefore, it is difficult to compare them with the species from earlier studies that were classified in the place holder genus '*Cercaria*' (Cawston, 1923; Faust, 1926; Porter, 1938; Fain, 1953).

Herein, echinostomatids are reported from *Burnupia transvaalensis* (Craven, 1881), *Burnupia trapezoidea* (Boettger, 1910), and *Burnupia mooiensis* (Walker, 1912) collected from the Vaal River (Orange River System) and Crocodile River (Limpopo River system), in South Africa. Morphological characterisation of the echinostomes was based on light and scanning electron microscopy (SEM). There is a paucity of data on the ultrastructural features of digenean parthenitae and cercariae (Pinheiro *et al*., 2004; Outa & Avenant-Oldewage, 2024). Therefore, in addition to optical data, the current study intended to assess the suitability of using tegumental features (observable only via SEM) for the differentiation of rediae and cercariae of closely related echinostomes. Taxonomic status of the echinostomes from this study were established using 28S rDNA sequences. The 28S rDNA gene possesses both variable and conserved regions and is useful for establishing boundaries between species and genera of diverse trematode families (Blasco-Costa *et al*., 2016). Hence, the gene is the most widely used marker for inferring phylogenetic relationships between echinostomatids (Tkach *et al*., 2016; Laidemitt *et al*., 2019; Izrailskaia *et al*., 2021). Additional genetic characterisations of the specimens were done using fragments of the ITS1-5.8S-ITS2 and 18S rDNA regions, and mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene. This follows the recommendation by Blasco-Costa *et al.* (2016) for a multi-loci characterisation of digeneans, to explore both interspecific and intraspecific variations, and provide a comprehensive reference database for future studies. Also, generation of new ITS and *cox1* sequences allowed for the comparison of the echinostomatids from the present study with isolates from Zimbabwe (Schols *et al*. 2020; Mudavanhu *et al*., 2024), Kenya (Outa *et al*., 2020), Tanzania (Chibwana & Katandukila, 2021) and Uganda (Hammoud *et al*. 2022), for which there are no 28S sequences.

Material and methods

Snail sampling and morphological analyses of digeneans

As shown in Fig. 1, the study was conducted at four sites, two each from the Vaal River (26.872364 °S, 28.117173 °E and 26.734854 °S, 27.634372 °E) and Crocodile River (25.959696 °S, 27.855555 °E and 25.957086 °S, 27.858308 °E), in South Africa. Snail sampling was done in summer (February and March) of 2022 and 2023 and in autumn (May 2023). Snails were picked by hand from submerged rocks and macrophyte stems, placed in plastic buckets containing pebbles and water from the sampling sites, and transferred to an onsite field laboratory. Identification of the snails was based on morphological features (Craven, 1881; Walker, 1912; Connolly, 1939; Brown, 1994) and the cytochrome c oxidase subunit 1 mitochondrial gene (*cox*1). DNA sequences of the snails have been published elsewhere (Outa & Avenant-Oldewage, 2024). Isolation of digenean parthenitae and cercariae followed the procedures outlined by Frandsen and Christensen (1984). Freshly isolated specimens were studied in temporary mounts; stained with Nile blue or unstained (Outa & Avenant-Oldewage, 2024). A drawing tube was used to make illustrations of each morphotype, followed by digitisation on Corel DRAW Graphics Suite X6 software (Corel Corporation, Ottawa, Canada). The specimens from the temporary mounts (representing different morphotypes from different snails) were transferred into 2-mL Eppendorf tubes containing 96% ethanol, for DNA analyses. Representative specimens of each morphotype from different snails (where possible) were preserved in 70% ethanol for morphometric analyses and SEM. Morphometric data of rediae and cercariae were obtained using a Zeiss Axioplan 2 epifluorescence microscope fitted with AxioVision 4.3 imaging software (Göttingen, Germany). Rediae and cercariae of each morphotype were prepared for SEM following the procedures provided by Nation (1983) and Outa and Avenant-Oldewage (2024). The specimens were dehydrated in graded series of ethanol and hexamethyldisilazane (Merck, Darmstadt, Germany), mounted on adhesive conductive carbon tape fixed on glass microscope slides, and dried for 24 h in a Sanpla dry keeper desiccator cabinet (Kitaku, Osaka, Japan). Gold coatings were applied on the mounted specimens using an Emscope SC500 (Quorum Technologies, Newhaven, UK) and a Vega 3 LMH, Tescan (Brno, Czech Republic) SEM was used to examine the specimens at 6 kV.

Genetic and phylogenetic analyses

An E.Z.N.A. Tissue DNA Kit (Omega, Bio-tek, Inc, Georgia, USA) was used to extract genomic DNA based on the manufacturer' s instructions. For each digenean morphotype, DNA was obtained from individual specimens of rediae and pooled samples of 10 cercariae per snail. Genetic characterisation was based on analyses of

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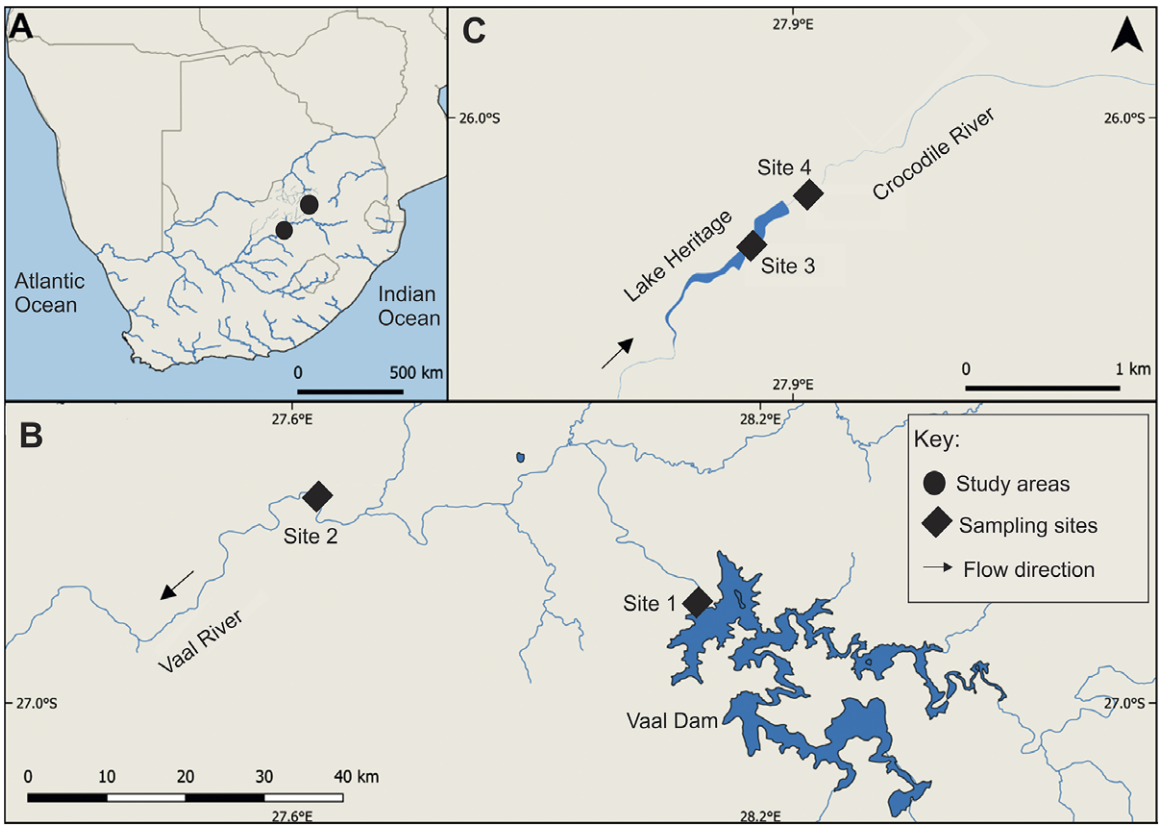

Figure 1. Map of the study area; adopted from Outa & Avenant-Oldewage (2024). A, Southern Africa; B, Vaal River; C, Crocodile River. Site 1: below the Vaal Dam (26.872364 °S, 28.117173 °E); site 2: below the Vaal River Barrage Reservoir (26.734854 °S, 27.634372 °E); site 3: Lake Heritage (25.959696 °S, 27.855555 °E); and site 4: below Lake Heritage (25.957086 °S, 27.858308 °E).

Table 1. Prevalence (%) of echinostomes in snails from the Vaal and Crocodile River systems

Sampling site	Host	Digenea	Prevalence
Vaal River, below Vaal Dam (S1)	*Burnupia transvaalensis*	*Petasiger radiatus*	0.85
		Petasiger sp. 3 ZA	2.03
Vaal River, below Vaal Barrage (S2)	*B. transvaalensis*	*Petasiger* sp. 3 ZA	0.76
	Burnupia mooiensis	Echinostomatidae gen. sp.	0.25
Lake Heritage, Crocodile River (S3)	*Burnupia trapezoidea*	*P. radiatus*	0.78
Crocodile River, below L. Heritage (S4)	*B. trapezoidea*	n.d.	

B. transvaalensis: S1, n = 590, S2, n =132; *B. mooiensis*: S2, n = 398; *B. trapezoidea*, S3, n = 128, S4 = 397; n.d., echinostomes not detected in the snails.

nuclear 18S, ITS and 28S rDNA, and *cox*1 gene. Polymerase chain reactions (PCRs) were performed in 30-µL volumes comprising 10 µL of DNA template, 3.8 µL of molecular grade water, 0.6 µL of each primer (forward and reverse), and 15 µL of Taq DNA Polymerase 2X Master Mix RED (Lasec) (Outa *et al.*, 2024). Nuclear 28S rDNA, primers dig12 (5′-AAGCATATCACTAAGCGG-3′) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) (Tkach *et al.*, 2003) were used, following the PCR conditions set by Outa *et al.* (2024). The Internal Transcribed Spacer (ITS) rDNA sequences consisting of ITS1-5.8S-ITS2 regions were amplified using BD1 (GTCGTAACAAGGTTTCCGTA) and BD2 (TATGCTTAAR TTCAGCGGGT) (Luton *et al.*, 1992), in accordance with the PCR profile provided by Luo *et al.* (2002). For 18S rDNA, amplification was done using primers JLR24 (5′-CGG AAT TCG CTA GAG GTG AAA TTC TTG G-3′) and JLR25 (5′-CCG AAT TCC GCA GGT TCA CCT ACG G-3′) (Campos *et al.*, 1998). The PCR profile (Mwita & Nkwengulila, 2010) was modified by increasing the annealing temperature to 50 °C. Fragments of *cox*1 were amplified using primers Dice1F (5′-ATTAACCCTCACTAAATTWCNTTRGATCATA AG-3′) and Dice14R (5′-TAATACGACTCACTATACCHACMRT AAACATATGATG-3′) following the PCR profile described by Van Steenkiste *et al.* (2015).

Successful amplification of the PCR products was verified visually in 1% agarose gel, loaded with Safeview FireRed (Applied Biological Materials) dye. Gel electrophoreses were performed by applying 80V in a SmartDoc 2.0 ultraviolet trans illuminator (Benchmark Scientific, NJ, USA) for 30 minutes. Dye-terminator sequencing (Applied Biosystems, Warrington, Cheshire, UK) was

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Table 2. List of cercariae of *Petasiger* spp. for which morphological descriptions are available, and their respective snail hosts and localities

Petasiger species	Reference	Snail host (s)	Locality
Petasiger sp. syn. *Cercaria bruynoghei* Fain, 1953	Fain, 1953	*Biomphalaria choanomphala* (Martens, 1879) and *Biomphalaria pfeifferi* (Krauss, 1848)	Congo (DRC)
Petasiger sp. syn. *Cercaria decora* Fain, 1953	Fain, 1953	*Bulinus natalensis* (Kuster, 1841) and *Bul. truncatus* (Audouin, 1827)	Congo (DRC)
Petasiger variospinosus (Odhner, 1910)	King & Van As, 2001	*Bulinus tropicus* (Krauss, 1848)	South Africa
***Petasiger radiatus* (Dujardin, 1845)**	Current study	*Burnupia transvaalensis* (Craven, 1880) and *Burnupia trapezoidea* (Boettger, 1910)	South Africa
Petasiger sp. 1 ZA syn Echinostomatidae sp.	Moema *et al*., 2008	*Radix natalensis* (Krauss, 1848)	South Africa
Petasiger sp. 2 ZA	Outa *et al*., 2024	*R. natalensis* and *Pseudosuccinea columella* (Say, 1817)	South Africa
***Petasiger* sp. 3 ZA**	Current study	*Bur. transvaalensis*	South Africa
Petasiger sp. 2	Laidemitt *et al*., 2019	*Bulinus globosus* (Morelet, 1866)	Kenya
Petasiger sp. 3	Laidemitt *et al*., 2019	*R. natalensis* and *Bulinus* sp.	Kenya
Petasiger sp. 4	Laidemitt *et al*., 2019	*Bi. pfeifferi* and *Biomphalaria sudanica (Martens, 1870)*	Kenya
Petasiger sp. 5	Laidemitt *et al*., 2019	*Bul. truncatus*, *Bul. globosus* and *Bulinus* sp.	Kenya
P. radiatus	Našincová *et al*., 1993	*Anisus leucostoma* (Millet, 1813), *Bathyomphalus contortus* (Linnaeus, 1758), *Gyraulus albus* (Müller, 1774), *Segmentina nitida* (Müller, 1774) and *Radix auricularia* (Linnaeus, 1758)	Czech Republic
Petasiger sp. (originally published as *Paryphostomum radiatum*)	Kiseliene, 1970	*Ampullaceana balthica* (Linnaeus, 1758) and *Planorbis planorbis* (Linnaeus, 1758)	Lithuania
Petasiger sp. (originally published as *Paryphostomum radiatum*)	Faltýnková *et al*., 2008b	*Anisus vortex* (Linnaeus, 1758), *G. albus* and *S. nitida*	Czech Republic
Petasiger segregatus (Dietz, 1909)	Lie & Basch, 1967	*Biomphalaria glabrata* (Say, 1818)	Brazil
Petasiger sp. syn. Echinocercaria III	Ostrowski de Núñez *et al*., 1991	*Biomphalaria occidentalis* Paraense, 1981	Argentina
Petasiger sp.	Fernández *et al*., 2016	*Bi. occidentalis*	Argentina
Petasiger sp.	Barton *et al*. 2022	*Isidorella hainesii* (Tryon, 1866)	Australia

Species from the current study are indicated in bold.

Table 3. Measurements (in μm) of rediae of *Petasiger* spp. from the current study (in bold) and previous studies, including species whose morphology correspond with *Petasiger* spp.

Measurement	*Cercaria bruynoghei*[a]	*Cercaria decora*[a]	*Petasiger segregatus*[b]	Echinocercaria III[c]	*Petasiger variospinosus*[d]	*Petasiger* sp.[e]	*Petasiger* sp. 2 ZA[f]	*Petasiger radiatus*[g]	*Petasiger radiatus*	*Petasiger* sp. 3 ZA
Body length	1000–1600	2600	750–3600	1300–2300	1808 (1412–2063)	1623 (1275–1995)	1623 (1255–2070)	1330–2650	1648 (1290–2343)	1362 (1154–1657)
Body width	150	280	112–395		232 (179–270)	247 (195–315)	361 (240–468)	220–300	305 (238–351)	308 (272–357)
Pharynx length	50			54–70	63 (54–67)	66 (51–99)	58 (47–62)	68–93	52 (46–57)	70 (63–76)
Pharynx width				54–60	47 (40–53)	64 (53–90)	41 (35–49)	60–75	49 (41–60)	55 (45–67)
Intestinal tube length	600	1400	400–2054		1055 (808–1260)	977 (705–1230)	1221 (932–1644)		1047 (850–1553)	751 (646–1001)
Collar from anterior			120–480		229 (200–264)		210 (107–353)		179 (131–247)	175 (147–228)
Procruscula from anterior					1184 (927–1375)		1100 (843–1425)		1115 (901–1610)	
Number of embryonic cercariae	11–20	20–25			1–9		14–17		6–9	5–8

[a]Fain (1953).
[b]Lie & Basch, 1967).
[c]Ostrowski de Núñez *et al*. (1991).
[d]King and Van As (2000).
[e]Fernández *et al*. (2016).
[f]Outa *et al*. (2024).
[g]Našincová *et al*. (1993).
Fixative/preservative: [a, c] and [g], 4% formaldehyde solution; [e, f] and current specimens 70% ethanol; [b] and [d], not stated.

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Table 4. Measurements (in μm) of cercariae of *Petasiger* spp. from the current study (in bold) and previous studies, including species whose morphology corresponds with *Petasiger* spp.

Measurement	*Cercaria bruynoghei*[a]	*Cercaria decora*[a]	*Petasiger segregatus*[b]	Echinocercaria III[c]	*Petasiger variospinosus*[d]	*Petasiger* sp. 1 ZA[e]	*Petasiger* sp.[f]	*Petasiger* sp.[g]	*Petasiger* sp. 2 ZA[h]	*Petasiger radiatus*[i]	*Petasiger radiatus*	*Petasiger* sp. 3 ZA
Body length	190	300–320	205–234	326 (270–440)	387 (300–440)	262 (232–292)	305 (290–320)	332 (255–380)	271 (240–311)	259 (233–277)	345 (301–397)	303 (273–327)
Body width	85	130–140	92–118	169 (120–280)	215 (172–265)	82 (52–99)	165 (150–190)		130 (110–152)	125 (110–145)	164 (137–188)	154 (136–169)
Oral sucker length	32–35	40–45	32–36	48 (40–70)	55 (37–68)	47 (36–60)	41 (37–46)	49 (40–60)	46 (38–50)	42 (37–47)	55 (44–59)	54 (49–59)
Oral sucker width			35–40	49 (40–70)	56 (37–67)	45 (26–67)	42 (39–44)		45 (39–53)	41 (37–46)	65 (58–71)	59 (54–62)
Prepharyngeal sac length					15 (11–21)	19 (16–24)	14 (11–17)		8.2 (7.5–9.1)		11 (8.2–12)	12 (9.0–14)
Prepharyngeal sac width					21 (16–26)	21 (14–25)	17 (14–21)		11 (10–12)		10 (8.9–12)	11 (8.7–12)
Prepharynx length		21	12.0–18		21 (15–25)	12 (9–15)	18 (14–21)		20 (15–24)		29 (24–36)	20 (19–21)
Pharynx length	17	17	12.0–15		30 (21–44)	18 (11–37)	18 (14–21)		27 (20–30)	17 (15–20)	28 (25–31)	26 (22–31)
Pharynx width	14				19 (14–23)	13 (10–15)	15 (14–18)		15 (13–18)	15 (13–17)	18 (14–22)	15 (13–18)
Oesophagus length			50–66		104 (66–154)	74 (45–82)					81 (73–93)	82 (78–87)
Ventral sucker (VS) length	38		37–48	57 (50–70)	67 (51–90)	50 (37–63)	44 (37–53)	70 (38–85)	50 (40–61)	55 (45–70)	81 (68–92)	74 (60–88)
VS width				57 (50–70)	75 (61–94)	55 (40–70)	54 (46–57)		56 (45–65)	61 (50–70)	92 (77–102)	89 (77–108)
VS from anterior end									155 (139–173)		180 (110–245)	162 (132–183)
Tail length	400	350	380–435	483 (360–600)	523 (397–669)	300 (297–306)	559 (520–610)	443 (385–500)	444 (376–514)	490 (422–548)	565 (453–649)	474 (427–542)
Tail width	35	45	34–40	49 (30–80)	63 (44–77)	37 (30–59)	52 (40–60)	44 (40–58)	49 (42–58)	45 (39–52)	58 (47–68)	61 (55–71)

[a]Fain (1953).
[b]Lie & Basch, 1967).
[c]Ostrowski de Núñez *et al.* (1991).
[d]King and Van As (2000).
[e]Moema *et al.* (2008).
[f]Fernández *et al.* (2016).
[g]Barton *et al.* (2022).
[h]Outa *et al.* (2024).
[i]Našincová *et al.* (1993).
Fixative/preservative: [a, b] and [c] – formaldehyde solution; [f, g, h] and current specimens 70% ethanol; [d], not stated and [e], live specimens.

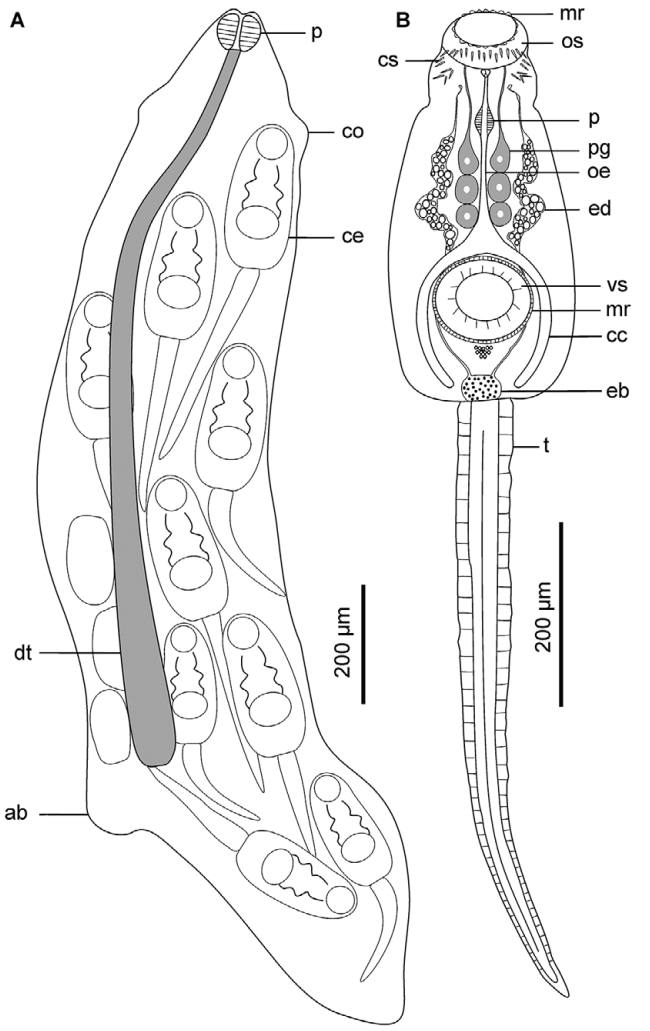

Figure 2. Schematic drawings of *Petasiger radiatus*. A, Redia and B, cercaria. Abbreviations: ab, ambulatory buds; cc, caecum; ce, cercaria; co, collar; cs, collar spines; dt, digestive tube; eb, excretory bladder; ed, main excretory duct; oe, oesophagus; os, oral sucker; mr, membranous rim; p, pharynx; pg, penetration gland cell; t, tail and vs, ventral sucker.

done using forward and reverse primers and the products were purified in an ABI 3137 automated sequencer (Applied Biosystems) (Avenant-Oldewage *et al.*, 2014). The forward and reverse sequences were visually inspected, trimmed, aligned and assembled using Geneious Prime 2023.0.1, following the guidelines provided by Kearse *et al.* (2012). To identify isolates with the closest similarities to the sequences generated in the current study, nucleotide searches were conducted on the GenBank database using the Basic Local Alignment Search Tool (BLASTn). Sequences of echinostomatids on GenBank with at least 50% query cover were downloaded and aligned with sequences from the present study using MUSCLE program on the MEGA7 software. The alignments were trimmed and genetic divergence was compared in accordance with the procedures outlined by Tamura *et al.* (2013). Lists of the sequences from GenBank that were compared with the current isolates are provided in Supplementary Tables S1-S4.

Phylogenetic trees were reconstructed using Bayesian inference (BI) and maximum likelihood (ML). The alignments that were used for phylogenetic analyses consisted of the sequences from the current study and representative sequences of echinostomatid genera, with species of the family Echinochasmidae as outgroup. In cases where multiple identical sequences of echinostomatids were available, only the sequences from adult worms (where present) or the longest sequences were included in the final alignments. Prior to the reconstructions, appropriate nucleotide substitution models were selected by running the final alignments through the model test tool in MEGA7. Accordingly, GTR+G (28S and ITS), JC +G+I (18S) and HKY+G (*cox*1) were applied. BI reconstructions were done in BEAST v2.5.0 (Bouckaert *et al.*, 2014) by applying 10 million Markov chain Monte Carlo analysis. Convergence and effective sample size were checked using Tracer v1.7.1 (Rambaut *et al.*, 2018) and the Maximum Clade Credibility tree (50% posterior probability limit) inferred using TreeAnnotator v2.5.0. The ML phylograms were reconstructed in MEGA7. In all reconstructions, five categories of discrete gamma (G) distribution were applied, and the reliability of the nodal support was tested using 1000 bootstrap replicates.

Results

Of the 1645 specimens of *Burnupia* spp. that were examined, 1.22% were infected with echinostomes. Three echinostomatids (*Petasiger radiatus* [Dujardin, 1845], *Petasiger* sp., and Echinostomatidae gen. sp.) were identified (Table 1). There was no co-occurrence of different digenean species in individual snails. Morphological descriptions of the specimens are provided below. The current *Petasiger* sp. has been designated *Petasiger* sp. 3 ZA, to distinguish it from two other *Petasiger* spp. that were reported from lymnaeid snails from South Africa (Moema *et al.*, 2008; Outa *et al.*, 2024); these are herein designated *Petasiger* sp. 1 ZA and *Petasiger* sp. 2 ZA (Table 2). Morphometric comparisons between the rediae (n = 10) and cercariae (n = 20) of the current *Petasiger* spp., with specimens of *Petasiger* from other studies are provided in Tables 3 and 4. For Echinostomatidae gen. sp., cercariae were not observed; hence, descriptions are based on rediae (n = 7) that were isolated from a single snail. All measurements are presented in micrometres as means, followed by the minimum and maximum values in parentheses.

Petasiger radiatus

Redia whitish to brown, elongated, slightly curved dorsally, contain 6–9 cercariae (Fig. 2A). Mouth surrounded by five rows of papillae, bearing short sensilla (Fig. 3B). Region between anterior extremity and collar has numerous spherical bodies and sparsely distributed papillae with long sensilla (Fig. 3C). Pharynx, nearly spherical; digestive tube dark brown, extends posteriorly from pharynx, runs ventrally, 64% (60%–69%) of body length (Fig. 2A). Collar bears four (dorsoventral and two lateral) inconspicuous processes. Birth pore slightly protruded (Fig. 3D), located on laterodorsal side of body, just posterior to collar. A pair of prominent ventral ambulatory buds (procruscula), located in posterior third of body.

Cercarial body elongate-oval, widest near middle part (Fig. 2B). Collar bears 27 spines. General body surface aspinous. Oral sucker oval-shaped, surrounded by tegumental membranous rim (Fig. 2A and 4B). Eight to nine rows of uniciliated papillae present on tegument of anterior end: three on rim of oral sucker (Fig. 4B), two on area between oral sucker and collar (Fig. 4C), 2–3 on collar and one posterior to collar (Fig. 5B). A pair of sub-apical multiciliated papillae (14–16 short cilia) present dorsolateral to oral sucker (Fig. 4D), cilia indistinct in some sensory receptors

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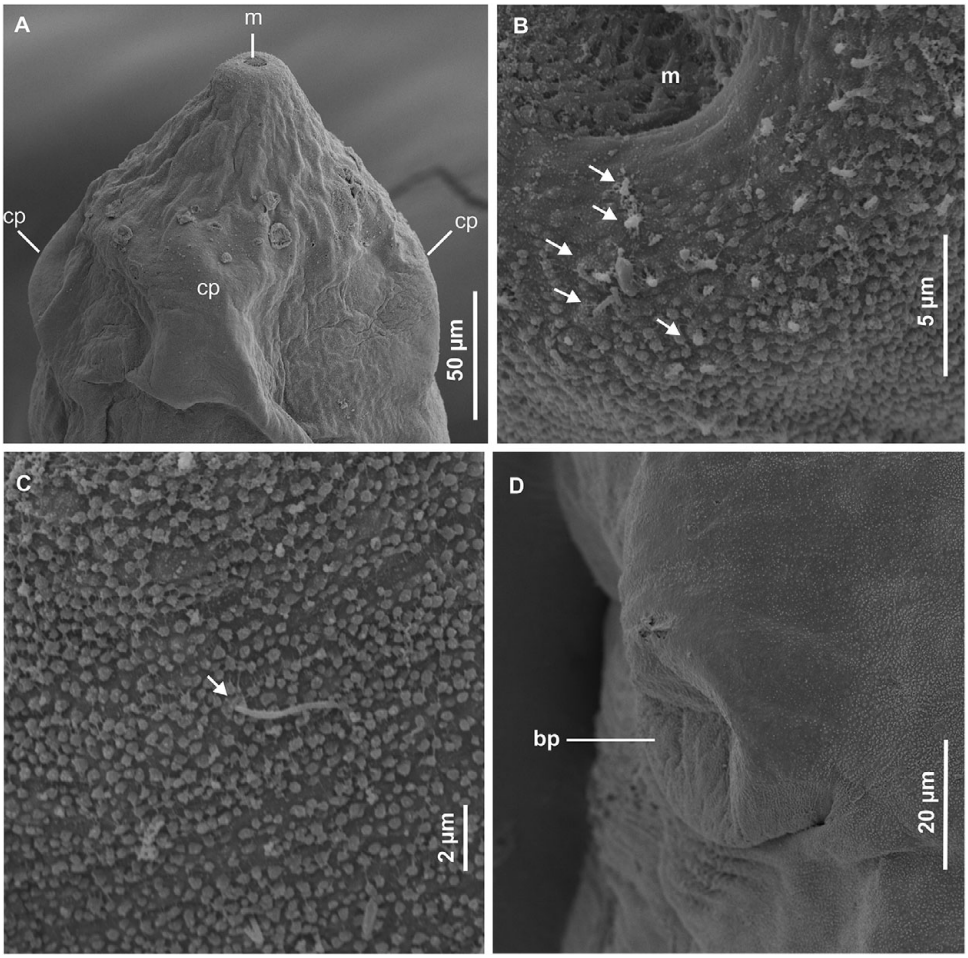

Figure 3. Scanning electron micrographs of redia of *Petasiger radiatus.* A, Lateral view of anterior end; B, rim of mouth; C, tegument structure on sub-apical end and D, birth pore. Arrows show uniciliated papillae. Abbreviations: bp, birth pore; cp, collar processes and m, mouth.

(Fig. 5A). Prepharynx characterised by a pair of prepharyngeal sacs immediately posterior to oral sucker. Pharynx ovoid; oesophagus long, bifurcates just anterior to ventral sucker; caeca terminate near posterior end of body (Fig. 2B). Six penetration gland cells present: three on each side of oesophagus. Ventral sucker post-equatorial, protrusible, transversely oval, larger than oral sucker, surrounded by tegumental membranous rim (Figs. 2B, 4A, and 5D). Genital primordium consists of an aggregation of cells posterior to ventral sucker. A pair of excretory ducts, each filled with 25–36 granules, extend anteriorly from excretory bladder (Fig. 2B). Tail simple, 1.7 (1.5–2.0) times longer than body; characterised by longitudinal furrow that extends from base and terminates near tip of tail. Tail tegument bears longitudinal rows of uniciliated papillae (Fig. 5C).

Petasiger *sp. 3 ZA*

Redia whitish to orange, elongated, contain 5–8 developed cercariae (Fig. 6A). Mouth surrounded by 5–6 rows of sensilla (Fig. 7B). Each lateral side of mouth bears three multiciliated papillae, each consisting of 4–6 short cilia (Fig. 7C). Sparsely distributed papillae, bearing long sensilla occur between apical end and collar (Fig. 7B). Pharynx ovoid; digestive tube dark brown to black, extends ventrally from pharynx to 64% (61%–66%) of body length (Fig. 6). Collar bears four (dorsoventral and two lateral) short processes. Birth pore dorsal, prominently protruded, just posterior to collar (Fig. 7A and D). A pair of prominent ambulatory buds located ventrally, 67% (63%–73%) from anterior extremity.

Cercarial body elongate-oval, widest near middle part; collar bears 27 spines (Fig. 6B). Entire body surface bears numerous minute spines, visible using SEM. Three rows of uniciliated papillae around oral sucker (Fig. 8B). A pair of sub-apical multiciliated papillae (18–22 cilia) present dorsolateral to oral sucker (Fig. 8B-E). Area between posterior margin of oral sucker and collar bears uniciliated papillae and unciliated pores (Fig. 8B-D). Lateral sides of body bear three rows of longitudinal uniciliated papillae. Oral sucker nearly spherical, surrounded by tegumental membranous rim (Figs. 6B and 8C). Prepharynx present, characterised by prepharyngeal sacs at posterior margin of oral sucker. Pharynx ovoid, oesophagus bifurcates into caeca at level of anterior margin of ventral sucker; each caecum terminates near posterior end of body (Fig. 6B). Penetration gland cells not clearly visible, appear to be five pairs along oesophagus. Ventral sucker post-equatorial, protrusible, transversely oval, larger than oral sucker (Figs. 6B, 8A, and 9D). Numerous cystogenous glands present, occurring from oesophageal region to posterior extremity. Secretions from glands visible (using SEM) on dorsal body surface on posterior part of some specimens (Fig. 9C). Two excretory ducts, filled with 30–42 granules, extend anteriorly from bladder towards pharyngeal region; flame cells pattern undiscernible. Tail, 1.5 (1.3–1.7) times longer than cercarial body, longitudinal furrow extends from tail base, terminates near tip. Tail tegument bears

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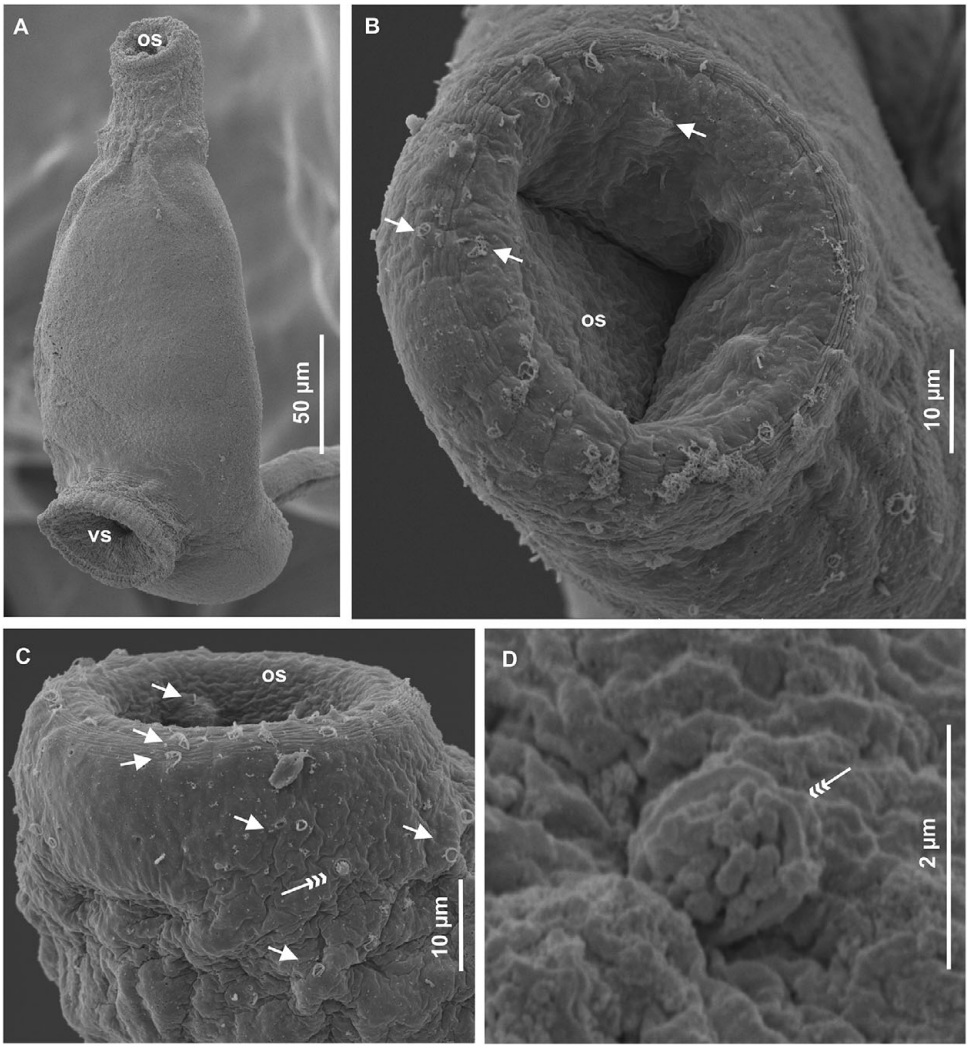

Figure 4. Scanning electron micrographs of cercaria of *Petasiger radiatus*. A, Ventral view of cercarial body; B, apical view of oral sucker; C, laterodorsal view of anterior end and D, close-up view of multiciliated papilla. Single arrows show uniciliated papillae and triple arrowheads show multiciliated papillae. Abbreviations: os, oral sucker and vs, ventral sucker.

numerous minute spines and longitudinal dorso-ventral rows of uniciliated papillae (Fig. 9E).

Remarks on rediae and cercariae of *Petasiger*

Redial morphological characteristics of the two species described previously: sensilla around the mouth, collar with processes, birth pore posterior to collar, conspicuous ambulatory buds in the posterior third of the body, correspond with species of the family Echinostomatidae (Pinheiro *et al.*, 2004; Keeler *et al.*, 2012; Outa *et al.*, 2024). Cercarial morphological features: collar with 27 spines, two prepharyngeal granular sacs located on the posterior margin of the oral sucker, post-equatorial ventral sucker, suckers surrounded by tegumental membranous rim (crista), presence of granules in the main excretory ducts and a simple tail without finfolds, correspond with the genus *Petasiger* Dietz, 1909 (Našincová *et al.*, 1993; Faltýnková *et al.*, 2008b; Fernández *et al.*, 2016; Outa *et al.*, 2024).

Redia of *Petasiger* sp. 3 ZA is distinguishable from *Pet. radiatus* by an ovoid pharynx and presence of multiciliated papillae around the oral aperture. The pharynx of *Pet. radiatus* is nearly round and multiciliated papillae were not observed. Cercaria of *Petasiger* sp. 3 ZA is distinguished by the presence of numerous tegumental spines on the body and tail and higher numbers of sensilla on the dorsolateral subapical papillae and penetration gland cells in the body, compared with *Pet. radiatus*. Morphological characteristics of the present *Petasiger* were compared with 16 cercarial morphotypes and redial data (where available) from 21 snail species in Africa, Europe, South America, and Australia (Table 2). This is inclusive of four echinostomatids whose cercarial features (collar with 27 spines, two prepharyngeal granular sacs on the posterior margin of the oral sucker, post-equatorial ventral sucker and presence of granules in the main excretory ducts) corresponds with *Petasiger*. They are: *Cercaria bruynoghei* and *Cercaria decora* (Fain, 1953), Echinocercaria III (Ostrowski de Núñez *et al.*, 1991) and Echinostomatidae sp. (Moema *et al.* (2008).

Rediae of different species are indistinguishable based on size due to overlap in body lengths between the species (Table 3). Rediae of the two species from the current study and *Pet. variospinosus* contain fewer cercariae compared with *Petasiger* sp. 2 ZA, *C. bruynoghei* and *C. decora* (Table 3). Differences were observed in the lengths of the intestinal tubes of some species. In *Petasiger* sp. from Argentina (Fernández *et al.* 2016) the intestinal tube extended only slightly into the posterior half of the body. For *Pet.*

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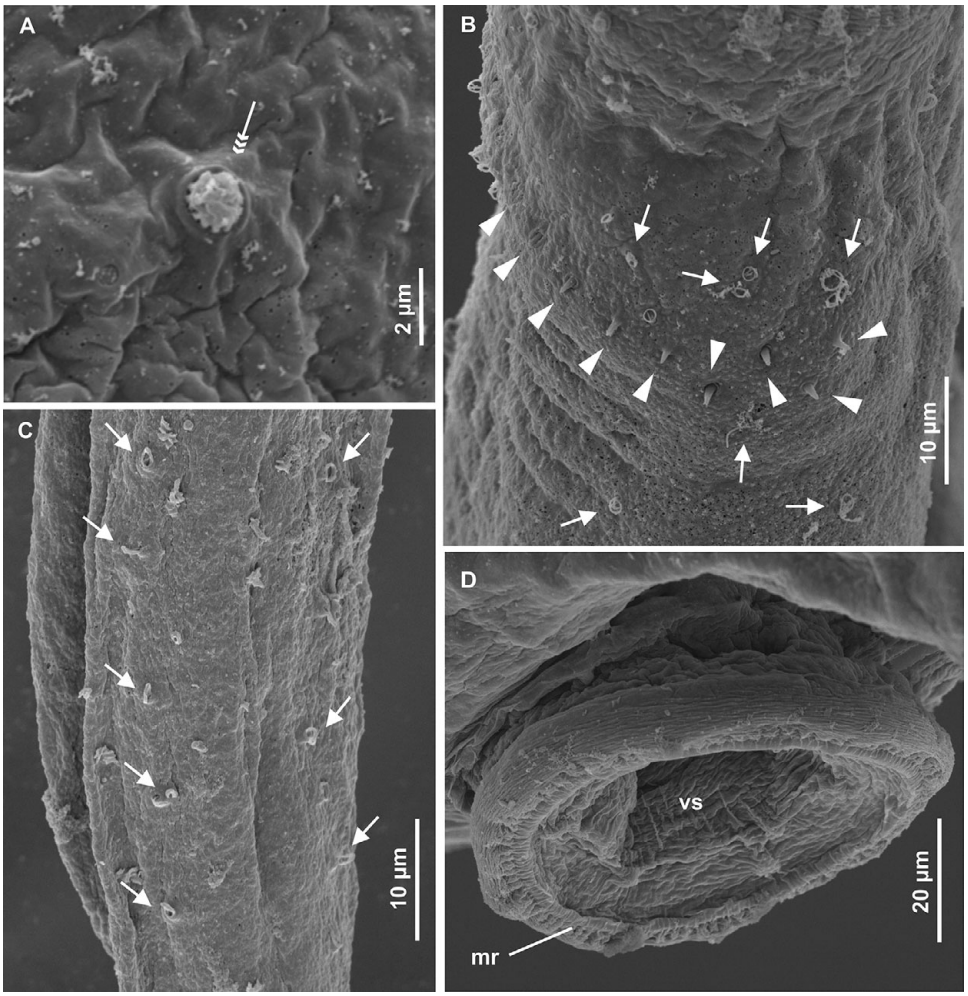

Figure 5. Scanning electron micrographs of cercaria of *Petasiger radiatus*. A, Close-up view of papilla with a cluster of indistinct cilia; B, lateral view of collar, showing spines and papillae; C, ventral side of mid-region of the tail stem and D, sub-ventral view of the ventral sucker. Single arrows show uniciliated papillae, triple arrowheads show multiciliated papilla and arrow heads without tails show collar spines. Abbreviation: vs, ventral sucker.

segregatus and the specimens in the current study, the intestine terminates in the posterior third of the body, just before the anterior margin of the ambulatory buds. In contrast, the intestinal tube extends to the level of the ambulatory buds in *Pet. variospinosus* (King & Van As, 2000) and terminates posterior to the ambulatory buds in *Petasiger* sp. 2 ZA (Outa *et al*., 2024). Also, rediae of *Petasiger* sp. 2 ZA were characterised by a distinct papilliform process at the posterior extremity of the body (Outa *et al*., 2024), while in the other species, the papilliform process was not apparent.

The length and width of cercarial body of *Petasiger* sp. 3 ZA were within the ranges of body dimensions of *Pet. radiatus* (current study) and four other species (Table 4). Only *C. bruynoghei* and *Pet. segregatus* were easily distinguished by their small sized bodies (Table 4). The morphology of *Pet. radiatus* cercaria from the current study is identical with the cercaria that was described by Našincová *et al.* (1993) following a complete life cycle study of *Pet. radiatus* in the Czech Republic. However, the current cercariae are bigger (Table 4). Two cercarial morphotypes from Europe that were putatively identified as *Paryphostomum radiatum* syn. *Pet. radiatus* (Kiseliene, 1970; Faltýnková *et al*., 2008b) show considerable distinctions from the current specimens and the one described by Našincová *et al.* (1993). The cercariae described by Kiseliene (1970) and Faltýnková *et al.* (2008b) were characterised by the presence of bifurcated excretory ducts in their tails. An excretory duct was not observed in the tail of the current cercaria nor in the specimens that were reported by Našincová *et al.* (1993). The cercaria reported by Faltýnková *et al.* (2008b) is further distinguished by at least 10 pairs of gland cells alongside the oesophagus. In contrast, the present specimens and those reported by Našincová *et al.* (1993) were characterised by only three pairs of penetration gland cells. Also, contrary to the current cercaria in which sensory hairs were not observed using light microscopy and SEM, the species reported by Kiseliene (1970) was characterised by tegumental sensilla that were visible using a light microscope. In this regard, the species described by Kiseliene (1970) resembles cercariae of *Pet. segregatus* (Lie & Basch, 1967) and *Petasiger* sp. (Fernández *et al*., 2016), both from South America, whose teguments are spinous.

The number of granules in each of the main excretory ducts of *Petasiger* sp. 3 ZA (30–42) is comparable with *Pet. radiatus* (25–36) (current study), *Pet. radiatus* (31–34) (Našincová *et al*., 1993), *Petasiger* sp. 2 ZA (27–38) (Outa *et al*., 2024), and *C. bruynoghei* (35) (Fain, 1953). These are distinct from other species which have fewer excretory granules, e.g. *Petasiger* sp. 2 (7–10), *Petasiger* sp. 4 (17) and *Petasiger* sp. 5 (19–20) (Laidemitt *et al*., 2019), *Pet. variospinosus* (19) (King & Van As, 2000) and *C. decora* (21) (Fain, 1953). *Petasiger* sp. 3 ZA is distinguished by five pairs

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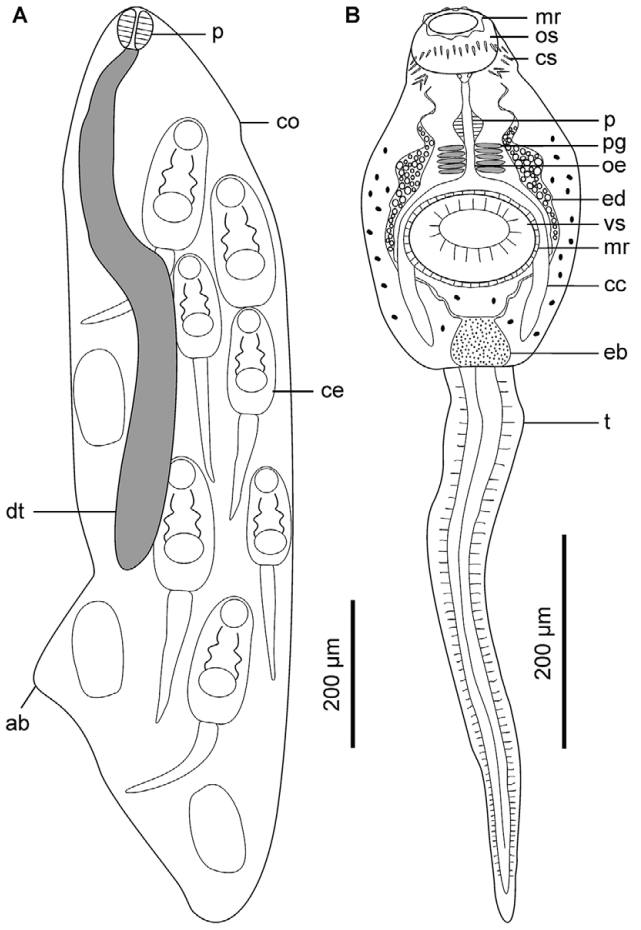

Figure 6. Schematic drawings of *Petasiger sp.* 3 ZA. A, Redia and B, cercaria. Abbreviations: am, ambulatory buds; cc, caecum; ce, cercaria; co, collar; cs, collar spines; dt, digestive tube; eb, excretory bladder; ed, main excretory duct; mr, membranous rim; oe, oesophagus; os, oral sucker; p, pharynx; t, pg, penetration gland cell; tail and vs, ventral sucker.

of gland cells along the oesophagus. Fewer penetration gland cells were observed in *Pet. radiatus* (three pairs) and more in *C. decora* (13 pairs). Penetration gland cells were not discernible in *Pet. segregatus*, Echinocercaria III, *Pet. variospinosus*, *Petasiger* sp., *Petasiger* sp. 1 ZA and *Petasiger* sp. 2 ZA (Lie & Basch, 1967; Ostrowski de Núñez *et al.*, 1991; King & Van As, 2000; Moema *et al.*, 2008; Fernández *et al.*, 2016; Outa *et al.*, 2024). The excretory systems of *C. decora*, Echinocercaria III, *Pet. variospinosus* and *Petasiger* sp. 1 ZA are characterised by 28 flame cells (Fain, 1953; Ostrowski de Núñez *et al.*, 1991; King & Van As, 2000; Moema *et al.*, 2008). This is higher than in *C. bruynoghei* (24) and lower than in *Pet. radiatus* (30). Flame cell patterns were not clearly discernible in *Pet. segregatus* (Lie & Basch, 1967), *Petasiger* sp. (Fernández *et al.*, 2016), *Petasiger* sp. 2 ZA (Outa *et al.*, 2024), and *Petasiger* sp. 3 ZA (current study). Data for penetration gland cells and flame cells patterns are not available for the *Petasiger* spp. from Kenya (Laidemitt *et al.*, 2019).

Apart from the current study, cercariae of only three other *Petasiger* spp. have been studied using SEM (King & Van As, 2000; Moema *et al.*, 2008; Outa *et al.*, 2024). *Petasiger variospinosus* is characterised by several short and long ciliated receptors surrounding the oral sucker, various groups of multiciliated papillae (3–23 short cilia) present dorsolateral to oral sucker and uniciliated papillae arranged bilaterally on both sides of the tail (King & Van As, 2000). *Petasiger* sp. 1 ZA is distinguished by multiple clusters of 6–12 short cilia surrounding the oral sucker (Figure 3G, Moema *et al.*, 2008). Cercaria of *Petasiger* sp. 2 ZA is distinguished by two subapical papillae with few sensilla (up to four) and minute spines, scattered on the rest of the body (Outa *et al.*, 2024). *Petasiger* sp. 3 ZA is characterised by numerous spines on the body and tail, and one pair of multiciliated papillae (18–22 cilia) on anterior end and numerous uniciliated papillae on the tail. *Petasiger radiatus* is distinguished by an aspinous tegument and a pair of anterior multiciliated papillae, each bearing 14–16 cilia.

Echinostomatidae gen. sp.

Redia orange, slightly curved dorsad (Fig. 10A and B), 1065 (915–1188) long, 241 (224–251) wide. Oral aperture surrounded by 6–7 rows of sensilla (Fig. 10C); lateral sides bear a pair of multiciliated papillae, each bearing 4–8 sensilla (Fig. 10D). Pharynx muscular, 52 (50–54) long, 47 (42–55) wide. Digestive tube dark brown to black, 511 (406–598) long, extends posteriorly, 53% (50%–55%) from anterior end. Collar, 149 (122–178) from the anterior end. Birth pore situated in pouch-like structure, on dorsal side just posterior to collar (Fig. 10B and E). Collar processes not observed; a pair of slightly protruded ambulatory buds located ventrally, 61% (58%–63%) from anterior end. Redia of this species is distinguished from *Petasiger* spp. by its short digestive tube (about half the body length) and a birth pore that is not elevated. What is more, the anterior end bears a pair of multiciliated papillae, contrary to three pairs in *Petasiger* sp. 3 ZA and *Pet. radiatus* in which multiciliated papillae were not observed.

Molecular and phylogenetic data

Usable rDNA sequences were obtained from seven, nine, and four isolates of *Pet. radiatus*, *Petasiger* sp. 3 ZA, and Echinostomatidae gen. sp., respectively. The newly generated sequences were 1214–1253, 1017–1029, and 871–898 bp for 28S, ITS, and 18S rDNA, respectively. The sequences have been submitted to GenBank: accession numbers PP738959-PP738964 (28S), PP738869-PP738871 (ITS), and PP738680- PP738682 (18S).

The 28S rDNA intraspecific variations for the sequences generated in the current study did not exceed 1 bp, corresponding to a p-distance of 0.1%. The 28S base pair differences and corresponding p-distances between the current sequences and other echinostomatids are shown in Supplementary Table S1. The p-distances between the current isolates of *Pet. radiatus* and sequences of *Pet. radiatus* obtained from adult worms (Tkach *et al.*, 2016; Cech *et al.*, 2017), ranged between 0% and 0.4%. The low variation between *Pet. radiatus* haplotypes was comparable to intraspecific variations between *Pet. exaeretus* isolates (0%–0.3%) published by Tkach *et al.* (2016) and Cech *et al.* (2017). *Petasiger* sp. 3 ZA sequences differed from *Pet. radiatus* by p-distances of 1.2%–1.3%. *Petasiger* sp. 3 ZA showed the highest similarity (99.3%–99.4%) with cercaria of *Petasiger* sp. 5 from *Bulinus globosus* (Morelet, 1866) from Kenya (Laidemitt *et al.*, 2019). *Petasiger* sp. 2 ZA (Outa *et al.*, 2024) varied from *Petasiger* sp. 3 ZA and *Pet. radiatus* by p-distances of 1.4–1.5 and 0.6–0.7 %, respectively. Echinostomatidae gen. sp. (current study) varied from other echinostomatids by p-distance ranges of 2.3%–5.7% (Supplementary Table S1). The 28S phylograms, consisting of 54 sequences of echinostomatids (1159–1170 bp), demonstrated that sequences of *Petasiger* occurred in 10 subclades (A–J) (Figs. 11 and 12). *Petasiger* spp. from South Africa clustered in three

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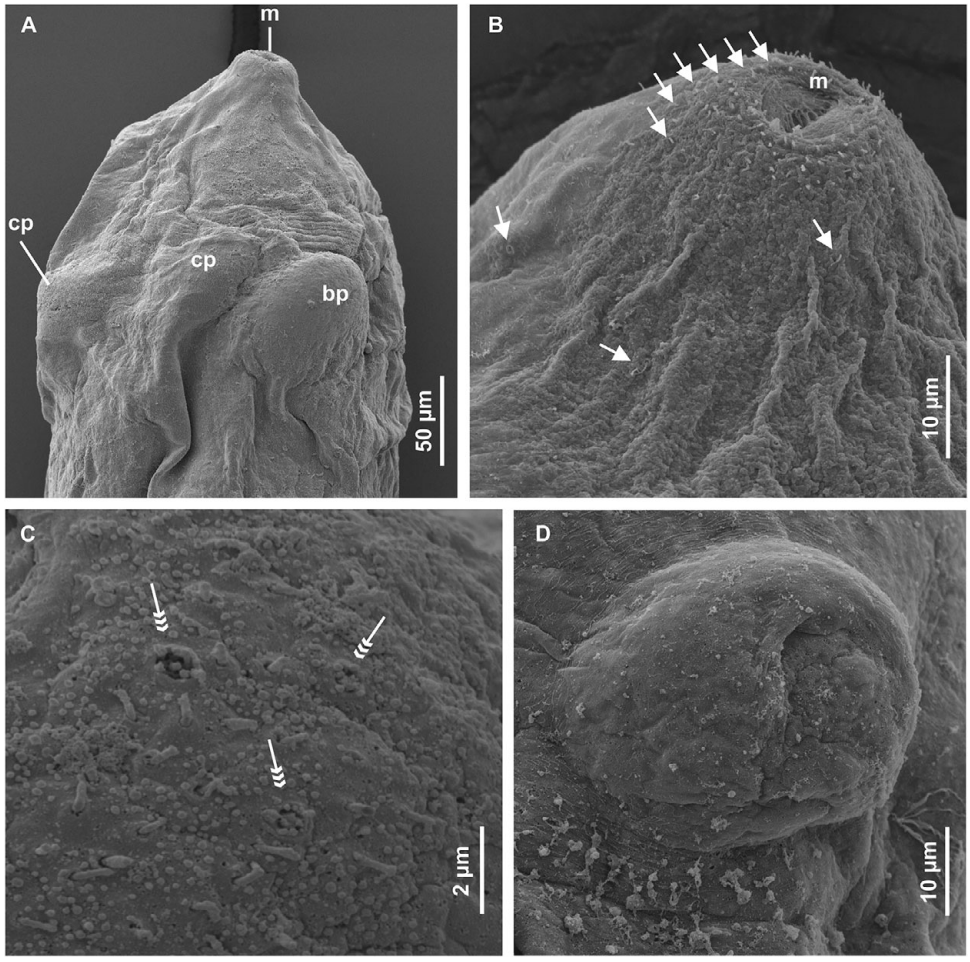

Figure 7. Scanning electron micrographs of redia of *Petasiger sp.* 3 ZA. A, Dorsal view of anterior end; B, lateral view of apical end; C, papillae on lateral side of mouth and D, enface view of protrusion bearing birth pore. Single arrows show uniciliated papillae and triple arrow heads show multiciliated papillae. Abbreviations: bp, birth pore; cp, collar processes and m, mouth.

separate subclades (A, C, and D). *Petasiger radiatus* sequences (subclade A) were monophyletic with cercarial isolates of *Petasiger* sp. 4 from Kenya and *Petasiger* sp. from Australia (subclade B). However, the branching between clades A and B was poorly supported (0.45) in the BI tree (Fig. 11). *Petasiger* sp. 2 ZA clustered with *Petasiger* sp. 3 from Kenya and *Petasiger* sp. from Hungary in subclade C. *Petasiger* sp. 3 ZA-*Petasiger* sp. 5 clade was basal to A, B, and C. In both the BI and ML phylograms, cercarial isolate of *Petasiger* sp. 1 from Kenya formed a strongly supported subclade (J) with *Pegosomum* sequences that were obtained from adult worms. *Petasiger exaeretus* sequences were sister to the subclade comprising *Pegosomum* and *Petasiger* sp. 1. The positions of *Petasiger* sp. 2 from Kenya and sequences of *Isthmiophora* did not resolve clearly between the ML and BI frameworks. In the BI tree, *Petasiger* sp. 2 formed a poorly supported branch that was basal to the clade comprising of *Pet. phalacrocoracis* and *Petasiger* sp. 6. Also, *Isthmiophora* was sister to the *Petasiger-Pegosomum* clade (Fig. 11). In ML, *Petasiger* sp. 2 was sister to sequences of *Isthmiophora* in a weakly supported subclade (H), which was nested within the larger *Petasiger* clade (Fig. 12). Four species from Germany (KM191799- KM191807) whose cercariae were 19-spined and large tailed and were initially thought to belong to *Petasiger* (Selbach *et al*., 2014), clustered with *Neopetasiger* sequences. Echinostomatidae gen. sp. was positioned in a poorly supported clade containing sequences of *Drepanocephalus*, *Chaunocephalus* and *Neopetasiger* (Figs. 11 & 12).

The ITS rDNA sequences for each species were identical. Sequence divergence (%) and nucleotide substitutions between the current isolates and echinostomatids from GenBank are shown in Supplementary Table S2. Sequences of *Pet. radiatus* from the current study were 99.9%–100% identical with sequences of adult *Pet. radiatus* from cormorants from Israel (Dzikowski *et al*., 2004) and metacercariae from fish in Hungary (Molnar *et al*., 2015). Also, the isolates from the present study showed a close relationship (98.8%–99% similarity) with cercarial isolates from *Bi. sudanica* in Kenya (Outa *et al*., 2020) and *Isi. hainesii* from Australia (Barton *et al*., 2022). *Petasiger* sp. 3 ZA differed from *Pet. radiatus* by p-distances of 5.3%–5.4%. The genetic distance between *Petasiger* sp. 2 ZA from South African lymnaeid snails (Outa *et al*., 2024) and the current *Petasiger* spp. ranged between 4.8% and 5.3%. Interestingly, *Petasiger* sp. 3 ZA had the highest similarity (98.8%–98.9%) with sequences from Tanzania (MZ412883) (Chibwana & Katandukila, 2021) and Zimbabwe (PP564877) (Mudavanhu *et al*., 2024) that were published as *Stephanoprora amurensis* Tatonova, Izrailskaia & Besprozvannykh, 2020 (Echinochasmidae). However, as shown in the supplementary Table S2 and Fig. 13, the two were distant (p-distance = 16%–19%) from other echinochasmid sequences. Therefore, the designation of MZ412883 and

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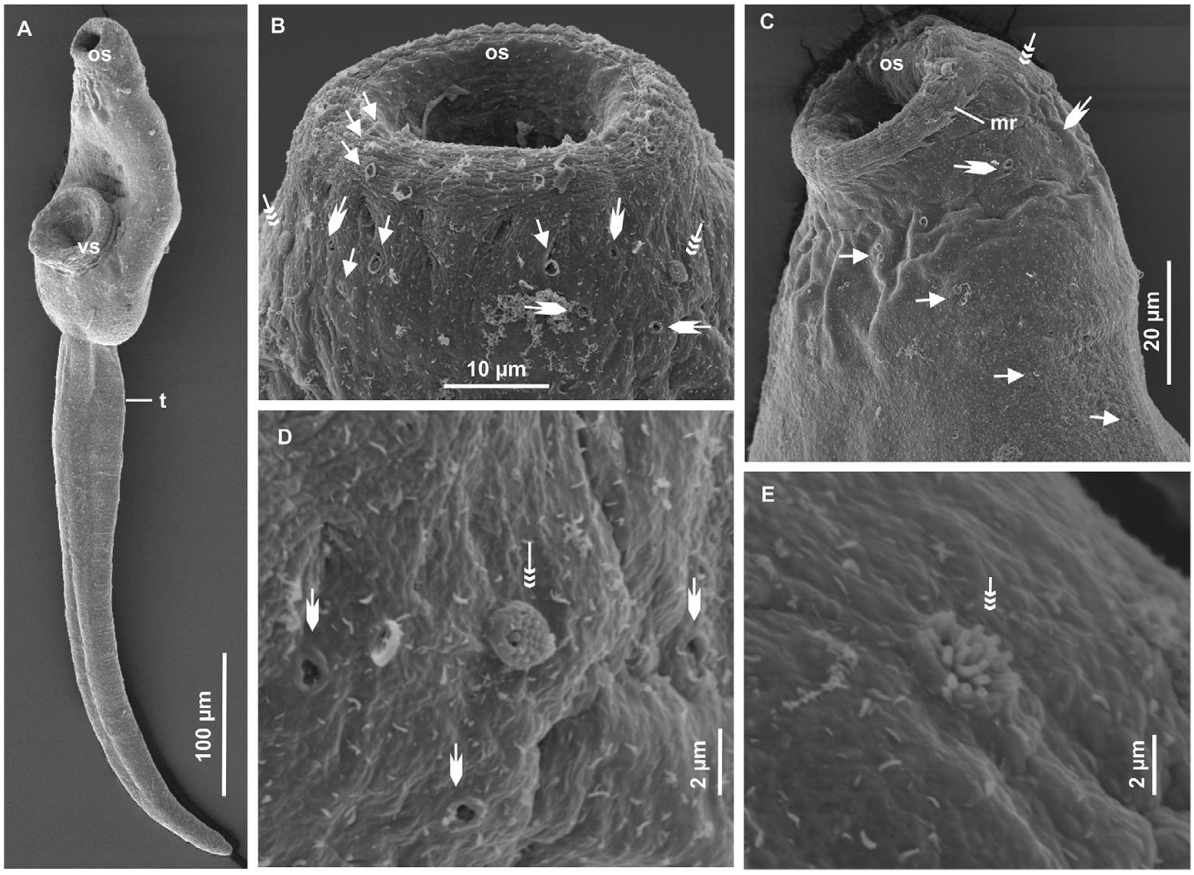

Figure 8. Scanning electron micrographs of cercaria of *Petasiger sp.* 3 ZA. A, Lateroventral view of cercaria; B, dorsal view of anterior end; C, lateral view of anterior end; D and E, close up view of multiciliated papillae on dorsolateral side of anterior end. Single arrows show uniciliated papillae, winged arrowheads indicate unciliated pores and triple arrowheads show multiciliated papillae. Abbreviations: mr, membranous rim; os, oral sucker; t, tail and vs, ventral sucker.

PP564877 as *S. amurensis* (Chibwana & Katandukila, 2021; Mudavanhu *et al.*, 2024) were erroneous. The p-distances between Echinostomatidae gen. sp. and *Petasiger* spp. ranged from 4.9% to 8.4% (Supplementary Table S2). ITS rDNA phylogenetic analyses of 39 isolates of echinostomatids (988–1042 bp) revealed that *Petasiger* spp. grouped in seven strongly supported subclades (Fig. 13). *Petasiger radiatus* from the present study clustered with three other sequences of *Pet. radiatus* (subclade A), and the four were sister to subclade B comprising of cercariae of *Petasiger* from Kenya (and Australia. *Petasiger phalacrocoracis* sequences formed a single cluster (C) that was basal to D, E, and F. *Petasiger* sp. 2 ZA from South Africa clustered with sequences of unnamed *Petasiger* from Hungary and Australia (subclade D). Subclade D was sister to the clade comprising *Petasiger* sp. 3 ZA and sequences from Tanzania and Zimbabwe which were incorrectly identified as *S. amurensis*. Sequences of *Isthmiophora hortensis* (Asada, 1926) and *Isthmiophora melis* (Schrank, 1788) (subclade G) were nested within the *Petasiger* clade. Echinostomatidae gen. sp. (subclade J) was monophyletic with sequences of *Rhopalias* in a moderately supported (0.70%) clade (fig. 13).

The 18S sequences for each species from the current study were identical. Until now, there were only four 18S rDNA sequences for *Petasiger* on GenBank, representing *Pet. radiatus*, *Pet. Phalacrocoracis*, and an unnamed species. Genetic distances were very low (0%–1.1%) between the present isolates and the representative sequences of *Petasiger* from GenBank. Consequently, interspecific boundaries were not apparent between some isolates (e.g. *Petasiger* sp. [Barton *et al.*, 2022] and *Pet. radiatus*). In contrast, as shown previously, the two species were clearly distinct in the 28S data. These findings echo previous concerns regarding the unsuitability of using 18S for systematic studies of lower digenean taxonomic groups (Blasco-Costa *et al.*, 2016). The p-distances between Echinostomatidae gen. sp. and *Petasiger* spp. ranged from 0.8% to 1.4% (Supplementary Table S3). Phylogenetic reconstruction comprising 23 sequences (876–879 bp) showed that *Petasiger* isolates were monophyletic. Echinostomatidae gen. sp. was basal to *Pegosomum*, *Isthmiophora* and *Petasiger* (Supplementary Figure S1).

Partial *cox*1 DNA fragments were generated from three isolates of *Petasiger* sp. 3 ZA (786–803 bp) and two for Echinostomatidae gen. sp. (632–644 bp). The sequences have been submitted to GenBank as accession numbers PP738976-PP738977 and PP738983-PP738984. Usable sequences were not obtained for *Pet. radiatus*. Genetic divergence and base pair differences between *Petasiger* sp. 3 ZA, Echinostomatidae gen. sp. and other echinostomatids, based on *cox*1 sequences are indicated in Supplementary Table S4. *Petasiger* sp. 3 ZA haplotypes varied by 0–5 bp, corresponding to p-distances of 0%–1.2%. The p-distances between *Petasiger* sp. 3 ZA and other *Petasiger* spp. ranged between 11% and 12%. Cercarial isolates from Zimbabwe that were published as Echinostomata sp. (MT994273-4) and 'Psilostomidae sp.' (MT013353) (Schols *et al.*, 2020), and '*Stephanoprora amurensis*' (PP556555) (Mudavanhu *et al.*, 2024), showed a close relationship (98.5%–99.8% similarity) with sequences of *Petasiger* sp. 5 from Uganda (Hammoud *et al.*, 2022). The small p-distance between the sequences (0.2%–1.5%) suggests that they belong to the same species. Echinostomatidae gen. sp. varied from other

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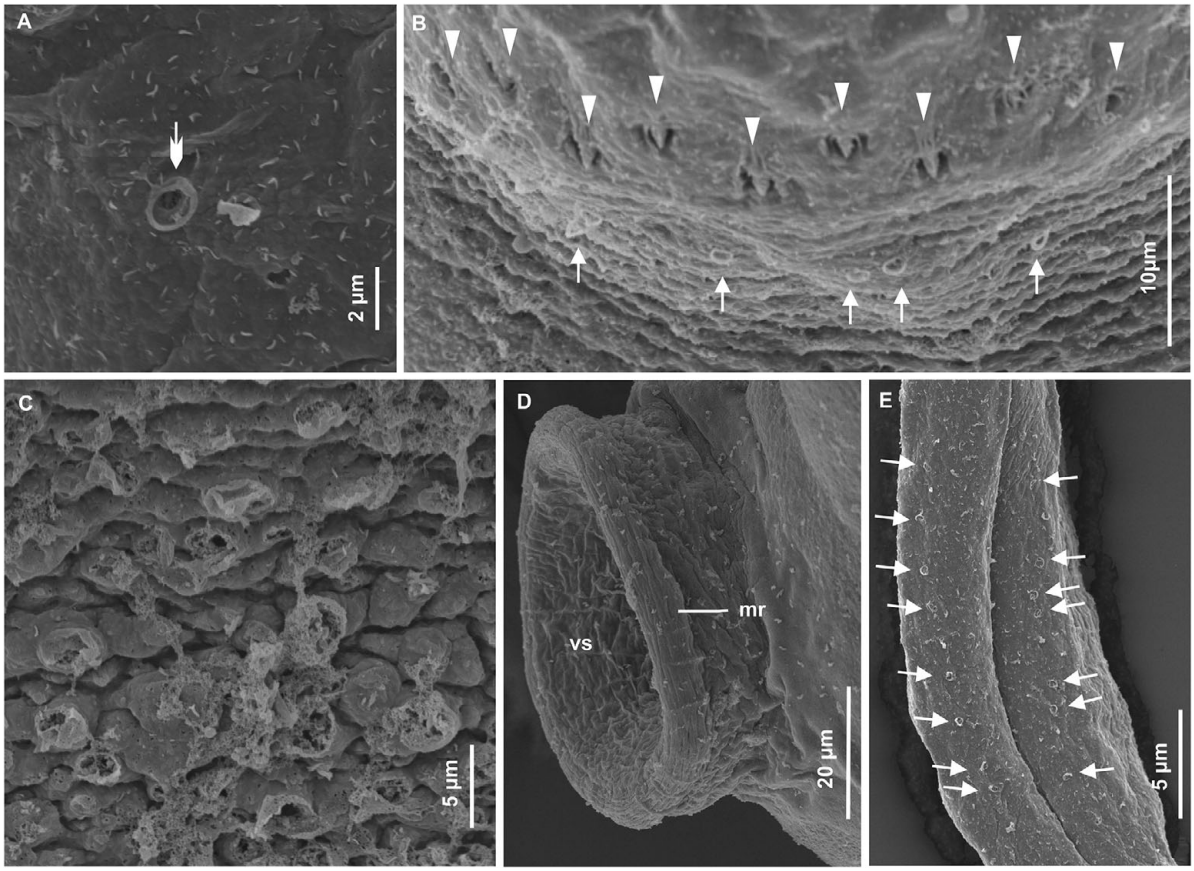

Figure 9. Scanning electron micrographs of cercaria of *Petasiger sp.* 3 ZA. A, Close-up view of unciliated pore; B, dorsal view of collar; C, dorsal surface on posterior part of body; D, lateral view of ventral sucker and E, anterior part of tail stem. Single arrows show uniciliated papillae, winged arrowheads indicate unciliated pores and arrow heads without tails show collar spines. Abbreviations: mr, membranous rim and vs, ventral sucker.

echinostomatids by 88–114 bp, corresponding to p-distances of 21.8%–28.3% (Supplementary Table S4). The BI and ML phylograms comprising of 41 echinostomatid sequences (403 bp), demonstrated that *Petasiger* sp. 3 ZA from South Africa (subclade B) was sister to a cluster (subclade A) comprising the sequences from Zimbabwe and Uganda (Fig. 14). The occurrence of cercarial isolates from Zimbabwe that were designated as 'Psilostomidae sp.' and '*S. amurensis*' (Mudavanhu *et al.*, 2024) within the strongly supported *Petasiger* clade (Fig. 14), shows that the two cercariae were misidentified. Similarly, sequences from Tanzania designated as *Pet. phalacrocoracis* (Chibwana & Katandukila, 2021) clustered with sequences of the family Echinochasmidae (subclades D and E). As mentioned in the ITS results, there appears to be mistakes in the identities of *Petasiger* and *Stephanoprora* sequences that were uploaded on GenBank by Chibwana & Katandukila (2021). Echinostomatidae gen. sp. formed a branch (subclade C) that was basal to A and B. An unnamed Echinostomata sp. (MT994275) from *Physella acuta* (Draparnaud, 1805) and *Bi. pfeifferi* from Zimbabwe showed a close genetic relationship (p-distance = 4.9%) with sequences of *Echinostoma miyagawai* Ishii, 1932 and they formed a strongly supported clade (F) with other *Echinostoma* spp. (Fig. 14).

Discussion

In general, morphological descriptions of intramolluscan stages of echinostomatids are often based on cercarial morphological features observed using light microscopy. For *Petasiger* cercariae, features such as overall body size, number, and arrangement of penetration gland cells and flame cells, number of granules in the main excretory ducts, visibility of the excretory duct in the tail stem, and the presence or absence of tegumental spines are considered when distinguishing between species (Našincová *et al.*, 1993; King & Van As, 2000; Fernández *et al.*, 2016; Laidemitt *et al.*, 2019; Outa *et al.*, 2024). The current study showed that apart from cercariae of *Petasiger* sp. (*Cercaria bruynoghei*) and *Pet. segregatus*, that were distinguishable by their small sized bodies, discrimination between the other species was difficult due to overlap in cercarial dimensions. These findings concur with some studies which showed that cercarial size can be an unreliable criterion for distinguishing between closely related species (Horák *et al.*, 2002; Podhorský *et al.*, 2009). Also, differences were observed in body dimensions of *Pet. radiatus* cercariae from the Czech Republic (Našincová *et al.*, 1993) and the current study. This variation in cercarial size might have been caused by differences in fixation techniques or it might be indicative of intraspecific variation. Specimens from the present study were fixed in 70% ethanol while the specimens described by Našincová *et al.* (1993) were fixed in 4% formalin. According to Blair & Islam (1983), fixation can influence the dimensions of cercariae, thereby making it difficult to compare specimens that were fixed using different techniques. In some species, intraspecific variation has been observed in cercariae obtained at different times or from different hosts. For instance, Porter (1938) reported the occurrence of two morphotypes of a sanguinicolid (that differed

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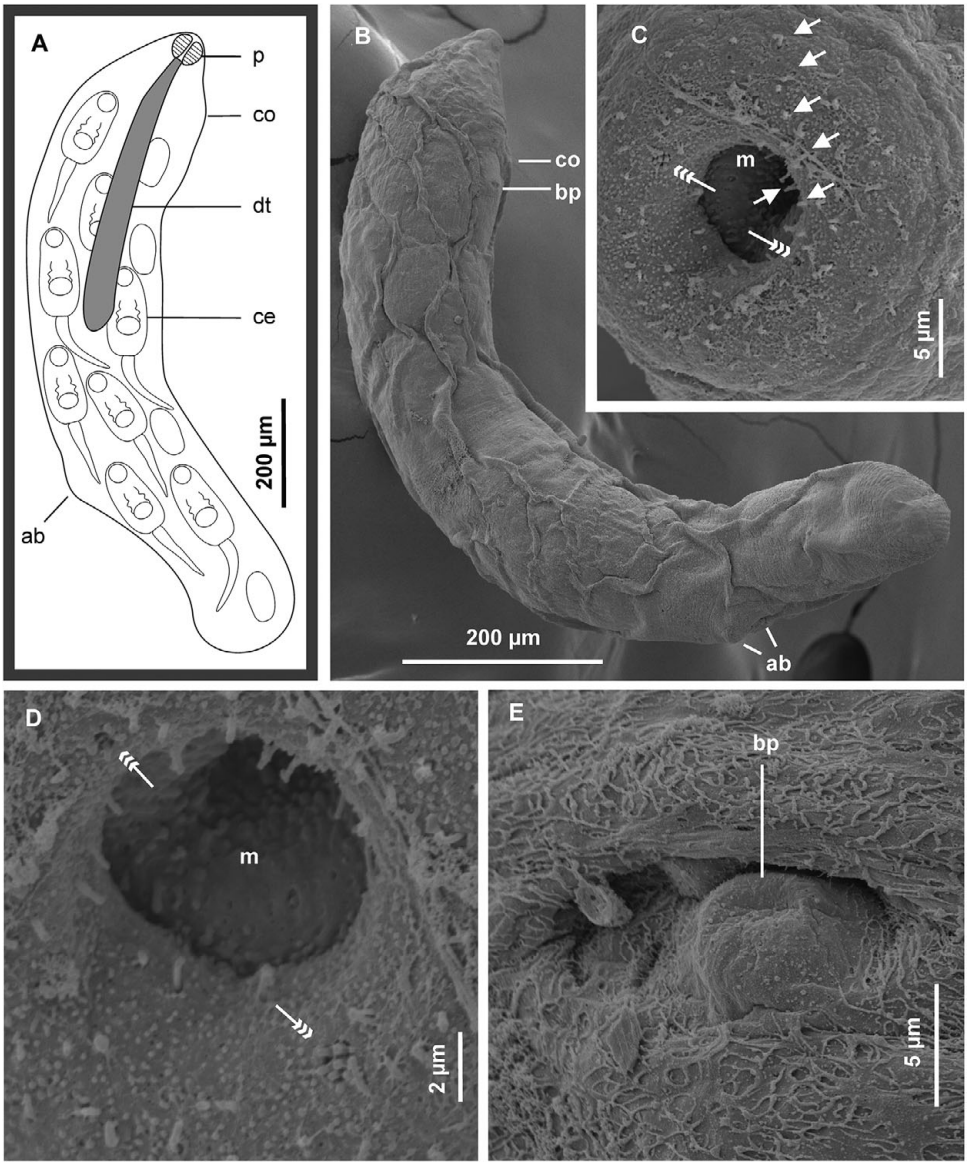

Figure 10. Redia of Echinostomatidae gen. sp. A, Schematic drawing of whole body; B, scanning electron micrograph of whole body; C, apical view of anterior end; D, close-up view of oral papillae and E, enface view of birth pore. Single arrows show uniciliated papillae, triple arrowheads show multiciliated papillae. Abbreviations: am, ambulatory buds; bp, birth pore; ce, cercaria; co, collar; dt, digestive tube; m, mouth and p, pharynx.

only in size) from *Bul. tropicus* collected in different months at the same locality in South Africa. Also, Neuhaus (1952) observed that cercariae of *Trichobilharzia szidati* Neuhaus, 1952 from two lymnaeid species differed significantly in size, despite being collected at the same time and place in Germany and using the same fixation and measurement techniques. Therefore, it is possible that host and environment related factors might have contributed to size differences between the specimens described by Našincová *et al.* (1993) and the current study. Indeed, the current cercariae were isolated from field collected samples of *Burnupia* spp. (Burnupiidae) while the specimens described by Našincová *et al.* (1993) were obtained from laboratory infected *R. auricularia* (Lymnaeidae).

In a survey of echinostomes from East Africa, Laidemitt *et al.* (2019) used the number of refractile granules in the main excretory ducts to distinguish between cercariae of four *Petasiger* spp. Based on that criteria, there seems to be three broad groups of cercariae. The first group has very few excretory granules (e.g., *Petasiger* sp. 2 from Kenya which has 7–10 granules) (Laidemitt *et al.*, 2019). The second group has approximately 17–23 granules (e.g., *C. decora* from DRC, *Pet. variospinosus* from South Africa, and *Petasiger* spp. 4 and 5 from Kenya) (Fain, 1953; King & Van As, 2000; Laidemitt *et al.*, 2019). We suggest the inclusion of *Petasiger* sp. 1 ZA in this second group. Although the number of excretory granules was not mentioned, the photomicrograph provided for *Petasiger* sp. 1 ZA cercaria showed 20 and 23 granules in the main excretory ducts in the paper by Moema *et al.* (2008). Based on the presence of 19–20 excretory granules, Laidemitt *et al.* (2019) implied that *C. decora*, *Pet. variospinosus*, and *Petasiger* sp. 5 might be identical. In addition, the excretory systems of *C. decora*, *Pet. variospinosus* and *Petasiger* sp. 1 ZA have 28 flame cells (Fain, 1953; King & Van As, 2000; Moema *et al.*, 2008). Data for flame cell patterns are not available for *Petasiger* spp. 4 and 5 (Laidemitt *et al.*, 2019). Despite the similarities within this second group, there are some differences that are worth noting. *Cercaria decora* is distinguished by a cluster of

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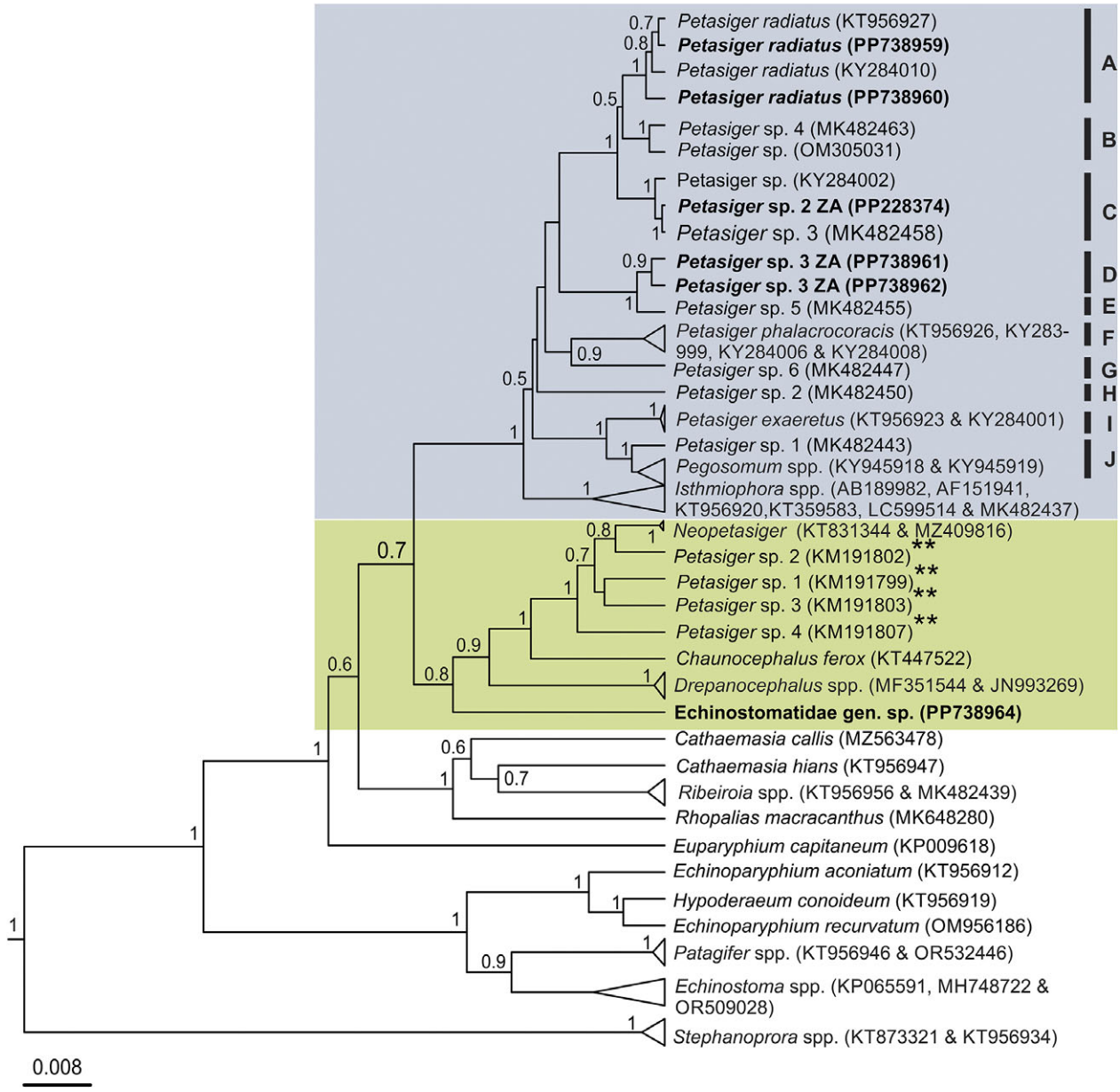

Figure 11. Bayesian inference 28S rDNA phylogram of Echinostomatidae spp. The clades containing *Petasiger* spp. and Echinostomatidae gen. sp. are highlighted, and isolates from South Africa are indicated in bold. Nodal support values lower than 0.5 are not shown. Isolates marked with asterisks (**) are for 19-spined and large-tailed cercariae belonging to the genus *Neopetasiger*.

numerous penetration gland cells along the oesophagus, whereas in the other species, penetration gland cells were not reported. *Petasiger* sp. 1 ZA and *Pet. variospinosus* are distinguishable based on the number of cilia on their oral papillae. Therefore, synonymity between the cercariae in this second group is unlikely. The third group is composed of *Petasiger* spp. with numerous granules (>25). For example, *Pet. radiatus* and *Petasiger* sp. 3 ZA from the current study, *Petasiger* sp. 2 ZA (Outa et al., 2024), *C. bruynoghei* (Fain 1953), *Pet.* segregatus (Lie & Basch, 1967), Echinocercaria III (Ostrowski de Núñez *et al.*, 1991) and *Petasiger* sp. (Fernández *et al.*, 2016). Flame cells were discernible in *C. bruynoghei* (24) and Echinocercaria III (28), and poorly visible in *Petasiger* sp. (Fernández *et al.*, 2016), *Pet. segregatus*, *Petasiger* sp. 2 ZA, and *Petasiger* sp. 3 ZA. Further distinctions were based on the visibility and number of penetration gland cells along the oesophagus. Poor visibility of cercarial internal structures usually corresponds to the presence of numerous cystogenous glands (Lie & Basch, 1967; Fernández *et al.*, 2016; Outa *et al.*, 2024). The close resemblance between *Petasiger segregatus* from Brazil (Lie & Basch, 1967) and *Petasiger* sp. from Argentina (Fernández *et al.*, 2016) suggests that they might be identical. Indeed, the number of granules in each excretory duct of *Pet. segregatus* (40–50) corresponds with *Petasiger* sp. (45–59). In addition, both species are characterised by sensory hairs (visible using light microscope) and the presence of numerous cystogenous gland cells.

Regarding caudal features, a bifurcated excretory duct near the base of the tail stem is a feature that seems to be limited to two unidentified species from Europe (Kiseliene, 1970; Faltýnková *et al.*, 2008b). It is also worth noting that Barton *et al.* (2022) mentioned the presence of finfolds along the cercarial tail of *Petasiger* sp. from Australia. However, several studies have shown that cercariae of *Petasiger* lack finfolds on their tails (Lie & Basch, 1967; Našincová

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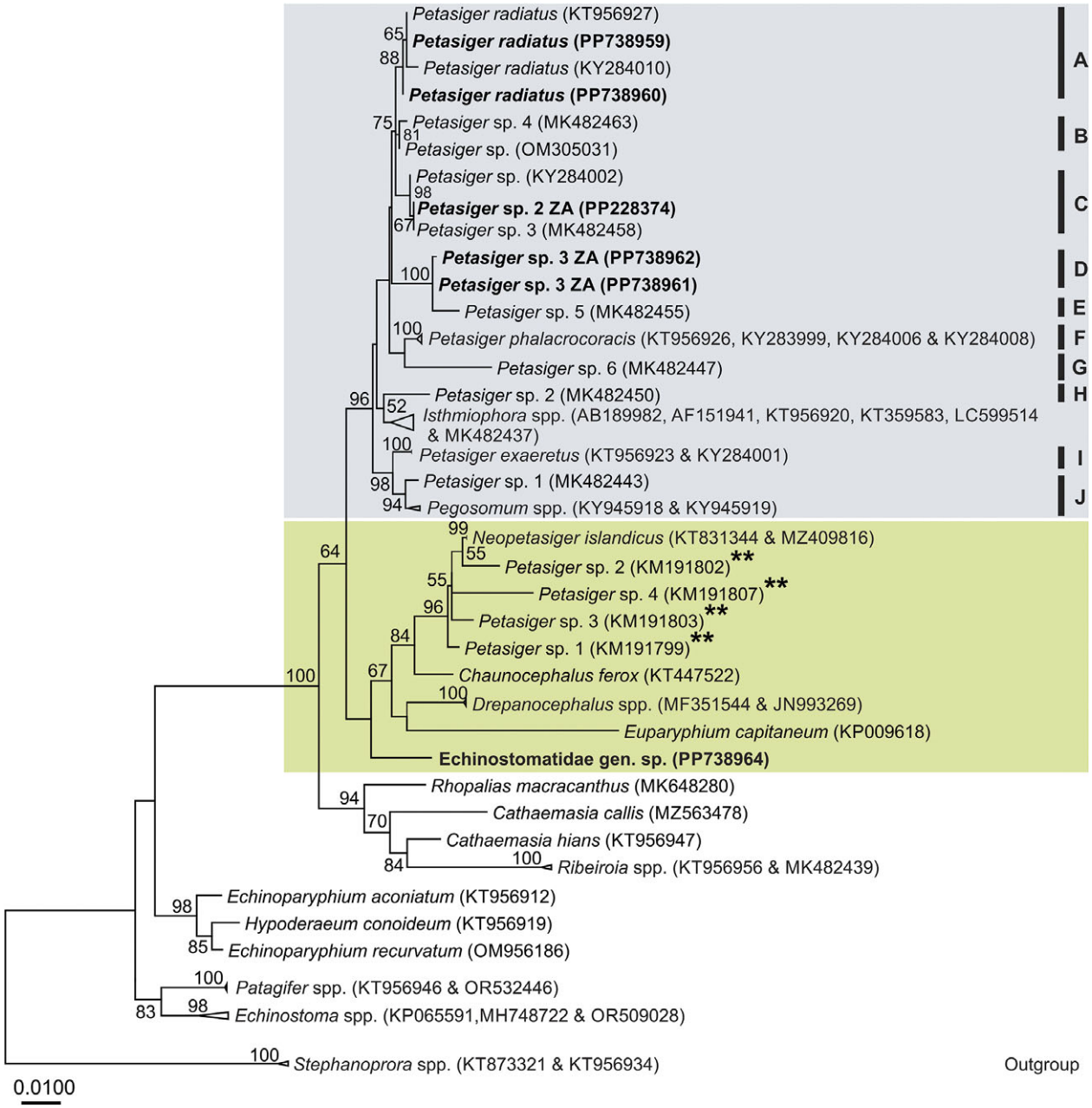

Figure 12. Phylogenetic relationships of Echinostomatidae spp. from the current study and from GenBank based on 28S rDNA inferred from maximum likelihood analyses. The clades containing *Petasiger* and Echinostomatidae gen. sp. are highlighted and isolates from South Africa are indicated in bold. Nodal support values lower than 50% are excluded. Isolates marked with asterisks (**) are for 19-spined and large-tailed cercariae belonging to the genus *Neopetasiger*.

et al., 1993; King & Van As, 2000; Faltýnková *et al*., 2008b; Fernández *et al*., 2016; Outa *et al*., 2024). Indeed, the photomicrograph and drawing provided by Barton *et al*. (2022) only show the lateral sides of the tail trunk which might have been confused for finfolds. Tegumental features of cercariae such as the presence (and density) or absence of sensory hairs, and the patterns of uniciliated and multiciliated papillae, proved to be important for *Petasiger* species characterisation. However, information of the papillary patterns is available only for *Pet. variospinosus* (King & Van As, 2000), *Petasiger* sp. 1 ZA (Moema *et al*., 2008), *Petasiger* sp. 2 ZA (Outa *et al*., 2024), and *Pet. radiatus* and *Petasiger* sp. 3 ZA (current study). Therefore, there is need to examine more *Petasiger* species to further demonstrate the usefulness of papillary patterns for species discrimination. Overall, the current study shows that differentiation between species of *Petasiger* based on cercarial morphology requires the consideration of multiple criteria. Hence, features such as the numbers of refractile granules in the excretory system and the patterns of flame cells, penetration gland cells and papillae, may not be useful for species discrimination when used in isolation.

Apart from the present study, surface features of rediae have been described only for *Petasiger* sp. 2 ZA (Outa *et al*., 2024). Rediae of *Petasiger* sp. 2 ZA and the current species are characterised by numerous sensilla around the mouth. The presence of oral sensilla seems to be a general feature of most echinostomatids since they have also been reported on *Echinostoma paraensei* Lie & Basch, 1967 (Pinheiro *et al*., 2004) and *Ribeiroia ondatrae* (Price, 1931) (Keeler *et al*., 2012). However, the presence and number of

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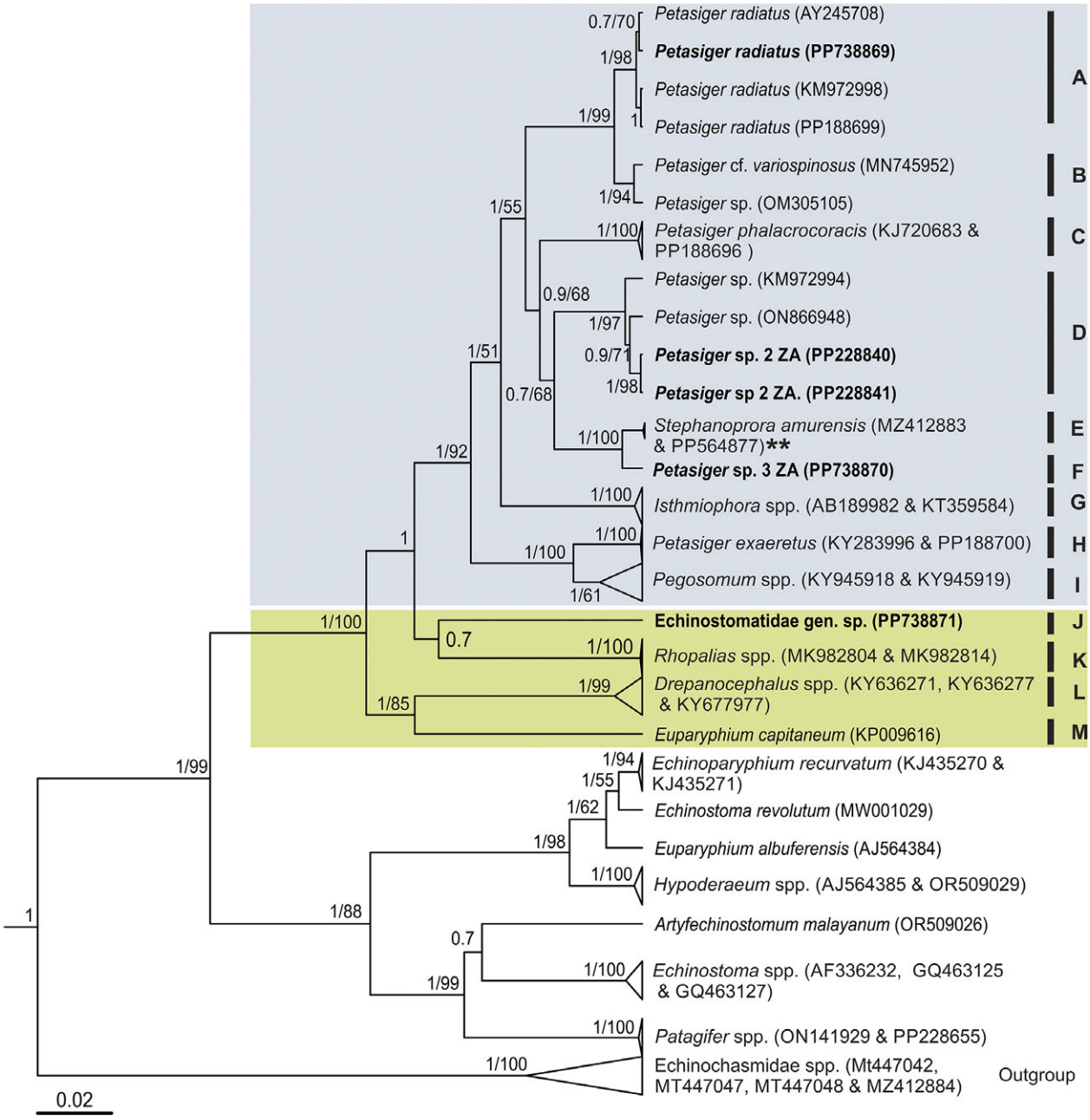

Figure 13. Phylogenetic tree based on Bayesian inference (BI) and maximum likelihood (ML) analyses of ITS sequences of Echinostomatidae spp. The clades containing *Petasiger* and Echinostomatidae gen. sp. are highlighted. Isolates from South Africa are indicated in bold. Nodal support values are given as BI/ML and values lower 0.5 (50%) are not shown. GenBank accession numbers of the sequences are given in parentheses. Isolates MZ412883 (Chibwana & Katandukila, 2021) and PP564877 (Mudavanhu *et al.*, 2024) marked with asterisks (**), indicate erroneous identification of an unknown *Petasiger* sp. as *Stephanoprora amurensis.*

multiciliated papillae around the mouth appears to be species specific. For instance, redia of *Petasiger* sp. 3 ZA was characterised by three pairs of multiciliated papillae while Echinostomatidae gen. sp. had only one pair. In contrast, multiciliated papillae were not reported on the teguments of *Ec. paraensei* and *Ri. ondatrae* rediae (Pinheiro *et al.*, 2004; Keeler *et al.*, 2012).

Molecular data based on 28S rDNA sequences confirmed the placement of the current specimens into the family Echinostomatidae. The identities of cercarial isolates of *Pet. radiatus* were confirmed based on the 99.9%–100% similarity to sequences of adult worms that were published by Tkach *et al.* (2016). *Petasiger* sp. 3 ZA showed a close genetic relationship with cercaria of *Petasiger* sp. 5 from Kenya (Laidemitt *et al.*, 2019). However, the two formed strongly supported divergent lineages with 28S p-distances of 0.6%–0.7%. The divergence was also seen in the *cox*1 sequences (11.4%–13.2%); hence, corroborating the distinction between *Petasiger* sp. 3 ZA and *Petasiger* sp. 5. Cercarial isolate of *Petasiger* sp. from Australia (OM305105) (Barton *et al.*, 2022) formed a strongly supported subclade with *Petasiger* sp. 4 from *Bi.* sudanica from Lake Victoria, Kenya (Laidemitt *et al.*, 2019). Based on this strong genetic relationship, we suggest that the two isolates are haplotypes of the same species. *Petasiger* sp. metacercariae from Hungary (Cech *et al.*, 2017), and cercariae of *Petasiger* sp. 3 from Kenya (Laidemitt *et al.*, 2019) and *Petasiger* sp. 2 ZA from

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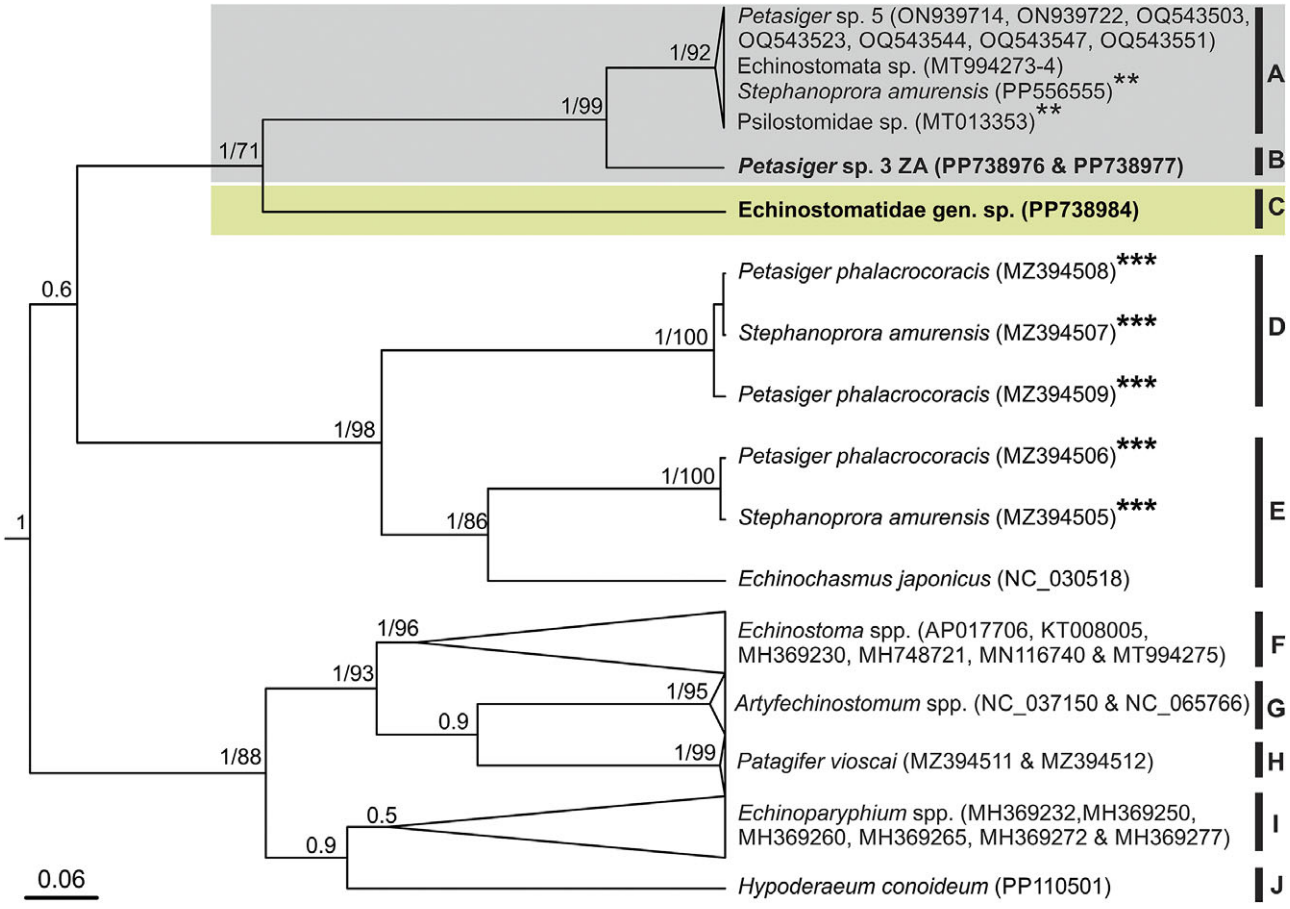

Figure 14. Bayesian inference (BI) and maximum likelihood (ML) phylograms of the relationships between Echinostomatidae spp., based on the cytochrome c oxidase subunit 1 mitochondrial gene (*cox*1) sequences. The clades containing *Petasiger* and Echinostomatidae gen. sp. are highlighted. Isolates from South Africa are indicated in bold. The branch length scale indicates the number of substitutions per site. Nodal support values lower than 0.5 (50%) are excluded. Sequences marked with asterisks (**) are for isolates from Zimbabwe (Mudavanhu *et al*., 2024) that have been synonymised with *Petasiger* sp. 5 and (***) are from Tanzania (Chibwana & Katandukila, 2021) whose identities are questionable.

South Africa (Outa *et al*., 2024) differed by only (0%–0.1%); hence, they are regarded to represent the same species. The current analyses also confirmed the designation of *Petasiger* spp. 2 and 6 from Kenya (Laidemitt *et al*., 2019) as distinct species. The high 28S similarity (99.3%–99.5%) between cercaria of *Petasiger* sp. 1 from Kenya (Laidemitt *et al*. (2019) and sequences of *Pegosomum asperum* and *Peg. saginatum* (adults) that were obtained from the gall bladder of egret *Ardea alba*, suggests that these three isolates belong to the same genus. According to Laidemitt *et al*. (2019), apart from the number of collar spines (27), other morphological features of *Petasiger* sp. 1 specimens were obscure since they had been preserved for many years. Similar to *Petasiger*, *Pegosomum* spp. are also characterised by 27 collar spines (Heneberg & Sitko, 2017). Since the morphological identification of *Petasiger* sp. 1 was based only on one cercarial feature, we suspect that the cercaria may have been misidentified.

In the present study, we also incorporated ITS and *cox*1 sequences of *Petasiger* (from GenBank), for which 28S data are lacking. For ITS, there is a sequence (MN745952) for cercaria (putatively identified as *Pet. variospinosus*) that was isolated from *Bi. sudanica* from Lake Victoria, Kenya (Outa *et al*., 2020). The sequence showed a high similarity (99.6 %) with cercaria of *Petasiger* sp. from Australia (OM305105) (Barton *et al*., 2022) and the two formed a strongly supported subclade. As shown in the 28S data (previous), sequence OM305105 (Barton *et al*. 2022) seems to be synonymous with *Petasiger* sp. 4, also from *Bi.* sudanica from Lake Victoria, Kenya (Laidemitt *et al*., 2019). Therefore, we suggest that *Petasiger* cf. *variospinosus* (Outa *et al*., 2020), *Petasiger* sp. (Barton *et al*., 2022) and *Petasiger* sp. 4 (Laidemitt *et al*., 2019), belong to the same species. The other ITS sequences (MZ412883 and PP564877) (Chibwana & Katandukila, 2021; Mudavanhu *et al*., 2024) are for isolates from Tanzania and Zimbabwe that were labelled as *S. amurensis*. However, phylogenetic data inferred that MZ412883 and PP564877 belong to *Petasiger*. As discussed in *cox1* data that follows, we suggest that those two sequences that were published by Chibwana & Katandukila (2021) and Mudavanhu *et al*. (2024) are synonymous with *Petasiger* sp. 5. Prior to the current study, *cox*1 sequences (on GenBank) designated as *Petasiger* spp. were available from two other investigations. The first study published three sequences that were assigned to *Pet. phalacrocoracis* (Chibwana & Katandukila, 2021). However, as mentioned in the Results, those sequences clustered with echinochasmids from the same study; hence, their valid identities are uncertain. The second study reported *Petasiger* sp. from *Bul. tropicus* in Uganda (Hammoud *et al*., 2022). Hammoud *et al*. (2022) noted that the isolates from Uganda were synonymous with *Petasiger* sp. 5 from Kenya (Laidemitt *et al*., 2019) based on *nad*1 sequences. The current study has shown that isolates that were published as Echinostomata sp. (MT994273-4) (Schols *et al*., 2020), '*S. amurensis*' (PP556555) (Mudavanhu *et al*., 2024) and 'Psilostomidae sp.' (MT013353), from *Bulinus* spp. from Zimbabwe, are haplotypes of *Petasiger* sp. 5. The sequences of echinostomes that were published by Schols *et al*. (2020) and Mudavanhu *et al*. (2024) were

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from cercariae for which morphological data were not provided. We echo the recommendations of previous studies on the importance of integrated characterisation of cercariae to increase the accuracy of identification and to provide adequate reference data for future studies (Pantoja *et al*., 2021; Outa *et al*., 2024). Based on the current findings, it appears that nuclear and mitochondrial DNA sequences of *Petasiger* on GenBank are representative of *Pet. exaeretus*, *Pet. phalacrocoracis*, *Pet. radiatus* and six unnamed *Petasiger* spp.

Data on the localities and genotypes of *Petasiger* indicate a wide geographical distribution of the genus. This concurs with previous studies regarding the cosmopolitan distribution of *Petasiger* (Faltýnková *et al*., 2008a; Tkach *et al*., 2016; Barton *et al*., 2022). Adults of *Petasiger* spp. inhabit the intestines of birds belonging to the families Phalacrocoracidae, Anhingidae, Ciconiidae, and Sulidae (Tkach *et al*., 2016). Although there are only a few reports of adults of *Petasiger* in Africa, data from the intramolluscan stages show the hidden diversity of *Petasiger* spp. It appears that the wide distribution of *Petasiger* is aided not only by the wide distribution of their definitive hosts, but also by their abilities to use diverse first and second intermediate hosts. Indeed, parthenitae and cercariae of *Petasiger* spp. have been reported from snails of the families Ampullariidae, Bulinidae, Lymnaeidae, and Planorbidae (King and Van As, 2000; Laidemitt *et al*., 2019; Outa *et al*., 2020; Hammoud *et al*., 2022; Outa *et al*., 2024) and Burnupiidae in the current study. Cercariae of *Petasiger* exit the first intermediate hosts and develop into encysted metacercariae in amphibians and fish, which are the second intermediate hosts (King and van As, 2000; Kostadinova, 2005; Cech *et al*., 2017).

Phylogenetic analyses demonstrated that Echinostomatidae gen. sp. was distinct from genera whose molecular data are available on GenBank. Its relationships with the other echinostomatids were best inferred using 28S and ITS rDNA, since there are more sequences on GenBank for these markers. That the present species could not be matched with any genus on GenBank confirms that genetic data is still lacking for some echinostomatid genera. According to the keys for the superfamily Echinostomatoidea that were provided by Tkach *et al*. (2016), the family Echinostomatidae is composed of 38 genera. However, our search through GenBank for sequences of the most widely used markers (28S and ITS) showed that genetic data is available for less than 20 genera. Therefore, in agreement with previous authors (Tkach *et al*. 2016; Izrailskaia *et al*., 2021; Pantoja *et al*., 2021), we suggest that an expansion of the genetic database of Echinostomatidae is necessary, to enable the validation of species identities and elucidation of suprageneric phylogenetic relationships.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000749.

Author contribution. J. O. O. and A. A.-O. conceptualized the study. J. O. O. conducted field sampling, morphological work, molecular and phylogenetic analyses, and wrote original draft of the article. J. O. O. and A. A.-O. reviewed and edited subsequent versions of the manuscript.

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Ethical standard. This research was undertaken following approval by the University of Johannesburg Ethics Committee (Reference Number: 2022-08-05/Outa_Oldewage) and complied with the South African national standard for care and use of animals for scientific purposes.

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