Morphological description and molecular analyses of *Tylodelphys* sp. (Trematoda: Diplostomidae) newly recorded from the freshwater fish *Gobiomorphus cotidianus* (common bully) in New Zealand

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Abstract

Among eyeflukes, *Tylodelphys* Diesing, 1850 includes diverse species able to infect the eyes, but also the brain, pericardial sac and body cavity of their second intermediate host. While the genus shows a cosmopolitan distribution with 29 nominal species in Africa, Asia, Europe and America, a likely lower research effort has produced two records only for all of Australasia. This study provides the first description of a species of *Tylodelphys* and the first record for a member of the Diplostomidae in New Zealand. *Tylodelphys* sp. metacercaria from the eyes of *Gobiomorphus cotidianus* McDowall, 1975 is distinguished from its congeners as being larger in all, or nearly all, metrics than *Tylodelphys clavata* (von Nordmann, 1832), *T. conifera* (Mehlis, 1846) and *T. scheuringi* (Hughes, 1929); whereas *T. podicipina* Kozicka & Niewiadomska, 1960 is larger in body size, ventral sucker and holdfast sizes and *T. ophthalmi* (Pandey, 1970) has comparatively a very small pharynx and body spination. *Tylodelphys* sp. exhibits consistent genetic variation for the 28S rDNA, internal transcribed spacer (ITS) and *Cox*1 genes, and phylogenetic analyses confirm that it represents an independent lineage, closely related to North American species. Morphological and molecular results together support the distinct species status of *Tylodelphys* sp. metacercaria, the formal description and naming of which await discovery of the adult. Furthermore, the validity of *T. strigicola* Odening, 1962 is restored, *T. cerebralis* Chakrabarti, 1968 is proposed as major synonym of *T. ophthalmi*, and species described solely on the basis of metacercariae are considered *incertae sedis*, except those for which molecular data already exist.

Introduction

Digeneans parasitizing the eyes of fish, known as eyeflukes, are metacercaria larval stages of species belonging to several genera of the Diplostomoidea Poirier, 1886, including *Tylodelphys* Diesing, 1850. However, *Tylodelphys* species can also infect the brain (or cranial cavity), the pericardial sac or the body cavity of their second intermediate hosts, which are typically fish, but sometimes amphibians. Like other diplostomids, *Tylodelphys* spp. have a complex three-host life cycle, with a variety of fish- and amphibian-eating birds such as ciconiiforms, suliforms, falconiforms and podicipediforms acting as definitive hosts, and lymnaeid or planorbid gastropods as first intermediate hosts (e.g. Dubois, 1970; Niewiadomska & Laskowski, 2002). *Tylodelphys* has a cosmopolitan distribution, with four nominal species in Africa (*T. aegyptius*...

The genus Tylodelphys was erected by Diesing (1850) for the metacercarial stages of two species, T. clavata (as original combination Diplastomum clavatum von Nordmann, 1832) and T. excavata (as original combination D. rhachiacont Henle, 1833), the adults of which were later discovered by Ciurea (1928) who erected another genus (Prodiplostomum Ciurea, 1933) to accommodate these adults with distinct metacercarial stages. Dubois (1937a) reinstated the name Tylodelphys according to the ICZN Principle of Priority. Since then a total of 17 species has been described morphologically from adult specimens, and 11 solely on the basis of the metacercarial stage (table 1). Clearly, the limitations of obtaining adults from birds and the difficulty of completing the life cycles in the laboratory have hampered taxonomic progress in this group. However, recent barcoding surveys have successfully characterized molecularly the cercariae, metacercariae and/or adults of T. aztecaec, T. clavata, T. excavata, T. immer, T. jenynsiae, T. masoniense and T. scheuringi (Locke et al., 2010, 2015; Behrmann-Godel, 2013; Georgieva et al., 2013; Chibwana et al., 2015; García-Varela et al., 2015). Moreover, they have discovered three distinct lineages of unidentified Tylodelphys metacercariae from Africa (Chibwana et al., 2013; Otachi et al., 2015) and four from North America (Locke et al., 2015). Only in a couple of studies was molecular characterization of metacercariae carried out in association with evaluation of morphological variation (Chibwana & Nkwengullia (2010) in conjunction with Chibwana et al. (2013); and Otachi et al. (2015)). In both cases, the authors found that morphological variation suggested a higher number of morphospecies than genetically identified units (lineages) in their samples. Taking into account these latter molecular findings and the fact that one-third of the known Tylodelphys spp. are distinguished based on the morphology of the metacercarial stage, we can expect that ‘true’ species diversity may be very different from what is currently known.

Materials and methods

Collection and examination of fish

Ten common bullies, Gobionymorphus cotidius McDowall (Actinopterygii: Electridae), were collected using a seine net from Lake Hayes in Otago (South Island, New Zealand) in May 2011 and 2013. Fish were euthanized by spinal severance and examined fresh. Metacercariae (devoid of cysts) were extracted from the vitreous humour of the eyes, fixed in absolute ethanol for molecular analyses, or 70% ethanol for whole-mount and scanning electron microscopy (SEM) examination.

Morphological data

Worms 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. Two specimens were dehydrated in a graded ethanol series, critical point-dried and sputter-coated with gold for SEM examination using a Zeiss DSM 940A (Zeiss AG, Oberkochen, Germany) at an accelerating voltage of 5 kV. All measurements are in micrometres unless otherwise stated in the text, and are given as the range, with the mean followed by standard deviation (SD) in parentheses. Voucher material is deposited as permanent mounts, SEM preparations, genophores (two specimens preserved in 70% ethanol) and molecular vouchers (extracted gDNA) at the Platyhelminthes collection of the Natural History Museum of Geneva.

Molecular data and analyses

Genomic DNA was extracted from seven single ethanol-fixed metacercariae specimens of Tylodelphys sp. in 200 μl of a 5% suspension of Chelex® in deionized water containing 0.1 mg/ml proteinase K, followed by incubation at 56°C for 5 min, boiling at 90°C for 8 min, and centrifugation at 14,000 g for 10 min. The following gene regions were amplified: partial fragment of the large ribosomal subunit (28S rDNA) [1800 bp]; primers U178F: 5′–GCA CCC GCT GAA YTT AAG–3′ and L1642R: 5′– CCA GGG CCA TTC ATT TTC A–3′ (Lockyer et al., 2003), the ITS1–5.8S–ITS2 ribosomal gene cluster (ITS) [900 bp]; primers D1: 5′–AGG ATT CCG TGG TAA GTG CAA G–3′ and D2: 5′–CGT TAC TGA GGA AGT CCT.
Table 1. *Tylodelphys* species described on the basis of adult or metacercariae specimens, status of the species, known life-cycle stages, diagnostic evidence for the species, site of infection in the second intermediate hosts and main references providing morphological or molecular information. Note that the reference list and synonyms for species are not exhaustive.

<table>
<thead>
<tr>
<th>Geographic region/species or genetic lineage</th>
<th>Species status</th>
<th>Developmental stages known</th>
<th>Diagnostic evidence</th>
<th>Site of infection in 2nd IH</th>
<th>Main references</th>
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<td>Fish cranial cavity</td>
<td>Nigrelli &amp; Maraventano (1944); Sudarikov (1974); Tinsley &amp; Sweeting (1974); King &amp; Van As (1997)</td>
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<td>Morphology/ molecular†</td>
<td>Fish cranial cavity</td>
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<td>Morphology/ molecular†</td>
<td>Fish cranial cavity</td>
<td>Chibwana &amp; Nkwengulila (2010); Chibwana et al. (2013); Chibwana &amp; Nkwengulila (2015)</td>
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<td>Otachi et al. (2015)</td>
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<td>Asia</td>
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<td>Morphology/ molecular†</td>
<td>Fish eye vitreous humour</td>
<td>Herein</td>
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<td>Europe</td>
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<td>Valid A, M, C</td>
<td>Morphology/ molecular†</td>
<td>Fish eye vitreous humour</td>
<td>von Nordmann (1832); Diesing (1850); Ciurea (1928); Dubois (1938); Bezuk (1956b); Dubois &amp; Fain (1956); Furmaga (1957); Kozicka &amp; Niewiadomska (1960a); Odening (1962); Niewiadomska (1963a); Faltykova et al. (2007); Behrmann-Godel (2013); Georgieva et al. (2013); Locke et al. (2015)</td>
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<td>Main references</td>
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<td><em>T. conifera</em> (Mehlis, 1846) syn. <em>Holostomum coniferum</em> Mehlis, 1846 in Creplin (1846); <em>Cercaria letifera</em> Fuhrmann, 1916; <em>Diplostomum gavium</em> (Guberlet, 1922) of Macko (1961)</td>
<td>Valid</td>
<td>A, M, C</td>
<td>Morphology</td>
<td>Fish eye vitreous humour</td>
<td>Fuhrmann (1916); Dubois (1937a, 1938); Bychovskaja-Pavlovskaja (1953); Sudarikov (1960); Macko (1961); Bychovskaja-Pavlovskaja (1962); Dubois (1964)</td>
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<td><em>T. craniaria</em> (Diesing, 1858)</td>
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<td>M</td>
<td>Morphology</td>
<td>Fish brain cerebrospinal fluid</td>
<td>Diesing (1858); Kozicka &amp; Niewiadomska (1960a)</td>
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<td><em>T. excavata</em> (Rudolphi, 1803)</td>
<td>Valid</td>
<td>A, M, C</td>
<td>Morphology/ molecular</td>
<td>Amphibian spinal cord</td>
<td>Diesing (1850); Ciurea (1933); Szidat (1935); Dubois (1938); Bezubik (1956b); Furmaga (1957); Niewiadomska (1960, 1963a); Odening (1970); Barsiene (1991); Faltynkova et al. (2008); Chibwana et al. (2013)</td>
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<td>A</td>
<td>Morphology</td>
<td></td>
<td>Dubois (1927, 1931); Kozicka &amp; Niewiadomska (1960b)</td>
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<td><em>T. podicipina</em> (Kozicka &amp; Niewiadomska, 1960)</td>
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<td>A, M</td>
<td>Morphology</td>
<td>Fish eye vitreous humour</td>
<td>Bezubik (1956a); Kozicka &amp; Niewiadomska (1960b); Dubois (1964); Odening &amp; Bockhardt (1961); Odening (1962)</td>
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<td><em>T. strigicola</em> Odening, 1962</td>
<td>Valid</td>
<td>A</td>
<td>Morphology</td>
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<td>Odening &amp; Bockhardt (1961); Odening (1962)</td>
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<td><em>T. pseudopectinosa</em> Dubois, 1969</td>
<td>Valid</td>
<td>A, M</td>
<td>Morphology</td>
<td>Fish eye vitreous humour</td>
<td>Hughes (1929); Van Cleave &amp; Mueller (1934); Locke et al. (2015)</td>
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<td><em>T. scheurigi</em> (Hughes, 1929) syn. <em>Diplostomum scheurigi</em> Hughes, 1929</td>
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<td>Morphology/ molecular</td>
<td>Fish eye humours</td>
<td>Locke et al. (2015)</td>
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<td>Molecular</td>
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<td>Locke et al. (2015)</td>
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<td>Molecular</td>
<td>Fish brain</td>
<td>Locke et al. (2015)</td>
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<td>Locke et al. (2015)</td>
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<td><em>Tylodelphys</em> sp. 6</td>
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<td>Molecular</td>
<td>Fish body cavity</td>
<td>Locke et al. (2015)</td>
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<td>South America</td>
<td><em>T. adulta</em> Lunaschi &amp; Drago, 2004</td>
<td>Valid</td>
<td>A</td>
<td>Morphology</td>
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<td><em>T. americanus</em> (Dubois, 1936) syn. <em>Prodiplostomum americanum</em> (Dubois, 1936); <em>T. elongata</em> of Caballero and Vogelsang (1949)</td>
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<td>A</td>
<td>Morphology</td>
<td></td>
<td>Dubois (1936, 1937b, 1938)</td>
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Table 1. (Cont.)

<table>
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<tr>
<th>Species</th>
<th>Main references</th>
<th>Developmental stages known</th>
<th>Diagnostic evidence</th>
<th>Site of infection in 2nd H</th>
<th>Morphology of infection site</th>
<th>Morphological information provided in association with molecular data, or morphological vouchers of the molecular samples deposited in Museum or University collections.</th>
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<td>T. argentinus</td>
<td>Quaggiotto &amp; Valverde, 1992</td>
<td>Incertae sedis</td>
<td>Morphology</td>
<td>Fish brain</td>
<td>Quaggiotto &amp; Valverde (1992); Flores (1997); Flores &amp; Baccalá (1998)</td>
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<td>T. barilochensis</td>
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<td>Incertae sedis</td>
<td>Morphology</td>
<td>Fish brain</td>
<td>Quaggiotto &amp; Valverde (1992); Flores (1997); Flores &amp; Baccalá (1998)</td>
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<td>T. brevis</td>
<td>Drago &amp; Lunaschi, 2008</td>
<td>Valid</td>
<td>Morphology</td>
<td>Fish pericardial cavity</td>
<td>Drago &amp; Lunaschi (2008); Drago et al. (2014)</td>
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<td>Morphology</td>
<td>Fish pericardial cavity</td>
<td>Fish pericardial cavity</td>
<td>Szidat (1969)</td>
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<td>T. crubensis</td>
<td>Quaggiotto &amp; Valverde, 1992</td>
<td>Incertae sedis</td>
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<td>Fish brain</td>
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<tr>
<td>T. destructor</td>
<td>Szidat &amp; Nani, 1951</td>
<td>Valid</td>
<td>Morphology</td>
<td>Fish body cavity</td>
<td>Szidat &amp; Nani (1951); Szidat (1969)</td>
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</tbody>
</table>

Abbreviations: A, adult; M, metacercaria; C, cercaria; IH, intermediate host.

Morphological information provided in association with molecular data, or morphological vouchers of the molecular samples deposited in Museum or University collections. GGT–3′ (Galazzo et al., 2002) and partial fragment of the mitochondrial cytochrome c oxidase subunit I gene (cox1) [600 bp; primers Plat-sexCOX1F: 5′–CGT GAT TAT ACG GAT CC–3′ and Plat-sexCOX1R: 5′–AGC ATA GTA ATM GCA GCA GC–3′ (Moszcynska et al., 2009)]. Polymerase chain reaction (PCR) amplifications were performed in 25 μl reactions containing 2.5 μl of extraction supernatant, 1× PCR buffer (16 mM (NH₄)₂SO₄, 67 mM Tris–HCl at pH 8.8), 2 μM MgCl₂, 200 μM of each deoxyribonucleoside triphosphate (dNTP), 0.5 μM each primer and 0.7 unit BIOTAQ™ DNA polymerase (Bioline (Aus) Pty Ltd, Alexandria, Australia). Thermocycling conditions used for amplification of the rDNA regions follow Blasco-Costa et al. (2009) for the 28S fragment and Chibwana et al. (2013) for the ITS region. Thermocycling conditions for the Cox1 fragment were as follows: initial denaturation at 95°C for 2 min followed by 40 cycles with denaturation at 94°C for 40 s, annealing at 50°C for 30 s and extension at 72°C for 45 s; with a final extension step at 72°C for 5 min. PCR amplicons were purified prior to sequencing using exonuclease I and shrimp alkaline phosphatase enzymes (Werle et al., 1994). Amplicons were cycle-sequenced from both strands, using, besides PCR primers for the 28S and Cox1 genes, an internal primer for the 28S fragment [L1200R: 5′–GCA TAG TTC ACC ATC TTT CCG–3′ (Littlewood et al., 2000)] and two other primers for the ITS fragment [BD1: 5′–GTC GTA ACA AGG TTT CCG TA–3′ and BD2: 5′–TAT GCT TAA ATT CAG CGG GT–3′ (Luton et al., 1992)]. Sequencing was performed at the commercial facility Macrogen (Seoul, Korea). Contiguous sequences were assembled and edited using Geneious® (v. 8.1 Biomatters Ltd, Auckland, New Zealand) and submitted to GenBank.

Newly generated sequences for the 28S rDNA, the ITS1–5.8S–ITS2 gene cluster and the Cox1 fragment were aligned in three independent datasets together with published sequences of identified strigeids and diplostomid species from GenBank. The Cox1 dataset was aligned using MUSCLE implemented in MEGA v. 6 (Tamura et al., 2011). The extremes of the alignments were trimmed to match the shortest sequence prior to phylogenetic analyses. The 28S and ITS datasets were aligned using MAFFT in Guidance (Sela et al., 2015); for the ITS dataset, positions in the alignment with a score below 0.93 were excluded. The 28S alignment (851 bp long) included two representative sequences of Diplostomum spp. and one of T. mahanense retrieved from GenBank (table 2). The ITS dataset (779 bp long of the ITS1–5.8S–ITS2 gene cluster) included two representative sequences of Diplostomum and eight of Tylodelphys spp. from GenBank. The Cox1 dataset (413 bp long) included two representative sequences of Diplostomum and 27 of Tylodelphys spp. Sequences of Apatemon and Australapatemon spp. belonging to the Strigeidae, sister family to the Diplostomidae, were included as outgroups in all analyses. Phylogenetic analyses were run on the Cox1 and ITS datasets individually under the maximum likelihood (ML) and Bayesian inference (BI) criteria, employing the model of nucleotide evolution GTR+Γ (estimated using jModelTest 2.1.1 (Guindon & Gascuel, 2003; Darriba et al., 2012)). ML analyses were conducted using the program RAxML v. 7.3 (Stamatakis, 2006; Stamatakis et al., 2008). All model parameters and bootstrap support values (1000 repetitions)

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Table 2. GenBank accession numbers for *Tylodelphys* species/lineages used as ingroup in the phylogenetic analyses.

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<th>Species</th>
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<td><em>Tylodelphys sp. 1 of Chibwana et al. (2013)</em></td>
<td>KT271494, KT271495</td>
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<tr>
<td><em>Tylodelphys sp. 2 of Chibwana et al. (2013)</em></td>
<td>KT271511, KT271512</td>
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<td><em>Tylodelphys sp. 3 of Locke et al. (2015)</em></td>
<td>KT271516, KT271519</td>
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<td><em>Tylodelphys sp. 4 of Locke et al. (2015)</em></td>
<td>KT271520, KT271521</td>
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<td><em>Tylodelphys sp. 5 of Locke et al. (2015)</em></td>
<td>KT271522, KT271523</td>
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<td><em>Tylodelphys sp. 6 of Locke et al. (2015)</em></td>
<td>KT271524, KT271525</td>
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<td><em>Tylodelphys sp. of Sokolov et al. (2013)</em></td>
<td>KT271526, KT271527</td>
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<tr>
<td><em>Tylodelphys sp.</em></td>
<td>KT271528, KT271529</td>
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In bold, accession codes for the newly obtained sequences for this study.

were estimated using RAxML. BI trees were constructed using MrBayes v. 3.2 (Ronquist et al., 2012), running two independent MCMC runs of four chains for 20 million generations and sampling tree topologies every 2000 generations. Burn-in periods were set to the first 2500 generations. A consensus topology and nodal support estimated as posterior probability values (Huelsenbeck et al., 2001) were calculated from the remaining trees. All MrBayes and RAxML analyses were performed on the computational resource CIPRES (Miller et al., 2010). Genetic divergences were calculated as uncorrected p-distances for each gene region/species using MEGA.

**Diplostomidae Poirier, 1886; *Tylodelphys* Diesing, 1850; *Tylodelphys* sp.**

References: *Tylodelphys* sp. in Lagrue & Poulin (2015)

**Description of metacercaria**

Based on 30 stained and mounted specimens and two specimens for SEM (figs 1, 2). Body linguiform, slightly concave ventrally, anterior end rounded, posterior end bluntly pointed, conical; length 858 – 1374 (1118 ± 167), width at level of holdfast organ 223 – 375 (259 ± 22). Pseudosuckers (lappets) either side of oral sucker, barely differentiated, only discernable on one specimen: length 143 (13% of total body length), width at widest (anterior margin) 161. Oral sucker sub-terminal, well-developed, smaller than ventral sucker (VS:OS width ratio 1:0.7); 52–65 (61 ± 5) × 41–73 (55 ± 7). Segments devoid of spines or papillae (fig. 2). Hindbody small and not well differentiated, only discernable on one specimen: length 143 (13% of total body length), width at widest (anterior margin) 161. Oral sucker sub-terminal, well-developed, smaller than ventral sucker (VS:OS width ratio 1:0.7–1.1 (1:0.9); 52–65 (61 ± 5) × 41–35–37. Ventral sucker sub-back, protrusive in lateral view; 55–78 (67 ± 7) × 51–98 (68 ± 10), and 464–756 (597 ± 80) from oral sucker. Ventral sucker situated 54–63 (59 ± 2)% length of body. Prepharynx absent. Pharynx longitudinal oval; 32–57 (43 ± 7) × 19–49 (32 ± 7). Oesophagus long; length 8–77 (46 ± 22). Intestinal caeca terminate posterior to holdfast organ. Holdfast organ strongly muscular, longitudinal oval, located midway between ventral sucker and posterior end of body; 113–220 (153 ± 26) × 63–134 (89 ± 20). Primordia of gonads directly posterior to holdfast organ within hindbody. Excretory bladder V-shaped with pore at posterior tip of body; three main excretory ducts united in region of pharynx by transverse duct about midway between oral sucker and ventral sucker. Minor ducts and flame cells not observed. Forebody of metacercaria filled with oval granular inclusions, average 10 × 5 in size.

**Second intermediate host:** common bully, *Gobiomorphus cotidianus* McDowall (Actinopterygii: Eleotridae).

**Site of infection in second intermediate host:** vitreous and aqueous humour of eye (see supplementary video V1).

**Locality:** Lake Hayes, South Island, New Zealand (44° 59’S, 168°48’E, freshwater, elevation 332 m).

**Prevalence in second intermediate host:** 80%.

**Mean intensity in second intermediate host:** 2.6.

**Material:** voucher specimens (30 mounted metacercariae on slides and two mounted metacercariae for SEM); paragenophores (two specimens preserved in 70% ethanol); and molecular voucher specimens (extracted gDNA of two specimens) MHNG-PLAT-92964.

**DNA sequences:** Cox1, KU588143–KU588149; 28S, KU588150–KU588151; ITS1, KU588152–KU588153; ITS1–5.8S–ITS2, KU588154–KU588158

**Remarks**

The above-described metacercaria conforms to the description of the ‘diplostomulum’ type of metacercaria according to Niewadowska (2002), and it resembles all described species of *Tylodelphys* metacercariae. Of the 30 or so species of *Tylodelphys*, 18 have been described at the metacercarial stage (table 1). Of these, *T. argentinus*, *T. barilocheensis*, *T. cardiophilus*, *T. cerebralis*, *T. craniaria*, *T. crubensis*, *T. destructor*, *T. excavata*, *T. grandis*,
T. jenynsiae and T. xenopi occur in the cranial cavity, brain, spinal cord, pericardial sac or body cavity.

The remaining species, T. clavata, T. conifera, T. ophthalmi, T. podicipina and T. scheuringi, in common with the above-described species, inhabit the eye of their second intermediate fish host. Of these, T. clavata, T. conifera and T. scheuringi are smaller in all, or nearly all, metrics than the present species, and T. podicipina is larger in body size, ventral sucker and holdfast sizes (table 3). Tyodelphys ophthalmi is reported to have a comparatively very small pharynx and tegumental spination. It is likely, therefore, that the metacercaria reported here from bullies in New Zealand is a hitherto undescribed species, the full description and naming of which will be completed either experimentally, or as and when the adult is discovered. This represents the first record for a species of Tyodelphys and for a member of the Diplostomidae in New Zealand.

At the molecular level, three unique Cox1 haplotypes were retrieved from sequences of seven metacercariae of Tyodelphys sp. from New Zealand (NZ). The three haplotype sequences formed a separate reciprocally monophyletic lineage, supporting the distinct species status of these metacercariae. Phylogenetically, Tyodelphys sp. from NZ appeared closely related to species from North America, sister to T. immer in a clade together with T. scheuringi and Tyodelphys sp. 3 of Locke et al. (2015) (fig. 3a). The relationships among Tyodelphys species/lineages were generally poorly supported. Phylogenetic analyses of the ITS region included sequences for eight Tyodelphys species besides our newly obtained sequences from NZ and depicted two clades with strong support (fig. 3a). One clade included sequences representative of American species together with that of our metacercariae and one sequence of Tyodelphys sp. metacercaria of Sokolov et al. (2013) from West Siberia. The other clade comprised sequences of the European and African species/lineages.

Intraspecific genetic divergence in the Cox1 fragment varied between 0.2 and 1.2% within Tyodelphys species; whereas Tyodelphys sp. from NZ showed 0.8% intraspecific variation. No intraspecific variability was detected in the ITS or 28S sequences of the two metacercariae of Tyodelphys sp. newly sequenced. Mean interspecific genetic divergence for the Cox1 sequences of Tyodelphys spp. showed a range of variation of 8–16.5%. Cox1 sequences of Tyodelphys sp. from NZ diverged 8.6–14.2% from the sequences of other Tyodelphys species/lineages. Mean interspecific genetic divergence for the ITS sequences of Tyodelphys species/lineages ranged from 0.7 to 8.3%, whereas Tyodelphys sp. from NZ diverged between 1.1–7.7% from other Tyodelphys species. Comparison of the partial sequences for the 28S rRNA gene of our metacercariae with the only available 28S sequence of Tyodelphys in GenBank (T. mashonense, referred to as T. mashonensis in GenBank; KF189071), revealed a genetic divergence of 3.1%; while divergence between the sequences for Tyodelphys spp. and those for Diplostomum spp. was 5.3–5.9%. Molecular information altogether confirms the generic affiliation of the newly sequenced specimens to Tyodelphys and their distinct status from all Tyodelphys species and lineages molecularly characterized so far.

Note on the taxonomic status of Tyodelphys spp.

Tyodelphys ophthalmi was first described as Diplostomulum ophthalmi Pandey, 1970, although it was...
cited as Pandey (1968) (an abstract) in Pandey & Tewari (1984). However, the name *D. ophthalmi* was used prior to the description of the species in Chakrabarti (1968). Therefore, *D. ophthalmi* should have been considered a *nomen nudum*. Pandey & Tewari (1984) redescribed *T. ophthalmi* and proposed *T. cerebralis* as a junior synonym of the former, based on the similarity of its morphological features. We do not question the synonymy of the two forms; however, since *D. ophthalmi* should have been considered as *nomen nudum* and, attending to the Principle of Priority, the name *T. cerebralis* should prevail over *T. ophthalmi*, and the latter should be considered as its junior synonym (table 1). Additionally, Yamaguti (1971) considered *T. strigicola* a *nomen nudum* because it lacked a description. However, Odening (1962) proposed *T. strigicola* for the material described as *Tylodelphys* sp. by Odening & Bockhardt (1961), for which type specimens were also deposited. Thus, we consider *T. strigicola* as a valid species for the time being notwithstanding the remarks regarding its validity made by Niewiadomska (1963b).

Fig. 2. *Tylodelphys* sp. metacercaria, scanning electron micrographs. Ventral view of the metacercaria (A), detail of the oral sucker (B) and detail of the ventral sucker (C).
Up to 14 reciprocally monophyletic lineages (putative species) of *Tylodelphys* have been characterized genetically so far (Moszczynska et al., 2009; Chibwana et al., 2013; Georgieva et al., 2013; García-Varela et al., 2015; Locke et al., 2015), but only half of them have been matched to already described species on the basis of either the adult or metacercarial stage (see Table 1). Recently, García-Varela et al. (2015) proposed to consider as *incertae sedis* all South American species known only from their metacercaria form. We agree with these authors that the relationships between these forms and described species from adults cannot be established with the available morphological information. Thus, we propose to consider all named species on the sole basis of their larval metacercaria stage as *incertae sedis*. We exclude, however, those named metacercariae for which molecular data have been provided (i.e. *T. jeysniae* and *T. scheuringi*), since evaluation of their phylogenetic relationship with other named species, either adults or metacercariae, is possible. Nonetheless, the adult stages of these recognized metacercaria species await formal description upon discovery or molecular matching to a known species when such data are obtained. Similarly, unnamed species/lineages of *Tylodelphys*, with molecular data supporting their uniqueness (at the molecular level at least), should be considered valid, especially when supplemented with morphological descriptions. Notwithstanding the *incertae sedis* status, these species should be taken into account in future morphological comparisons of metacercariae in order to eventually uncover their life cycle and clarify their status. Therefore, we recognize 21 nominal species of *Tylodelphys* and eight additional genetic lineages that we consider as valid.

Almost certainly, *Tylodelphys* species diversity is very different from what we know today, given that one-third of the known *Tylodelphys* spp. are distinguished solely based on the morphology of the metacercarial larval stage. Often a higher number of morphospecies than genetically identified units (lineages) has been found when morphological variation of metacercaria forms is assessed in combination with molecular data (Chibwana & Nkwengulila, 2010; Chibwana et al., 2013; Otachi et al., 2015). Host-induced variation in the metacercariae of other diplostomids has also been documented (Niewiadomska & Szymanski, 1991, 1992). This leads us to think that the two subspecies of *T. excavata* and *T. podicipina* recognized by Dubois (1964, 1969) may not be valid; in particular *T. excavata spinnata* (Gupta, 1962), which was already suggested as a likely junior synonym of *T. excavata excavata* (Rudolphi, 1903) by Dubois (1964). We refrain from proposing a change in taxonomy until molecular sequence data are obtained from the original host and geographical region of the above subspecies. Nevertheless, we strongly recommend no further description and naming of new *Tylodelphys* species on the basis of morphological differences of the metacercarial forms alone.

Supplementary material

Video V1. Live movement of *Tylodelphys* sp. metacercariae in the vitreous humour of the eye of *Gobiomorphus costidiansus* immediately after eye removal. Observation made from a dissecting microscope.
Fig. 3. Phylogenetic relationships inferred by maximum likelihood analysis for representatives of *Tylodelphys* based on *Cox1* (A) and ITS (B) sequence data, with posterior probability values followed by bootstrap percentages above the branches (posterior probabilities <0.90 and bootstrap values <60 not reported). Species in bold were newly sequenced in this study. Abbreviations: hap, haplotype (followed by a number); (L), Locke *et al.* (2015); (C), Chibwana *et al.* (2013); (O), Otachi *et al.* (2015); and (S), Sokolov *et al.* (2013).
To view supplementary material for this article, please visit [http://dx.doi.org/10.1017/S0022149X16000298](http://dx.doi.org/10.1017/S0022149X16000298)

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**Conflict of interest**

None.

**Ethical standards**

The sampling in this paper complies with the current laws and animal ethics regulations of New Zealand.

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