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# Four marine digenean parasites of *Austrolittorina* spp. (Gastropoda: Littorinidae) in New Zealand: morphological and molecular data

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**Abstract** Littorinid snails are one particular group of gastropods identified as important intermediate hosts for a wide range of digenean parasite species, at least throughout the Northern Hemisphere. However nothing is known of trematode species infecting these snails in the Southern Hemisphere. This study is the first attempt at cataloguing the digenean parasites infecting littorinids in New Zealand. Examination of over 5,000 individuals of two species of the genus *Austrolittorina* Rosewater, *A. cincta* Quoy & Gaimard and *A. antipodum* Philippi, from intertidal rocky shores, revealed infections with four digenean species representative of a diverse range of families: Philophthalmidae Looss, 1899, Notocotylidae Lühe, 1909, Renicolidae Dollfus, 1939 and Microphallidae Ward, 1901. This paper provides detailed morphological descriptions of the cercariae and intramolluscan stages of these parasites. Furthermore, partial sequences of the 28S rRNA gene and the mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox*1) for varying numbers of isolates of each species were obtained. Phylogenetic analyses were carried out at the superfamily level and along with the morphological data were used to infer the generic affiliation of the species.

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## Introduction

Digenean trematode parasites typically infect a gastropod as the first intermediate host in their complex life-cycles. They are common in the marine environment, particularly in the intertidal zone (Mouritsen & Poulin, 2002). One abundant group of gastropods in the marine intertidal environment is the littorinids (i.e. periwinkles), which are characteristic organisms of the high intertidal or littoral zone and have a global distribution (Davies & Williams, 1998). In the Northern Hemisphere, species rich digenean faunas have been reported for littorinids such as *Littorina littorea* L., *L. obtusata* L. and *L. saxatilis* Olivi (e.g. James, 1968; Granovitch & Mikhailova, 2004; Thieltges et al., 2006, 2009; Blakeslee & Byers, 2008; Granovitch & Maximovich, 2013). These include more than 30 species from the families Echinostomatidae Looss, 1899, Microphallidae Ward, 1901, Notocotylidae Lühe, 1909, Heterophyidae Leiper, 1909, Renicolidae Dollfus, 1939 and Opecoelidae Ozaki, 1925 (James, 1968; Granovitch & Mikhailova, 2004; Blakeslee & Byers, 2008; Thieltges et al., 2009; Skirnísson & Galaktionov, 2002) with up to 12 species per sampling site

(Skirnísson & Galaktionov, 2002). However, for the Southern Hemisphere no parasitological investigations of periwinkles could be sourced in the literature, in stark contrast to that available for the Northern Hemisphere.

*Austrolittorina* Rosewater includes five species, all distributed in the Southern Hemisphere: *Austrolittorina antipodum* Philippi and *A. cincta* Quoy & Gaimard in New Zealand, *A. unifasciata* Gray in Australia, and *A. fernandezensis* Rosewater and *A. araucana* d'Orbigny in western South America (Reid & Williams, 2004). During 2012 and 2013 extensive sampling of *A. antipodum* and *A. cincta* was carried out in the high intertidal zone around the Otago Harbour and Kaikoura, South Island, New Zealand and Paihia, North Island, New Zealand. We found frequent infections with larval stages of four digeneans of the families Philophthalmidae Looss, 1899, Notocotylidae, Renicolidae and Microphallidae. Here we provide detailed morphological descriptions of the cercariae and intramolluscan stages of the four species and molecular evidence based on phylogenetic analyses at the superfamily level using newly-generated 28S rDNA sequences and, where possible, sequences of the mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox*1).

## Materials and methods

*Austrolittorina antipodum* and *A. cincta* were collected in 2012 and 2013 off New Zealand, primarily around Otago Harbour (−45.80, 170.66) (four sites: Aramoana, Portobello Marine Laboratory, Lower Portobello Bay and Weller's Rock) and off Kaikoura (−42.43, 173.69) and Paihia (−35.28, 174.09). Snails were dissected in the laboratory under a dissecting microscope and mature cercariae, sporocysts and rediae were photographed live, under natural conditions and following staining with Neutral Red under slight coverslip pressure, using a compound microscope. Metacercarial cysts were photographed and measured following encystment on a glass slide. Samples of cercariae killed in hot seawater were photographed for morphological study and subsamples were fixed in 100% ethanol for molecular analysis and in 2.5% glutaraldehyde, 4% formalin or 70% ethanol for scanning electron microscopy (SEM) examination. Photomicrographs were taken using an Olympus CX41 microscope, Olympus DP25 camera and DP2-BSW v. 1.4 software. Measurements were obtained from digital photographs using ImageJ (Schneider et al., 2012). Samples for SEM fixed in glutaraldehyde and formalin were washed in 0.1 M phosphate buffer (three washes, 15 min each), post-fixed in 1% osmium tetroxide, washed in 0.1 M phosphate buffer (three washes, 15 min each), and dehydrated in a graded ethanol series. Samples fixed in 70% ethanol were dehydrated in a graded ethanol series. After dehydration all samples were critical point-dried and splutter-coated with gold. Samples were examined using a JEOL JSM 7401-F at an accelerating voltage of 4 kV. The nomenclature of Bayssade-Dufour (1979) was generally accepted for describing the chaetotaxy reconstructed from series of SEM photomicrographs.

All measurements are in micrometres and are presented as the range followed by the mean in parentheses. Metrical data in the descriptions are based on live specimens under slight coverslip pressure; measurements of fixed material are provided separately (where available). Voucher material (in alcohol and permanent mounts in Canada balsam) has been deposited at the Institute of Parasitology, Academy of Sciences of the Czech Republic under accession numbers HCIP D-700; D-701; D-702 and D-703.

DNA was extracted from single ethanol-fixed cercariae/sporocysts/rediae in 150–500 µl of 5% suspension of Chelex® in deionised water containing 0.1 mg/ml proteinase K, followed by incubation overnight at 60°C, boiling at 90°C for 8 min and centrifugation at 15 000 *g* for 10 min.

Polymerase chain reaction (PCR) amplifications (total volume 20 µl) were carried out using 1 µl extraction supernatant, 4 µl MyTaqRed buffer (5 mM dNTPs), 10 µM of each primer and 0.5 units MyTaq (Bioline Ltd). Partial (domains D1–D3) 28S rDNA sequences were amplified using primers U178 (forward: 5′-GCA CCC GCT GAA YTT AAG-3′) and L1642 (reverse: 5′-CCA GCG CCA TCC ATT TTC A-3′) (Lockyer et al., 2003). The following thermocycling profile was used: DNA denaturation at 95°C for 3 min; 40 amplification cycles (94°C for 40 sec, 56°C for 30 sec, 72°C for 1 min 20 sec); and a final extension step for 4 min at 72°C. Amplification of partial *cox*1 gene fragments was performed using the primer combination JB3 (forward: 5′-TTT TTT GGG

CAT CCT GAG GTT TAT-3′) (Bowles et al., 1993) and Plag16S-COIdR (reverse: 5′-TCG GGG TCT TTC CGT CT-3′) (Blasco-Costa et al., 2012). The following thermocycling profile was used: DNA denaturation at 94°C for 3 min; 40 amplification cycles (94°C for 40 sec, 54°C for 35 sec, 72°C for 2 min); and a final extension step for 4 min at 72°C. The thermocycling profile for microphallid and renicolid samples was run at a higher annealing temperature (57°C).

The PCR products were purified using exonuclease I, and shrimp alkaline phosphatase, enzymes (Werle et al., 1994). For 20 µl PCR product we added 1.5 U of Exo1 and 1 U of SAP. PCR amplicons were cycle-sequenced from both strands using the PCR primers [plus 1200R (5′-GCA TAG TTC ACC ATC TTT CGG-3′) for 28S rDNA (Lockyer et al., 2003)] with BigDye® Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit, alcohol-precipitated and run on an ABI 3730XL Analyser (Applied Biosystems, Foster City, California). Contiguous sequences were assembled and edited using Sequencher™ (GeneCodes Corp. v. 5) and submitted to GenBank under accession numbers KJ868206–KJ868217 (28S rDNA) and KJ868192–KJ868205 (*cox*1).

The newly-generated partial sequences for the 28S rRNA gene (931–1,608 bp) were aligned using MAFFT (default progressive FFT alignment with two-tree building cycles) (Katoh & Standley, 2013) and the ends of each sequence were trimmed to match the shortest sequences in the alignments. The alignments included members of the four corresponding superfamilies, i.e. the Echinostomatoidea Looss, 1902, Pronocephaloidea Looss, 1899 and Microphalloidea Ward, 1901 (GenBank numbers included in Figs. 4, 7, 10). Phylogenetic relationships were assessed *via* Bayesian inference (BI) analyses. Prior to analyses, the best-fitting substitution models were estimated with jModelTest 2.1.4 (Darriba et al., 2012; Guindon & Gascuel, 2003) using the Akaike Information Criterion (AIC); the general time reversible model with estimates of invariant sites and gamma-distributed among-site rate variation (GTR+I+G) was the model estimated for all alignments. Bayesian inference analyses were carried out in MrBayes 3.2.2 (Ronquist et al., 2012) using Markov chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains over 10,000,000 generations. Trees were sampled every 1,000 generations. "Burn-in" was applied by discarding the first 25% of the sampled trees for each dataset. Estimation of consensus tree topology and nodal support was obtained as posterior probability values from the remaining samples.

The newly-generated *cox*1 sequences were aligned using Muscle implemented in MEGA v6 (Tamura et al., 2013) with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code (Telford et al., 2000). Genetic distances (uncorrected p-distance) were calculated for each gene/species using MEGA v6.

## Results

Examination of over 5,000 individuals of two species of the genus *Austrolittorina* Rosewater, *A. cincta* Quoy & Gaimard (1,955 individuals) and *A. antipodum* Philippi (3,424 individuals), from intertidal rocky shores at six localities in New Zealand revealed infections with four digenean species. Periwinkle collections for *A. cincta* numbered 173–993 individual snails per site (across four sites), and 193–633 per site (across five sites) for *A. antipodum*. Both littorinid species were found infected with the philophthalmid *Parorchis* sp. NZ and an unidentified notocotylid (Notocotylidae gen. sp. 1 NZ). Three other parasite species were also recovered: the renicolid *Renicola* sp. NZ and a second unidentified notocotylid (Notocotylidae gen. sp. 2 NZ) in *A. antipodum* and the microphallid *Microphallus* sp. NZ in *A. cincta*. Detailed descriptions of the mature cercariae and intramolluscan stages of these digeneans are provided below.

### Superfamily Echinostomatoidea Looss, 1902
### Family Philophthalmidae Looss, 1899

### *Parorchis* sp. NZ

*First intermediate hosts*: *Austrolittorina antipodum* Philippi, *Austrolittorina cincta* Quoy & Gaimard (Gastropoda: Littorinidae).
*Locality*: Otago Harbour, Dunedin (New Zealand).
*Prevalence*: *A. antipodum*: 0.56% (Aramoana; n = 716); 7.45% (Weller's Rock; n = 993);

12.14% (Portobello Marine Laboratory; n = 725); 69.36% (Lower Portobello Bay; n = 173); 5.08% (Kaikoura; n = 394). *A. cincta*: 0.32% (Aramoana; n = 633); 3.22% (Weller's Rock; n = 622); 2.96% (Portobello Marine Laboratory; n = 507); 64.77% (Lower Portobello Bay; n = 193).
*Voucher material*: HCIP D-700.
*Representative sequences*: KJ868206–KJ868209 (28S rDNA); KJ868192–KJ868197 (*cox*1).

Description (Figs. 1, 2, 3A)

*Redia*
[Measurements based on 13 small and 15 large live specimens.] Two types (small and large) of white, opaque rediae present (Fig. 1B). Small rediae occasionally containing germinal balls, cylindrical, tapering at both ends, 354–683 × 51–96 (484 × 68), with 2 slender prominent locomotory processes at posterior fifth of body. Pharynx large, 44–64 × 30–52 (52 × 41); intestine broad, extends beyond locomotory processes, close to posterior extremity. Large rediae containing 1–7 (4) cercariae, sausage-shaped, 768–1,694 × 176–471 (1,335 × 321), with indistinct locomotory processes, slender intestine extending to one third of body length; pharynx 40–75 × 40–70 (60 × 53).

*Cercaria*
[Measurements based on 40 live specimens under slight coverslip pressure; not all specimens contributed a data point to all metrical variables.] Distome megalurous cercaria (Fig. 1A). Body elongate-pyriform, 364–498 (418) long, with maximum width 173–284 (224) at anterior level of ventral sucker, narrowing posterior to its mid-level, with deep transverse pit just anterior to ventral sucker. Forebody 149–305 (217) long, 41–58 (50)% of body length. Tegument thick, spineless, with regularly alternating minute tubercles and depressions on ventral surface (appearance of knitted sweater under SEM; Fig. 2A, B) and giving a false impression of spined tegument under light microscopy), covered with sponge-like matter on lateral and dorsal surfaces probably released from cystogenous gland-cells after cercarial emergence (Fig. 2A, B).

Tail 330–579 (438) long, 45–85 (58) wide at base, 72–119 (94)% of body length, containing spherical parenchymatous cells for *c.*2/3 of length; posterior part [1/3 of length, 80–137 (107)] contains 10–12 club-shaped nucleated gland-cells staining intensely with Neutral Red (probably involved in adhesion to substrate), ends with a cup-shaped tip (adhesive organ), 15–29 (21) long.

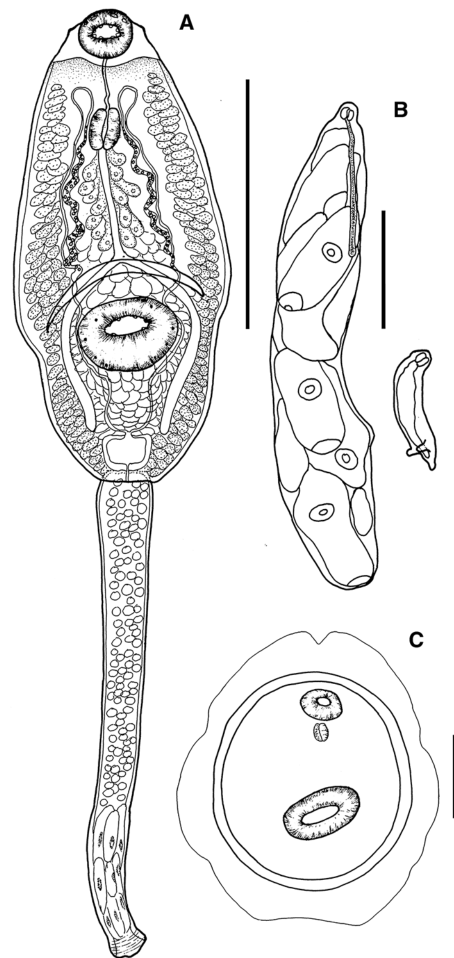


**Fig. 1** *Parorchis* sp. NZ ex *Austrolittorina cincta*. A, Cercaria; B, Rediae; C, Encysted metacercaria. *Scale-bars*: A, 250 µm; B, 400 µm; C, 100 µm

Oral sucker ventro-subterminal, muscular, transversely-elongate, 61–82 × 56–97 (67 × 77), followed by muscular 'neck' (narrow area with smooth tegument, see Figs. 1, 2A, C), clearly delineated by the absence of gland-cells (lack of staining with Neutral Red). 'Neck', may be highly contracted or extended [distance from posterior margin of 'neck' to anterior extremity 42–55 (47); width at posterior margin 92–155 (114)]. Collar-like thickening with 'collar' spines at anterior extremity

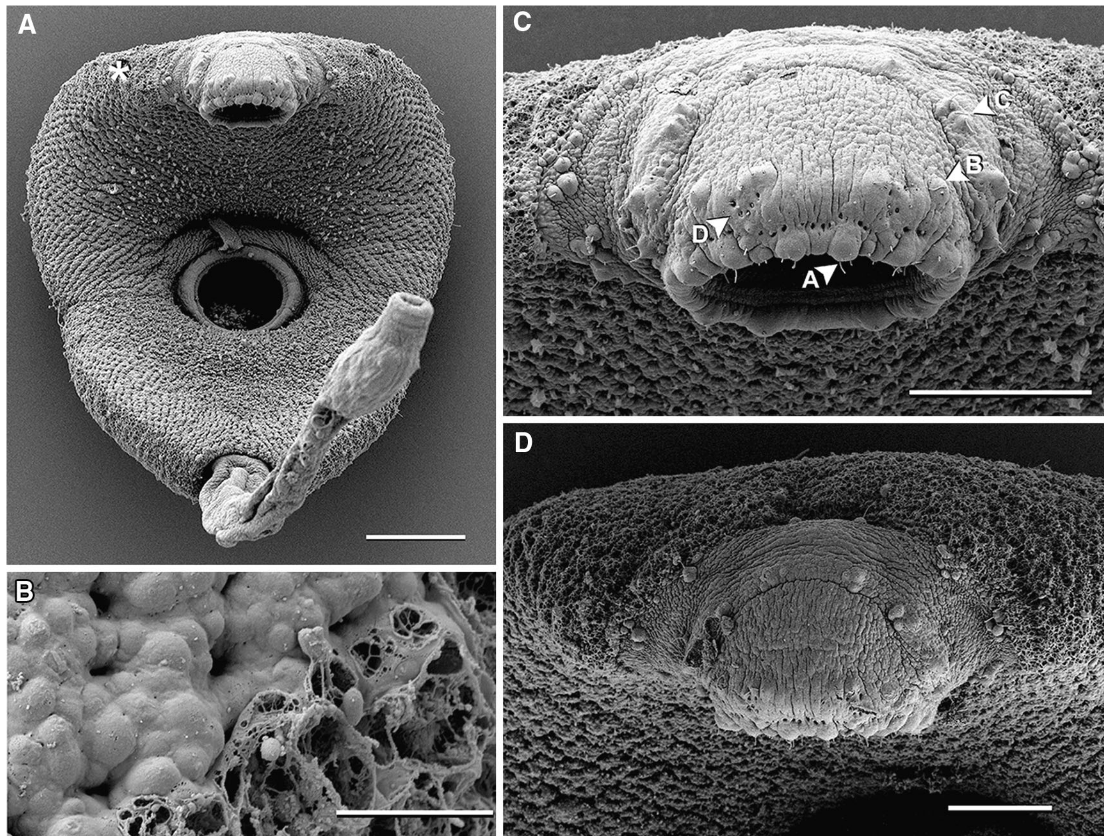


**Fig. 2** *Parorchis* sp. NZ, scanning electron micrographs. A, Cercaria, ventral view (* indicates the area at the margin between ventral and lateral tegument detailed in B); B, Detail of ventral (upper left) and lateral tegumental surface (lower right); C, Detail of the cephalic region with four types of sensory papillae (A–D) indicated by arrowheads; D, Detail of the cephalic region. *Scale-bars*: A, 50 µm; B, 4 µm; C, 30 µm; D, 50 µm

not observed. Ventral sucker post-equatorial, larger than oral, strongly muscular, subspherical, 77–118 × 70–129 (93 × 99), surrounded by smooth tegument delineated anteriorly by deep tegumental fold (Figs. 1A, 2A); opening bears 6 small papillae (2 median and 2 sublateral on anterior rim and 2 sublateral on posterior rim). Four small papillae with long cilia on posterior rim of ventral sucker, outer circle of 2 medio-lateral anterior papillae, 2 lateral and 4 sublateral posterior papillae. Sucker length ratio 1:1.20–1.73 (1:1.38); sucker width ratio 1:1.06–1.86 (1:1.28). Prepharynx long, pharynx muscular, elongate-oval, 30–43 × 21–29 (36 × 25). Oesophagus long, rather wide, bifurcates at short distance from ventral sucker, anterior to ventral pit. Caeca long, wide, extend nearly to level of excretory vesicle.

Cystogenous gland-cells abundant, from level of 'neck' to posterior extremity, narrow area (single to double row of cells) on both lateral sides stained most intensely with Neutral Red. Two groups of *c.*6–7 nucleated gland-cells on either side of oesophagus, ducts open in a row of 12–14 pores on dorsal surface of oral sucker, just posterior to circle $C_I$ of sensory receptors (Fig. 2C, D).

Excretory vesicle thick-walled, sub-rectangular in ventral aspect; main collecting ducts extend to level of prepharynx, forming a tight loop; filled with small spherical refractive granules, smallest 1–2 × 1–2 (1 × 1), largest 3–4 × 3–4 (3 × 3). Caudal excretory tubule not observed. Flame-cell formula not determined (flame-cells obscured by cystogenous gland-cells).

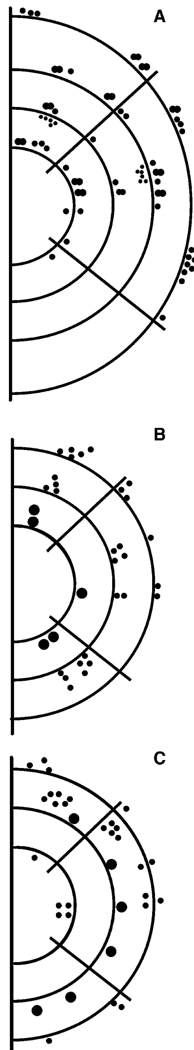


**Fig. 3** Cercarial chaetotaxy patterns (cephalic region). A, *Parorchis* sp. NZ; B, Notocotylidae gen. sp. 1 NZ; C, *Renicola* sp. NZ

Measurements based on material killed in hot seawater. Body 442–525 (480) × 164–195 (181); tail 359–409 (387) long, 47–53 (49) wide at base; oral sucker 55–65 × 58–67 (62 × 62); ventral sucker 67–77 × 35–53 (72 × 45).

Sensory receptors. Four new types of sensory receptors, all lacking tegumentary collar or tegumentary sheath (see Bogéa, 2004), were observed in the cephalic region under SEM (Fig. 2C): (i) large, double, papilla-like receptors with dome-like base, each with long cilium (type A); (ii) large and medium-sized single papilla-like receptors with dome-like base, each with single long cilium (type B); (iii) small dome-like receptors each with single very short cilium (type C); (iv) small deep depressions with single very short cilium (type D). Four cephalic ($C_I$, $C_{II}$, $C_{III}$ and $C_{IV}$) and one ventral sucker (S) circle were identified from SEM reconstructions. A total of 106 sensory receptors was found on the tegument in the cephalic region (Type A = 24; Type B = 60; Type C = 12; Type D = 10) and 4 and 2 non-ciliated papillae were located on the anterior and posterior inner rim of the ventral sucker, respectively. Breakdown of the four types of receptors by cephalic circle was as follows: ($C_I$: 6A + 14B; $C_{II}$: 4A + 6B + 10D; $C_{III}$: 8A + 12B + 12C; $C_{IV}$: 6A + 28B). The chaetotaxy of the cephalic region is summarised as follows (double papillae indicated by "×2"; see also Fig. 3A):

Circle $C_I$: $1C_IV + 1C_IL_1 + 2C_IL_2 + (2 \times 2 + 1)\ C_{II}L_3 + 3C_ID_1 + (1 \times 2)\ C_ID_2$
Circle $C_{II}$: $(1 \times 2 + 1)\ C_{II}L_1 + 1C_{II}L_2 + (1 + 1 \times 2)\ C_{II}D_1 + 5C_{II}D_2$
Circle $C_{III}$: $(1 + 1 \times 2)\ C_{III}L_1 + (1 + 1 \times 2 + 1)\ C_{III}L_2 + 6C_{III}L_3 + 2C_{III}L_4 + (1 \times 2)C_{III}D_1 + (1 + 1 \times 2)\ C_{III}D_2$
Circle $C_{IV}$: $1C_{IV}L_1 + 7C_{IV}L_2 + (3 + 1 \times 2)\ C_{IV}L_3 + (2 \times 2)\ C_{IV}D_1 + 3C_{IV}D_2$

*Metacercaria*

[Measurements based on 5 live specimens, following encystment on a glass slide, after proliferation of the snail tissue during dissection.] Cyst oval (Fig. 1C), dome-shaped, flattened at base, 227–237 × 182–208 (232 × 195), with 2-layered wall, outer layer 56–65 (61) wide at widest point, with anterior and posterior indentations, inner layer 7–8 (7).

## Molecular analysis

The alignment of 28S rDNA sequences for the Echinostomatoidea comprised 1,208 characters for analysis and included one sequence (four replicates) for *Parorchis* sp. NZ together with ten sequences for species of eight genera from four families (Philophthalmidae, Echinostomatidae, Fasciolidae Railliet, 1895 and Cyclocoelidae Stossich, 1902), available on GenBank (Olson et al., 2003; Lotfy et al., 2008; Griffin et al., 2012; Church et al., 2013), with the

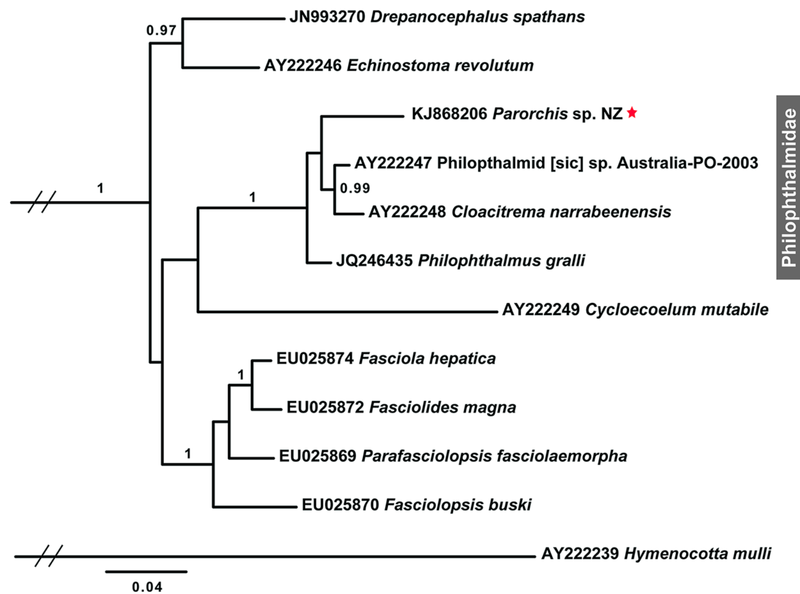


**Fig. 4** Phylogenetic relationships inferred by Bayesian analysis based on 28S rDNA sequence data for representatives of the superfamily Echinostomatoidea. The newly-generated sequence is indicated by a star. Only posterior probabilities >0.95 are shown; the scale-bar indicates the expected number of substitutions per site

haplosplanchnid *Hymenocotta mulli* Manter, 1961 as an outgroup (Olson et al., 2003) (accession numbers provided in Fig. 4). There was good support at the family level within the Echinostomatoidea with the newly-generated sequence clustering with strong support within the family Philophthalmidae. However, the relationships among the representative taxa within the Philophthalmidae were poorly resolved due to the small number of sequences. The sequence for *Parorchis* sp. NZ did not show affiliation to *Philophthalmus gralli* Looss, 1899 or *Cloacitrema* spp. The genetic divergence between *Parorchis* sp. NZ and the remaining philophthalmids was 4.6–4.8% (55–58 bp) whereas the latter differed by 1.8–3.0% (22–36 bp).

The six *cox*1 sequences generated (791 bp) represented six haplotypes. Of these one was widespread among four sites, three within Otago Harbour (Lower Portobello Bay, Weller's Rock and Aramoana) and one at Kaikoura. The remaining five haplotypes were recorded once each, three haplotypes at Portobello Marine Laboratory, one at Weller's Rock and one at Kaikoura. The intraspecific divergence was 0.1–0.3% (0–2 bp) thus confirming the conspecificity of all isolates. Comparisons with the only available philophthalmid sequences on GenBank [*Philophthalmus* sp. SMS-2011 ex *Melanoides tuberculata* in Iran; *Philophthalmus* sp. DBK-2009 and *Philophthalmus* sp. TLFL-2009, both ex *Zeacumantus subcarinatus* (Sowerby II) in Otago Harbour (Keeney et al., 2009; Leung et al., 2009)] revealed a range of genetic divergence of 15.9–16.6%.

Remarks

The present cercaria bears all characteristics of megalurous cercariae *sensu* Cort (1914) of species of the Philophthalmidae: elongate-pyriform body, well-developed suckers, thick, granular tegument, tail comprised of anterior region with parenchymatous cells followed by nucleated gland-cells and a cup-shaped adhesive organ, and encystment on the substrate. The cercaria further possesses characteristics of the cercariae of *Parorchis* spp. such as the large number of sensory papillae in the region of oral and ventral suckers, long oesophagus and encystment in a dome-shaped flattened cyst (see Angel, 1954; Yamaguti, 1975; Gilardoni et al., 2012).

The cercaria of *Parorchis* sp. NZ resembles in many aspects the cercaria of *Parorchis acanthus* (Nicoll, 1906) described by Rees (1937) as *Cercaria purpurae* Lebour, 1911 from the muricid *Nucella lapillus* (L.) (as *Purpura lapillus*) at Aberystwyth, UK, especially in the presence of distinct papillae around the openings of the suckers, but differs in their

number (six double and 14 single *vs* 16 around mouth opening) and in the lack of 'collar' spines (64 in *P. acanthus*). The metrical data for live cercariae of *Parorchis* sp. NZ generally fall within the rather wide range given for *P. acanthus* (body 364–498 × 173–284 *vs* 360 × 250 and 1,000 × 90 µm in contracted and expanded cercariae, respectively; tail 330–579 *vs* 180–820 µm).

Angel (1954) completed the life-cycle of *Parorchis acanthus* var. *australis* Angel, 1954 starting with cercariae from the littorinind *Bembicium auratum* (Quoy & Gaimard) collected from a tidal mud-flat at the mouth of the Patawalonga Creek, Glenelg, South Australia; three other snail species [the littorininds *Bembicium nanum* (Lamarck), *B. melanostoma* (Gmelin) and the muricid *Lepsiella flindersi* (Adams & Angas) (as *Emozamia flindersi* (Adams & Angas)] in the region were also found to be infected. The present cercaria is consistent with the morphological description of Angel (1954) in the shape and structure of the body and tail as well as in the thinner tegument of the cephalic region (Fig. 2C, D) that also stains poorly with vital stains ('neck' region in the description above, see Fig. 1A) and in the presence of a number of large papillae in the cephalic region and on the ventral sucker (six on the inner rim as in the present cercaria). However, we failed to observe 'collar' spines, excretory tubule in the tail in live cercariae and a ventral excretory pore (as illustrated in figure 1 of Angel, 1954) in specimens examined either live or under SEM. Further, although no detail on the number of the papillae was provided by this author, we counted *c.*10 papillae in the outer rim of the oral sucker in figure 1 of Angel (1954) (*vs* six double plus 14 single papillae in the cercaria of *Parorchis* sp. NZ). Comparisons of data from fixed cercariae revealed that the cercaria of *Parorchis* sp. NZ exhibits lower upper limits for body length and width (442–525 × 164–195 *vs* 370–670 × 130–225 µm) but is on average longer and wider (means 480 × 181 *vs* 450 × 170 µm) than the cercaria of *P. acanthus* var. *australis*. The tail length and width at base in *Parorchis* sp. NZ vary within the range given for *P. acanthus* var. *australis* (359–409 × 47–53 *vs* 265–595 × 45–60 µm) but the means are lower (387 × 49 *vs* 450 × 52 µm).

The cercaria of *Parorchis* sp. NZ also resembles morphologically *Cercaria caribbea* LIX Cable, 1963 developing in *Thais rustica* (Lamarck) in Curaçao (Cable, 1963) but differs in the absence of 'collar' spines. Moreover, *C. caribbea* LIX is a much longer form (690–752 *vs* 364–498 µm) with a tail of similar length (470–502 *vs* 330–579 µm), smaller oral sucker (width 67–72 *vs* 56–97 µm) and with a ventral sucker falling within the range of the present material (width 91–94 *vs* 70–129 µm) but generally below the mean (99 µm).

Recently Gilardoni et al. (2012) described a cercaria of *Parorchis* sp. emerging from the muricid snail *Trophon geversianus* Pallas on the Patagonian coast of the South West Atlantic and provided morphological data from both light and SEM study. Their description resembles the cercaria of *Parorchis* sp. NZ in the general structure of body and tail, the presence of poorly stained 'neck' region, the appearance of the tegumental surface in light and SEM micrographs, and the presence of numerous papillae in the region of suckers. However, the cercaria of *Parorchis* sp. NZ possesses two and 20 papillae on the inner and outer rim of the oral sucker, respectively (*vs* eight and 12) and 18 (*vs* eight) papillae on the ventral sucker.

The major consistent difference of the cercaria of *Parorchis* sp. NZ from the cercariae of *Parorchis* spp. in the comparisons above, is the lack of tegumental and 'collar' spines. However, a crown of 'collar' spines may be present or absent in *Parorchis* spp. (see Kanev et al., 2005); the morphological similarities and the specific dissimilarities depicted above indicate that we may have discovered a species without 'collar' spines at least at the stage of cercaria. It is also possible that we failed to observe these spines because the tegument was already covered by the substance excreted by the cystogenous gland-cells, especially on the lateral and dorsal surfaces of the body (Fig. 2A, B). The presence of tegumental spines in all species of *Parorchis* appears questionable since the only other SEM study of *Parorchis* sp. reveals similar structure of the tegument that appears as spined under light microscopy (see Gilardoni et al., 2012). Under SEM we have observed small, shrunken follicles scattered on the tegumental surface; these are likely misinterpreted as spines under light microscopy.

The only record of larval philophthalmids in New Zealand is that of *Philophthalmus* sp., tentatively assigned to *P. burrili* Howell & Bearup, 1967 by Martorelli et al. (2008), infecting the mudsnail *Z. subcarinatus* (see Howell, 1965; Martorelli et al., 2008; Leung et al., 2009 and references therein). Adults of three philophthalmid species have been reported from New Zealand: *Cloacitrema* sp. in *Haematopus finschi*

Martens and *H. unicolor* Forster; *Parorchis acanthus* in *Chroicocephalus scopulinus* Forster, *H. finschi*, *Larus dominicanus* Lichtenstein and *Limosa lapponica baueri* L.; and *Philophthalmus* sp. in *L. dominicanus* (see McKenna et al., 2010). The above comparisons indicate that *Parorchis* sp. NZ may represent an as yet undescribed species of the genus. The detailed morphological and molecular data provided here will help the discovery of the adult stage of this species.

**Superfamily Pronocephaloidea Looss, 1899**
**Family Notocotylidae Lühe, 1909**

### Notocotylidae gen. sp. 1 NZ

*First intermediate hosts*: *Austrolittorina antipodum* Philippi and *Austrolittorina cincta* Quoy & Gaimard (Gastropoda: Littorinidae).
*Localities*: Otago Harbour, Dunedin; Kaikoura; Paihia (New Zealand).
*Prevalence*: *A. antipodum*: 1.73% (Lower Portobello Bay; n = 173); 0.01% (Kaikoura; n = 560); 0.39% (Paihia; n = 257). *A. cincta*: 1.55% (Lower Portobello Bay; n = 193).
*Voucher material*: HCIP D-701.
*Representative sequences*: KJ868210–KJ868213 (28S rDNA); KJ868198–KJ868201 (*cox*1).

Description (Figs. 3B, 5, 6)

*Redia*

[Measurements based on 12 live specimens.] Rediae (Fig. 5B) orange, saccular, large, muscular, with contractile posterior third, 654–1,464 × 141–349 (992 × 239); contain 1–14 (6) mostly immature cercariae; with constrictions when containing few (0–1) cercariae. Locomotory processes lacking; pharynx 36–56 × 32–47 (44 × 37); intestine sac-like, extends to 3/4 of body length in young rediae, to 1/4–1/3 of body length in mature rediae packed with cercariae, contain yellow refractive particles.

*Cercaria*

[Measurements based on 16 live specimens; not all specimens contributed a data point to all metrical variables.] Monostome triocellate cercaria (Fig. 5A). Body greyish, opaque, almost round, 194–298 × 155–240 (243 × 207), with 2 indistinct posterior-lateral glands opening dorsally. Tegument spineless, densely covered with minute (*c*.0.5 long) projections (presumably microvilli, see Fig. 6B–C). Eye-spots 3: 2 lateral, black, measuring 5–10 × 7–10 (7 × 8) and 1 diffuse, brownish, median. Tail simple, 115–194 (162) long, 30–60 (46) wide at base, shorter than body [50–79 (68)% of body length], with tegument covered with microvilli, central axis comprising cells densely stained with Neutral Red, and strongly muscular contractile tip.

Oral sucker terminal, muscular, subglobular, 30–44 × 31–51 (37 × 42). Pharynx and ventral sucker absent. Oesophagus short, bifurcation posterior to anterior collecting duct loop, just posterior to median eye-spot. Caeca long, narrow, extend to posterior body, extremities not observed (obscured by main excretory collecting ducts).

Cystogenous gland-cells densely distributed throughout body, staining intensely with Neutral Red along lateral margins. Anlagen of reproductive organs a chain of transparent cells along median line of body.

Excretory vesicle thin-walled, transversely oval; main collecting ducts wide, extend to and unite just posterior to oral sucker, forming small median anterior loop ventral to median eye-spot, filled with irregular refractive granules with variable size [smaller posteriorly, 2–5 × 3–4 (4 × 3), larger anteriorly 8–14 × 3–7 (11 × 5)]. Bifurcation of caudal excretory tubule not observed. Flame-cell formula not determined (flame-cells obscured by cystogenous gland-cells).

Measurements based on material killed in hot seawater. Body 257–334 (290) × 139–176 (159); tail 214–272 (241) long, 22–27 (24) wide at base; oral sucker 30–42 × 32–40 (34 × 36).

Sensory receptors. Three types of sensory receptors, all lacking tegumentary collar or tegumentary sheath, were observed in the cephalic region under SEM (Fig. 6B): (i) large dome-like receptors, each with single long cilium (type B); (ii) small dome-like receptors each with single very short cilium (type C); medium-sized knob-like non-ciliated receptors (type E, new type). Three body circles were observed ventrally ($1A_{I} + 1A_{II} + 1A_{III}$) but the rest of the chaetotaxy was not resolved successfully. A total of 56 sensory receptors were found on the tegument in

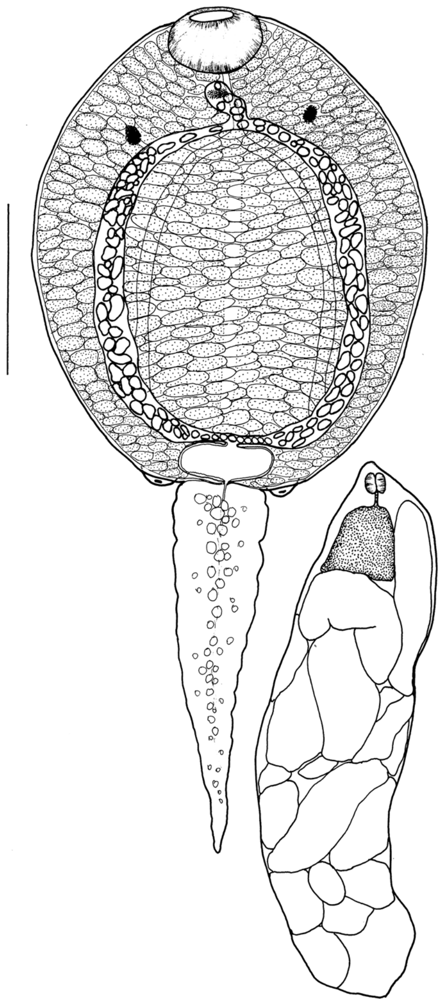

**Fig. 5** Notocotylidae gen. sp. 1 NZ ex *Austrolittorina antipodum.* A, Cercaria; B, Redia. *Scale-bars*: A, 100 μm; B, 200 μm

the cephalic region (Type B = 10; Type C = 40; Type E = 6). The chaetotaxy of the cephalic region is summarised as follows (see Fig. 3B):

Circle $C_I$: $2C_IV + 1C_IL + 2C_ID$

Circle $C_{II}$: $3C_{II}V_1 + 4C_{II}V_2 + 2C_{II}L_1 + 4C_{II}L_2 + 4C_{II}D$

Circle $C_{III}$: $2C_{III}L_1 + 1C_{III}L_2 + 3C_{III}L_3 + 5C_{III}D$

Molecular analysis

The alignment of 28S rDNA sequences (940 bp) for Pronocephaloidea included two sequences, one (four replicates) for the cercaria identified as Notocotylidae gen. sp. 1 NZ and one for Notocotylidae gen. sp. 2 NZ (see below), plus eight sequences for representatives of eight genera from four families, available on GenBank (Tkach et al., 2001; Olson et al., 2003; Boyce et al., 2012; Detwiler et al., 2012); the fellodistomid *Fellodistomum fellis* (Olsson, 1868) was used as an outgroup (Olson et al., 2003) (accession numbers in Fig. 7). The Notocotylidae was strongly supported in the analysis but the relationships among genera within this family were poorly resolved. The newly-generated sequences exhibited close relationship in a well-supported clade within the Notocotylidae but did not associate with other representatives of *Notocotylus* Diesing, 1839, *Quinqueserialis* Barker & Laughlin, 1911 or *Catatropis* Odhner, 1905. The four *cox*1 sequences generated (646 bp) for Notocotylidae gen. sp. 1 NZ represented different haplotypes: two from isolates from Lower Portobello Bay and one each from isolates sampled at Kaikoura and Paihia. The intraspecific difference between the isolates of Notocotylidae gen. sp. 1 NZ ranged between 0.2 and 1.1% thus confirming their conspecificity whereas the *cox*1 sequence divergence between Notocotylidae gen. sp. 1 NZ and Notocotylidae gen. sp. 2 NZ was 5.7–6.2%. These data provide additional molecular evidence for their distinct species status.

Remarks

The monostome triocellate cercaria, with densely packed cystogenous gland-cells, main excretory collecting ducts united just posterior to oral sucker and two indistinct posterior-lateral glands opening dorsally, is also characterised by the absence of prepharynx and pharynx, the presence of a short oesophagus and long caeca reaching to posterior extremity of body. These features are consistent with the morphology of cercariae of the family Notocotylidae (see Yamaguti, 1975). Furthermore, few mature cercariae were observed in the rediae thus suggesting that final maturation occurs outside the redia. The microvilli-like projections of the tegument in the present cercaria suggests similarities with *Paramonostomum philippinense* Velasquez, 1969, *P. caeci* Smith & Hickman, 1983, *P. bursae* Smith & Hickman, 1983 and *Catatropis johnstoni* Martin, 1956, the only notocotylid species described as having “spined” tegument (Martin, 1956;

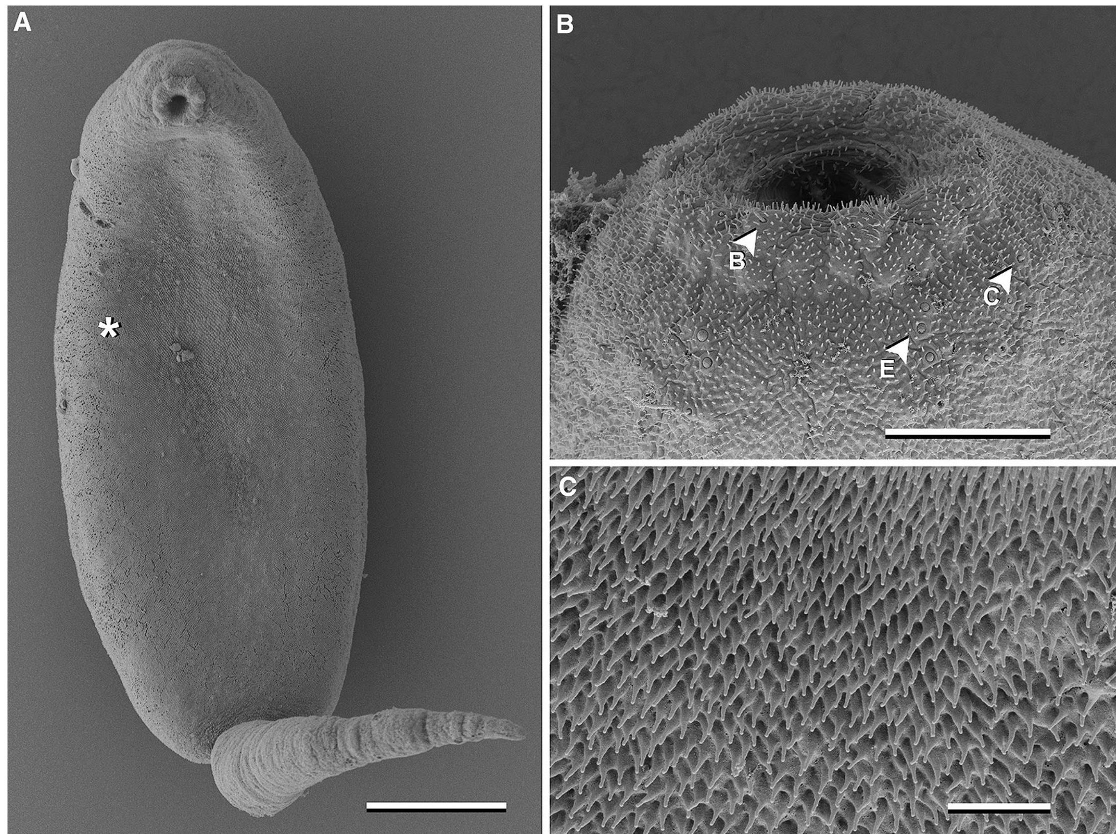


**Fig. 6** Notocotylidae gen. sp. 1 NZ, scanning electron micrographs. A, Cercaria, ventral view (* indicates the area of the tegument detailed in C); B, Detail of the cephalic region with three types of sensory papillae (B, C and E) indicated by arrowheads; C, Detail of tegumental surface. *Scale-bars*: A, 50 µm; B, 10 µm; C, 5 µm

Velasquez, 1969; Smith & Hickman, 1983); it is possible that "spines" represent similar structures in these species (as indicated by Smith & Hickman, 1983: "tegument is speckled with minute papillae or spines") but this would require a SEM examination.

A number of notocotylid records in a range of birds in New Zealand exist (summarised by McKenna et al., 2010 and Bisset, 1977): *Catatropis* sp. ex *Tadorna variegata* Gmelin, *Anas superciliosa superciliosa* Gmelin; *A. platyrhynchos platyrhynchos* L.; *A. rhynchotis variegata* Gould, *Himantopus novaezelandiae* Gould and *H. himantopus* L.; *Notocotylus attenuatus* Rudolphi, 1809 ex *T. variegata* and *Branta canadensis* L.; *Notocotylus tadornae* Bisset, 1977 ex *T. variegata* and *Anser anser* L.; *Notocotylus* sp. ex *H. finschi* and *H. unicolor*; and *Uniserialis gippyensis* Beverley-Burton, 1958 ex *T. variegata*, *A. superciliosa superciliosa*, *A. rhynchotis variegata*, *B. canadensis* and *A. anser*. Considering the result of the phylogenetic analysis and existing records of species with predominantly fresh water life-cycles from New Zealand, it is currently impossible to suggest a generic affiliation for the notocotylid cercariae described here.

### Notocotylidae gen. sp. 2 NZ

*First intermediate host*: *Austrolittorina antipodum* Philippi (Gastropoda: Littorinidae).
*Locality*: Kaikoura (New Zealand).
*Prevalence*: *A. antipodum*: 0.002% (n = 560).
*Voucher material*: None.
*Representative sequences*: KJ868214 (28S rDNA); KJ868202 (*cox*1)

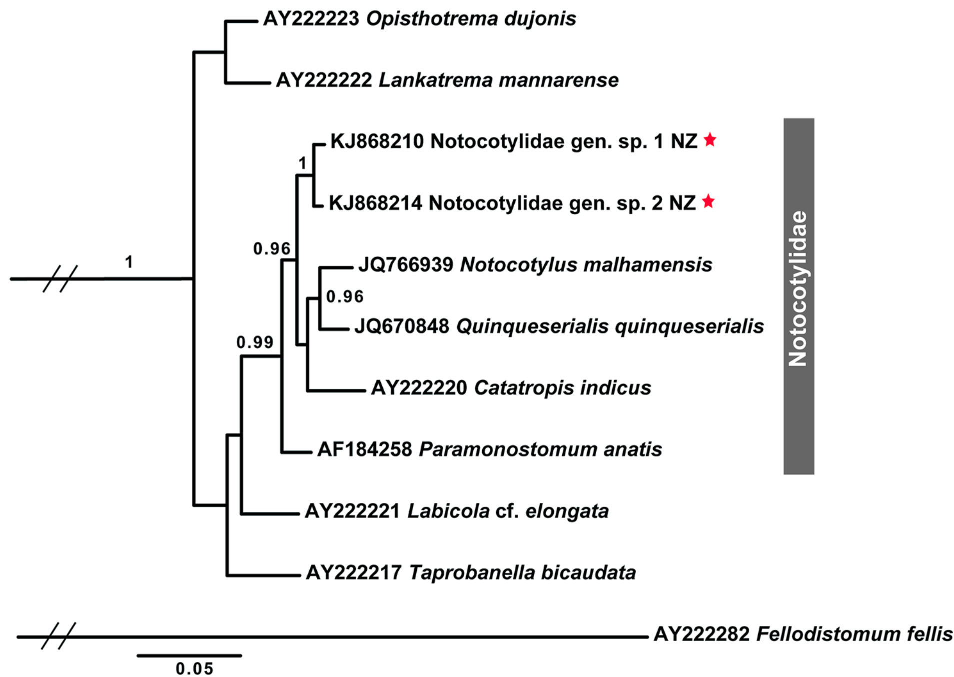


**Fig. 7** Phylogenetic relationships inferred by Bayesian analysis based on 28S rDNA sequence data for representatives of the superfamily Pronocephaloidea. The newly-generated sequences are indicated by stars. Only posterior probabilities >0.95 are shown; the scale-bar indicates the expected number of substitutions per site

Remark

A second notocotylid genotype was detected in a sample collected at Kaikoura. However, no morphological data were obtained for cercariae of this isolate since the infection was prepatent. The 28S rDNA sequence for this isolate comprised 940 bp and differed by seven base pairs from the isolate Notocotylidae gen. sp. 1 NZ. We, therefore, consider that this isolate represents a second species of notocotylid.

**Superfamily Microphalloidea Ward, 1901**
**Family Renicolidae Dollfus, 1939**

### *Renicola* sp. NZ

*First intermediate host*: *Austrolittorina antipodum* Philippi (Gastropoda: Littorinidae).
*Locality*: Paihia, New Zealand.
Prevalence: 1.56% (n = 257).
*Voucher material*: HCIP D-703.
*Representative sequences*: KJ868215 (28S rDNA); KJ868205 (*cox*1).

Description (Figs. 3C, 8C, D, 9)

*Sporocyst*
[Measurements based on 12 live specimens.] Sporocysts (Fig. 8D) greyish, elongate-oval, small, 256–627 × 150–277 (397 × 198), containing 1–5 cercariae.

*Cercaria*
[Measurements based on 13 live specimens; not all specimens contributed a data point to all metrical variables.] Distome leptocercous xiphidiocercaria (Fig. 8C). Body elongate-oval, 205–264 × 77–101 (240 × 86). Forebody long, 95–128 (114), 46–56 (49)% of body length. Tegument armed with simple sharp spines; spines dense on ventral surface anteriorly, less abundant posteriorly and dorsally; oral sucker opening surrounded by narrow area devoid of spines (Fig. 9A, C, D). Tail simple, with smooth tegument, 150–207 (166) long, 16–24 (19) wide at

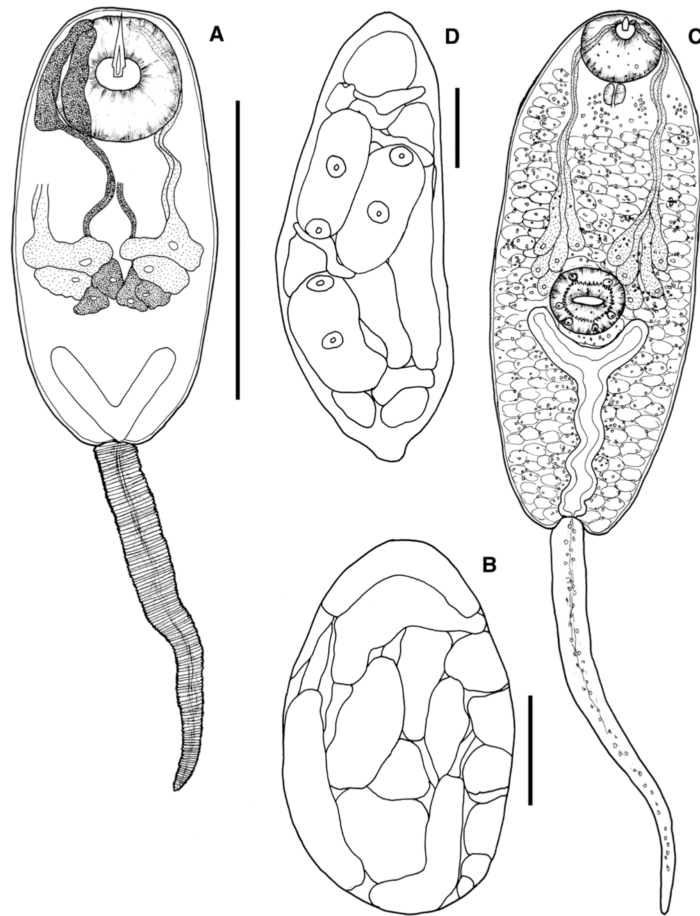


**Fig. 8** Microphalloidean larval stages ex *Austrolittorina cincta* (A, B) and *A. antipodum* (C, D). A, B, Cercaria and sporocyst of *Microphallus* sp. NZ; C, D, Cercaria and sporocyst of *Renicola* sp. NZ. *Scale-bars*: 100 µm

base, shorter than body, 61–81 (70)% of body length.

Oral sucker subterminal, muscular, subglobular, 33–40 × 29–37 (37 × 33), armed with single row of 29–30 spines, surrounded by smooth tegument bearing numerous sensory receptors (see below). Stylet simple, small, 10–12 × 1 (n = 2), dorsal to mouth opening, terminates in a single point. Ventral sucker subspherical, just post-equatorial, 30–36 × 26–36 (33 × 32), armed with single row of 41–46 spines, with outer circle of 6 large non-ciliated papillae (2 anterior and 4 posterior) and inner circle of 6 small dome-shaped ciliated papillae (4 anterior and 2 posterior) (Fig. 9B). Sucker length ratio 1:0.81–1.00 (0.90), width ratio 1:0.76–1.13 (0.98). Prepharynx absent; pharynx very small, spherical, 12 × 12 (n = 1); intestine and caeca obscured by cystogenous cells.

Cystogenous gland-cells numerous throughout body. Penetration gland-cells 5 pairs, antero-lateral to ventral sucker, with inconspicuous ducts opening in two groups on either side of stylet (Fig. 9D). Numerous small, refractive granula scattered throughout body, aggregated in places. Anlagen of reproductive organs just anterior to ventral sucker.

Excretory vesicle thick-walled, Y-shaped, stem undulating, longer than arms, the latter reaching to mid-level of ventral sucker. Flame-cell formula not determined (flame-cells obscured by cystogenous gland-cells).

Sensory receptors. Two types of sensory receptors, all lacking tegumentary collar or tegumentary sheath,

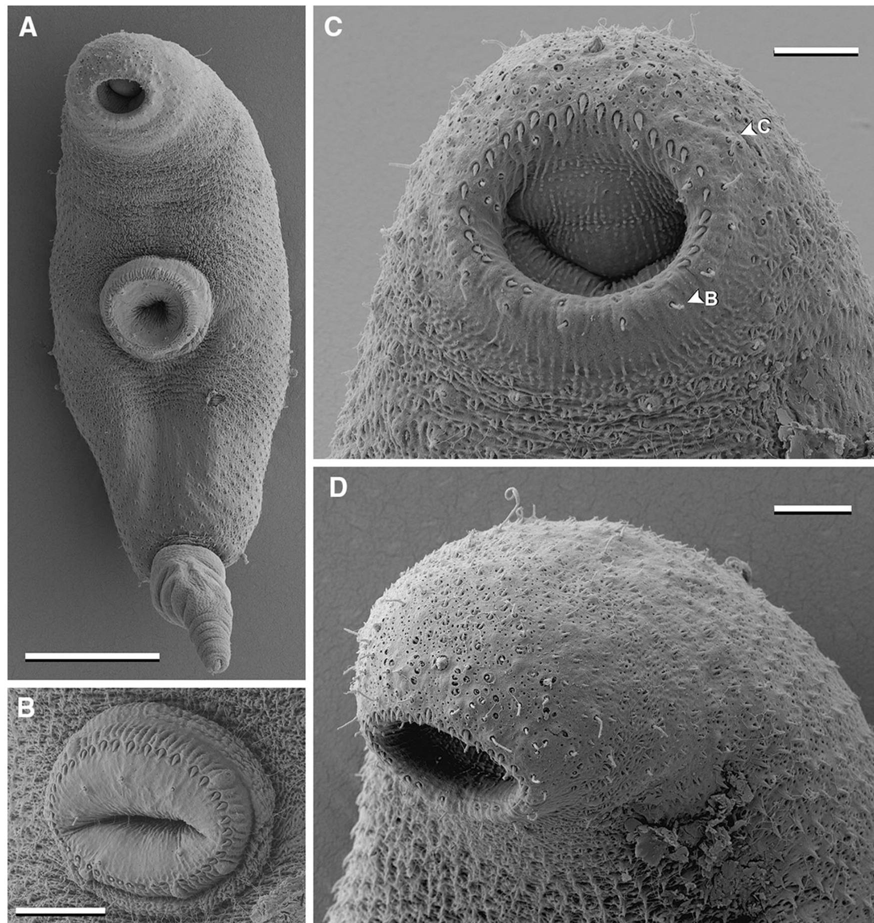


**Fig. 9** *Renicola* sp. NZ, scanning electron micrographs. A, Cercaria, ventral view; B, Detail of ventral sucker; C, Detail of cephalic region with two types of sensory papillae (B and C) indicated by arrowheads; D, Detail of cephalic region, dorsoventral view. *Scale-bars*: A, 30 µm; B, 10 µm; C, 5 µm; D, 10 µm

were observed in the cephalic region under SEM: (i) large and medium-sized single papilla-like receptors with dome-like base, each with single long cilium (type B, Fig. 9C, D); (ii) small dome-like receptors each with single short cilium (type C, Fig. 9C, D). Three cephalic ($C_I$, $C_{II}$ and $C_{III}$), two ventral sucker (S), four anterior and two posterior body circles were identified from SEM reconstructions. A total of 66 sensory receptors were found on the tegument in the cephalic region; six non-ciliated and six ciliated papillae (type C) were located on the ventral sucker (Fig. 9B), and 32 sensory papillae were observed on body surface. The chaetotaxy is summarised as follows (see also Fig. 3C for schematic representation of the papillae in the cephalic region):

Circle $C_I$: $4C_IL + 1C_ID$

Circle $C_{II}$: $1C_{II}V_1 + 1C_{II}V_2 + 1C_{II}L_1 + 1C_{II}L_2 + 1C_{II}L_3 + (1 + 5)\ C_{II}D$

Circle $C_{III}$: $1C_{III}V_1 + 2C_{III}V_2 + 3C_{III}L_1 + 2C_{III}L_2 + (5 + 1)\ C_{III}L_3 + 3C_{III}D$

Circle $A_I$: $1A_IV + 1A_IL + 1A_ID$

Circle $A_{II}$: $1A_{II}V + 1A_{II}L + 1A_{II}D$

Circle $A_{III}$: $1A_{III}V + 1A_{III}L + 1A_{III}D$

Circle $A_{IV}$: $1A_{IV}V + 1A_{IV}L$

Circle S: $6S_I + 6S_{II}$

Circle $P_I$: $1P_IV + 2P_IL + 1P_ID$

Circle $P_{II}$: $1P_{II}V + 2P_{II}L$

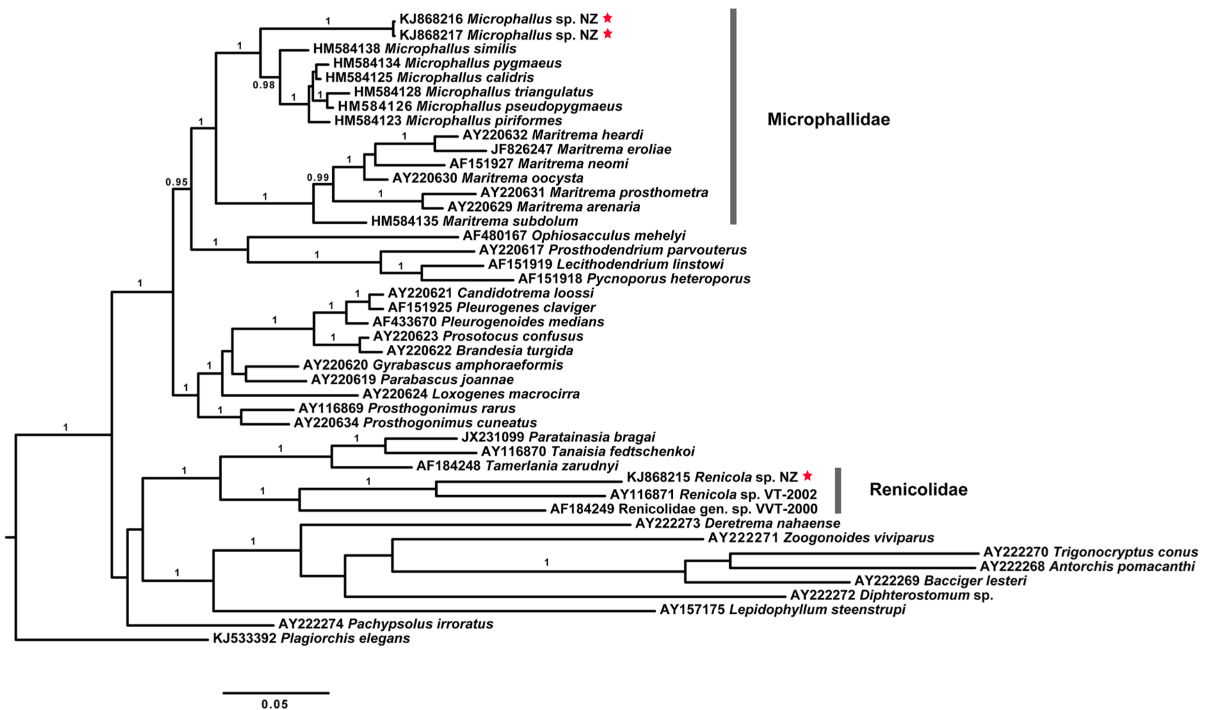


**Fig. 10** Phylogenetic relationships inferred by Bayesian analysis based on 28S rDNA sequence data for representatives of the superfamily Microphalloidea. The newly-generated sequences are indicated by stars. Only posterior probabilities > 0.95 are shown; the scale-bar indicates the expected number of substitutions per site

### Molecular analysis

One 28S rDNA sequence for cercariae of *Renicola* sp. NZ was incorporated in an alignment (1,180 bp) together with representatives of the superfamily Microphalloidea comprising two sequences for cercariae identified as *Microphallus* sp. NZ (see below) and 40 sequences for species of 29 genera from nine families, available on GenBank (Tkach et al., 2000, 2001, 2003; Lockyer et al., 2003; Olson et al., 2003; Al-Kandari et al., 2011; Galaktionov et al., 2012; Unwin et al., 2013), plus a sequence for the plagiorchiid *Plagiorchis elegans* Rudolphi, 1802 as an outgroup (Zikmundová et al., 2014) (accession numbers in Fig. 10). Support for almost all families was high in the phylogenetic hypothesis depicted for the Microphalloidea. The newly-generated renicolid sequence exhibited a well-supported affiliation with *Renicola* sp. VT-2002 ex *Numenius arquata* L. from the Ukraine (AY116871; Olson et al., 2003) within the Renicolidae. Only one *cox*1 sequence (679 bp) was obtained for *Renicola* sp. described above; the divergence between this isolate and the isolates ex *Z. subcarinatus* described by Martorelli et al. (2008) and sequenced by Leung et al. (2009), ranged between 16.9 and 17.2% indicating the distinct status of the two renicolids from New Zealand.

### Remarks

The morphology of the present cercaria agrees well with the diagnosis of renicolid cercariae by Hechinger & Miura (2014) based on the following features: spined tegument and ventral sucker, eye-spots and prepharynx absent, cystogenous gland-cells conspicuous, abundant, mesostomate excretory system with epithelial Y-shaped excretory vesicle. Renicolid cercariae represent a heterogeneous assemblage represented by the "rhodometopa" group of cercariae with large finned tails, exemplified by *Cercaria rhodometopa* Pérez, 1924 and a group with non-finned tails. The latter is further subdivided into a sub-group of cercariae with large tails lacking stylets, exemplified by *Cercaria buchanani* Martin & Gregory, 1951 and a sub-group of cercariae with small tails with or without stylets, exemplified by *Renicola*

*roscovita* (Stunkard, 1932) (see Martin, 1971). The cercaria of *Renicola* sp. NZ belongs to the latter subgroup.

The present cercaria exhibits similarities with the cercaria of *Renicola* sp. described from *Z. subcarinatus* by Martorelli et al. (2008) in having spined tegument, short tail, Y-shaped excretory vesicle with arms reaching to mid-level of ventral sucker. However, it differs in the number (5 *vs* 6 pairs) and the more posterior location of the penetration gland-cells, as well as in the shape (anterior thickening absent *vs* present) and the size (10–12 × 1 *vs* 10–14 × 4–6 µm) of the stylet. Detailed SEM examination of the cercariae revealed a specific armament of the oral and ventral suckers and a complex chaetotaxy pattern with two new types of sensory receptors; this adds a range of new characters that, in association with sequence data, may be useful in characterisation of renicolid larval stages in *Austrolittorina* spp. There is a single record of an unidentified *Renicola* sp. from New Zealand, recovered from *Phalacrocorax punctatus punctatus* (Sparrman), a species associated with the coastal environment, including the Otago coastline (Szabo, 2013).

## Family Microphallidae Ward, 1901

### *Microphallus* sp. NZ

*First intermediate host*: *Austrolittorina cincta* Quoy & Gaimard (Gastropoda: Littorinidae).
*Locality*: Weller's Rock, Otago Harbour, Dunedin (New Zealand).
*Prevalence*: 0.32% (n = 312).
*Voucher material*: HCIP D-702.
*Representative sequences*: KJ868216–KJ868217 (28S rDNA); KJ868203–KJ868204 (*cox*1).

#### Description (Figs. 8A, B)

*Sporocyst*
[Measurements based on 16 live specimens.] Sporocysts (Fig. 8B) oval, yellowish, contain 5–9 (7) cercariae, 133–385 × 101–169 (216 × 123).

*Cercaria*
[Measurements based on 6 live specimens under slight coverslip pressure, not all specimens contributed a data point to all metrical variables.] Monostome anenteric, leptocercous xiphidiocercaria (Fig. 8A). Body small, elongate-oval, 140–177 × 36–64 (156 × 54). Tegument covered with minute spines. Tail simple, 94–132 (115) long, 13–18 (16) wide at base, shorter than body [TL/BL = 53–88 (74)%], with fine tegumental annulations and pointed tip.

Oral sucker subterminal, muscular, subspherical, 31–38 × 27–34 (34 × 32). Stylet simple, sharply-pointed, 19–21 (20) long, 2–3 (2) wide at base, with long triangular tip comprising *c.*2/3 of length and rectangular base in ventral view; widest point 3–4 (3) wide (Fig. 8A). No anlagen of ventral sucker observed.

Penetration gland-cells 4 pairs: 2 anterior pairs of larger gland-cells with narrow undulating lateral ducts opening close to stylet tip; 2 posterior pairs of smaller gland-cells with medial ducts, widely expanding at level of oral sucker (staining intensely with Neutral Red), opening on its antero-lateral margin.

Excretory vesicle V-shaped. Other details of excretory system not observed; flame-cell formula not determined.

#### Molecular analysis

The newly-generated sequences for *Microphallus* sp. (1,315 bp) clustered with strong support together with representatives of six species of the genus *Microphallus* Ward, 1901 within the family Microphallidae (Fig. 10). The second strongly supported clade within the family represented five species of *Maritrema.* Two *cox*1 haplotypes of *Microphallus* sp. NZ (656 bp) were identified from two locations in Otago Harbour with an intraspecific difference of 0.2%. The availability of *cox*1 sequences for other microphallids sampled in New Zealand allowed a comparison with the newly-generated sequences (Fig. 11). These formed a monophyletic lineage associated with strong support with sequences for *Microphallus* sp. from the mudsnail *Z. subcarinatus* and "crabs", described as a "larval stage of

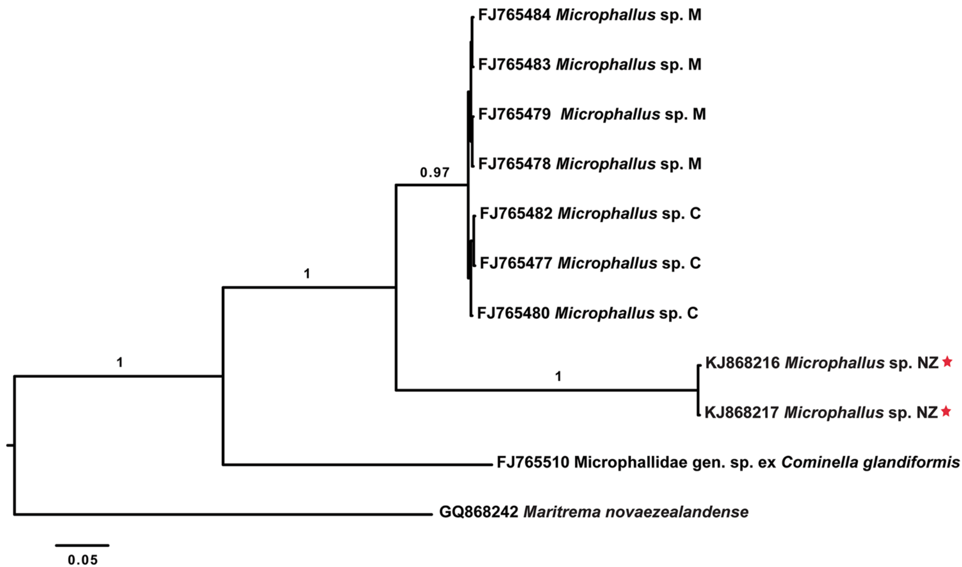


**Fig. 11** Phylogenetic relationships of microphallid larval stages from New Zealand inferred by Bayesian analysis based on *cox*1 sequence data. The newly-generated sequences are indicated by stars. Only posterior probabilities > 0.95 are shown; the scale-bar indicates the expected number of substitutions per site. *Abbreviations*: C, cercaria; M, metacercaria

*Microphallus* sp. or *Megallophalus* sp.” by Martorelli et al. (2008) and sequenced by Leung et al. (2009). The sequence divergence between the isolates of the two lineages of *Microphallus* from New Zealand ranged between 20.1–20.5% thus confirming a specific distinction.

Remarks

The monostome, anenteric, leptocercous xiphidiocercaria described above clearly belongs to a species of the Microphallidae (see Deblock, 2008). This form further exhibits features typical for cercariae of the species of *Microphallus* Ward, 1901 i.e. two pairs of large penetration gland-cells with narrow ducts opening just next to the stylet tip and two pairs of smaller, more difficult to observe penetration gland-cells with ducts conspicuously enlarged at the level of oral sucker (their content staining intensely with Neutral Red) and opening laterally to stylet (see Deblock, 1980).

The only marine cercaria of a species of *Microphallus* described from New Zealand is that recorded from *Z. subcarinatus* by Martorelli et al. (2008) under the names “*Microphallus* sp. or *Megalophallus* sp.”. The dubious identification of this material is associated with the similarities in the shape and location of the penetration gland-cells with *Microphallus claviformis* (Brandes, 1888) and *Megalophallus carcini* Prévot & Deblock, 1970 as described by Deblock & Rose (1965) and Prévot (1972), respectively. No comparison of the metrical data is possible since we do not possess sample of fixed cercariae but the cercaria of *Microphallus* sp. NZ clearly differs from the cercaria described by Martorelli et al. (2008) in having a much longer and thinner stylet (19–21 × 2–3 *vs* 9–13 × 3–4 µm) that also exhibits a different shape. Further, the enlarged ducts in the cercaria described here are clearly connected with the two posterior pairs of small penetration gland-cells and do not represent “very short anterior pairs of cephalic glands” restricted only to the region of oral sucker. It is worth noting that Galaktionov & Skirnísson (2000) in their description of *Cercaria littorina saxatilis* VII Newell, 1986, considered morphologically similar with the form described by Martorelli et al. (2008), indicated that two pairs of penetration gland-cells degenerate by the final stages of larval morphogenesis within the sporocysts so that only their enlarged ducts were visible at the level of oral sucker in fully formed cercariae. It is possible that a similar process

may have resulted in the morphology observed by Martorelli et al. (2008).

Finally, the phylogenetic analyses clearly indicated the close relationships of *Microphallus* sp. NZ and other *Microphallus* spp. (28S rDNA data) and supported its distinct species status relative to the forms sequenced from New Zealand including "*Microphallus* sp. or *Megalophallus* sp." of Martorelli et al. (2008). Obtaining 28S rDNA sequences from the latter species would be essential for clarifying its generic affiliation. Because of the few diagnostic characters of the microphallid cercariae the use of molecular methods for identification is indispensable. Regarding the possible definitive hosts of *Microphallus* sp. NZ, only unidentified *Microphallus* spp. (also as *Spelotrema* sp.) have been reported from *Anas chlorotis* Grey (as *A. aucklandica chlorotis*) and "wild duck" in New Zealand (McKenna et al., 2010). It is likely that shore birds feeding upon small crustaceans in the high intertidal zone may represent the definitive hosts of the species reported here.

## Concluding remark

In conclusion, this study provides descriptions of the cercariae and intramolluscan stages of four digenean parasites of New Zealand littorinid snails. The observation of five distinct species in the relatively geographically confined sample highlights, for the first time, the potential parasite diversity of this group in the Southern Hemisphere. The provision of both morphological and molecular data enables future studies to compare and contrast their findings with the parasites found here, hence enabling better distinctions of parasite taxonomy and phylogeny. Although the gastropod hosts studied inhabit the harsh upper intertidal zone, they remain an important transmission step in the life-cycles of these digeneans, all of which require a bird definitive host.

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