


Pronocephaloid cercariae (Platyhelminthes: Trematoda) from gastropods of the Queensland coast, Australia

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Research Paper

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Abstract

The superfamily Pronocephaloidea Looss, 1899 comprises digeneans occurring in the gut and respiratory organs of fishes, turtles, marine iguanas, birds and mammals. Although many life cycles are known for species of the Notocotyliidae Lühe, 1909 maturing in birds and mammals, relatively few are known for the remaining pronocephaloid lineages. We report the cercariae of five pronocephaloid species from marine gastropods of the Queensland coast, Australia. From Lizard Island, northern Great Barrier Reef, we report three cercariae, two from *Rhinochloris vertagus* (Cerithiidae) and one from *Nassarius coronatus* (Nassariidae). From Moreton Bay, southern Queensland, an additional two cercariae are reported from two genotypes of the gastropod worm shell *Thylacodes* sp. (Vermetidae). Phylogenetic analysis using 28S rRNA gene sequences shows all five species are nested within the Pronocephaloidea, but not matching or particularly close to any previously sequenced taxon. In combination, phylogenetic and ecological evidence suggests that most of these species will prove to be pronocephalids parasitic in marine turtles. The Vermetidae is a new host family for the Pronocephaloidea.

Introduction

The Pronocephaloidea Looss, 1899 is a superfamily of trematodes that mainly infect the intestines of marine and freshwater turtles (Pronocephalidae Looss, 1899), a wide range of birds and mammals (Notocotyliidae Lühe, 1909; Nudacotyliidae Barker, 1916) and sirenians (dugong and manatees) (Labicolidae Blair, 1979; Opisthotrematidae Poche, 1926; Rhabdiopoeidae Poche, 1926); a few pronocephalids infect marine iguanas and fishes (Blair, 2005). Although there has been only limited molecular exploration of the relationships within the group (Olson *et al.*, 2003), they are convincingly considered the sister taxon to the Paramphistomoidea. Life cycles are known only for notocotyliids, one nudacotyliid and a handful of pronocephalids. As far as it is known, these always involve the infection of gastropods by ingestion of the egg (Murrills *et al.*, 1985), the development of ‘monostome’ cercariae in rediae, cercariae which emerge from the gastropod and encyst as metacercariae on the surface of potential food of the definitive host and infection of the definitive host when that food is consumed. There are many life cycles reported for the Notocotyliidae (e.g. Herber, 1942; Stunkard, 1960; Pike, 1969; Kanev *et al.*, 1994) involving a wide range of gastropods: caenogastropods (Bithyniidae, Cerithiidae, Hydrobiidae, Littorinidae, Pleuroceridae, Pomatiopsidae, Tateidae, Thiaridae and Viviparidae); neritimorphs (Neritidae); and heterobranchs (Lymnaeidae, Physidae and Planorbidae). In contrast, relatively few pronocephalid life cycles are known. They are known for entirely freshwater species infecting Pleuroceridae or Thiaridae (Horsfall, 1935; Hsü, 1937) and one species that infects the semi-marine terrapin *Malaclemys terrapin* (see Hunter, 1967), which has a nassariid (Caenogastropoda) as intermediate host. The single reported nudacotyliid life cycle (Ameel, 1944) involves a pomatiopsid (Caenogastropoda). There have so far been no intermediate hosts identified for Labicolidae, Opisthotrematidae, Pronocephalidae or Rhabdiopoeidae that infect marine turtles and sirenians, which, between them, harbour an exceptionally rich fauna of these trematodes.

Here we report five new pronocephaloid cercariae based on combined morphological and molecular data. These species were found in specimens of *Rhinochloris vertagus* (Cerithiidae), *Nassarius coronatus* (Nassariidae) and *Thylacodes* sp. (Vermetidae).

Materials and methods

Collection and morphological study

Cerithiid and nassariid gastropods were collected by hand from rocks and sand flats surrounding Lizard Island on the northern Great Barrier Reef (14°40'S, 145°27'E). These were identified

without difficulty by reference to Wilson (1993a, b). Vermetids were collected by hammer and chisel from intertidal rocks off Dunwich (27°24'S, 153°20'E) and Amity (27°23'S, 153°26'E), North Stradbroke Island, Moreton Bay, Queensland, Australia. The specimens could not be readily identified by reference to any available guide and were characterized by 16S rDNA data. Sequence data were analysed by Dr Timothy Rawlings relative to his unpublished database of vermetid sequences. Two vermetid genotypes, differing by 6 bp and found off Dunwich and Amity respectively, possibly represent two closely related species. These genotypes were nested among species of the genus *Thylacodes* Guettard, 1770, but did not correspond to any species for which sequence data were available. Sequence data for these specimens are lodged on GenBank (MN574296–97), and morphological vouchers relating to the two genotypes are lodged in the Queensland Museum (MO85861–2).

Cerithiids and nassariids were held in seawater for 24 h and monitored for emerged cercariae; no emerged cercariae were detected, so infections were found only by dissection. Vermetids were held in aerated seawater prior to dissection. Because these gastropods formed large tangled masses encrusting rocks, they could not be monitored individually for cercarial emergence, and all were dissected. Asexual trematode stages were fixed by pipetting into near-boiling saline followed by preservation in 80% ethanol to allow for either morphological or molecular analysis. Specimens for morphology were stained in Mayer's haematoxylin, destained in dilute HCl, neutralized in dilute ammonia solution, dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted on slides in Canada balsam. Mounted specimens were measured using CellSens software (<https://www.olympus-lifescience.com/en/software/cellsens>) connected to an Olympus SC50 camera mounted onto an Olympus BX53 compound microscope (Olympus, Notting Hill, Australia). Drawings were made with a camera lucida mounted on an Olympus BX53 compound microscope and were digitized in Adobe Illustrator (<https://www.adobe.com/au/products/illustrator.html>).

Molecular sequencing and phylogenetic analysis

Sequence fragment amplifications of pronoccephaloids were mostly obtained from individual cercariae or rediae through direct polymerase chain reaction (PCR) (see Huston *et al.*, 2018), but in some cases total genomic DNA was extracted using phenol/chloroform extraction techniques (Sambrook & Russell, 2001). Pronoccephaloids were genetically characterized by complete ITS2 and partial 28S rDNA, and vermetid hosts by partial 16S rDNA. The ITS2 rDNA region was amplified using the primers 3S (Morgan & Blair, 1995) and ITS2.2 (Cribb *et al.*, 1998), the partial D1-D3 28S rDNA region using LSU5 (Littlewood, 1994) and 1500R (Snyder & Tkach, 2001) and the partial 16S rDNA region using 16SARL-CBU (5'-CGC CTG TWT ADC AAA AAC ATG-3') and 16SBRH (5'-CCG GTC TGA ACT CAG ATC ACG-3') (both modified from Palumbi, 1996). Direct PCR for the ITS2 and 28S regions was performed following Huston *et al.* (2018). Genomic DNA-based PCR for all regions was performed following Cutmore *et al.* (2016). Amplification was carried out on an MJ Research PTC-150 thermocycler (Thermo Fisher Scientific, Brisbane, Australia). The following profile was used to amplify the 16S region for vermetid gastropods: an initial 94°C denaturation for 3 min, followed by 40 cycles of 94°C denaturation for 30 s, 50°C annealing for 30 s and 72°C extension for 30 s, with a final extension at 72°C for 10 min. Amplified DNA was purified using a Bioline ISOLATE II PCR and Gel Kit (Bioline,

Alexandria, Australia) according to the manufacturer's protocol. Cycle sequencing of purified DNA was carried out using ABI Big Dye™ version 3.1 (Thermo Fisher Scientific) chemistry following the manufacturer's recommendations, using the same primers used for PCR amplification as well as the additional 28S primers 300F (Littlewood *et al.*, 2000) and ECD2 (Littlewood *et al.*, 1997). Cycle sequencing was carried out at the Australian Genome Research Facility. Geneious® version 10.2.3 (Kearse *et al.*, 2012) was used to assemble and edit contiguous sequences, and the start and end of the ITS2 rDNA region were determined by annotation through the ITS2 Database (Keller *et al.*, 2009; Ankenbrand *et al.*, 2015) using the 'Metazoa' model.

The partial 28S rDNA sequences generated during this study were aligned with sequences of related species from GenBank (table 1) using MUSCLE (Edgar, 2004), with ClustalW sequence weighting and UPGMB clustering for iterations 1 and 2. The resultant alignment was refined by eye using MESQUITE (Maddison & Maddison, 2018); no ambiguously aligned sites were identified (those constituting more than three bases and present in two or more of the sequences in the dataset). Bayesian inference analysis of the 28S dataset was performed using MrBayes version 3.2.6 (Ronquist *et al.*, 2012), run on the CIPRES portal. The best nucleotide substitution model was estimated using jModelTest version 2.1.10 (Darriba *et al.*, 2012). The Akaike Information Criterion and Bayesian Information Criterion predicted the GTR + I + Γ model as the best estimator; Bayesian inference analyses were conducted using the closest approximation to this model and run over 10,000,000 generations (ngen = 10,000,000), with two runs each containing four simultaneous Markov Chain Monte Carlo chains (nchains = 4) and every 1000th tree saved. Analysis used the following parameters: 'nst = 6', 'rates = invgamma', 'ngammacat = 4', and the priors parameters of the combined dataset were set to 'ratepr = variable'. Samples of substitution model parameters, and tree and branch lengths, were summarized using the parameters 'smp burnin = 3000' and 'sumt burnin = 3000'. Species of *Hexangium* Goto & Ozaki, 1929 and *Neohexangitrema* Machida, 1984 were designated as functional outgroup taxa.

Results

Infections of pronoccephaloid-type cercariae (oculate, monostome, paired dorsal adhesive pockets and simple tail) were found in three gastropod species, two in *R. vertagus* (Cerithiidae) from off Lizard Island, one in *N. coronatus* (Nassariidae) from off Lizard Island and two in the two genotypes of *Thylacodes* sp. (Vermetidae), one in both genotypes from off Dunwich and Amity, the second only from off Dunwich.

Morphological characterization

Because cercariae could not be assigned to any named species, they are herein assigned a temporary name consisting of an alphabetical (A–E) and geographical identifier: LI for Lizard Island, Great Barrier Reef; NSI for North Stradbroke Island.

Superfamily: Pronoccephaloidea Looss, 1899

Pronoccephaloid sp. A-LI (fig. 1a, b; measurements in table 2).

Taxonomic summary

Intermediate host. *Rhinoelavis vertagus* (Linnaeus, 1767) (Cerithiidae).

Prevalence. Two of 138 (1.45%).

Table 1. Collection data and GenBank accession numbers for pronoccephaloid species analysed in this study.

Species	Host species	GenBank accession #	Reference
Pronoccephaloidea Looss, 1899			
Family Labicolidae Blair, 1979			
<i>Labicola</i> cf. <i>elongata</i>	<i>Dugong dugon</i> (Müller, 1776)	AY222221	Olson <i>et al.</i> (2003)
Family Notocotylidae Lühe, 1909			
<i>Catartropis indicus</i> Srivastava, 1935	<i>Cairina moschate</i> (Linnaeus, 1758)	AY222220	Olson <i>et al.</i> (2003)
<i>Catartropis</i> sp.	<i>Anas platyrhynchos</i> Linnaeus, 1758	MH750019	Izrailskaia <i>et al.</i> (2019)
<i>Notocotylus attenuatus</i> (Rudolphi, 1809)	<i>Aythya ferina</i> (Linnaeus, 1758)	AF184259	Tkach <i>et al.</i> (2001)
<i>Notocotylus intestinalis</i> Tubangui, 1932	<i>Parafossarulus striatulus</i> (Benson, 1842)	JQ890563	Besprozvannykh <i>et al.</i> (2013)
<i>Notocotylus malhamensis</i> Boyce, Hide, Craig, Harris, Reynolds, Pickles & Rogan, 2012	<i>Myodes glareolus</i> (Schreber, 1780)	JQ766939	Boyce <i>et al.</i> (2012)
<i>Notocotylus</i> sp. UK-PO-2003	<i>Stagnicola palustris</i> (Müller, 1774)	AY222219	Olson <i>et al.</i> (2003)
<i>Notocotylus</i> sp. AK-2017	<i>Ampullaceana balthica</i> (Linnaeus, 1758)	KY513158	Soldanova <i>et al.</i> (2017)
<i>Ogmogaster antarctica</i> Johnston, 1931	<i>Balaenoptera borealis</i> Lesson, 1828	KM258675	Fraija-Fernandez <i>et al.</i> (2015)
<i>Paramonostomum anatis</i> Garkawi, 1965	<i>Tringa erythropus</i> (Pallas, 1764)	AF184258	Tkach <i>et al.</i> (2001)
<i>Pseudocatartropis</i> sp.	<i>Anas platyrhynchos</i>	MH750024	Izrailskaia <i>et al.</i> (2019)
Notocotylidae gen. sp. 1	<i>Austrolittorina antipodum</i> (Philippi, 1847)	KJ868212	O'Dwyer <i>et al.</i> (2014)
Notocotylidae gen. sp. 2	<i>Austrolittorina unifasciata</i> (Gray, 1826)	KP903410	O'Dwyer <i>et al.</i> (2015)
Pronoccephaloidea sp. S5M1A5(M)	<i>Potamopyrgus antipodarum</i> (Gray, 1843)	EU371602	Adema <i>et al.</i> (2009)
Family Nudacotylidae Barker, 1916			
<i>Nudacotyle undicola</i> Dailey, Vogelbein & Forrester, 1988	<i>Trichechus manatus</i> Linnaeus, 1758	MF538578	Vélez <i>et al.</i> (2018)
Opisthotrematidae Poche, 1926			
<i>Lankatrema mannarensis</i> Cruz & Fernand, 1954	<i>Dugong dugon</i>	AY222222	Olson <i>et al.</i> (2003)
<i>Opisthotrema dujonis</i> (Leuckart, 1874)	<i>Dugong dugon</i>	AY222223	Olson <i>et al.</i> (2003)
Pronoccephalidae Looss, 1899			
<i>Macravestibulum</i> sp.	<i>Trachemys scripta</i> (Thunberg in Schoepff, 1792)	AY116877	Olson <i>et al.</i> (2003)
Family Rhabdiopoeidae Poche, 1926			
<i>Rhabdiopoes taylori</i> Johnston, 1913	<i>Dugong dugon</i>	AY222218	Olson <i>et al.</i> (2003)
<i>Taprobanella bicaudata</i> Cruz & Fernand, 1954	<i>Dugong dugon</i>	AY222217	Olson <i>et al.</i> (2003)
Outgroup taxa			
<i>Hexangium</i> sp.	<i>Siganus fuscescens</i> (Houttuyn, 1782)	AY222215	Olson <i>et al.</i> (2003)
<i>Neohexangitrema zebrasomatis</i> Machida, 1984	<i>Zebrasoma scopas</i> (Cuvier, 1829)	AY222214	Olson <i>et al.</i> (2003)

Locality. Off Lizard Island, Great Barrier Reef, Queensland, Australia (14°40'S, 145°27'E).

Voucher material. Seven slides (QM G238198–238204).

Representative DNA sequences. ITS2, four identical replicates (one submitted to GenBank: MN574291); 28S, two identical replicates (one submitted to GenBank: MN574298).

Description

Redia. Based on ten excised specimens. Body elongate, allantoid, attenuating posteriorly, typically containing fewer than five well-developed cercarial bodies in anterior region and fewer than 12 germinal balls in posterior region. Pharynx doliiform to subglobular. Intestine elongate, ellipsoidal, with margins indented, occupying up to one-half of body length. Birth pore distinct, adjacent to pharynx.

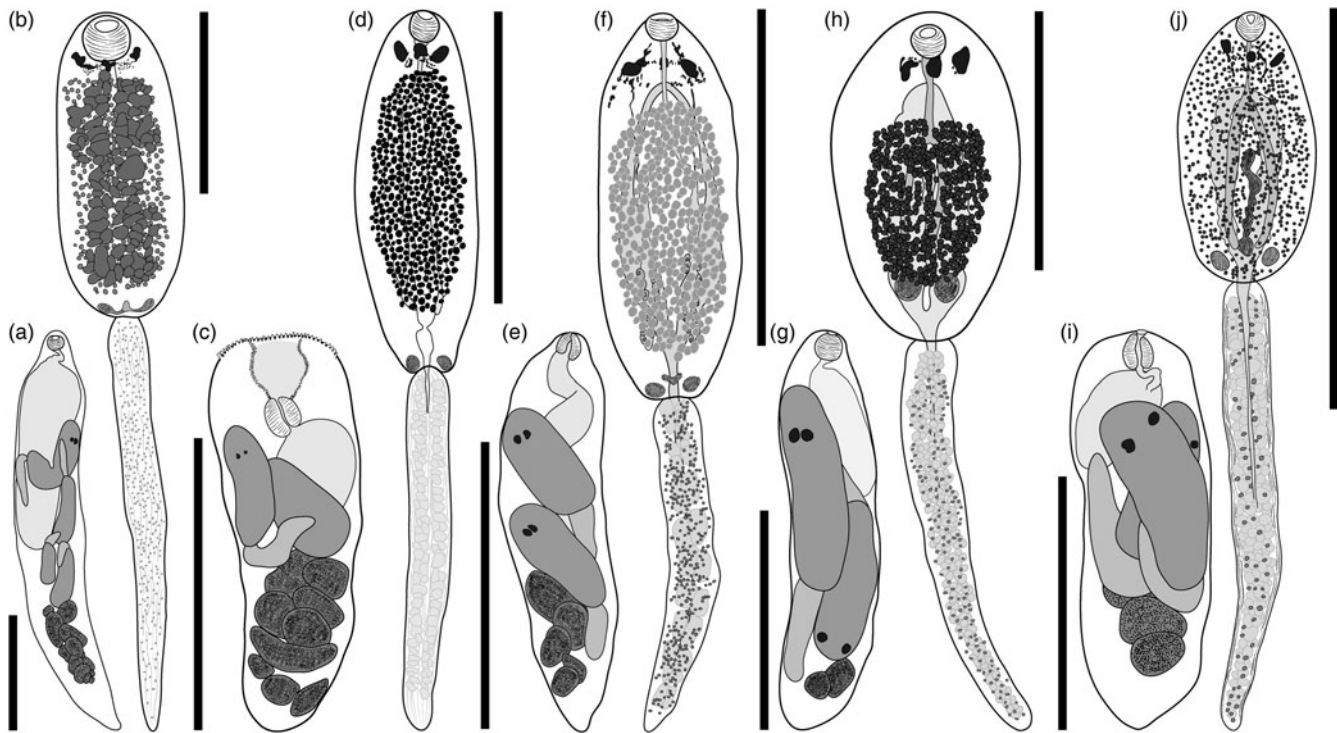


Fig. 1. Rediae and cercariae of five putative pronocephaloids from Queensland marine gastropods. (a, b) Species A-LI, ex *Rhinoclavis vertagus*, Lizard Island; (c, d) species B-LI, ex *R. vertagus*, Lizard Island; (e, f) species C-LI, ex *Nassarius coronatus*, Lizard Island; (g, h) species D-NSI, ex *Thylacodes* sp., Moreton Bay; (i, j) species E-NSI, ex *Thylacodes* sp., Moreton Bay. Cephalic collar and adhesive pockets of cercariae not illustrated. Scale bars: 500 μ m.

Cercaria. Based on ten excised specimens. Trioculate monostome. Body elongate, ellipsoidal. Cephalic collar indistinct. Tegument smooth. Cystogeneus gland-cells extensive in body, filled with opaque, granular substance, extending from pharynx to testicular masses, with distinct gap between gland-field and body margins, obscure most of internal anatomy. Dorsal adhesive pockets inconspicuous, bowl-shaped, on postero-lateral extremities of body. Eyespots three (two in immature cercariae), in anterior portion of pre-equatorial region of body, closely associated with ganglionic mass, often irregular and diffused, conical, with proximal portion near dorsal surface, expanding ventrally; central eyespot slightly posterior to parallel lateral eyespots. Additional pigment scattered in pre-equatorial body region. Oral sucker terminal, subglobular. Oesophagus bifurcates near posterior margin of cephalic collar; caeca terminate blindly in posterior third of body. Reproductive primordia indistinct. Testicular masses two, parallel, joined by U-shaped group of cells, near posterior extremity, at level of adhesive pockets. Ovarian complex indistinct. Excretory bladder variable in shape; consists of two extensively diverticulated lateral arms, uniting anteriorly near central eyespot and posteriorly near testicular primordia. Tail simple, longer than body, attenuating posteriorly.

Pronocephaloid sp. B-LI (fig. 1c, d; measurements in table 2).

Taxonomic summary

Intermediate host. *Rhinoclavis vertagus* (Linnaeus, 1767) (Cerithiidae).

Prevalence. One of 138 (0.72%).

Locality. Off Lizard Island, Great Barrier Reef, Queensland, Australia (14°40'S, 145°27'E).

Voucher material. Seven slides (QM G238205–238211).

Representative DNA sequences. ITS2, three identical replicates (one submitted to GenBank: MN574292); 28S, two identical replicates (one submitted to GenBank: MN574299).

Description

Redia. Based on ten excised specimens. Body elongate, blunted anteriorly, tapering posteriorly, typically containing fewer than five well-developed cercarial bodies in anterior region and fewer than 12 germinal balls in posterior region. Mouth cavity large, at anterior extremity, lined with noticeable annulations. Pharynx at base of mouth cavity, ellipsoidal. Intestine sac-like, occupying up to one-half of body length. Birth pore inconspicuous, adjacent to pharynx.

Cercaria. Based on ten excised specimens. Trioculate monostome. Body elongate, ellipsoidal. Cephalic collar indistinct. Tegument smooth. Cystogeneus gland-cells extensive in body, filled with opaque, granular substance, extending from pharynx to testicular masses, with distinct gap between gland-field and body margins. Dorsal adhesive pockets conspicuous, bowl-shaped, each on distinct projection on postero-lateral extremities of body. Eyespots three (two in immature cercariae), in anterior portion of pre-equatorial region of body, closely associated with ganglionic mass, laterally parallel, often irregular and diffused, conical, with proximal portion near dorsal surface, expanding ventrally. Additional pigment scattered in pre-equatorial body region. Oral sucker terminal, spherical. Oesophagus bifurcates near posterior margin of cephalic collar; caeca terminate blindly in posterior third of body. Reproductive primordia distinct. Testicular masses two, well-differentiated, parallel, near posterior extremity. Ovarian complex a conspicuous longitudinal mass of

Table 2. Morphometric data for pronocephaloid cercariae expressed in micrometres or as percentages.

Feature	Pronocephaloid sp. A-LI			Pronocephaloid sp. B-LI			Pronocephaloid sp. C-LI			Pronocephaloid sp. D-NSI			Pronocephaloid sp. E-NSI		
	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean
Cercariae															
BL	707	882	794	363	630	496	474	572	540	544	691	610	397	444	422
BW	292	358	325	172	216	197	196	232	214	286	341	315	168	211	197
EspL	22	46	35	18	39	29	14	32	22	23	47	36	10	20	16
EspW	15	48	28	15	33	23	14	36	26	17	48	30	10	16	13
OSL	109	133	121	37	59	49	32	43	38	62	79	71	34	46	40
OSW	130	144	138	47	59	52	41	50	46	57	65	63	32	44	38
OesL	94	208	126	64	113	87	119	154	135	99	188	136	100	128	113
Pcs	81	205	120	30	83	66	56	81	69	106	159	123	45	79	61
TL	820	1218	1046	463	716	598	451	633	540	725	1165	918	596	832	725
TW	93	113	104	65	83	74	67	80	73	86	108	96	74	99	83
BL/BW	2.26	2.55	2.44	2.06	3.10	2.50	2.12	2.79	2.53	1.66	2.20	1.94	1.97	2.40	2.15
OSL/OSW	0.79	0.92	0.88	0.63	1.13	0.94	0.68	0.93	0.82	1.00	1.25	1.13	0.95	1.14	1.04
OesL % BL	0.12	0.25	0.16	0.13	0.24	0.18	0.22	0.27	0.25	0.16	0.28	0.22	0.25	0.29	0.27
Pcs % BL	0.10	0.23	0.15	0.08	0.18	0.13	0.12	0.14	0.13	0.16	0.28	0.20	0.11	0.18	0.14
Rediae															
BL	1328	1712	1508	457	929	457	628	919	690	510	889	707	632	827	738
BW	250	381	319	218	278	218	169	196	186	172	213	197	246	311	273
PhL	43	71	64	49	65	49	30	46	39	46	62	54	54	80	62
PhW	53	74	69	54	64	54	38	44	41	53	64	58	54	76	60
PhL/PhW	0.81	1.00	0.93	0.91	1.05	0.91	0.70	1.12	0.94	0.80	1.00	0.92	0.86	1.20	1.03
InL	516	792	664	191	301	191	114	336	271	222	323	273	153	282	227
InW	139	290	242	120	181	120	65	317	110	75	128	112	141	199	158
IL % BL	0.30	0.49	0.44	0.42	0.42	0.42	0.12	0.52	0.40	0.32	0.45	0.39	0.20	0.36	0.31

B, body; L, length; W, width; Esp, eyespot (data combined for all three eyespots); OS, oral sucker; Oes, oesophagus; Pcs, post-caecal space; T, tail; Ph, pharynx; I, intestine.

cells, passing from testes to mid-body. Excretory bladder variable in shape, consists of two extensively diverticulated lateral arms, uniting anteriorly near central eyespot and posteriorly near testicular primordia. Numerous large, lightly stained gland-cells associated with excretory bladder. Tail simple, longer than body, attenuating posteriorly.

Pronocephaloid sp. C-LI (fig. 1e, f; measurements in table 2).

Taxonomic summary

Intermediate host. *Nassarius coronatus* (Brugvière, 1789) (Nassariidae).

Prevalence. One of 43 (2.33%).

Locality. Off Lizard Island, Great Barrier Reef, Queensland, Australia (14°40'S, 145°27'E).

Voucher material. Seven slides (QM G238212–238218).

Representative DNA sequences. ITS2, five identical replicates (one submitted to GenBank: MN574293); 28S, two identical replicates (one submitted to GenBank: MN574300).

Description

Redia. Based on ten excised specimens. Body elongate, allantoid, typically containing fewer than five well-developed cercarial bodies in anterior region and fewer than 12 germinal balls in posterior region. Pharynx subglobular. Intestine elongate, ellipsoidal, occupying up to one-half of body length. Birth pore distinct, posterior to pharynx.

Cercaria. Based on ten excised specimens. Trioculate monostome. Body elongate, ellipsoidal. Cephalic collar inconspicuous. Tegument smooth. Cystogeneus gland-cells dense and extensive, extending from region just posterior to eyespots to bifurcation of excretory vesicle, with distinct gap between gland-field and body margins, containing opaque, granular substance. Dorsal adhesive pockets, inconspicuous, conical, each on postero-lateral extremities of body. Eyespots three (two in immature cercariae), in anterior portion of pre-equatorial region of body, closely associated with ganglionic mass, often irregular and diffused, conical, with proximal portion near dorsal surface, expanding ventrally; central eyespot indistinct, smaller than and situated anterior to parallel, lateral eyespots. Additional pigment extensive in pre-equatorial body region, forming web-like pattern around eyespots. Oral sucker terminal, cup-shaped. Oesophagus bifurcates near posterior margin of cephalic collar; caeca terminate blindly in posterior third of body. Reproductive primordia distinct. Testicular masses two, well-differentiated, parallel, near posterior extremity. Ovarian complex a conspicuous longitudinal mass of cells, passing from testes to mid-body. Excretory bladder elongate, balloon-like; consists of two extensively diverticulated lateral arms, uniting anteriorly near central eyespot and posteriorly near testicular primordia. Numerous large, lightly stained gland-cells associated with excretory bladder. Tail simple, longer than body, attenuating posteriorly.

Pronocephaloid sp. D-NSI (fig. 1g, h; measurements in table 2).

Taxonomic summary

Intermediate host. *Thylacodes* sp. Genotypes 1 (Dunwich) and 2 (Amity) (Vermetidae).

Prevalence. Dunwich: three of 400 (0.75%); Amity: one of 355 (0.28%).

Locality. Off Dunwich (27°24'S, 153°20'E) and Amity (27°23'S, 153°26'E), North Stradbroke Island, Queensland, Australia.

Voucher material. Four slides (QM G238219–238222).

Representative DNA sequences. ITS2, seven identical replicates (one submitted to GenBank: MN574294); 28S, three identical replicates (one submitted to GenBank: MN574301).

Description

Redia. Based on ten excised specimens. Body elongate, allantoid, typically containing fewer than five well-developed cercarial bodies in anterior region and fewer than 12 germinal balls in posterior region. Pharynx subglobular. Intestine elongate, ellipsoidal, occupying up to one-half of body length. Birth pore distinct, adjacent to pharynx.

Cercaria. Based on ten excised specimens. Trioculate monostome. Body elongate, ellipsoidal. Cephalic collar inconspicuous; lobes extend about one-third of body length. Tegument smooth. Cystogeneus gland-cells extending from oesophageal bifurcation to testicular masses, with distinct gap between gland-field and body margins, filled with an opaque, granular substance. Dorsal adhesive pockets inconspicuous, bowl-shaped, each on postero-lateral extremities of body. Eyespots three (two in immature cercariae), in anterior portion of pre-equatorial region of body, closely associated with ganglionic mass, laterally parallel, often irregular and diffused, conical, with proximal portion near dorsal surface, expanding ventrally. Additional pigment scattered in pre-equatorial body region. Oral sucker subterminal, spherical. Oesophagus bifurcates near posterior margin of cephalic collar; caeca terminate blindly in posterior third of body. Reproductive primordia distinct. Testicular masses two, well-differentiated, parallel, near posterior extremity. Ovarian complex a conspicuous longitudinal mass of cells, passing from testes to mid-body. Excretory bladder variable in shape; consists of two extensively diverticulated lateral arms, uniting anteriorly near central eyespot and posteriorly near testicular primordia. Numerous large, lightly staining gland-cells associated with excretory bladder. Tail simple, longer than body, attenuating posteriorly.

Pronocephaloid sp. E-NSI (fig. 1i, j; measurements in table 2).

Taxonomic summary

Intermediate host. *Thylacodes* sp. Genotype 1 (Vermetidae).

Prevalence. One of 400 (0.25%).

Locality. Off Dunwich, North Stradbroke Island, Queensland, Australia (27°24'S, 153°20'E).

Voucher material. Five slides (QM G238223–238227).

Representative DNA sequences. ITS2, four identical replicates (one submitted to GenBank: MN574295); 28S, two identical replicates (one submitted to GenBank: MN574302).

Description

Redia. Based on ten excised specimens. Body elongate, allantoid, blunted anteriorly, typically containing fewer than five well-developed cercarial bodies in anterior region and fewer than 12 germinal balls in posterior region. Mouth cavity large, at anterior extremity, lined with cilia-like projections. Pharynx ellipsoidal, at base of mouth cavity. Intestine elongate, ellipsoidal, occupying up to one-half of body length. Birth pore distinct, adjacent to pharynx.

Cercaria. Based on ten excised specimens. Trioculate monostome. Body elongate, ellipsoidal. Cephalic collar inconspicuous; lobes extend about one-third of body length. Tegument smooth. Cystogeneus gland-cells sparse, extending throughout body, filled with opaque, granular substance. Dorsal adhesive pockets conspicuous, cup-shaped, each on postero-lateral extremities of body. Eyespots three (two in immature cercariae), in anterior portion of pre-equatorial region of body, closely associated with ganglionic mass, laterally parallel, often irregular and diffused, conical, with proximal portion near dorsal surface, expanding ventrally. Additional pigment scattered in pre-equatorial body region. Oral sucker terminal, infundibuliform. Oesophagus bifurcates near posterior margin of cephalic collar; caeca terminate blindly in posterior third of body. Reproductive primordia distinct. Testicular masses two, well-differentiated, parallel, near posterior extremity. Ovarian complex a conspicuous longitudinal mass of cells, passing from testes to mid-body. Excretory bladder variable in shape; consists of two extensively diverticulated lateral arms, uniting anteriorly near central eyespot and posteriorly near testicular primordia. Tail simple, thick-walled, longer than body, attenuating posteriorly.

Molecular data

28S rDNA sequences were generated for all five morphotypes. BLAST analysis for all five returned closest matches with pronocephaloids. Alignment of the new sequence data with those of related taxa (table 1) yielded 1266 characters for analysis. Sequences representing all six pronocephaloid families formed individual clades, but nodal support was poor for many major nodes (fig. 2). The best-represented family (Notocotylidae) formed a well-supported clade, as did the two species of Opisthotrematidae and the two species of Rhabdiopoeidae. The five cercarial infections are scattered in the phylogeny, none forming a well-supported clade with any other taxon. Species A, B, C and E formed a clade with the sole pronocephalid in the analysis, but this relationship was poorly supported. ITS2 rDNA sequences are provided principally to assist future matching of lifecycle stages. The ten combinations of comparison between the five sequences differed by 64–91 bp.

Discussion

The combined morphology and molecular phylogenetic analyses of the five species reported here demonstrate that they belong to the superfamily Pronocephaloidea. As currently conceived, the Pronocephaloidea comprises six families: Labicolidae Blair, 1979 has one species parasitic in the dugong; Notocotylidae Lühe, 1909 has approximately 20 species parasitic in marine birds and mammals, and many more transmitted in freshwater habitats; Nudacotylidae Barker, 1916 has two genera with species parasitic in both marine and terrestrial mammals; Opisthotrematidae Poche, 1926 has 11 species in six genera, all parasites of sirenians; Pronocephalidae Looss, 1899 is a large family mainly parasitizing aquatic turtles (marine and fresh water; species of about ten genera in marine turtles), but with a few species in fishes and marine iguanas; Rhabdiopoeidae Poche, 1926 comprises four monotypic genera, all parasites of sirenians. As far as we can determine, life cycles are known only for species of Notocotylidae, Nudacotylidae and Pronocephalidae. There are many life cycles known for Notocotylidae (e.g. Martin, 1956; Rohde & Onn, 1967; Pike, 1969; Kanev *et al.*, 1994), for

one nudacotylid (Ameel, 1944) and a handful for Pronocephalidae (e.g. Hsü, 1937; Hunter, 1967). The cercariae described in these studies are (essentially universally) characterized as monostomes (possessing only a single anterior muscular structure, debatably either a pharynx or an oral sucker), possessing eyespots, a simple tail and, diagnostically, paired dorsal adhesive pockets. These pockets have been reported repeatedly, and under many names, but we consider the designation of 'dorsal adhesive pockets' proposed by Krupenko & Gonchar (2017) to be appropriate for them. These structures were present in all five of the cercariae reported here.

Although the identity of the five cercariae reported here as pronocephaloids does not appear to be in doubt, it is not clear to which family they belong. Moreton Bay has a rich fauna of shorebirds and passing baleen whales, which may act as hosts to notocotylids, a major population of green turtles (*Chelonia mydas*), and smaller numbers of some other turtle species known to harbour a rich (though unreported) fauna of pronocephalids, and a major population of dugongs (*Dugong dugon*), which can be expected to harbour Labicolidae, Opisthotrematidae and Rhabdiopoeidae. Lizard Island has fewer shorebirds, but turtles (especially *C. mydas*) and dugongs both occur there regularly. Probably the only pronocephaloid family absent from the two collecting sites is the Nudacotylidae, which are known only from North and South America.

Our phylogenetic analysis included representatives of all six pronocephaloid families. Although support for many of the clades is weak, several observations and inferences can be made. The single sequence of a nudacotylid was sister to all other pronocephaloids. The two species of Rhabdiopoeidae form a well-supported clade that is the next most-basal clade in the Pronocephaloidea. The single sequenced labicolid species is the next most-basal taxon and is isolated from all other taxa. The two opisthotrematid species form a moderately supported clade. The multiple notocotylid taxa formed a strongly supported clade to the exclusion of all other taxa including the five cercarial species reported here. There is no strong evidence of a relationship between any of these families and the cercariae reported here. We predict that most, if not all, will prove to be pronocephalids on the basis that the phylogenetic analysis appears least consistent with relationship to Nudacotylidae, Rhabdiopoeidae, Labicolidae or Notocotylidae. The Pronocephalidae is almost certainly the richest pronocephaloid taxon in the areas of study given the abundance of turtles near the collection sites. The pronocephalid fauna of marine turtles in Australia has not yet been characterized, but Santoro *et al.* (2006) reported 15 pronocephalid species in *C. mydas* from Costa Rica. As such, it is perhaps inherently most likely that most, if not all, the present species will ultimately match with species of that family. We also suspect that this diverse assemblage of species will ultimately prove to be paraphyletic relative to the specialized families parasitic in sirenians. It is striking that the available molecular data for the Pronocephalidae is so poor; only one 28S gene sequence is available – that of *Macravestibulum obtusicaudum* from a freshwater turtle (Olson *et al.*, 2003).

Cerithiids have been reported as hosts for pronocephaloids twice previously. Cable (1956) reported an unidentified cercaria, which he predicted would prove to be that of a pronocephalid. Martin (1956) reported a cerithiid as the intermediate host of the notocotylid *Catatropis johnstoni* Martin, 1956. For the Nassariidae, there is just one report of a pronocephaloid infection, that of *Pleurogonius malacaclemys* Hunter, 1961 by Hunter (1967). Intriguingly, this species infects the semi-marine terrapin

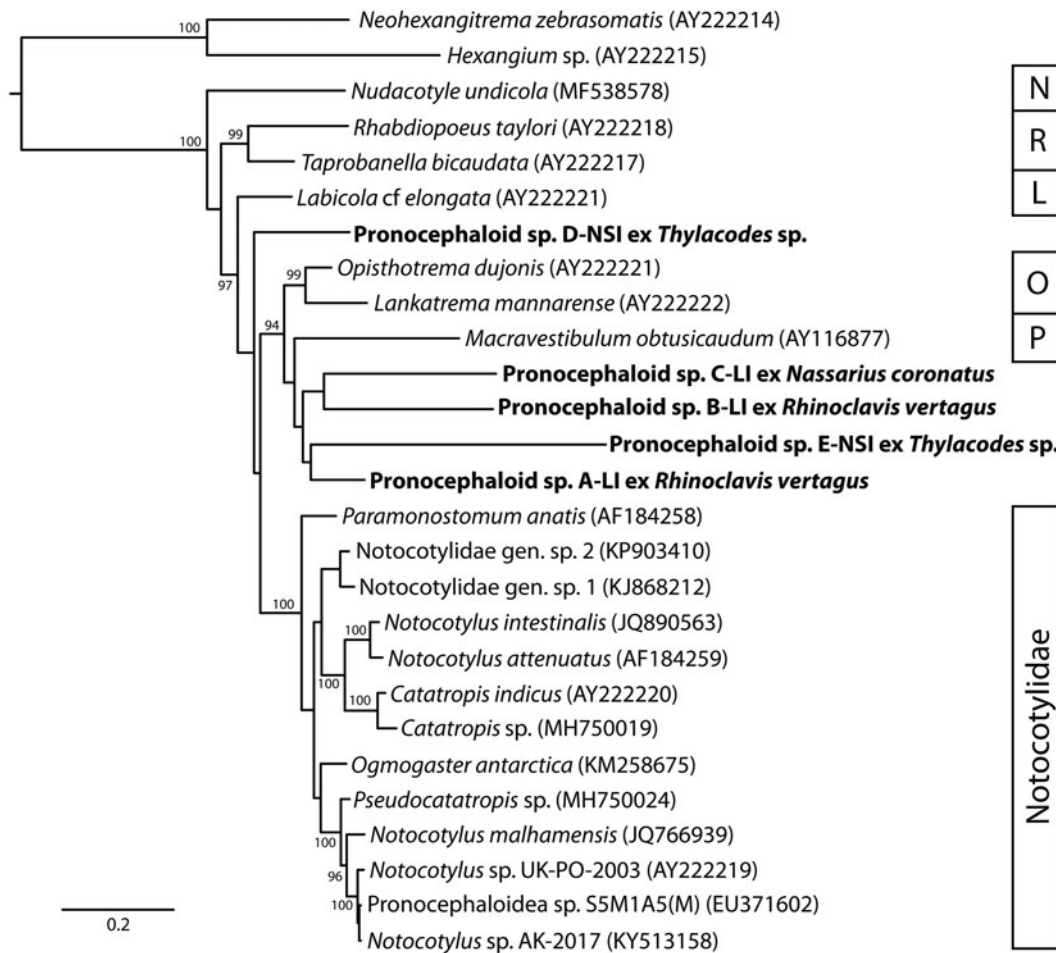


Fig. 2. Phylogenetic position of five pronocephaloid cercariae (in bold) relative to known pronocephaloid taxa based on Bayesian inference analyses of the partial 28S rDNA dataset. Posterior probabilities are shown above the nodes; values <85 are not shown. The scale bar indicates the expected number of substitutions per site. Abbreviations: L, Labicolidae; N, Nudacotylidae; O, Opisthotrematidae; P, Pronocephalidae; R, Rhabdiopoeidae.

M. terrapin, rather than one of the seven fully marine turtle species.

The Vermetidae (order Littorinimorpha: Vermetoidea) is a new host family and superfamily for the Pronocephaloidea. However, other families of the order Littorinimorpha (Bithyniidae, Hydrobiidae, Littorinidae, Pomatiopsidae and Tateidae) have been reported extensively as hosts for notocotylids (e.g. Stunkard, 1960; Dollfus, 1966; Rohde & Onn, 1967; Graefe, 1968; Smith & Hickman, 1983). Vermetids have the form of tube-worms, forming asymmetrical calcareous tubes encrusting on rocks or among other substrates. They may occur in large numbers but are easily overlooked as part of the gastropod community because their morphology is so unusual. Indeed, several species were initially described as polychaetes given the close resemblance of their tubes to those of Serpulidae. Vermetids have been reported as hosts to several other lineages of trematodes – an echinostomatid, a hemiurid, a mesometrid, a microphallid and a possible opoecoid (Prévôt, 1969, 1971a, b; Jousson *et al.*, 1999). Most recently, and of special relevance to the present findings, Cribb *et al.* (2017) reported an infection of a turtle-infecting spirorchiid from a vermetid in Spain. Indeed, the genesis of the current study of Moreton Bay vermetids was to seek further infections of spirorchids, which are known to be abundant in marine turtles there (Chapman

et al., 2015, 2017). If one or both of the present species from vermetids should prove to be a pronocephalid, it will be intriguing that species of two distantly related trematode families that infect turtles should both infect vermetid gastropods. Whether such a shared host group might be coincidental or related to an aspect of transmission ecology remains to be explored. Cribb *et al.* (2017) discussed the possibility that vermetids might be carried on the carapaces of turtles, creating a situation where the definitive host carries the intermediate hosts of their parasites with them. We think that vermetids are worthy of further study for parasites, especially in areas where turtles are abundant.

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Conflicts of interest. None.

Ethical standards. All applicable institutional, national and international guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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