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Two new *Stephanostomum*-like cercariae (Digenea: Acanthocolpidae) from *Nassarius dorsatus* and *N. olivaceus* (Gastropoda: Nassariidae) in Central Queensland, Australia

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Abstract

Two new *Stephanostomum*-like cercariae, *Cercaria capricornia* VII and *Cercaria capricornia* VIII (Digenea: Acanthocolpidae), are described from the nassariid gastropods *Nassarius dorsatus* and *Nassarius olivaceus* collected from the intertidal zone in the Capricornia region of Central Queensland, Australia. Morphological analysis of these new cercariae was augmented with DNA sequence data from the large subunit (LSU) ribosomal DNA region to aid in identification. Bayesian inference analysis of the LSU rDNA revealed that these putative acanthocolpid cercariae nested well within a clade containing species of *Stephanostomum*, which along with morphological data, suggests they are species of *Stephanostomum*. Comparative analysis of LSU rDNA sequences also indicates that these two cercariae are not *S. adlardi*, *S. bicoronatum*, *S. tantabiddii* or *S.* cf. *uku*, all species known from Australian fishes. The secondary structure of the internal transcribed spacer 2 (ITS2) rDNA region was inferred for these two cercariae using minimum free energy modelling algorithms. Both cercarial types displayed a four helix ITS2 secondary structure model and differed from each other by two compensatory base changes (CBCs) and nine hemi-CBCs.

Key words: *Cercaria capricornia*; Capricornia; *Nassarius dorsatus*; *Nassarius olivaceus*; LSU rDNA; large subunit ribosomal DNA; CBC; compensatory base change; ITS2; internal transcribed spacer 2

Introduction

The Acanthocolpidae Lühe, 1906 is a large family of digenean trematodes that infects the gastrointestinal tract of marine fishes. Asexual reproduction occurs in a range of gastropods and metacercariae infect the tissues of fishes which are then eaten by piscivores to complete the life cycle. Although 17 genera were recognised in the family by Bray (2005), *Stephanostomum* Looss, 1899, with over one hundred species, is by far the largest in the family and one of the largest in marine fishes (Cribb *et al*. 2002). Despite the size of the genus, the life cycles and cercarial stages have been characterised for only five species (Madhavi & Shameem 1993). A single further *Stephanostomum*-like cercariae, *Cercaria bengalensis* VII Gnana Mani, 1994, has been described (Gnana Mani 1994). For such a large and important genus, the range of hosts infected and the morphological consistency of the intra-molluscan stages is of considerable interest.

The nassariid gastropods *Nassarius dorsatus* (Röding) and *Nassarius olivaceus* (Bruguière) are common scavengers in the intertidal mangroves of the Capricornia region in Central Queensland, Australia. There are ten probable acanthocolpid cercariae reported previously from nassariid gastropods. There are three species of *Stephanostomum*: those of *Stephanostomum tenue* (Linton, 1898) Linton, 1934 and *Stephanostomum dentatum* (Linton, 1900) Linton, 1940 (syn. *Cercaria dipterocerca* Miller and Northup, 1926) from *Nassarius obsoletus* Say (see Martin 1939; Stunkard 1961) and *Stephanostomum cloacum* (Srivastava, 1938) Manter &

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Van Cleave, 1951 from *Nassarius orissaensis* (Preston) (see Madhavi & Shameem 1993). *Cercaria bengalensis* VII was reported from *Nassarius stolatus* (Gmelin), and was described as closely resembling the cercariae of *S. tenue* and *Stephanostomum caducum* (Looss, 1901) Manter, 1934 (Gnana Mani 1994). Other cercariae reported from nassariids are *Cercaria capricornia* I–IV Barnett, Smales & Cribb, 2008 from *N. olivaceus* and *Cercaria capricornia* V–VI Barnett, Smales & Cribb, 2008 from *N. dorsatus*. This is a complex of six unusual cercariae which are morphologically very different from cercariae of *Stephanostomum* in that they have an unusual body shape including a ventral keel (Barnett *et al.* 2008). Other *Stephanostomum* cercariae are *Stephanostomum baccatum* (Nicoll, 1907) Manter, 1934 (syn. *Cercaria neptunae* Lebour, 1911) from *Buccinum undatum* Linnaeus, *Neptunea decemcostata* (Say) and *Neptunea antiqua* (Linnaeus) (see MacKenzie & Liversidge 1975; Wolfgang 1955) and *S. caducum* from *Euspira pulchella* (Risso) (syn. *Natica alderi* Forbes) (see Køie 1978).

Here, we present two new acanthocolpid cercariae from *N. dorsatus* and *N. olivaceus* found as part of a study of the parasite fauna of these molluscs in Capricornia. Bayesian inference analysis of partial large subunit (LSU) ribosomal DNA (rDNA) was performed with the aim of exploring the integrity of the putative new cercarial types and their phylogenetic relationships with other acanthocolpid taxa. In addition, we sequenced the internal transcribed spacer 2 (ITS2) rDNA region for these two types and inferred their secondary structures to explore the homology of these structures to each other and to that known for other digeneans.

Material and methods

Host gastropod and parasite collection. Molluscs were collected by hand from mudflats at Sandy Point, Corio Bay (22°58' S, 150°46' E), Ross Creek, Yeppoon (23°8' S, 150°45' E) and the mouth of Cawarral Creek, Keppel Sands (23°19' S, 150°47' E) in Central Queensland, Australia. A total of 1766 *N. dorsatus* and 1908 *N. olivaceus* were collected between August 2004 and May 2006. Molluscs were held in filtered seawater at room temperature (20–28°C) and examined for naturally emerged cercariae every 1–3 days for up to one month. Freshly emerged cercariae were transferred to a cavity block in a small volume of seawater and heat-killed by pouring several volumes of near boiling seawater into the dish. Specimens for morphological analysis were fixed in 5% formalin and specimens for molecular analysis were stored in 70% ethanol or in phosphate buffered saline at -20°C. One or more infected molluscs were dissected to determine the nature of intra-molluscan stages for each cercarial species. Rediae were placed into near boiling seawater and fixed in 5% formalin or 70% ethanol. Representative wholemounts were stained with Mayer's haematoxylin, dehydrated through a graded series of ethanol, cleared in methyl salicylate and mounted in Canada balsam. Wholemounts and wet specimens are deposited in the Queensland Museum, Brisbane, Queensland, Australia.

Morphological analysis. Fixed cercariae were examined as temporary wet mounts without flattening. Live specimens and rediae were also observed as temporary wet mounts. Measurements were taken using an eyepiece micrometer on an Olympus BX-41 microscope and are given in micrometres as the range followed by the mean in parentheses. Standard deviations were calculated where more than 30 measurements were taken. Drawings were prepared with the aid of a drawing tube. Line drawings of *C. capricornia* VII and *C. capricornia* VIII were deposited in MorphBank (http://www.morphbank.net/).

Confirmation of prevalence. Of 17 infections, mature cercariae for morphological analysis were not available from one *N. olivaceus* and this specimen was omitted from the calculation of prevalence. A subset of putatively uninfected molluscs was dissected to confirm the absence of infection. Of 556 *N. dorsatus* (31.5% of 1766) and 399 *N. olivaceus* (20.9% of 1908) dissected, there were just two *N. dorsatus* infected with a trematode similar to those described here, but the cercariae were too immature for morphological comparison. This result suggests that the emergence prevalence reported here closely approximates the actual prevalence.

Molecular analysis. Total genomic DNA was extracted from fresh, frozen or alcohol fixed specimens using a QIAGEN® DNeasy® Blood and Tissue Kit following the manufacturer’s protocol, except that the incubation step with Proteinase K was extended to overnight in a rotating incubator at 56°C. Final elution volume was 30–200 µL. Amplification of the partial LSU rDNA region was performed with the primers LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3' Littlewood *et al.* 2000) and ECD2 (5'-CCTTGGTCCGTGTTTCAAGACGGG-3' Littlewood *et al.* 2000) and the ITS2 region with the primers 3S (5'-GGTACCGGTGGATCACGTGGCTAGTG-3' Morgan & Blair 1995) and ITS2.2 (5'-CCTGGTTAGTTTCTTTTCCTCCGC-3' Cribb *et al.* 1998). PCR amplification (total volume 50 µL) was performed using 1X iTaq Buffer (Bio-Rad), 1.25 U iTaq™ DNA polymerase (Bio-Rad), 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each primer and 1–10 µL genomic extract, with the following thermocycling profile: 5 min denaturation at 95°C; 30 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C; and 4 min extension at 72°C. PCR amplicons were purified using a QIAGEN QIAquick® PCR Purification Kit (with 30 µL elution buffer). Purified amplicons were sequenced (in both directions) using the same primers with ABI BigDye™ v3.1 chemistry at the Australian Genome Research Facility in Brisbane, Queensland. The resulting sequences were edited by eye and contigs constructed using Sequencher™ version 4.5 (GeneCodes Corp.). The sequences for each cercarial type were constructed from multiple replicates (each replicate contig being both a forward and reverse sequence from a single individual) from different host/locality combinations whenever possible.

Phylogenetic analyses. The partial LSU rDNA sequences for *C. capricornia* VII and *C. capricornia* VIII were aligned with data reported for the species of Acanthocolpidae and Brachycladiidae listed in Table 1. Species of Brachycladiidae were included as this family is considered closely related to the Acanthocolpidae (Bray *et al.* 2005). *Cableia pudica* Bray, Cribb & Barker, 1996 was omitted from this analysis as recent molecular phylogenetic analyses have indicated that this species is a member of the Monorchiidae (Bray *et al.* 2005). *Paragonimus westermani* (Kerbert, 1878) and *Paragonimus iloktsuenensis* Chen, 1940 were chosen as outgroup taxa.

The partial LSU rDNA dataset was initially aligned using ClustalX version 2.0.9 (Larkin *et al.* 2007) under the following parameters: pairwise alignment parameters = gap opening 10.00, gap extension 0.10, DNA weight matrix International Union of Biochemistry (IUB); and multiple alignment parameters = gap opening 10.00, gap extension 0.20, delay divergent sequences 30%, DNA weight matrix IUB. The resulting sequence alignment was exported from ClustalX in FASTA and NEXUS formats, and refined by eye using MacClade version 4.08 (Maddison & Maddison 2005). After alignment of the LSU dataset was edited, the ends of each fragment were trimmed to match the shortest sequence in the alignment. Bayesian inference analysis of the LSU dataset was performed using MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003) to explore relationships between these taxa. Modeltest version 3.7 (Posada & Crandall 1998) was used to estimate the best substitution model for the LSU dataset. Bayesian inference analysis was conducted on the combined dataset using the GTR+I+G model predicted as the best estimator by the Akaike Information Criterion (AIC) in Modeltest. Bayesian inference analysis was run over 1,000,000 generations (ngen=1000000) with two runs each containing four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains=4) and every 100th tree saved (samplefreq=100). Bayesian analysis used the following parameters: nst=6, rates=invgamma, ngammacat=4, and the MCMC parameters were left at the default settings, and the priors parameters of the combined dataset were set to ratepr=variable. Samples of substitution model parameters, and tree and branch lengths were summarised using the parameters 'sump burnin=3000' and 'sumt burnin=3000'. These 'burnin' parameters were chosen because the log likelihood scores 'plateaued' well before 300,000 replicates in the Bayesian inference analysis.

ITS2 rDNA secondary structure prediction. The secondary structure of the ITS2 rDNA region for *C. capricornia* VII and *C. capricornia* VIII was predicted using minimum free energy folding algorithms with Mfold software version 3.2 (Zuker 2003). The ITS2 sequence was treated as linear and the folding temperature set at 37ºC for analysis using Mfold. Alignment of the predicted ITS2 secondary structures for these two cercariae was performed using 4SALE (Seibel *et al.* 2008). Compensatory base change (CBC) analysis was then conducted on the aligned secondary structures using CBCAnalyzer software (Wolf *et al.* 2005). The predicted folding results from the Mfold analyses were viewed with Pseudoviewer version 3 (Byun & Han 2006).

TABLE 1. List of taxa and partial LSU rDNA sequences from GenBank used in this study.

Species	Source: host/locality	GenBank accession
Acanthocolpidae		
Cercaria capricornia VII	*Nassarius dorsatus*, Capricornia, Australia	FJ809037
Cercaria capricornia VIII	*Nassarius dorsatus*, Capricornia, Australia	FJ809036
Monostephanostomum nolani Bray & Cribb, 2007	*Carangoides plagiotaenia*, Lizard Island, Australia	EF506763
Pleorchis polyorchis (Stossich, 1889)	*Sciaena umbra*, Scandola, Corsica	DQ248215
Pleorchis uku Yamaguti, 1970	*Aprion virescens*, Lizard Island, Australia	DQ248216
Stephanostomum adlardi Bray, Cribb, Waeschenbach & Littlewood, 2007	*Plectropomus leopardus*, Lizard Island, Australia	EF506761
Stephanostomum baccatum (Nicoll, 1907)	*Hippoglossus hippoglossus*, North Sea, UK	DQ248218
Stephanostomum baccatum (Nicoll, 1907)	*Eutrigla gurnadus*, North Sea, UK	AY222256
Stephanostomum bicoronatum (Stossich, 1883)	*Sciaena umbra*, Scandola, Corsica	DQ248225
Stephanostomum cesticillus (Molin, 1858)	*Lophius piscatorius*, Scandola, Corsica	DQ248226
Stephanostomum gaidropsari Bartoli & Cribb, 2001	*Gaidropsarus mediterraneus*, Marseille, France	DQ248221
Stephanostomum interruptum Sparks & Thatcher, 1958	*Menticirrhus americanus*, Gulf of Mexico, USA	DQ248223
Stephanostomum minutum (Looss, 1901)	*Uranoscopus scaber*, Scandola, Corsica	DQ248224
Stephanostomum pristis (Deslongchamps, 1824)	*Phycis phycis*, Scandola, Corsica	DQ248222
Stephanostomum tantabiddii Bray & Cribb, 2004	*Carangoides fulvoguttatus*, Ningaloo, Australia	DQ248220
Stephanostomum cf. *uku* Yamaguti, 1970	*Aprion virescens*, Lizard Island, Australia	DQ248219
Tormopsolus orientalis (Yamaguti, 1934)	*Seriola dumerili*, Scandola, Corsica	DQ248217
Brachycladiidae		
Zalophotrema hepaticum Stunkard & Alvey, 1929	*Zalophus californianus*, California, USA	AY222255
Paragonimidae		
Paragonimus iloktsuenensis Chen, 1940	*Rattus norvegicus*, Amami Island, Japan	AY116875
Paragonimus westermani (Kerbert, 1878)	*Canis familiaris*, Hyogo, Japan	AY116874

Results

Two different types of acanthocolpid cercariae with morphology similar to described *Stephanostomum* cercariae emerged naturally from *N. dorsatus* and *N. olivaceus*.

Morphological data – intramolluscan stages

Diagnosis: Distomate cercariae. Dorsoventrally flattened body, longer than wide. Tegument spinose. Eye-spots present. Oral sucker ventrally subterminal. Excretory vesicle Y-shaped. Tail simple, lacking spines, chaetae or fins.

***Cercaria capricornia* VII**
(Fig. 1)

Host: *Nassarius dorsatus* (Röding), (Gastropoda, Nassariidae).

Other host: *Nassarius olivaceus* (Bruguière), (Gastropoda, Nassariidae).

Locality: Cawarral Creek, Keppel Sands, Queensland (23°19' S; 150°47' E).

Habitat: Intertidal mudflats.

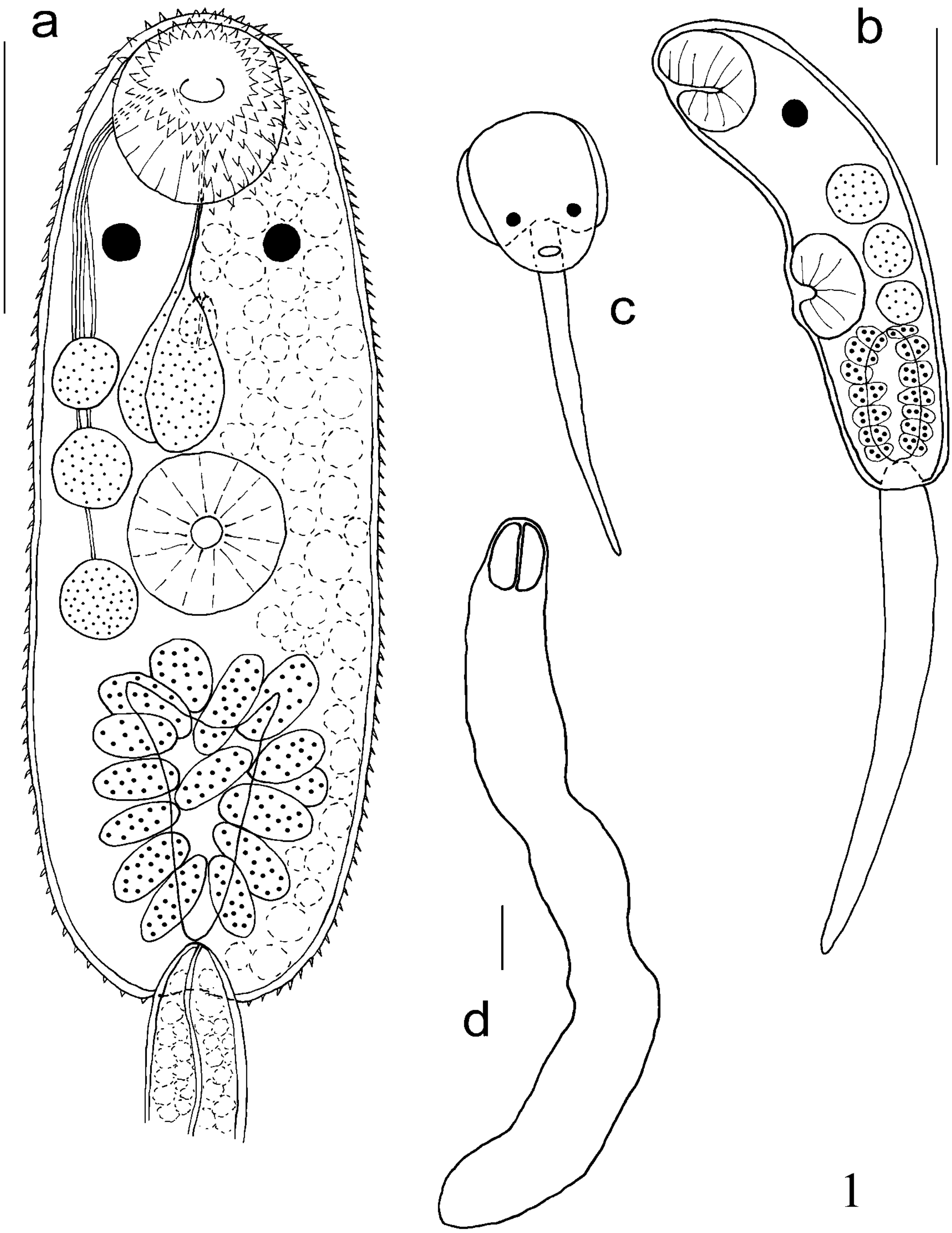

FIGURE 1. a–d. *Cercaria capricornia* VII, naturally emerged from *Nassarius dorsatus* and *N. olivaceus*. **a.** Body, heat-killed, in ventral view; **b.** Entire cercaria, heat-killed, from side view; **c.** Live cercaria, body folded for swimming, from ventral view; **d.** Redia, fixed. Scale bars a, b, d = 50 µm; c not to scale.

TABLE 2. Morphometric comparison between *Cercariae capricornia* VII and VIII and published *Stephanostomum* and *Stephanostomum*-like cercariae (Gnana Mani 1994; Køie 1978; Madhavi & Shameem 1993; Martin 1939; Stunkard 1961; Wolfgang 1955).

	C. capricornia		*S. dentatum*	*S. tenue*	*S. caducum*	*S. cloacum*	*S. baccatum*	*C. bengalensis* VII
	VII	VIII						
Total body length	155–250 (195.2)	182–240 (205.3)	135–145	145–380 (240)	220–260 (240)	480–500	320–500	160–195 (181)
Maximum body width	50–85 (69.2)	62–83 (70.5)	46–53	45–86 (64)	115–140 (125)	154–166	90–140	59–74 (62)
Forebody length	75–125 (97.2)	95–138 (112.4)						
Tail length	142–210 (175.6)	215–270 (253.6)	220–240	183	250–300	300–320	540–640	210–244 (194)
Tail width	17–28 (20.6)	20–30 (22.1)	16–20	31	25–35	28–32		20–23 (20)
Oral sucker length	30–40 (34.4)	35–43 (38.2)	24–25	31	42–50 (48)	72–78	60–80	31–35
Oral sucker width	27–38 (33.4)	32–38 (35.4)	24–25	30		72–78	60–80	31–35
Ventral sucker length	17–33 (29.1)	27–35 (30.6)	23–24	33	35–38 (37)	62	50–70	31–35
Ventral sucker width	20–35 (30.4)	27–35 (31.3)	23–24	30	35–40 (38)	66	50–70	31–35
Pharynx length	10		25–48	25–52	15	40–44		31–34
Excretory vesicle length	37–75 (52.1)	42–55 (49.7)				88–100		
Excretory vesicle width	32–58 (45.1)	32–48 (39.3)				58–70		
Eyes	7–10 (diam.)	13 x 10–13	9–12 (diam.)					8 (diam.)
Glands (central + lateral)	2 + 3	2 + 3	2 + 3	2 + 2	2 + 3	2 + 2	2 + 0	5 + 5
Oral sucker–eye distance	32–55 (46.6)	37–68 (53.6)						
Eye–eye distance	12–28 (20.3)	7–23 (14.4)						
Stylet (length)	no	no	no	yes (12)	yes (6)	yes	no	yes (5)

Other locality: Sandy Point, Corio Bay (22°58' S, 150°46' E), Ross Creek, Yeppoon, Queensland (23°8' S, 150°45' E).

Location in host (redia): Digestive gland, gonads.

Prevalence of emergence: 0.57% (10 of 1766 *N. dorsatus*), 0.21% (4 of 1908 *N. olivaceus*).

Material deposited: Queensland Museum; G231698–G231720.

Molecular sequence data: ITS2, 6 replicates; LSU, 6 replicates.

GenBank accession numbers: ITS2 (FJ809039); LSU (FJ809037).

MorphBank accession number: 515656.

Dates of collection: August/November 2004, February/May–June/November 2005 and March/May 2006.

Description: *Redia.* (Fig. 1d, based on 10 unflattened specimens): Body elongate, cylindrical with posterior extremity tapering, 530–1210 (703.0) long by 60–120 (75.5) wide. Mouth opens terminally. Pharynx 25–32.5 (29.3) long by 20–25 (23.8) wide. In reproductive and digestive glands.

Cercaria. (Figs 1a–c, based on 140 naturally emerged specimens): Body elliptical, longer than wide, widest point posterior to ventral sucker, 155–250 (195.2 ± 16.1) long by 50–85 (69.2 ± 5.9) wide; length/width 2.27–4.17 (2.84 ± 0.3). Tegument spinose; spines arranged in regular rows. Tail simple, long, cylindrical, gradually tapering terminally, lacking spines, chaetae, fins, 142–210 (175.6 ± 15.2) long by 17–28 (20.6 ± 2.1) wide. Oral sucker 30–40 (34.4 ± 2.2) long by 27–38 (33.4 ± 2.0) wide. Ventral sucker 17–33 (29.1 ± 2.2) long by 20–35 (30.4 ± 1.9) wide. Mouth subterminal, opening anteroventrally, surrounded by spines; spines around mouth aperture slightly larger than on surrounding tegument. Forebody 75–125 (97.2 ± 8.9) long; forebody/hindbody 0.80–1.34 (0.99 ± 0.07). Pharynx generally obscured by central glands, pyriform, 10 long by 12 wide (n=1). Penetration glands in 5 pairs; 2 pairs median and immediately anterior to ventral sucker and 3 pairs lateral or anterolateral to ventral sucker. Excretory vesicle 37–75 (52.1 ± 6.1) long by 32–58 (45.1 ± 4.7) wide; V-shaped when full, Y-shaped when empty; excretory duct appears to continue length of tail. Eye-spots dense, spherical, 12–28 (20.3 ± 2.9) apart, 32–55 (46.6 ± 5.0) from anterior end; 7–10 diameter (n=24).

Remarks: Large numbers of cercariae emerge, sometimes on consecutive days, with pauses between emergences (few days to weeks). Cercariae generally emerge before 8 am. Naturally emergent cercariae are free-swimming and strongly positively phototactic. When swimming, the hindbody is folded ventrally (Fig. 1c) and the tail lashes from side to side for movement. When resting, the hindbody remains folded ventrally. When first emerged, cercariae swim actively towards light, frequently forming swarms near the surface. After about 24 hours, cercariae become less active. Cercariae were never observed to encyst.

Cercaria capricornia VII can be distinguished from the cercariae of *S. tenue*, *S. cloacum, S. caducum* and *C. bengalensis* VII by the absence of a stylet (Gnana Mani 1994; Køie 1978; Madhavi & Shameem 1993; Martin 1939). *Stephanostomum baccatum* is larger and has no lateral penetration glands (Wolfgang 1955). *Stephanostomum dentatum* has a tail with lateral cuticular fins and is photonegative (Stunkard 1961). Morphometric comparison between *C. capricornia* VII and *C. capricornia* VIII, *S. tenue*, *S. cloacum*, *S. caducum*, *C. bengalensis* VII, *S. baccatum* and *S. dentatum* is given in Table 2.

Cercaria capricornia VIII
(Fig. 2)

Host: *Nassarius dorsatus* (Röding) (Gastropoda, Nassariidae).

Other host: None.

Locality: Cawarral Creek, Keppel Sands, Queensland (23°19' S; 150°47' E).

Habitat: Intertidal mudflats.

Location in host (redia): Digestive gland, gonads.

Prevalence of emergence: 0.11% (2 of 1766 *N. dorsatus*).

Material deposited: Queensland Museum; G231721–G231724.

Molecular sequence data: ITS2, 1 replicate; LSU, 1 replicate.

GenBank accession numbers: ITS2 (FJ809038); LSU (FJ809036).

MorphBank accession number: 515658.

Dates of collection: August and November 2004.

Description: *Redia.* (Fig. 2c, based on 10 unflattened specimens): Body elongate, cylindrical with posterior extremity tapering, 240–580 (416.0) long by 35–50 (41.5) wide. Mouth opens terminally. Pharynx 27–30 (28.5) long by 20–25 (23.3) wide. In reproductive and digestive glands.

Cercaria. (Figs 2a–b, based on 30 naturally emerged specimens): Body elliptical, longer than wide, widest point anterior to ventral sucker, 182–240 (205.3 ± 13.1) long by 62–83 (70.5 ± 4.7) wide; length/width ratio 2.48–3.31 (2.92 ± 1.2). Tegument spinose; spines arranged in regular rows. Tail simple, long, cylindrical, gradually tapering terminally, lacking spines, chaetae, fins, 215–270 (253.6 ± 13.4) long by 20–30 (22.1 ± 2.2) wide. Oral sucker 35–43 (38.2 ± 1.8) long by 32–38 (35.4 ± 1.9) wide. Ventral sucker 27–35 (30.6 ± 1.7) long by 27–35 (31.3 ± 1.8) wide. Mouth subterminal, opening anteroventrally, surrounded by spines; spines around mouth aperture slightly larger than on surrounding tegument. Forebody 95–138 (112.4 ± 9.8) long; forebody/

hindbody ratio 1.08–1.34 (1.21 ± 0.07). Pharynx obscured by central glands. Penetration glands in 5 pairs; 2 pairs median and immediately anterior to ventral sucker and 3 pairs lateral or anterolateral to ventral sucker. Excretory vesicle 42–55 (49.7 ± 2.8) long by 32–48 (39.3 ± 3.5) wide; V-shaped when full, Y-shaped when empty; excretory duct appears to continue length of tail. Eye-spots dense, oblong, 7–23 (14.4 ± 2.9) apart, 37–68 (53.6 ± 6.1) from anterior end; 13 long by 10–13 wide (n=7).

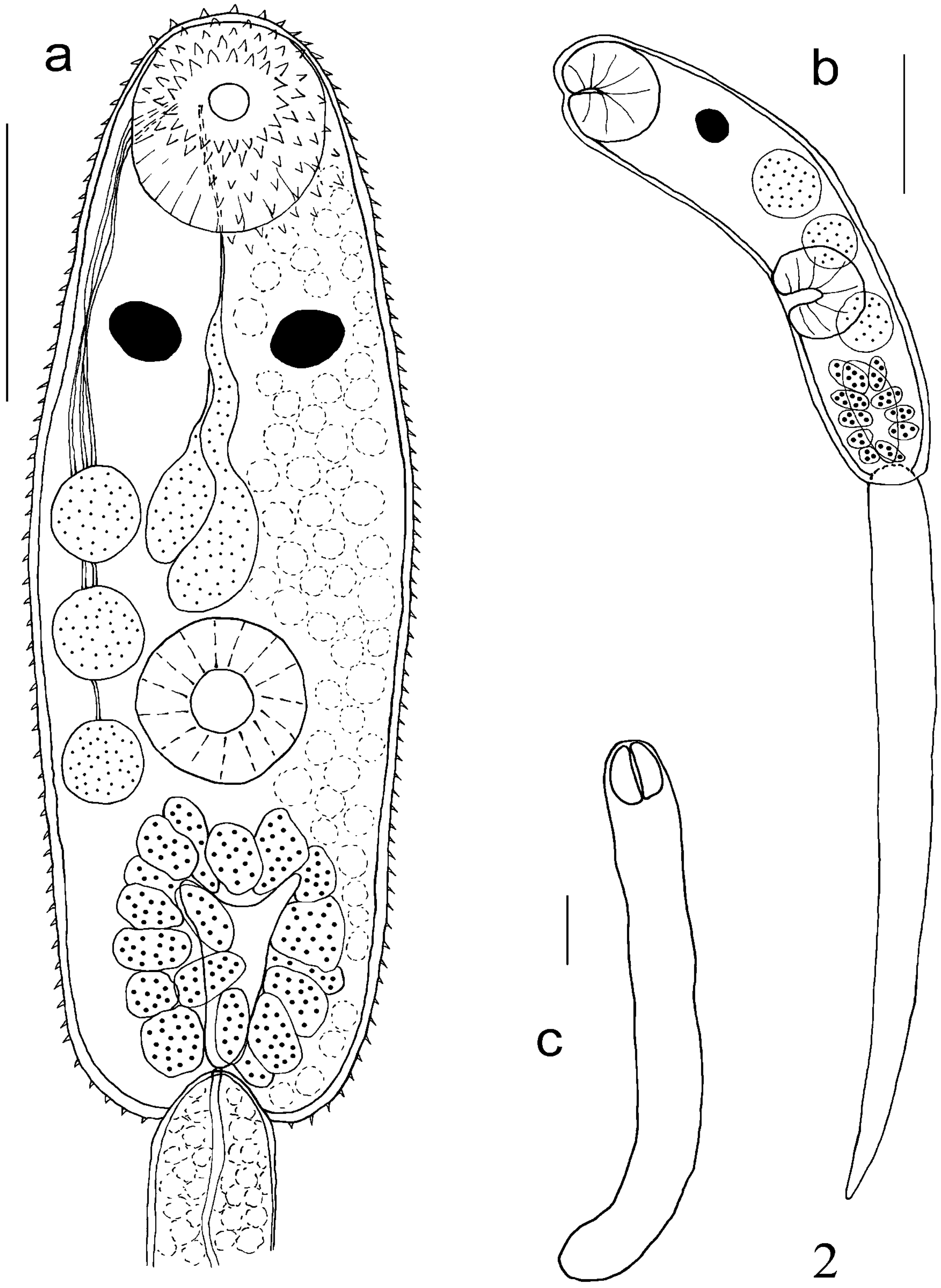

FIGURE 2. a–c. *Cercaria capricornia* VIII, naturally emerged from *Nassarius dorsatus*. **a.** Body, heat-killed, in ventral view; **b.** Entire cercaria, heat-killed, from side view; **c.** Redia, fixed. Scale bars a, b, c = 50 µm.

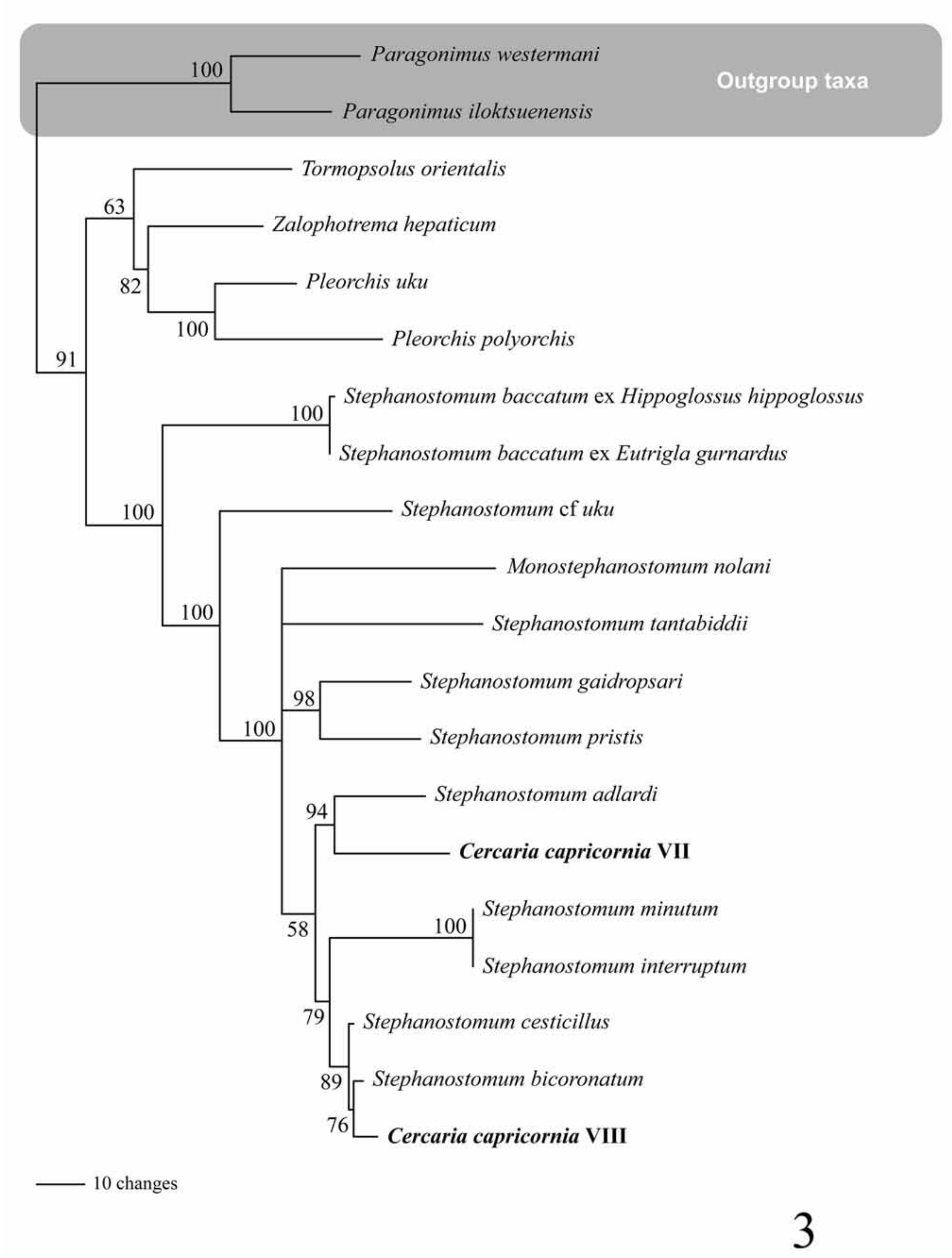

FIGURE 3. Relationships between *Cercaria capricornia* VII and *C. capricornia* VIII and the remainder of the taxa examined based on Bayesian inference analysis of partial LSU rDNA. Posterior probabilities are shown at the nodes with values < 50 not shown.

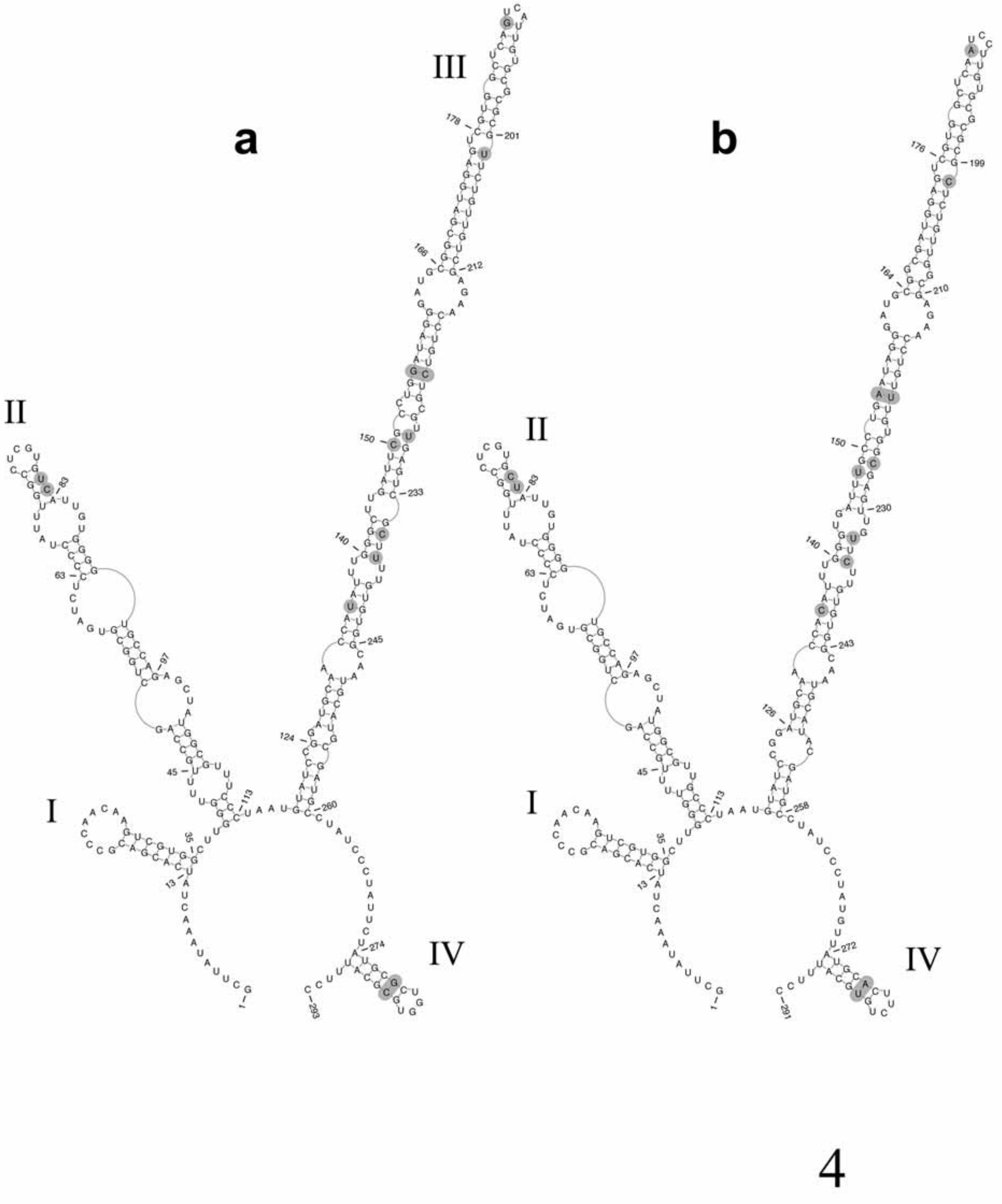

FIGURE 4. Inferred secondary structure of the ITS2 rDNA region for based on minimum free-energy modelling for **a.** *Cercaria capricornia* VII and **b.** *C. capricornia* VIII. Helices one through four are indicated with Roman numerals. Complete compensatory base changes (CBCs) and hemi-CBCs are indicated in grey.

Remarks: Large numbers of cercariae emerge, sometimes on consecutive days, with pauses between emergences (few days to weeks). Cercariae generally emerge before 8 am. Naturally emergent cercariae are free-swimming and strongly positively phototactic. When swimming, the hindbody is folded ventrally and the tail lashes from side to side for movement, similar to *C. capricornia* VII (see Fig. 1c). When resting, the hindbody remains folded ventrally. When first emerged, cercariae swim actively towards light, frequently forming swarms near the surface. After about 24 hours, cercariae become less active. Cercariae were never observed to encyst.

Cercaria capricornia VIII can also be distinguished from *S. tenue*, *S. cloacum*, *S. caducum* and *C. bengalensis* VII by the absence of a stylet, from *S. baccatum* by size and the presence of lateral penetration glands, and from *S. dentatum* by the absence of lateral cuticular fins on the tail.

Cercaria capricornia VIII has a longer tail relative to body length than *C. capricornia* VII, and the eye-spots are oblong in shape and larger than the spherical eye-spots of *C. capricornia* VII. The hindbody of *C. capricornia* VIII tapers inward towards the tail and the widest point of the body is anterior to the ventral sucker, while the hindbody of *C. capricornia* VII is broader with the widest point posterior to the ventral sucker. Morphometric comparison between *C. capricornia* VIII and *C. capricornia* VII, *S. tenue*, *S. cloacum*, *S. caducum*, *C. bengalensis* VII, *S. baccatum* and *S. dentatum* is given in Table 2.

Molecular data

Comparative LSU rDNA analysis: Alignment of the partial LSU rDNA region for *C. capricornia* VII and *C. capricornia* VIII and the remainder of the taxa examined yielded 884 characters for analysis. Neither cercaria showed any intraspecific variation over the LSU rDNA region sequenced here.

Bayesian inference analysis of the LSU dataset resulted in a phylogram with *C. capricornia* VII and *C. capricornia* VIII nested well within a clade containing species of *Stephanostomum* (Fig. 3). *Cercaria capricornia* VII formed a well-supported clade with *Stephanostomum adlardi* Bray, Cribb, Waeschenbach & Littlewood, 2007, while *C. capricornia* VIII was observed as sister to *Stephanostomum bicoronatum* (Stossich, 1883) Fuhrmann, 1928. Most genera were well resolved with strong posterior probability support in the Bayesian inference analysis. The only exception was that *Monostephanostomum nolani* Bray & Cribb, 2007 nested within the well-supported clade containing species of the *Stephanostomum*. The Acanthocolpidae was resolved here as paraphyletic, with the brachycladiid *Zalophotrema hepaticum* Stunkard & Alvey, 1929 nested within a clade containing species of *Tormopsolus* Poche, 1926 and *Pleorchis* Railliet, 1896.

ITS2 rDNA sequencing and secondary structure prediction: The ITS2 rDNA regions sequenced for *C. capricornia* VII and *C. capricornia* VIII were 293 and 291 nucleotides long (respectively). No intraspecific variation was observed in this region for these cercarial types. The putative secondary structure predicted for the ITS2 region of *C. capricornia* VII using Mfold software resulted in a four helix model with a calculated free-energy minimum of -108.2 kcal/mol (Fig. 4a). The putative ITS2 secondary structure predicted for *C. capricornia* VIII also resulted in a four helix model, which had a calculated free-energy minimum of -115.0 kcal/mol (Fig. 4b). Helices I and IV of these putative models are both relatively short, each containing fewer than 25 nucleotides. Helix III in both secondary structure prediction models was the longest, comprising approximately 140 nucleotides.

Compensatory base change analysis for the two cercariae revealed the presence in each of two CBCs (one in Helix III and the other near the distal end of Helix IV) and nine hemi-CBCs (two near the distal end of Helix II and seven in Helix III) (Fig. 4).

Discussion

Systematics

The combined morphological and molecular data for *C. capricornia* VII and *C. capricornia* VIII suggest strongly that these are cercariae of species of *Stephanostomum*. These cercariae are morphologically similar to previously described acanthocolpid cercariae, but can be readily distinguished using morphometric data (Table 2).

Cercaria capricornia VII and *C. capricornia* VIII have a body shape similar to those of the cercariae of *S. cloacum* and *S. baccatum*, but are smaller and can also be differentiated from *S. baccatum* by molecular analysis (Fig. 3) and the number of penetration glands (Table 2). They are also similar in body shape to *C. bengalensis* VII, but in that cercaria the suckers were of equal size, whereas in *C. capricornia* VII and *C. capricornia* VIII the oral sucker is larger than the ventral sucker. They have a simple tail unlike *S. dentatum* which has a tail with lateral cuticular fins. The cercaria of *S. tenue* has a longer body, with an elongated hindbody compared to *C. capricornia* VII and *C. capricornia* VIII. The body of *S. caducum* is broader and with larger oral and ventral suckers. In addition, no stylet was seen in *C. capricornia* VII or *C. capricornia* VIII, while a stylet was reported for *S. tenue*, *S. cloacum S. caducum* and *C. bengalensis* VII.

TABLE 3. List of species of Acanthocolpidae reported from Australian and South Pacific waters and partial LSU rDNA sequences from GenBank.

Species	Publication	GenBank accession
Acanthocolpidae		
Monostephanostomum nolani Bray & Cribb, 2007	(Bray & Cribb 2007a)	EF506763
Monostephanostomum krusei Reimer, 1983	(Bray & Cribb 2007a)	
Monostephanostomum georgianum Bray & Cribb, 2002	(Bray & Cribb 2002)	
Monostephanostomum manteri Kruse, 1979	(Bray & Cribb 2002)	
Ningalooia psammopercae Bray & Cribb, 2007	(Bray & Cribb 2007b)	
Pleorchis uku Yamaguti, 1970	(Bray *et al.* 2005)	DQ248216
Stephanostomum aaravi Bray & Cribb, 2003	(Bray & Cribb 2003)	
Stephanostomum adlardi Bray, Cribb, Waeschenbach & Littlewood, 2007	(Bray *et al.* 2007)	EF506761
Stephanostomum bicoronatum (Stossich, 1883) Fuhrmann, 1928	(Bray & Cribb 2003)	DQ248225
Stephanostomum carangi Liu, 1998	(Bray & Cribb 2008)	
Stephanostomum cobia Bray & Cribb, 2003	(Bray & Cribb 2003)	
Stephanostomum ditrematis (Yamaguti, 1939) Manter, 1947	(Bray & Cribb 2008)	
Stephanostomum hawaiiense Yamaguti, 1970	(Bray & Cribb 2008)	
Stephanostomum lamothei Bray & Cribb, 2008	(Bray & Cribb 2008)	
Stephanostomum madhaviae Bray & Cribb, 2003	(Bray & Cribb 2003)	
Stephanostomum nyoomwa Bray & Cribb, 2003	(Bray & Cribb 2003; Bray & Cribb 2008)	
Stephanostomum pagrosomi (Yamaguti, 1939) Manter, 1947	(Bray & Cribb 2003)	
Stephanostomum petimba Yamaguti, 1970	(Bray & Cribb 2003; Hutson *et al.* 2007)	
Stephanostomum talakitok Bray & Cribb, 2006	(Bray & Cribb 2006)	
Stephanostomum tantabiddii Bray & Cribb, 2004	(Bray & Cribb 2004)	DQ248220
Stephanostomum tupatupa Bray & Cribb, 2008	(Bray & Cribb 2008)	
Stephanostomum cf. *uku* Yamaguti, 1970	(Bray *et al.* 2005)	DQ248219
Stephanostomum pacificum (Yamaguti, 1951) Yamaguti, 1953	(Bray & Cribb 2003)	
Stephanostomum votonimoli Bray & Cribb, 2003	(Bray & Cribb 2003)	
Tormopsolus orientalis Yamaguti, 1934	(Bartoli *et al.* 2004; Hutson *et al.* 2007)	DQ248217
Tormopsolus attenuatus Bray & Cribb, 2001	(Bray & Cribb 2001)	
Venusicola inusitatus Bray & Cribb, 2000	(Bray & Cribb 2000)	

Cercariae capricornia I–VI, also reported from nassariid gastropods, have an expanded hindbody and an unusual body shape, including a keel, strikingly different to that of these cercariae.

Cercaria capricornia VII and *C. capricornia* VIII can also be differentiated from other acanthocolpid cercariae. *Cercaria caribbea* XXXIV Cable, 1956, *Cercaria portosacculus* Holliman, 1961, *Cercaria caribbea* LXXII Cable, 1963, and *Cercaria caribbea* LXXIII Cable, 1963, have unusual caudal features which differentiate them from *C. capricornia* VII and *C. capricornia* VIII (Cable 1956; Cable 1963; Holliman 1961), while *Cercaria itoi* Shimura, 1984 and a probable *Tormopsolus* cercaria described by Bartoli and Gibson have an expanded hindbody and are larger than *C. capricornia* VII and *C. capricornia* VIII (Bartoli & Gibson 1998; Shimura 1984).

Neophasis anarrhichae (Nicoll, 1909) Bray, 1987 (syn. *Neophasis lageniformis* (Lebour, 1910) Miller, 1941) was reported with 7–8 pairs of glands, and was suggested to lose its tail whilst in the redia and not emerge from the snail (Chubrik 1966). The tail of *Neophasis oculatus* (Levinsen, 1881) Miller, 1941 is much longer and tegumental spines are restricted to the anterior end (Chubrik 1966). Another probable *Neophasis* cercaria from *Neptunea despecta* (Linnaeus), *Neptunea borealis* (Philippi) and *Cryptonatica affinis* (Gmelin) (syn. *Cryptonatica* [*Tectonatica*] *clausa* Broderip & Sowerby) was spinose anterior to the eyes only and was larger than *C. capricornia* VII and *C. capricornia* VIII (Marasaev 1984).

Cercaria criollisima V Nasir, 1976, a cercaria suggested by Madhavi and Shameem (1993) to have characters which fit those of acanthocolpids, was described as having an anterior organ more as a penetration device than a sucker, while both *C. capricornia* VII and *C. capricornia* VIII have obvious oral suckers (Madhavi & Shameem 1993; Nasir 1976). In addition, *C. criollisima* V was reported to develop in sporocysts, while *C. capricornia* VII and *C. capricornia* VIII develop in rediae.

The acanthocolpid fauna of fishes from Australian and South Pacific waters is rich (Table 3). There are presently four species of *Monostephanostomum*, one species of *Ningalooia*, one species of *Pleorchis*, 18 species of *Stephanostomum*, two species of *Tormopsolus* and one species of *Venusicola* known. Species of *Stephanostomum* reported from eastern Australian waters include *S. adlardi*, *S. aaravi*, *S. bicoronatum*, *S. carangi*, *S. cobia*, *S. ditrematis*, *S. hawaiiense*, *S. lamothei*, *S. madhaviae*, *S. nyoomwa*, *S. pagrosomi*, *S. petimba*, *S. talakitok*, *S. tantabiddii*, *S. tupatupa*, and *S.* cf. *uku*. Of these, only *S. adlardi*, *S. bicoronatum*, *S. tantabiddii* and *S.* cf. *uku* have published partial LSU rDNA sequences available for comparison. Molecular analysis indicates that *C. capricornia* VII and *C. capricornia* VIII are not cercariae from these four species. *Cercaria capricornia* VII resolved as a sister to *S. adlardi* and *C. capricornia* VIII to *S. bicoronatum*, both species reported from fishes of eastern Australian waters.

First intermediate hosts

We are now aware of 25 apparently distinct cercariae that have been referred to the Acanthocolpidae, either definitively by experimental infection, by molecular phylogenetic analysis as here, or by shared general morphology. We find all these identifications broadly convincing except for *C. criollisima* V, which, uniquely for this group, was reported from a bivalve (Veneridae) rather than a gastropod. There are nine families of trematodes that routinely infect bivalves as first intermediate hosts and a few others (e.g. Lepocreadiidae, Hemiuridae) that infect them exceptionally (e.g. Hassanine 2006; Wardle 1975). The combination of prominent eye-spots, absence of a stylet, simple tail and parasitism of a marine rather than a freshwater bivalve distinguish *C. criollisima* V from eight of these, the Allocreadiidae, Aporocotylidae, Bucephalidae, Faustulidae, Fellodistomidae, Gorgoderidae, Gymnophallidae and Tandanicolidae. The morphology of *C. criollisima* V is broadly consistent with the cercariae of some Monorchiidae in which the tail is well-developed such as *C. caribbea* XXXV Cable, 1956, *C. caribbea* LXIII Cable, 1963 and that of *Paratimonia gobii* Prevot & Bartoli, 1967 (Cable 1956; Cable 1963; Maillard 1975). We will therefore not consider this cercaria further as a possible acanthocolpid.

If *C. criollisima* V is excluded, then all the other putative acanthocolpid cercariae develop in gastropods (Table 4). All infections are in the Clade Hypsogastropoda (Bouchet & Rocroi 2005) which incorporates numerous superfamilies of which just two, Buccinoidea and Naticoidea, are infected by putative acanthocolpids. Naticoidea has just one family, Naticidae, and this has been shown convincingly to harbour species of *Neophasis* and *Stephanostomum* (Chubrik 1966; Køie 1978; Marasaev 1984; Yamaguti 1975). Acanthocolpids are known from four families of Buccinoidea. Cribb *et al.* (2001) reviewed the overall pattern of specificity of trematodes in fishes and first intermediate hosts. They found that no well-studied taxon has a relatively narrower range of vertebrate definitive hosts than of molluscan intermediate hosts, but they did not consider the Acanthocolpidae in any detail. The present results suggest that this family is consistent with the

TABLE 4. First intermediate hosts of putative Acanthocolpidae.

Host species	Parasite species	Reference
Superfamily Buccinoidea		
Buccinidae		
Buccinum undatum Linnaeus	*Neophasis anarrhichae* (Nicoll, 1909) Bray, 1987 syn. *Neophasis lageniformis* (Lebour, 1910) Miller, 1941	(Chubrik 1966; Køie 1971; Køie 1973)
	Stephanostomum baccatum (Nicoll, 1907) Manter, 1934 syn. *Cercaria neptunae* Lebour, 1911	(MacKenzie & Liversidge 1975; Wolfgang 1955; Yamaguti 1971; Yamaguti 1975)
Cantharus dorbignyi Payraudau	*Tormopsolus* sp.	(Bartoli & Gibson 1998)
Neptunea antiqua (Linnaeus)	*Stephanostomum baccatum* (Nicoll, 1907) Manter, 1934 syn. *Cercaria neptunae* Lebour, 1911	(MacKenzie & Liversidge 1975)
Neptunea borealis (Philippi)	*Neophasis* sp.	(Marasaev 1984)
Neptunea decemcostata (Say) syn. *Neptunea decemcostatum* (Say)	*Stephanostomum baccatum* (Nicoll, 1907) Manter, 1934 syn. *Cercaria neptunae* Lebour, 1911	(Wolfgang 1955; Yamaguti 1971; Yamaguti 1975)
Neptunea despecta (Linnaeus)	*Neophasis* sp.	(Marasaev 1984)
Columbellidae		
Anachis obesa C. B. Adams	*Cercaria caribbea* LXXII Cable, 1963	(Cable 1963)
	Cercaria portosacculus Holliman, 1961	(Holliman 1961)
Columbella mercatoria (Linnaeus)	*Cercaria caribbea* LXXIII Cable, 1963	(Cable 1963; Yamaguti 1975)
	Cercaria caribbea XXXIV Cable, 1956	(Cable 1956)
Fasciolariidae		
Fusinus perplexus A. Adams	*Cercaria itoi* Shimura, 1984	(Shimura 1984)
Nassariidae		
Nassarius dorsatus (Röding)	*Cercaria capricornia* V Barnett, Smales & Cribb, 2008	(Barnett *et al.* 2008)
	Cercaria capricornia VI Barnett, Smales & Cribb, 2008	(Barnett *et al.* 2008)
	Cercaria capricornia VII	This study
	Cercaria capricornia VIII	This study
Nassarius obsoletus, Say	*Stephanostomum dentatum* (Linton, 1900) Linton, 1940 syn. *Cercaria dipterocerca* Miller & Northup, 1926	(Miller & Northup 1926; Stunkard 1961; Yamaguti 1971; Yamaguti 1975)
	Stephanostomum tenue (Linton, 1898) Linton, 1934	(Martin 1939; Yamaguti 1971; Yamaguti 1975)
Nassarius olivaceus (Bruguière)	*Cercaria capricornia* I Barnett, Smales & Cribb, 2008	(Barnett *et al.* 2008)
	Cercaria capricornia II Barnett, Smales & Cribb, 2008	(Barnett *et al.* 2008)
	Cercaria capricornia III Barnett, Smales & Cribb, 2008	(Barnett *et al.* 2008)
	Cercaria capricornia IV Barnett, Smales & Cribb, 2008	(Barnett *et al.* 2008)
	Cercaria capricornia VII	This study
Nassarius orissaensis (Preston)	*Stephanostomum cloacum* (Srivastava, 1938) Manter & Van Cleave, 1951	(Madhavi & Shameem 1993)
Nassarius stolatus (Gmelin)	*Cercaria bengalensis* VII Gnana Mani, 1994	(Gnana Mani 1994)
Superfamily Naticoidea		
Naticidae		
Cryptonatica affinis (Gmelin) syn. *Cryptonatica* [*Tectonatica*] *clausa* Broderip & Sowerby	*Neophasis oculatus* (Levinsen, 1881) Miller, 1941	(Bray & Gibson 1991; Chubrik 1966)
	Neophasis sp.	(Marasaev 1984)
Euspira pulchella (Risso) syn. *Natica alderi* (Forbes)	*Stephanostomum caducum* (Looss, 1901) Manter 1934	(Køie 1978)

overall pattern. Our records suggest that acanthocolpids have been reported as sexual adults from 24 orders of fishes but as intra-molluscan stages from just two superfamilies. Of course future collecting may modify this pattern but overall it appears that acanthocolpids have a narrow range of first intermediate hosts. However, range is not so restricted as to suggest that cophyly has occurred at the species level. We suspect that the overall pattern of host-specificity for gastropods suggests that the Acanthocolpidae radiated in association with the Buccinoidea and have subsequently transferred, perhaps at least twice, into the Naticoidea. However, far more host-parasite records will need to be accumulated and far better knowledge of the phylogenetic relationships in the Acanthocolpidae are needed for these ideas to be more than speculative.

Cribb *et al*. (2002) observed that *Stephanostomum* was the second largest genus of trematodes of fishes (second to *Phyllodistomum* from urinary bladders). The success of this genus is evident from the huge range of fishes (over 70 families) that are infected as adults. The genus also appears to have low specificity for first intermediate hosts, having been identified (or inferred) from three families and two superfamilies of molluscs. Given the current size of the genus there are clearly many more intermediate hosts to be found and we predict that further families of Buccinoidea and perhaps of other Hypsogastropoda will prove to be infected.

Relationships between *Cercariae capricornia* VII and VIII and other acanthocolpid taxa

Phylogenetic analysis of the taxa examined here showed that *C. capricornia* VII and *C. capricornia* VIII were nested within a clade containing species of *Stephanostomum*, suggesting that these cercariae are species of this relatively large acanthocolpid genus. The results of our Bayesian inference analysis using partial LSU rDNA sequences agree broadly with the phylogenetic hypothesis of relationships among the Acanthocolpidae based on complete small subunit (SSU) and partial LSU rDNA sequence data reported by Bray *et al.* (2005, 2007). One of the distinct differences in topology observed between the phylograms reported by Bray *et al*. (2005, 2007) and here is the position of *Z. hepaticum*, which forms a clade with species of *Pleorchis* to the exclusion of *Tormopsolus* in our analysis rather than sister to these genera (Fig. 3).

Inferred secondary structure of the ITS2 rDNA region

The inferred secondary structure of the ITS2 rDNA region for *C. capricornia* VII and *C. capricornia* VIII using minimum free energy modelling agrees with the core four helix domain structure (with helix III being the longest) that has been recently identified as common to almost all eukaryote taxa (Coleman 2003; Coleman 2007; Schultz *et al*. 2005). The optimal secondary structures inferred here for these putative acanthocolpid cercariae also agree with the four domain model of the ITS2 rDNA region using minimum free energy folding algorithms reported for other digenean families (Morgan & Blair 1998). All four of the helices in the models for these two cercariae are of similar length and structure to that of the general digenean structure reported by Morgan & Blair (1998) and Miller & Cribb (2009).

Differences of as little as a single compensatory base change in the secondary structure pairing of the ITS2 rDNA region has recently been shown to be strongly correlated with sexual incompatibility and, therefore, possibly indicative of distinct “biological species” (Coleman 2009; Müller *et al.* 2007; Wolf *et al.* 2005). When compared, the two putative acanthocolpid cercariae reported here had two CBCs and nine hemi-CBCs, which correlates well with our conclusion that two distinct species are present in this system based on phylogenetic analysis of LSU rDNA and the results of morphometric analysis. While it is clear that incorporating analysis of the secondary structure of the ITS2 rDNA region as well as phylogenetic analysis of raw sequence data will help in resolving relationships among difficult or cryptic taxonomic groups within the Acanthocolpidae, more comparative ITS2 secondary structure data is needed to address how the numbers of CBCs and hemi-CBCs observed may correlate with distinct ‘biological species’ within this family and the Digenea as a whole.

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