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Galactosomum otepotiense n. sp. (Trematoda: Heterophyidae) infecting four different species of fish-eating birds in New Zealand: genetically identical but morphologically variable

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Research Paper

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Hydroprogne caspia; *Larus dominicanus*; *Chroicocephalus scopulinus*; *Eudyptula novaehollandiae*; New Zealand; *Galactosomum* new species; host-induced variability

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Abstract

Trematodes of the genus *Galactosomum* are cosmopolitan parasites that infect the intestines of fish-eating birds and mammals. Adults of named *Galactosomum* species have not been recorded from bird hosts in New Zealand, despite their cercarial stage being known from various studies of the first intermediate host, *Zeacumantus subcarinatus*. Here we describe a new species of *Galactosomum* infecting four different piscivorous birds in New Zealand: Caspian terns, red-billed and black-backed gulls and little blue penguins. Specimens from each of these hosts are genetically identical in the genes sequenced, but show considerable morphological variability. *Galactosomum otepotiense* n. sp. is distinguished from most other members of the '*bearupi*-group' in having a single circle of spines on the ventral sucker, and spines, as opposed to scales, over most of the body. It is most similar to *G. bearupi* and *G. angelae*, both from Caspian terns in Australia, but differs in the relative sizes of the reproductive organs and in the possession of a very long forebody. Molecular data confirm that *G. otepotiense* is not conspecific with *G. bearupi*, but 28S and ITS2 phylogenies show its close relationship to *G. bearupi* and other Australian species. We use the *cox1* sequence to confirm identity with the larval stage infecting *Z. subcarinatus*, as previously described in the literature. We discuss briefly the relationships between Australian and New Zealand *Galactosomum* spp. and their hosts, variability between genetically identical specimens found in different hosts and their potential for harm to mariculture economy.

Introduction

The genus *Galactosomum* Looss, 1899 (Plagiorchiida: Heterophyidae) is a widespread group of parasite species that infect the gastrointestinal tract of fish-eating birds and marine mammals. Their life cycle includes gastropod and fish intermediate hosts, and all species so far reported inhabit marine or brackish environments. Species of *Galactosomum* have been recorded from all continents except Antarctica, and five of the 22 species are found in Australia (Fischthal & Kuntz, 1972; Pearson, 1973; Dailey *et al.*, 2002).

Adults of *Galactosomum* spp. have only once been recorded from New Zealand: specimens of an unnamed species were recovered from a little blue penguin on Tiritiri Matangi Island, off North Island (McKenna, 2009). However, larval stages (rediae and cercariae) have been known for some years to be infecting the intertidal mud snail, *Zeacumantus subcarinatus* (Sowerby, 1855), and Martorelli *et al.* (2008) concluded from morphology that the cercariae belonged to a species closely related to *Galactosomum bearupi* Pearson, 1973 (Martorelli *et al.*, 2008).

During a survey of the helminth parasites of birds in South Island, New Zealand, we found specimens of adult *Galactosomum* sp. in the intestines of Caspian terns (*Hydroprogne caspia* (Pallas, 1770)), red-billed gulls (*Chroicocephalus scopulinus* (Forster, 1844)), black-backed gulls (*Larus dominicanus* Lichtenstein, 1823) and little blue penguins (*Eudyptula novaehollandiae* (Stephens, 1826)). The birds all came from the same geographical and ecological region as the snails, so it was suspected that, if these specimens represented a single species, it was likely to be that found as larval stages in *Z. subcarinatus* from Otago Harbour.

The aim of this study is to give a scientific name, describe and add to our knowledge of the life cycle of the *Galactosomum* sp. previously known to infect New Zealand *Z. subcarinatus*. We use molecular tools to compare the cercarial stage of *Galactosomum* sp. infecting *Z. subcarinatus* with the adult infecting fish-eating birds, and present a preliminary phylogeny for the genus based on both 28S and ITS2 available sequences. We use morphological methods to compare the adult *Galactosomum* specimens from New Zealand to other described *Galactosomum* species, and conclude that the specimens belong to a hitherto undescribed species, which we here describe and name.

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Materials and methods

Bird collection and trematode sampling

A total of 87 birds of four species, two Caspian terns (*H. caspia*), 30 red-billed gulls (*C. scopulinus*), 20 black-backed gulls (*L. dominicanus*) and 35 little blue penguins (*E. novaehollandiae*), were examined for gastrointestinal helminths between October and November 2018. Birds were found dead, or donated after death or euthanasia by the Dunedin Wildlife Hospital, and were frozen less than 12 h after death. Birds were defrosted, the intestines removed and examined under a dissecting microscope, and the worms preserved in 70% ethanol for whole-mount and 96% ethanol for genetic analyses.

All little blue penguins were collected from around the Otago coast; therefore, we have assigned these specimens to *E. novaehollandiae* (Otago and Australian little blue penguin) as opposed to *E. minor* (Forster, 1781) (New Zealand little blue penguin) (see Grosser *et al.*, 2015, 2017).

Morphological data

Trematodes fixed for whole mounts were stained using acetic acid iron carmine stain, dehydrated through a graded ethanol series, cleared in clove oil and mounted in permanent preparations with Canada balsam. A few specimens were used to trial Nile blue and haematoxylin stains, which proved useful for highlighting muscle fibres and vitellaria. Measurements were made using ImageJ software (Wayne Rasband, NIH, USA) from photographs taken on an Olympus BX51 compound microscope mounted with DP25 camera attachment. All measurements are in micrometres unless otherwise indicated, with the mean followed by the range. Drawings were made with the aid of a drawing tube mounted on an Olympus compound microscope. For scanning electron microscopy (SEM), five specimens were fixed overnight in 2.5% aldehyde in 0.1 M cacodylate buffer. They were then post-fixed in 1% osmium tetroxide for 1 h prior to being dehydrated through a gradient series of ethanol, critical-point dried in a CPD030 BalTec critical-point dryer (BalTec AG, Balzers, Liechtenstein) using carbon dioxide, mounted on aluminium stubs using double-sided adhesive carbon tape and sputter coated with gold/palladium (60:40) to a thickness of 10 nm in an Emitech K575X Peltier-cooled high-resolution sputter coater (EM Technologies, Ashford, Kent, UK). The specimens were viewed with a JEOL 6700 F field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan) at the Otago Centre for Electron Microscopy (OCEM, University of Otago, New Zealand). Type specimens were deposited in Te Papa Museum, Wellington (accession numbers W.003498–W.003502) and the Otago Museum, Dunedin (accession numbers IV107611–IV107616). Comparative material examined comprised paratype and vouchers of *G. angelae* (South Australia Museum; accession numbers 20107–20112, 41029) and *G. bearupi* paratype (South Australia Museum; accession number AHC41030).

Molecular data and analysis

Genomic DNA was extracted from five ethanol-fixed specimens (one from each of black-backed gull, red-billed gull, Caspian tern and two from little blue penguins) using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To compare the sequence of larval *Galactosomum* sp. infecting *Z. subcarinatus*, we amplified a partial fragment of cytochrome c oxidase subunit 1 gene (*cox1*) of an adult *Galactosomum* sp. from little blue penguin using primers JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) (Bowles *et al.*, 1993) and Trem.Cox.1 (5′AAT CAT GAT GCA AAA GGT A-3′) (Králová-Hromadová *et al.*, 2008). Polymerase chain reactions (PCRs) were run in 25 µl reaction mixtures using an Eppendorf Mastercycler Pro thermal cycler (Eppendorf, New York) and conditions consisted of an initial denaturation phase (2 min at 95°C); 40 cycles of denaturation (30 s at 95°C), primer annealing (40 s at 48°C), extension (1 min at 72°C) and a 10 min final extension (72°C).

To compare the identity of *Galactosomum* specimens among hosts sampled, two partial gene fragments were amplified; 28S rRNA gene (28S), using primers T16 (5′-GAG ACC GAT AGC GAA ACA AGT AC-3′) and T30 (5′-TGT TAG ACT CCT TGG TCC GTG-3′) (Harper & Saunders, 2001) and ITS2 rDNA region (ITS2), using primers 3s (5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′) (Bowles *et al.*, 1993) and ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) (Cribb *et al.*, 1998). The 28S marker was selected for its previous use in heterophyid phylogenies (e.g. Thaenkham *et al.*, 2011). The ITS2 region was selected because although not suitable at family-level analyses (Nolan & Cribb, 2005; Thaenkham *et al.*, 2012), it has been useful for distinguishing species and population-level diagnostics between digenean species (e.g. Nolan & Cribb, 2005). The amplification protocol for 28S consisted of an initial denaturation phase (2 min at 94°C); 38 cycles of denaturation (30 s at 94°C), annealing (30 s at 50°C), extension (2 min at 72°C) and a 7 min final extension (72°C). The amplification protocol for ITS2 region consisted of an initial denaturation phase (3 min at 95°C); one cycle of annealing (2 min at 45°C) and extension (1 min 30 s at 72°C); followed by four cycles of denaturation (45 s at 95°C), annealing (45 s at 50°C) and extension (1 min 30 s at 72°C); followed by 30 cycles of denaturation (20 s at 95°C), annealing (20 s at 52°C) and extension (1 min 30 s at 72°C) and a 5 min final extension (72°C). All PCR products were cleaned using EXOSAP-IT™ Express PCR Product Cleanup Reagent (USB Corporation, Cleveland, OH, USA), following manufacturer's instructions. Sanger sequencing by capillary electrophoresis was performed by the Genetic Analysis Service, Department of Anatomy, University of Otago (Dunedin, New Zealand).

All sequences were imported into Geneious v8.1.9 (Kearse *et al.*, 2012), trimmed using the trim function with default parameters and manually edited for incorrect or ambiguous base calls. The generated sequences were aligned together with published heterophyid sequences, as found on GenBank. The datasets were aligned using MAFFT algorithm implemented in Geneious v8.1.9 (auto algorithm using default settings). For outgroups, an echinostomatid outgroup was selected based on previous phylogenetic studies of Heterophyidae (e.g. Thaenkham *et al.*, 2011) for the 28S dataset, and an opisthorchiid outgroup was used as one of the closest to the ITS2 heterophyid sequences. GenBank accession numbers of sequences used in each phylogenetic analysis are shown in tables 1 and 2, respectively. The *cox1* sequence was used only to confirm identity with the *Galactosomum* sp. infecting *Z. subcarinatus*. Genetic divergences were calculated in MEGA v7 (Kumar *et al.*, 2016) using uncorrected pairwise genetic distances and, as there was no genetic difference between the sequences from each different host, a single representative sequence of adult *Galactosomum* for 28S and ITS2 was included in the final datasets.

To infer the phylogenetic positioning of the new *Galactosomum* specimens within the Heterophyidae, Bayesian

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Table 1. Taxa included in the dataset for partial 28S phylogenetic analysis, including ID, GenBank accession numbers and references.

Taxon ID	GenBank accession number	Reference
Apophallus zalophi	MG806918	Kuzmina *et al.* (2018)
Ascocotyle cameliae	MK359080	Hernández-Orts *et al.* (2019)
Ascocotyle longa	MF980613	Santos & Borges (2017) (unpublished)
Ascocotyle pindoramensis	KJ094561	Borges *et al.* (2014) (unpublished)
Ascocotyle pindoramensis	MF980609	Santos & Borges (2017) (unpublished)
Ascocotyle sp.	KU559561	Masala *et al.* (2016)
Centrocestus formosanus	KY351633	Le *et al.* (2017)
Cryptocotyle lingua	AY222228	Olson *et al.* (2003)
Galactosomum bearupi	MH257773	Huston *et al.* (2018)
Galactosomum lacteum	AY222227	Olson *et al.* (2003)
***Galactosomum otepotiense* n. sp.**	MN227729	This study
Galactosomum ubelakeri	MG806920	Kuzmina *et al.* (2018)
Galactosomum sp.	MH257775	Huston *et al.* (2018)
Galactosomum sp.	MH257774	Huston *et al.* (2018)
Haplorchis popelkae	EU883584	Snyder & Tkach (2009)
Haplorchis pumilio	HM004173	Thaenkham *et al.* (2011)
Haplorchis pumilio	HM004186	Thaenkham *et al.* (2011)
Haplorchis taichui	HM004181	Thaenkham *et al.* (2011)
Haplorchis yokogawai	HM004177	Thaenkham *et al.* (2011)
Haplorchoides maiwariensis	MG747501	Hostettler *et al.* (2018)
Haplorchoides sp.	AY222226	Olson *et al.* (2003)
Heterophyes sp.	KU559560	Masala *et al.* (2016)
Metagonimus takahashii	HQ832636	Pornruseetairatn *et al.* (2016)
Metagonimus yokogawai	HQ832639	Pornruseetairatn *et al.* (2016)
Metagonimus miyatai	HQ832633	Pornruseetairatn *et al.* (2016)
Metagonimus suifunensis	KX387456	Shumenko *et al.* (2017)
Metagonimus katsuradai	KM061391	Pornruseetairatn *et al.* (2016)
Metagonimus otsurui	KM061394	Pornruseetairatn *et al.* (2016)
Metagonimus pusillus	MF407172	Tatonova *et al.* (2018)
Metagonimus sp.	LC422950	Nakao (2018) (unpublished)
Metagonimus hakubaensis	KM061388	Pornruseetairatn *et al.* (2016)
Metagonimoides oregonensis	JQ995473	Belden *et al.* (2012)
Phocitrema fusiforme	MG806921	Kuzmina *et al.* (2018)
Procerovum cheni	HM004179	Thaenkham *et al.* (2011)
Procerovum varium	HM004182	Thaenkham *et al.* (2011)
Stellantchasmus falcatus	HM004174	Thaenkham *et al.* (2011)
Stellantchasmus falcatus	HM004176	Thaenkham *et al.* (2011)
Echinostoma revolutum OUTGROUP	AY222246	Olson *et al.* (2003)

inference was conducted in MrBayes version 3.2.6 (Huelsenbeck & Ronquist, 2001) using the online interface, Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway (Miller *et al.*, 2010). The analyses used random starting trees for two runs each (one cold and three heated chains), employing a Markov Chain Monte Carlo approach for sampling the joint posterior probability distribution across 10,000,000 generations, sampling every 1000 generations. A heating chain value of 0.01 was selected and the first and last 25% of samples were discarded as burn-in. The resulting phylogenies were summarized in a 50% majority-rule consensus tree with clade credibility support values (Bayesian posterior probability (BPP)) and branch length

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Table 2. Taxa included in the dataset for ITS2 phylogenetic analysis, including ID, GenBank accession numbers and references.

Taxon ID	GenBank accession number	Reference
Apophallus donicus	MF447672	Sándor *et al.* (2017)
Apophallus muehlingi	MF438069	Sándor *et al.* (2017)
Apophallus sp.	MF438051	Sándor *et al.* (2017)
Apophallus sp.	MF438073	Sándor *et al.* (2017)
Apophallus sp.	MF438075	Sándor *et al.* (2017)
Euryhelmis costaricensis	AB521800	Sato *et al.* (2010)
Euryhelmis zelleri	KM594133	Heneberg *et al.* (2015)
***Galactosomum otepotiensis* n. sp.**	MN227730	This study
Galactosomum bearupi	MH257764	Huston *et al.* (2018)
Galactosomum sp.	MH257766	Huston *et al.* (2018)
Haplorchis popelkae	EU883584	Snyder & Tkach (2009)
Haplorchis pumilio	KX815125	Le *et al.* (2017)
Haplorchis taichui	KJ630831	Chontananarth & Wongsawad (2014) (unpublished)
Haplorchoides daguilarensis	MG747500	Hostettler *et al.* (2018)
Haplorchoides maiwariensis	MG747502	Hostettler *et al.* (2018)
Haplorchoides sp.	KJ630832	Chontananarth & Wongsawad (2014) (unpublished)
Opisthorchis pedicellata OUTGROUP	KU688153	Choudhary & Agrawal (2016) (unpublished)

information. BPP higher than 0.95 was considered strong support for nodal positions.

Results

Galactosomum otepotiense n. sp. (figs 1 and 2; table 3)

Synonyms. Galactosomum sp. adult ex little blue penguin from Tiritiri Matangi Island, of McKenna (2009); *Galactosomum* sp. rediae and cercariae ex *Z. subcarinatus* from Otago Harbour, of Martorelli *et al.* (2008); Leung *et al.* (2009); Lloyd & Poulin (2011); Studer & Poulin (2012); MacLeod & Poulin (2015); Guilloteau *et al.* (2016); Lawrence & Poulin (2016).

Description

Based on 18 specimens from Caspian terns: Heterophyidae Odhner, 1914; Galactosominae Looss, 1899. Body elongate, more-or-less cylindrical, lateral margins parallel or slightly wider in hindbody; 2.11 (1.59–2.46) mm long by 249 (209–300) wide at widest point. Tegument spinose, although spines often lost; spines *c.* 7 µm long around anterior end, becoming smaller posteriorly, extending approximately to level of first testis; density

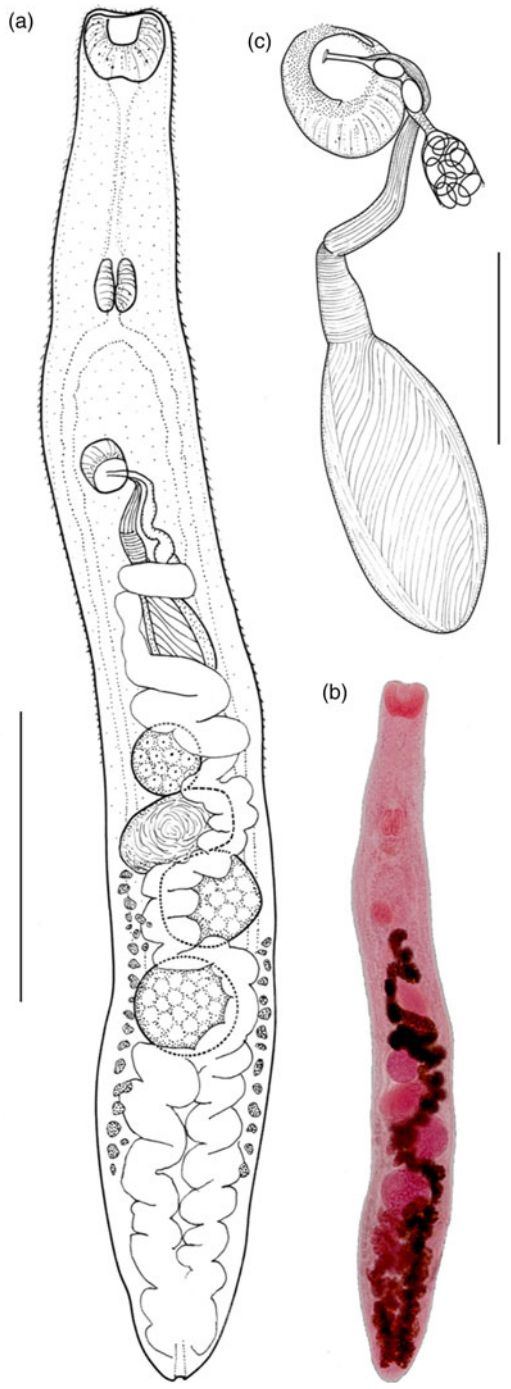

Fig. 1. *Galactosomum otepotiense* n. sp. (a) Holotype (Te Papa W.003498) ex Caspian tern; (b) photomicrograph of holotype; (c) ventrogenital complex. Scale bars: (a) 500 µm; (c) 100 µm.

c. 115 per 50 µm^2 on forebody. Oral sucker subterminal, 110 (95–131) long by 130 (102–150) wide; tegumental spines surrounding edges of sucker. Prepharynx long, 253 (145–342) in length. Pharynx 85 (66–93) long by 75 (56–94) wide. Oesophagus short, 29 (18–38) in length. Caecal bifurcation 376 (354–395) from oral sucker; caeca extend almost to posterior extremity, usually longer on right side, usually obscured by eggs. Pigment granules scattered in area between ventrogenital complex and oral sucker. Ventrogenital sac median or slightly dextral, 24–33%

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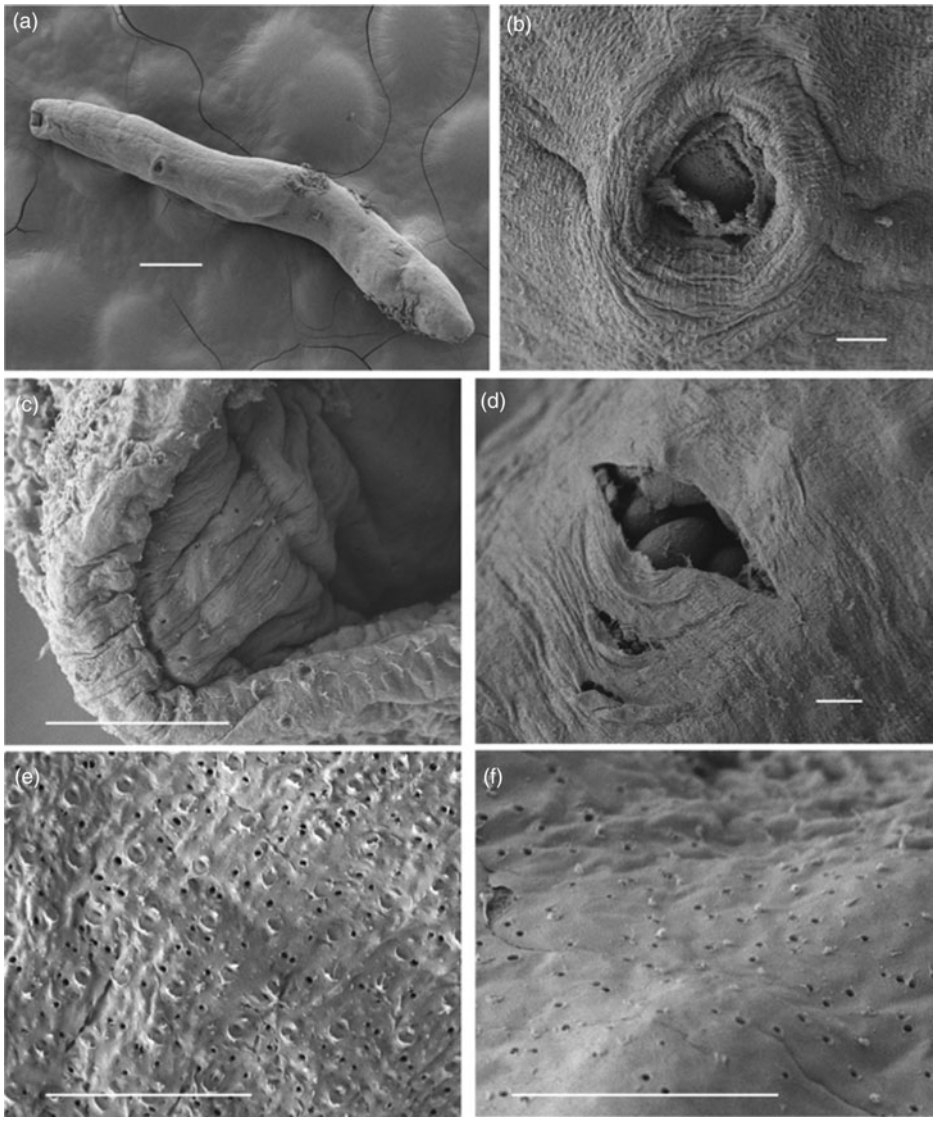

Fig. 2. *Galactosomum otepotiense* n. sp. ex Caspian tern, SEM photomicrographs. (a) Entire worm having lost tegumental spines; (b) ventrogenital complex; (c) mouth and oral sucker showing pores around lip and inside oral opening; (d) a split in hindbody wall showing mature eggs inside; (e) tegument at testicular level showing pores and scars of spine placements; (f) similar tegument from posterior end showing pores but no spine scars. Scale bars: (a) 200 µm; (b–f) 10 µm.

distant from anterior end, unarmed, without lateral pocket; tegumental spines missing in area around sac mouth. Ventral sucker dextral, often overlapping right branch of caeca; oval, 81 (71–93) by 65 (53–74), long axis oblique, tilted posteriorly on the left, lip enlarged ventrally on right, armed with complete circle of *c.* 340 min spines, ten rows wide on raised dextral lip, narrowing to 3–5 rows on sinistral lip; widest part of band *c.* 10 µm. Ratio oral sucker to ventral sucker (widths) 1:2.0 (1:1.7–2.2). Gonotyl unarmed, roughly circular in outline, axis oblique, 31 (28–33) long, arises sinistrally and posteriorly, overlies ventral sucker ventrally; traversed by genital atrium, genital pore a subterminal transverse slit or circular. Seminal vesicle fusiform, single-chambered, unconstricted, 237 (160–331) by 91 (75–108), with thick wall of diagonal fibres, except at anterior end where muscle fibres are strongly circular; terminal papilla not observed. Prostatic ejaculatory duct elongate, with conspicuous outer longitudinal muscle-fibres, at junction with seminal vesicle sharply twisted ventrally. Ejaculatory duct opens into genital atrium dorsal to uterus; muscular papilla and prostatic gland cell bodies not observed. Testes 2, entire, usually tandem, occasionally slightly offset, in which case anterior, located slightly to left of posterior; both testes posterior to midbody; round to transverse or longitudinal oval, anterior testis 148 (124–178) long by 137 (118–165) wide, posterior testis 161 (141–202) long by 141 (116–173) wide. Ovary rounded, dextral, located between posterior of seminal vesicle and anterior of seminal receptacle, 107 (88–143) by 116 (98–136). Seminal receptacle very large and circular when full of sperm, transversely oval when not full; contiguous with ovary and anterior testis when full, with gaps between when not full, 113 (66–201) by 138 (76–210). Course of uterus typical (see Pearson, 1973, p. 351), except that loops of ascending arm lie ventral to seminal vesicle in cylindrical specimens. Vitellaria follicular; reaching anteriorly to level of seminal receptacle or ovary, posteriorly about level with ends of caeca; right and left fields often unequal in length; rosettes discernible in less mature specimens, distributed irregularly over ventral side of ovary to posterior testis. Eggs 22 (21–23) by 13 (12–14). Excretory pore terminal; excretory bladder does not reach posterior margin of posterior testis.

Taxonomic summary

Type host. Caspian tern, *H. caspia* (Pallas, 1770) (Charadriiformes: Sternidae).

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Table 3. Comparative metrics for *Galactosomum otepotiense* n. sp. from four different hosts.

	Caspian tern				Black-backed gull				Red-billed gull				Little blue penguin			
	Hydroprogne caspia				*Larus dominicanus*				*Chroicocephalus scopulinus*				*Eudyptula novaehollandiae*			
	min	max	av	*n*	min	max	av	*n*	min	max	av	*n*	min	max	av	*n*
Body length	1590	2455	2114.8	18	1598	2070	1821.0	20	1510	1522	1516	3	1145	1597	1379	8
Width at widest	209	300	248.8	18	144	295	232.9	20	253	298	277.7	3	162	215	201.3	8
Length/width	5.6	10.6	8.6	18	5.9	13.5	8.0	20	5.1	6.0	5.5	3	5.4	7.5	6.9	8
Oral sucker length	95	131	110.1	18	88	131	114.1	18	109	132	121.3	3	96	124	117.0	7
Oral sucker width	102	150	130.3	16	104	136	120.6	9	104	136	115.0	3	101	124	112.7	6
Prepharynx length	145	342	253.4	14	77	138	113.8	13	73	103	87.7	3	47	179	90.3	6
Pharynx length	66	93	85.4	18	80	98	84.6	16	82	90	86.0	2	78	97	82.3	6
Pharynx width	56	94	74.7	18	57	75	65.9	17	61	63	62.0	2	50	59	55.0	6
Oesophagus	18	38	28.6	14	14	34	20.8	10	12	21	16.5	2	17	20	18.5	2
Oral sucker to caecal bifurcation	354	395	376.2	5	194	247	219.9	8	165	170	168.0	3	152	165	160.0	3
Anterior to VGS	452	763	640.3	17	351	516	430.6	18	302	426	346.0	3	310	372	340.4	5
Anterior to VGS, % body length	23.9	33.2	29.8	17	20.4	27.3	23.4	18	20.0	28.0	22.8	3	22.3	27.1	24.3	5
Gonotyl length	28	33	31.7	7	32	34	32.8	6	36	36	36.0	2	34	34	34.0	1
Ventral sucker length	71	93	80.9	17	77	96	87.3	12	75	85	81.0	3	81	87	83.7	6
Ventral sucker width	53	74	65.0	17	56	70	62.9	12	62	66	64.7	3	55	67	63.2	6
OS/VS width	1.7	2.2	2.0	16	1.5	1.9	1.7	4	1.6	2.1	1.8	3	1.6	1.9	1.8	5
Ovary length	88	143	107.2	17	90	123	110.6	18	90	90	90	1	38	55	45.4	5
Ovary width	98	136	115.6	17	96	132	118.2	18	112	112	112	1	36	56	47.8	5
Ant. testis length	124	178	148.1	17	121	164	136.8	18	101	112	106.5	2	44	101	65.8	5
Ant. testis width	118	165	137.4	17	117	147	138.4	17	97	116	106.5	2	55	82	73.4	5
Post. testis length	141	202	160.5	17	131	162	150.7	15	117	117	117	1	64	90	77.6	5
Post. testis width	116	173	140.7	17	128	162	144.7	16	100	115	107.5	2	78	89	83.6	5
Seminal vesicle length	160	331	237.5	15	151	282	211.3	19	–	–	–	–	79	199	141.2	6
Seminal vesicle width	75	108	91.3	15	81	112	95.9	19	–	–	–	–	56	83	70.7	6
Seminal receptacle length	66	210	113.3	16	68	181	108.8	19	84	96	90	2	46	73	57.4	5
Seminal receptacle width	76	210	137.9	16	91	196	138.4	19	85	105	95	2	67	105	90.8	5
Eggs length	21	23	22.0	20	21	22	21.5	20	22	24	23	10	21	23	22.1	10
Eggs width	12	14	12.7	20	12	12	12.0	20	11	13	12.2	10	11	13	12.3	10

min, minimum; max, maximum; av, average; VGS, ventrogenital sac; OS, oral sucker; VS, ventral sucker.

Other hosts. Red-billed gull, *C. scopulinus* (Forster, 1844) (Charadriiformes: Laridae); southern black-backed gull, *L. dominicanus* Lichtenstein, 1823 (Charadriiformes: Laridae); little blue penguin, *E. novaehollandiae* (Stephens, 1826) (Sphenisciformes: Spheniscidae).

Site of infection in definitive host. Intestine.

Prevalence in definitive hosts. 2/2 Caspian terns (100%); 4/30 red-billed gulls (13%); 1/20 black-backed gulls (5%); 3/35 little blue penguins (9%).

Intensity in definitive hosts. Caspian terns, nine and 50; red-billed gulls, 1–8; black-backed gull, 20; little blue penguins, 15–40.

Intermediate hosts. Marine intertidal mud whelk, *Z. subcarinatus* (Sowerby, 1855) (Prosobranchia: Batillariidae).

Type locality. Portobello Bay, Otago Harbour, South Island, New Zealand (45°52′S, 107°42′E).

Other localities. Hampden Beach, Otago (45°19′S, 170°49′E); Dunedin, Otago (45°52′S, 170°30′); Aramoana, Otago (45°46′S, 170°40′E).

Deposited specimens. Museum of New Zealand Te Papa Tongarewa, holotype W.003499, paratypes W.003499–W.003502; Otago Museum, paratypes IV107611–IV107616.

Zoobank registration. urn:lsid:zoobank.org:act:ED1CB047-8272-4F77-B0D3-E968C794D491.

Etymology. The species name is inspired by the Maori name for Dunedin, Ōtepoti, and is in recognition of the Dunedin Wildlife Hospital, which plays such an invaluable role in the welfare and conservation of New Zealand birds, and the staff of which were kind enough to donate their dead birds to the cause of parasitology.

Remarks

In having an elongate, spinose body, an oval, dextral ventral sucker with spines on external face, a bipartite seminal vesicle, expulsor, permanent ventro-genital sac with unspined gonotyl bearing the genital pore and a tubular excretory vesicle, the specimens described herein clearly belong to the genus *Galactosomum* (sPearson, 1964, 2008).

Pearson (1973) assigned the species of *Galactosomum* to four morphological groups. *Galactosomum otepotiense* n. sp. morphologically falls into his '*bearupi*-group', in having a one-chambered seminal vesicle with an additional layer of diagonal fibres over the major proximal portion and prominent circular fibres in the distal portion, and a short excretory bladder. There are ten species in this group, of which eight (*G. darbyi* Price, 1934; *G. dollfusi* Pearson, 1973; *G. fregatae* Prudhoe, 1949; *G. johnsoni* Price, 1934; *G. puffini* Yamaguti, 1941; *G. timondavidi* Pearson & Prévot, 1971 *G. ussuriense* Oshmarin, 1963; and *G. yehi* (Dissanaike, 1961)) are distinguishable from *G. otepotiense* n. sp. by a number of features, including the form and spination of the gonotyl and ventrogenital sac, and the form of the seminal vesicle. In particular, all of these species have zero, two or three patches of spines on the ventral sucker, as opposed to *G. otepotiense* n. sp., which has a single circle of spines. The new species is unique among this group in having tegumental spines from the anterior tip to the level of the testes, as opposed to anterior scales grading to posterior spines.

The new species is morphologically closest to *G. bearupi* and *G. angelae* Pearson, 1973, with which it shares a symmetrical ventral sucker armed with a complete circle of minute spines, the absence of a lateral pocket, similar distribution of vitellaria and a one-chambered seminal vesicle in two parts, the smaller with circular muscle fibres and the larger with spiral outer fibres. It differs from *G. bearupi* in having a larger, oval, ventral sucker (length 81(71–93)–versus–51(43–56)) and a larger pharynx (length 85(66–93)–v–51(45–60)). In addition, *G. bearupi* has ventral sucker spines in a circular band of uniform number (8–9 rows), whereas that in *G. otepotiense* n. sp. is narrower on the right (reduced to 2–3 rows). The new species differs from *G. angelae* in having a smaller gonotyl (length 32(28–33)–v–53(39–68)), and a narrower ventral sucker (width 65(53–74)–v–113(110–120)). Where *G. angelae* has no scales at the anterior tip but a row of three scales on the anterior margin of the oral sucker, *G. otepotiense* n. sp. has spines that extend to the anterior tip of the worm. Additionally, *G. otepotiense* n. sp. can be distinguished from these two similar species by overall size (maximum length 2455–v–1900 and 1740), by the long forebody, exemplified by the very long prepharynx (2.2–3.7 times length of pharynx, as opposed to the same length as pharynx), and by the size of the ovary and testes, which are all larger in the new species than in *G. bearupi* and *G. angelae*. The genetic evidence confirms that *G. otepotiense* n. sp. is not conspecific with *G. bearupi*, but no genetic data are available for *G. angelae*.

The specimens recovered from all four piscivorous birds were found to be genetically identical for the partial 28S and ITS2 markers, confirming that they were the same species. In addition, a short (230 bp), but variable, region of the *cox1* was sequenced (GenBank accession number MN233790), which confirmed identity with the cercarial and redial stages of *Galactosomum* sp. from *Z. subcarinatus* (sequence provided by Leung *et al.*, 2009; Genbank accession number FJ765489). A phylogeny inferred using all available heterophyid ITS2 sequences confirmed the close relationship between the specimens herein and *G. bearupi* from Australia (fig. 3), with strong support (BPP > 0.95). A larger number of heterophyid sequences were available for 28S, and the phylogeny placed the new species as sister to both *G. bearupi* and a species of *Galactosomum* that inhabits the same snail in the Great Barrier Reef (fig. 3) (BPP > 0.95), with *G. lacteum* (from Ukraine) as sister to these species. Genetic divergence was relatively low between *G. otepotiense* n. sp. and both closely related Australian species in the '*bearupi*-group' (uncorrected p-distance 1.6–1.8%) compared to divergence between *G. otepotiense* n. sp and the other three representatives of *Galactosomum* (uncorrected p-distance 5.5–11.6%) for the 28S marker.

Discussion

Intermediate hosts

This study provides a morphological description of a new species of *Galactosomum*, *G. otepotiense* n. sp., and matches two lifecycle stages (adult and redia/cercaria) using molecular tools, following the approach encouraged by Blasco-Costa & Poulin (2017). The redial and cercarial stages of this parasite were described by Martorelli *et al.* (2008) and compared morphologically to other cercariae described in the literature, and those authors concluded that the cercaria from the intertidal mud snail, *Z. subcarinatus*, in New Zealand, was a close relative of *G. bearupi*. We have herein confirmed their speculation with both our molecular phylogeny and morphometric comparison of the adults. In other species of *Galactosomum*, the large, worm-like cercariae are actively ingested by a fish, where they encyst as metacercariae (Kearn, 1998). Although *Z. subcarinatus* is the only known first intermediate host for *G. otepotiense* n. sp. and it is unknown at this stage what fish species fill the role of second intermediate host, we have shown that at

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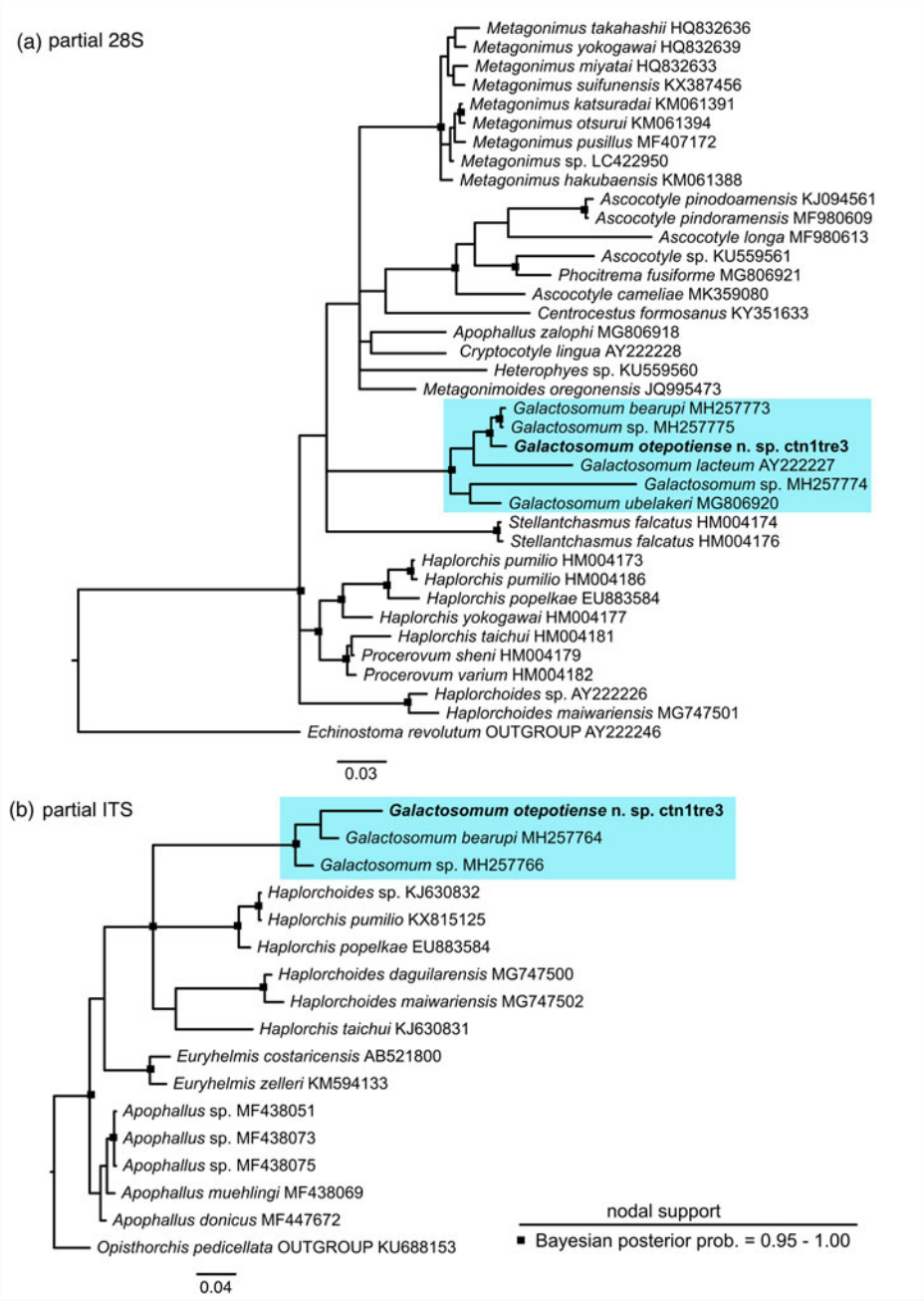

Fig. 3. Bayesian 50% majority-rule inference tree for (a) partial 28S dataset and (b) ITS2 dataset. Scalebars indicate the number of substitutions per site. Outgroup for the 28S phylogeny is *Echinostoma revolutum* (Echinostomatidae) and outgroup for the ITS2 phylogeny is *Opisthorchis pedicellata* (Opisthorchiidae).

least four species of fish-eating bird act as definitive hosts (Caspian tern, red-billed gull, black-backed gull and little blue penguin). All four of these birds prey on small inshore fish species (New Zealand Birds Online: www.nzbirdsonline.org.nz). In the case of little blue penguins at Oamaru, Graham's gudgeon (*Grahamichthys radiata*) and slender sprat (*Sprattus antipodum*) are predominant in the diet (Fraser & Lalas, 2004; Flemming *et al.*, 2013), and these fish species may be a good starting point in a search for the second intermediate host.

Comments on closely related species

In this study, both morphometric and DNA sequence data suggest that *G. otepotiense* n. sp. is most closely related to species from Australia. This pattern is seen in several species of parasitic helminth (Presswell *et al.*, 2012; Cribb *et al.*, 2014b; Georgieva *et al.*, 2017; Huston *et al.*, 2018), and is perhaps unsurprising when considering that Australia is the closest large land mass and much of New Zealand's marine avifauna has evolved from Australian immigrant species or *vice versa* (e.g. Diamond, 1984; Given *et al.*, 2005). It is interesting that the two closest species to *G. otepotiense* n. sp. (*G. angelae* and *G. bearupi*) have also been described from Caspian terns in Australia; indeed, the Caspian tern is type host for all three species. The Australian *G. angelae* has also been found in the little blue penguin and both Australian *Galactosomum* species have been found in the silver gull (*Chroicocephalus novaehollandiae* (Stephens, 1828)), which is sister species to the New Zealand red-billed gull (Given *et al.*, 2005). Two further species of *Galactosomum* are found on the Queensland coast, *G. ussuriense* Oshmarin, 1963 and *G. renincola* Pearson, 1973, the former sharing

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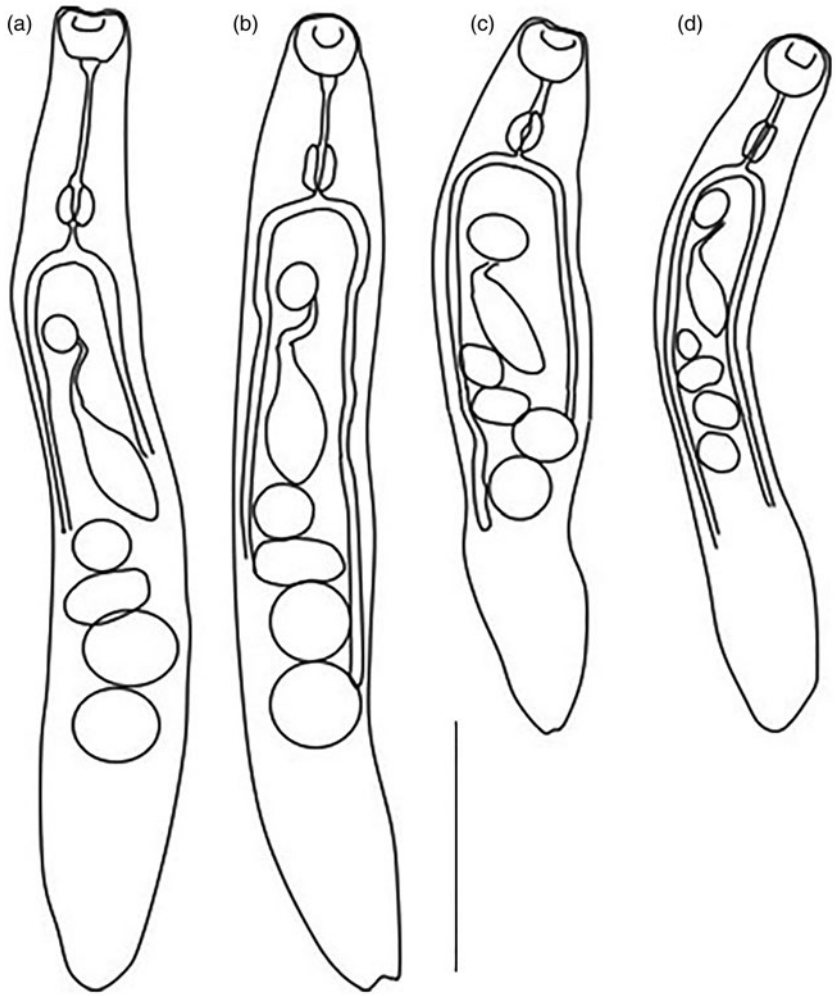

Fig. 4. *Galactosomum otepotiense* n. sp. from four different hosts showing morphological variation: (a) Ex Caspian tern; (b) ex black-backed gull; (c) ex red-billed gull; (d) ex little blue penguin. Scale bar = 500 μm.

a host with *G. bearupi*, *G. angelae* and *G. otepotiense* n. sp. (Caspian tern), and all of which have hosts with a diet of small inshore fish. With several species using the same hosts, there has obviously been strong selection pressure for speciation in this genus. It is not within the remit of this study, but elucidating the life cycles of each of the Australasian species and the historical biogeography of their hosts, may clarify the driving force for this level of speciation.

Although we have been able clearly to distinguish *G. otepotiense* n. sp. from *G. bearupi*, we do not have genetic data for *G. angelae*, which appears morphologically close to the new species. A combination of morphological similarity and host preference, added to the fact that *G. angelae* was described from South Australia, leads us to speculate that the two species will be found to be very closely related genetically. Genetic data for *G. angelae*, and a description of the cercariae, ideally genetically identified, will demonstrate the relationships between these three closely related Australasian species.

Variability in different hosts

We have chosen *H. caspia* as the type host for this species because similar members of the genus (*G. angelae*, *G. bearupi* and *G. ussuriense*) are found in the same host in the Australasian region, and because the specimens recovered from terns were in the best condition. In addition, one of the Caspian terns was found at Portobello Bay, the locality of the larval stages from *Z. subcarinatus*. We note, however, that there appears to be considerable morphological variability between the specimens found in the four different bird hosts (fig. 4). A range of comparative measurements is given in table 3 to illustrate the variability between specimens from different hosts. In particular, those from gulls appeared more flattened than cylindrical when recovered, and the length of the forebody (exemplified by the prepharynx length and distance between the anterior tip and the ventrogenital complex) was much shorter than in the specimens from the Caspian tern. The little blue penguin parasites were considerably smaller, although all were ovigerous (considered 'mature'), and in body shape and proportions resembled the gull specimens more than those from the tern (see fig. 4). These differences appeared to be consistent within the different hosts, rather than an artefact induced by the state of preservation of each individual bird. It is true that some birds were in better condition of preservation than others, and the length of time between death and freezing is often a factor in the shape and condition of parasites recovered from their intestines. But, as worms from different individuals of the same host seem consistent, we suggest that this is an example of host-induced variability, where the worms are genetically identical in the genes sampled. Although there are plenty of examples in the literature of cryptic species of trematode (i.e. morphologically indistinguishable but genetically divergent) (e.g. León-Régagnon *et al.*, 1999;

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Georgieva *et al.*, 2013; Cribb *et al.*, 2014a), and reports of host-induced variability within species (e.g. Stunkard, 1957; Blankespoor, 1974; Pérez Ponce de León, 1995), records of host-induced variability supported by proof of genetic identity are rarer (Cutmore *et al.*, 2010; Hildebrand *et al.*, 2015). During this study we examined slides of *G. angelae* from five different host species, and found that they, too, showed considerable variability. These observations lend weight to the argument that apparent host specificity is not a reliable criterion with which to delineate a species, and that both morphological and molecular analyses should be used in combination when investigating parasites infecting multiple hosts.

Comments on specimens

As Pearson (1973) observed, we found that the tegumental spines were frequently lost after death, even on the most well-preserved specimens (measurements noted herein were taken from a few stained specimens that had retained their spines). The scanning electron micrograph shows that not a single tegumental spine seems to persist on the specimen from the Caspian tern, but we interpret the small tears in the outer tegument to be the scars of the spines, based on their distribution and frequency when compared to other specimens where the spines were *in situ*. Also visible in the SEMs are tegumental pores, or pits, that are arranged in apparently random fashion on the visible ventral surface. Such pores have been observed on the surface of other trematodes, and have been assumed to be secretory (Bakke, 1976; Smales & Blankespoor, 1984; Otubanjo, 1985; El Abdou *et al.*, 2001).

Importance of Galactosomum *species*

Galactosomum spp. are potentially of future interest because, although they probably do little harm to their definitive hosts, the metacercarial stages of many species inhabit the optic lobe of the second intermediate host's brain, where they affect the behaviour of the fish, causing it to flip and whirl at the water surface, thus making the host highly visible to piscivorous birds, and more likely to be predated (Kimura & Endo, 1979; Bartoli & Boudouresque, 2007; Ogawa, 2015). Concern has been expressed that the parasite, causing 'trematode whirling disease', could have a marked negative effect on the mariculture of food fish, as it has been found in farmed populations of *Seriola quinqueradiata* (Japanese amberjack), *Takifugu rubripes* (Japanese puffer fish), *Oplegnathus fasciatus* (barred knifefish) and *Chrysophrys major* (red seabream) (Kimura & Endo, 1979; Yasunaga, 1981; Ogawa, 2015). Currently, aquaculture provides over half of all seafood consumed globally, and mariculture makes up one-third of this production (Liu *et al.*, 2018). With an ever-growing human population and ever-decreasing natural marine fish stock this can only increase, and any threat to production, including parasites, will need to be taken seriously.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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