Journal of Helminthology (2017) **91**, 332–345
© *Cambridge University Press 2016*

doi:10.1017/S0022149X16000298

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

I. Blasco-Costa[1]*, R. Poulin[2] and B. Presswell[2]

[1]Natural History Museum of Geneva, PO Box 6434, CH-1211 Geneva 6, Switzerland: [2]Department of Zoology, University of Otago, PO Box 56, Dunedin, New Zealand

(Received 27 January 2016; Accepted 20 April 2016; First published online 26 May 2016)

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. *Tylodephys* 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 *Tylodephys* 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, *Tylodeplphys* 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*

---

*E-mail: isa.blasco.costa@gmail.com

El-Naffar, Khalifa & Sakla, 1980, *T. grandis* Zhokov, Morozova & Tessema 2010, *T. mashonense* Beverley-Burton, 1963 and *T. xenopi* (Nigrelli & Maraventano, 1944)); seven in Asia (*T. cerebralis* (Chakrabarti, 1968), *T. chandrapali* Jain & Gupta, 1970, *T. darteri* Mehra, 1962, *T. duboisilla* Mehra, 1962, *T. excavata spinnata* (Gupta, 1962), *T. ophthalmi* (Pandey, 1970), *T. rauschi* (Singh, 1956)); six in Europe (*T. clavata* (von Nordmann 1832), *T. conifera* (Mehlis, 1846), *T. craniaria* (Diesing, 1858), *T. excavata excavata* (Rudolphi, 1803), *T. glossoides* (Dubois, 1928) and *T. podicipina podicipina* Kozicka & Niewiadomska, 1960); four in North America (*T. aztecae* Garcia-Varela *et al.*, 2015, *T. immer* Dubois, 1961, *T. podicipina robrauschi* Dubois, 1969, *T. scheuringi* (Hughes 1929)) and ten in South America (*T. adulta* Lunaschi & Drago, 2004, *T. americana* (Dubois, 1936), *T. argentinus* Quaggiotto & Valverde, 1992, *T. barilochensis* Quaggiotto & Valverde, 1992, *T. brevis* Drago & Lunaschi 2008, *T. cardiophilus* Szidat 1969, *T. crubensis* Quaggiotto & Valverde, 1992, *T. destructor* Szidat & Nani, 1951, *T. elongata* (Lutz, 1928), *T. jenynsiae* Szidat, 1969). Unexpectedly, only two records of this genus exist in the whole Australasian region, *Tylodelphys* sp. and *T. podicipina* (Dubois & Angel, 1972; Kennedy, 1995), which is most likely due to a more limited research effort rather than a distribution gap.

The genus *Tylodelphys* was erected by Diesing (1850) for the metacercarial stages of two species, *T. clavata* (as original combination *Diplostomum clavatum* von Nordmann, 1832) and *T. excavata* (as original combination *D. rhachiaeum* Henle, 1833), the adults of which were later discovered by Ciurea (1928) who erected another genus (*Prodiplostomum* Ciurea, 1933) to accommodate these adults with distinctive 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. aztecae*, *T. clavata*, *T. excavata*, *T. immer*, *T. jenynsiae*, *T. mashonense* 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 & Nkwengulila (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 maybe very different from what is currently known.

The aim of this study was to describe morphologically the first displostomid, *Tylodephys* sp. metacercaria, found in New Zealand and to distinguish it from its congeners for which the metacercarial stage is known. Additionally, we provide sequences for the 28S, internal transcribed spacer (ITS) and *Cox*1 genetic markers in order to characterize the species molecularly, distinguish it from other *Tylodelphys* genetic lineages and allow future discovery of its life cycle or new distribution areas via matching molecular sequence data. Furthermore, we discuss and provide an updated list on the taxonomic status of *Tylodephys* species. Finally, we provide video images showing the movements of *Tylodephys* sp. metacercaria within the eye of its fish host.

Materials and methods

Collection and examination of fish

Ten common bullies, *Gobiomorphus cotidianus* McDowall (Actinopterygii: Eleotridae), 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 metacercaria 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 GCG CCA TCC ATT TTC A–3′ (Lockyer et al., 2003)], the ITS1–5.8S–ITS2 ribosomal gene cluster (ITS) [900 bp; primers D1: 5′–AGG AAT TCC TGG TAA GTG CAA G–3′ and D2: 5′–CGT TAC TGA GGG AAT 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.

Geographic region/ species or genetic lineage	Species status	Developmental stages known	Diagnostic evidence	Site of infection in 2nd IH	Main references
Africa					
T. aegyptius El-Naffar, Khalifa & Sakla, 1980	Valid	A	Morphology		El-Naffar *et al.* (1980)
T. grandis Zhokov, Morozova & Tessema, 2010	*Incertae sedis*	M	Morphology	Fish cranial cavity and adipose tissue surrounding the brain	Zhokhov *et al.* (2010)
T. xenopi (Nigrelli & Maraventano, 1944)	Valid	A, M, C	Morphology	Fish pericardial cavity	Nigrelli & Maraventano (1944); Sudarikov (1974); Tinsley & Sweeting (1974); King & Van As (1997)
T. mashonense Beverley-Burton, 1963	Valid	A, M, C	Morphology/ molecular[†]	Fish cranial cavity	Beverley-Burton (1963); Mashego & Saayman (1989); Mwita & Nkwengulila (2008); Chibwana & Nkwengulila (2010); Chibwana *et al.* (2013); Moema *et al.* (2013); Mwita (2014); Chibwana & Nkwengulila (2015); Chibwana *et al.* (2015)
Tylodelphys sp. 1 (or sp. X)	Valid	M	Morphology/ molecular[†]	Fish cranial cavity	Chibwana & Nkwengulila (2010); Chibwana *et al.* (2013); Chibwana & Nkwengulila (2015)
Tylodelphys sp. 2 (or sp. Y)	Valid	M	Morphology/ molecular[†]	Fish cranial cavity	Chibwana & Nkwengulila (2010); Chibwana *et al.* (2013); Chibwana & Nkwengulila (2015)
Tylodephys sp. 2	Valid	M	Morphology/ molecular[†]	Fish eye vitreous humour	Otachi *et al.* (2015)
Asia					
T. cerebralis (Chakrabarti, 1968) syn. *Diplostomulum cerebralis* Chakrabarti, 1968; *T. ophthalmi* (Pandey, 1970)	*Incertae sedis*	M	Morphology	Fish cranial cavity, body cavity and eyes	Chakrabarti (1968); Pandey (1970); Sudarikov (1974); Pandey & Tewari (1984)
T. chandrapali Jain & Gupta, 1970 syn. *Tylodelphis onkari* Jain & Gupta, 1970	Valid	A	Morphology		Jain & Gupta (1970)
T. darteri Mehra, 1962 syn. *T. indiana* Gupta, 1962; *T. indica* Mehra, 1962	Valid	A	Morphology		Gupta (1962); Mehra (1962a); Dubois (1964)
T. duboisilla (Mehra, 1962) syn. *Glossodiplostomum duboisilla* Mehra, 1962	Valid	A	Morphology		Mehra (1962b); Yamaguti (1971)
T. excavata spinnata (Gupta, 1962) syn. *T. spinnata* Gupta, 1962	Valid	A	Morphology		Gupta (1962); Dubois (1964)
T. rauschi (Singh, 1956) syn. *Diplostomum rauschi* Singh, 1956	Valid	A	Morphology		Singh (1956); Odening (1970)
Australasia					
Tylodelphys sp.	Valid	M	Morphology/ molecular[†]	Fish eye vitreous humour	Herein
Europe					
T. clavata (von Nordmann, 1832) syn. *Diplostomum clavatum* (von Nordmann, 1832); *Proalaria clavata* Ciurea, 1928; *Prodiplostomum clavatum* Ciurea, 1933; *T. excavata* of Bezubik (1956b); *Tylodelphys circibuteonis* Odening, 1962	Valid	A, M, C	Morphology/ molecular	Fish eye vitreous humour	von Nordmann (1832); Diesing (1850); Ciurea (1928); Dubois (1938); Bezubik (1956b); Dubois & Fain (1956); Furmaga (1957); Kozicka & Niewiadomska (1960a); Odening (1962); Niewiadomska (1963a); Faltynkova *et al.* (2007); Behrmann-Godel (2013); Georgieva *et al.* (2013); Locke *et al.* (2015)

Table 1. (*Cont.*)

Geographic region/ species or genetic lineage	Species status	Developmental stages known	Diagnostic evidence	Site of infection in 2nd IH	Main references
T. conifera (Mehlis, 1846) syn. *Holostomum coniferum* Mehlis, 1846 in Creplin (1846); *Cercaria letifera* Fuhrmann, 1916; *Diplostomum gavium* (Guberlet, 1922) of Macko (1961)	Valid	A, M, C	Morphology	Fish eye vitreous humour	Fuhrmann (1916); Dubois (1937a, 1938); Bychovskaja-Pavlovskaja (1953); Sudarikov (1960); Macko (1961); Bychovskaja-Pavlovskaja (1962); Dubois (1964)
T. craniaria (Diesing, 1858) syn. *Diplostomulum craniarius* Diesing, 1858; *Diplostoma craniarium* Cobbold, 1860; *Tetracotyle craniaria* (Diesing, 1858)	*Incertae sedis*	M	Morphology	Fish brain cerebrospinal fluid	Diesing (1858); Kozicka & Niewiadomska (1960a)
T. excavata (Rudolphi, 1803) syn. *Fasciola excavata* (Rudolphi, 1803); *Diplostomum excavatum* (Rudolphi, 1803); *Proalaria excavata* (Rudolphi, 1803); *Prodiplostomum excavatum* (Rudolphi, 1803); *Distoma excavatum* Rudolphi, 1809; *Holostomum excavatum* (Rudolphi, 1803); *Holostoma excavatum* (Rudolphi, 1803); *Distomum excavatum* Rudolphi, 1803 of Creplin (1829); *Tylodelphys rhachiaea* Henle, 1833; *Hemistomum excavatum* Diesing, 1850; *Hemistomum pileatum* Hausmann, 1899	Valid	A, M, C	Morphology/ molecular	Amphibian spinal cord	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)
T. glossoïdes (Dubois, 1927) syn. *Hemistomum glossoïdes* (Dubois, 1927), *Glossodiplostomum glossoïdes* (Dubois, 1927)	Valid	A	Morphology		Dubois (1927, 1931); Kozicka & Niewiadomska (1960b)
T. podicipina (Kozicka & Niewiadomska, 1960) syn. *Tylodelphys excavata* of Bezubik (1956a)	Valid	A, M	Morphology	Fish eye vitreous humour	Bezubik (1956a); Kozicka & Niewiadomska (1960b); Dubois (1964)
T. strigicola Odening, 1962	Valid	A	Morphology		Odening & Bockhardt (1961); Odening (1962)
North America					
T. aztecae Garcia-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2015	Valid	A, M	Morphology/ molecular†	Fish body cavity	García-Varela *et al.* (2015)
T. immer Dubois, 1961 syn. *Diplostomum gavium* of Dubois (1938); *Tylodelphys gavium* [in part] of Sudarikov (1960)	Valid	A, M	Morphology/ molecular†	Fish eye vitreous humour	Dubois & Rausch (1950); Dubois (1961); Dubois (1964, 1968); Locke *et al.* (2015)
T. podicipina robrauschi Dubois, 1969	Valid	A, M	Morphology		Dubois (1969); Yamaguti (1971)
T. scheuringi (Hughes, 1929) syn. *Diplostomulum scheuringi* Hughes, 1929	Valid	M	Morphology/ molecular†	Fish eye humours	Hughes (1929); Van Cleave & Mueller (1934); Locke *et al.* (2015)
Tylodelphys sp. 3	Valid	M	Molecular†	Fish eye vitreous humour	Locke *et al.* (2015)
Tylodelphys sp. 4	Valid	M	Molecular†	Fish brain	Locke *et al.* (2015)
Tylodelphys sp. 5	Valid	M	Molecular†	Fish brain	Locke *et al.* (2015)
Tylodelphys sp. 6	Valid	M	Molecular†	Fish body cavity	Locke *et al.* (2015)
South America					
T. adulta Lunaschi & Drago, 2004	Valid	A	Morphology		Lunaschi & Drago (2004); Drago *et al.* (2011)
T. americana (Dubois, 1936) syn. *Prodiplostomum americanum* (Dubois, 1936); *T. elongata* of Caballero and Vogelsang (1949)	Valid	A	Morphology		Dubois (1936, 1937b, 1938)

Continued

Table 1. (*Cont.*)

Geographic region/ species or genetic lineage	Species status	Developmental stages known	Diagnostic evidence	Site of infection in 2nd IH	Main references
T. argentinus Quaggiotto & Valverde, 1992	*Incertae sedis*	M	Morphology	Fish brain	Quaggiotto & Valverde (1992); Flores & Baccalá (1998)
T. barilochensis Quaggiotto & Valverde, 1992	*Incertae sedis*	M	Morphology	Fish brain	Quaggiotto & Valverde (1992); Flores (1997); Flores & Baccalá (1998)
T. brevis Drago & Lunaschi, 2008	Valid	A	Morphology		Drago & Lunaschi (2008); Drago *et al.* (2014)
T. cardiophilus Szidat, 1969	*Incertae sedis*	M	Morphology	Fish pericardial cavity	Szidat (1969)
T. crubensis Quaggiotto & Valverde, 1992	*Incertae sedis*	M	Morphology	Fish brain	Quaggiotto & Valverde (1992); Flores (1997); Flores & Baccalá (1998)
T. destructor Szidat & Nani, 1951	*Incertae sedis*	M	Morphology	Fish brain	Szidat & Nani (1951); Szidat (1969)
T. elongata (Lutz, 1928) syn. *Alaria elongata* Lutz, 1928; *Diplostomum brevisegmentatum* Pérez Vigueras, 1944	Valid	A	Morphology		Dubois (1937a, 1938)
T. jenynsiae Szidat, 1969	Valid	M	Morphology/ molecular[†]	Fish body cavity	Szidat (1969)

[†] Morphological information provided in association with molecular data, or morphological vouchers of the molecular samples deposited in Museum or University collections.
Abbreviations: A, adult; M, metacercaria; C, cercaria; IH, intermediate host.

GGT–3′ (Galazzo *et al.*, 2002)] and partial fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (*Cox*1) [600 bp; primers Plat-diploCOX1F: 5′–CGT TTR AAT TAT ACG GAT CC–3′ and Plat-diploCOX1R: 5′–AGC ATA GTA ATM GCA GCA GC–3′ (Moszczynska *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 $(NH_4)_2SO_4$, 67 mM Tris–HCl at pH 8.8), 2 mM $MgCl_2$, 200 µM of each deoxynucleoside 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 *Cox*1 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 *Cox*1 genes, an internal primer for the 28S fragment [L1200R: 5′–GCA TAG TTC ACC ATC TTT CGG–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 *Cox*1 fragment were aligned in three independent datasets together with published sequences of identified strigeids and diplostomids from GenBank. The *Cox*1 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. mashonense* 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 *Cox*1 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 *Cox*1 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)

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

	GenBank accession numbers		
Species	*Cox*1	ITS1–5.8S–ITS2	28S
Tylodelphys aztecae	KT175316, KT175368	KT175377	
Tylodelphys clavata	KR271480, JX986908	JQ665459	
Tylodelphys excavata	KC685344		
Tylodelphys immer	KR271491, KR271492	KT186804	
Tylodephys jenynsiae	KR271494, KR271495		
Tylodelphys mashonense	KC685327, KC685328	KC685362	KF189071
Tylodelphys scheuringi	FJ477223, HM064914	FJ469596	
Tylodelphys sp. 1 of Chibwana *et al.* (2013)	KC685346, KC685348	KC685365	
Tylodelphys sp. 2 of Chibwana *et al.* (2013)	KC685356, KC685358	KC685367	
Tylodelphys sp. 2 of Otachi *et al.* (2015)	KF809488, KF809494		
Tylodelphys sp. 3 of Locke *et al.* (2015)	KR271511, KR271512		
Tylodelphys sp. 4 of Locke *et al.* (2015)	KR271516, KR271519		
Tylodelphys sp. 5 of Locke *et al.* (2015)	KR271520, KR271521		
Tylodelphys sp. 6 of Locke *et al.* (2015)	KR271522, KR271523		
Tylodephys sp. of Sokolov *et al.* (2013)		KF477191	
Tylodelphys sp.	**KU588143-KU588149**	**KU588152-KU588153**	**KU588150-KU588151**

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 223–429 (315 ± 54). Tegument 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) × 41–73 (55 ± 7). Pseudosuckers (lappets) either side of oral sucker, barely differentiated, only discernable on one specimen; 45–46 × 35–37. Ventral sucker sub-round, 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: Cox*1, KU588143–KU588149; 28S, KU588150–KU588151; ITS1, KU588152–KU588153.

Remarks

The above-described metacercaria conforms to the description of the ‘diplostomulum’ type of metacercaria according to Niewadomska (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. barilochensis, T. cardiophilus, T. cerebralis, T. craniaria, T. crubensis, T. destructor, T. excavata, T. grandis,*

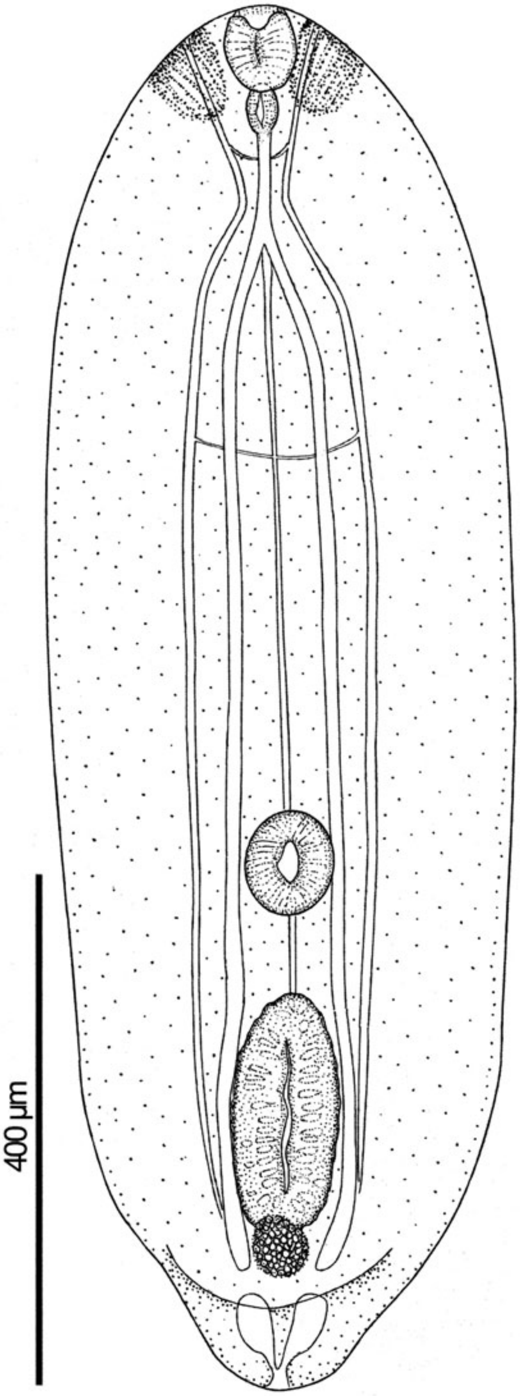

Fig. 1. *Tylodelphys* sp. metacercaria ex *Gobiomorphus cotidianus*.

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). *Tylodelphys 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 *Tylodelphys* and for a member of the Diplostomidae in New Zealand.

At the molecular level, three unique *Cox*1 haplotypes were retrieved from sequences of seven metacercariae of *Tylodelphys* sp. from New Zealand (NZ). The three haplotype sequences formed a separate reciprocally monophyletic lineage, supporting the distinct species status of these metacercariae. Phylogenetically, *Tylodelphys* sp. from NZ appeared closely related to species from North America, sister to *T. immer* in a clade together with *T. scheuringi* and *Tylodelphys* sp. 3 of Locke *et al.* (2015) (fig. 3A). The relationships among *Tylodelphys* species/lineages were generally poorly supported. Phylogenetic analyses of the ITS region included sequences for eight *Tylodelphys* species besides our newly obtained sequences from NZ and depicted two clades with strong support (fig. 3B). One clade included sequences representative of American species together with that of our metacercariae and one sequence of *Tylodelphys* 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 *Cox*1 fragment varied between 0.2 and 1.2% within *Tylodelphys* species; whereas *Tylodelphys* sp. from NZ showed 0.8% intraspecific variation. No intraspecific variability was detected in the ITS or 28S sequences of the two metacercariae of *Tylodelphys* sp. newly sequenced. Mean interspecific genetic divergence for the *Cox*1 sequences of *Tylodelphys* spp. showed a range of variation of 8–16.5%. *Cox*1 sequences of *Tylodelphys* sp. from NZ diverged 8.6–14.2% from the sequences of other *Tylodelphys* species/lineages. Mean interspecific genetic divergence for the ITS sequences of *Tylodelphys* species/lineages ranged from 0.7 to 8.3%, whereas *Tylodelphys* sp. from NZ diverged between 1.1–7.7% from other *Tylodelphys* species. Comparison of the partial sequences for the 28S rRNA gene of our metacercariae with the only available 28S sequence of *Tylodelphys* 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 *Tylodelphys* spp. and those for *Diplostomum* spp. was 5.3–5.9%. Molecular information altogether confirms the generic affiliation of the newly sequenced specimens to *Tylodelphys* and their distinct status from all *Tylodelphys* species and lineages molecularly characterized so far.

Note on the taxonomic status of Tylodelphys *spp.*

Tylodelphys ophthalmi was first described as *Diplostomulum ophthalmi* Pandey, 1970, although it was

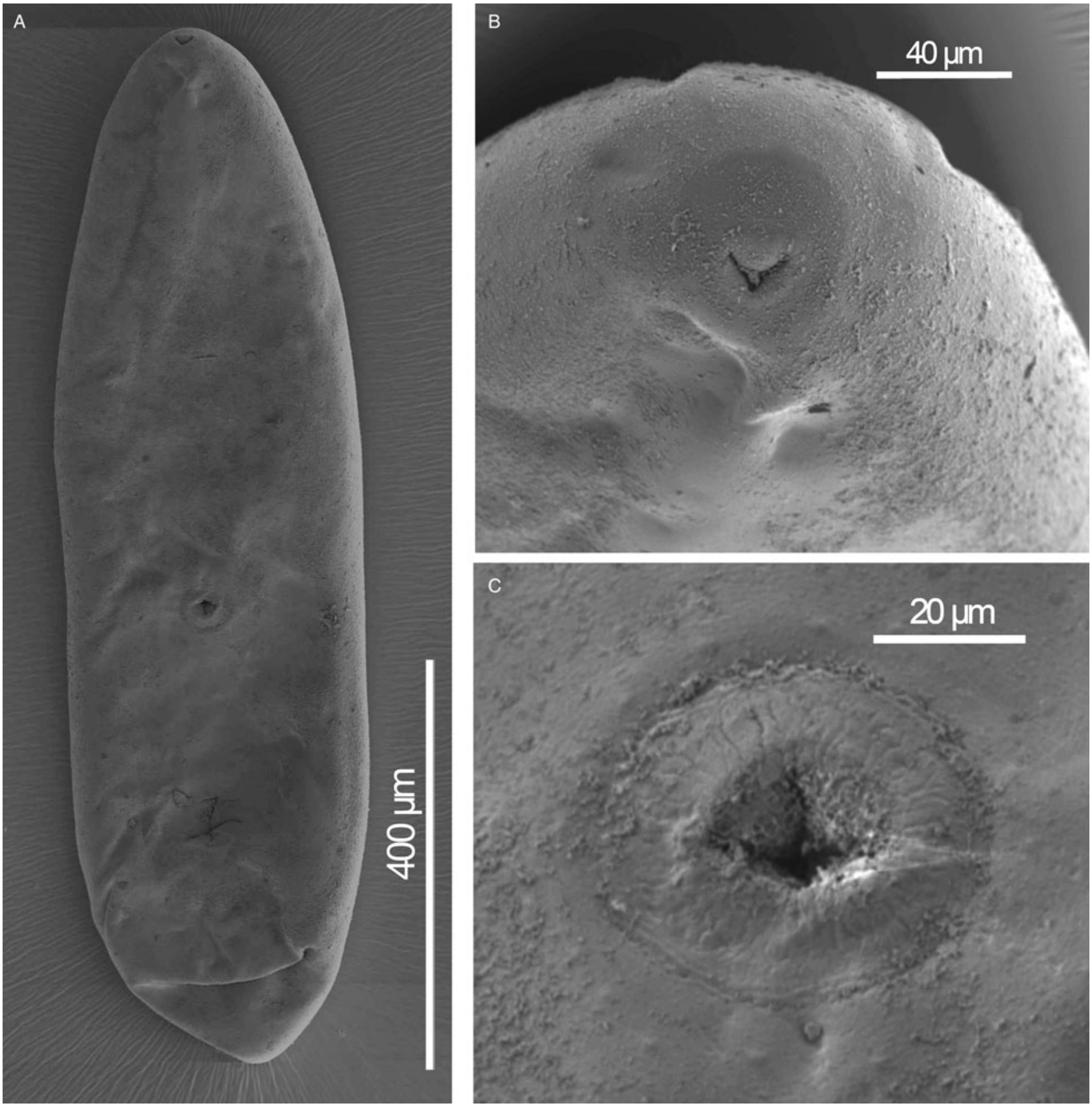

Fig. 2. *Tylodelphys* sp. metacercaria, scanning electron micrographs. Ventral view of the metacercaria (A), detail of the oral sucker (B) and detail of the ventral sucker (C).

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).

Table 3. Comparative metrical data for metacercariae of *Tylodelphys* species parasitizing the eye of fish. The range is followed by the mean in parentheses when available.

Species	*Tylodelphys* sp.	*Tylodelphys clavata*	*Tylodelphys conifera*	*Tylodelphys ophthalmi*	*Tylodelphys podicipina*	*Tylodelphys scheuringi*
Source	This study	Dubois (1938), after Ciurea (1928)	Dubois (1968), after Kozicka & Niewiadomska (1960a)	Pandey (1970)	Kozicka & Niewiadomska (1960b)	Hughes (1929)
Body length	858–1374 (1118)	310–520 (440)	300–680	650–1120	1140–1820 (1450)	885–1155 (1026)
Body width	223–429 (315)	100–150 (110)	100–190	250–300	340–570 (490)	210–270 (249)
Pharynx length	32–57 (43)	24	20		32–52 (40)	*c.* 25*
Pharynx width	19–41 (32)	15	12	10–30d	26–40 (32)	*c.* 16*
Oral sucker length	52–65 (61)	41	28–44		52–80 (59)	36–45 (41)
Oral sucker width	41–73 (58)	33	24–36	40–60d	60–72 (58)	30–39 (33)
Ventral sucker length	55–78 (67)		18–24		72–104 (95)	
Ventral sucker width	51–98 (67)	24d	20–28	70–80d	72–104 (88)	36–45 (40)d
Ventral sucker position (%)	54–63 (59)	56	51*	60*	42*	61*
Holdfast organ length	113–220 (153)	59	52–84	*c.* 80*	190–300 (212)	96–150 (117)
Holdfast organ width	63–134 (89)	37	28–40	*c.* 45*	105–190 (144)	42–75 (56)
Body spines	No spines	Stages only	Stages	Blunt spines	Not stated	Not stated

*Data inferred from illustrations in original descriptions or redescriptions. Blank cells are missing data and not possible to infer from literature.
Abbreviation: d, diameter.

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. jenynsiae* 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 metacercaria 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 cotidianus* immediately after eye removal. Observation made from a dissecting microscope.

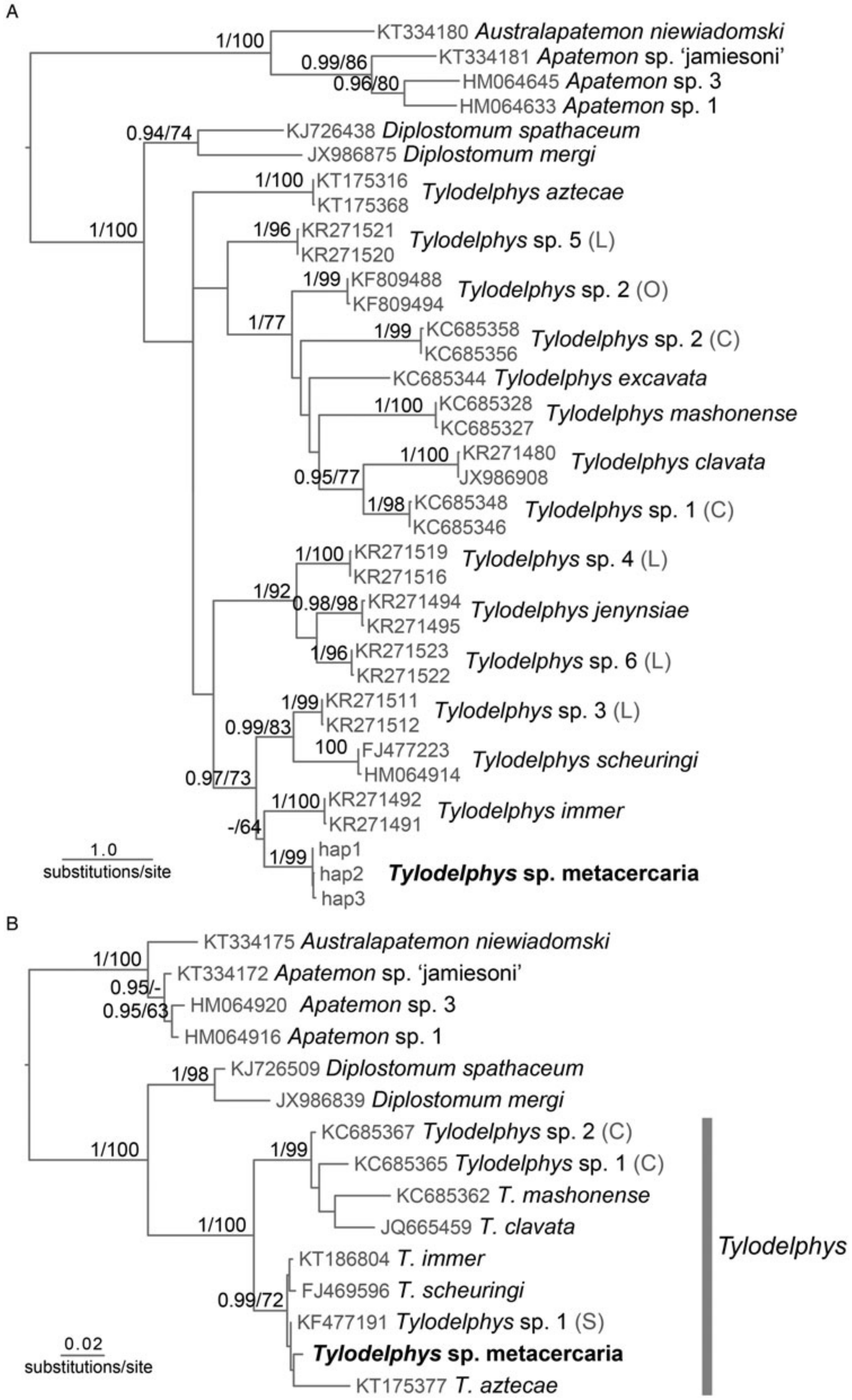

Fig. 3. Phylogenetic relationships inferred by maximum likelihood analysis for representatives of *Tylodelphys* based on *Cox*1 (A) and ITS (B) sequence data; with posterior probability values followed by bootstrap percentages above the branches (posterior probabilities <0.90 and bootstrap values <60 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

Acknowledgements

We are thankful to Dr Carlos Rouco (University of Cordoba, Spain) and Dr Clement Lagrue (University of Otago, New Zealand) for their assistance in the field, and Dr André Piuz (Natural History Museum of Geneva, Switzerland) for his help with scanning electron microscopy. We are also grateful to two anonymous reviewers for their helpful suggestions and detailed corrections.

Financial support

This work has been supported indirectly by a University of Otago Research Grant and through a Marie Curie Outgoing International Fellowship for Career Development (IB-C, grant number PIOF-GA-2009–252124) within the 7th Framework Programme (FP7/2007–2013) of the European Commission.

Conflict of interest

None.

Ethical standards

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

References

Barsiene, J. (1991) Chromosome sets of *Tylodelphys clavata, T. excavata* and *Posthodiplostomum cuticola* (Diplostomidae, Trematoda) from freshwater snails. *Angewandte Parasitologie* **32**, 87–92.

Behrmann-Godel, J. (2013) Parasite identification, succession and infection pathways in perch fry (*Perca fluviatilis*): new insights through a combined morphological and genetic approach. *Parasitology* **140**, 509–520.

Beverley-Burton, M. (1963) A new strigeid, *Diplostomum* (*Tylodelphys*) *mashonense* n. sp., (Trematoda: Diplostomatidae), from the grey heron, *Ardea cinerea* L. in Southern Rhodesia, with an experimental demonstration of part of the life cycle. *Revue de Zoologie et de Botanique Africaines* **68**, 291–308.

Bezubik, B. (1956a) Materials to the helminthofauna of aquatic birds of Poland. *Acta Parasitologica Polonica* **4**, 59–88.

Bezubik, B. (1956b) The helminth fauna of wild ducks (subfam. Anatinae). *Acta Parasitologica Polonica* **4**, 407–510.

Blasco-Costa, I., Balbuena, J.A., Kostadinova, A. & Olson, P.D. (2009) Interrelationships of the Haploporinae (Digenea: Haploporidae): a molecular test of the taxonomic framework based on morphology. *Parasitology International* **58**, 263–269.

Bychovskaja-Pavlovskaja, I.E. (1953) [The fauna of trematodes of birds from occidental Siberia and their dynamics]. *Parasitological collection of the Zoological institute of the Academy of Sciences of the URSS* **17**, 5–116.

Bychovskaja-Pavlovskaja, I.E. (1962) *[Trematodes of birds of the URSS]*. Moscow-Leningrad, Academy of Sciences of the URSS.

Caballero, C.E. & Vogelsang, E.G. (1949) Fauna helmintológica venezolana II. Algunos tremátodos de aves y mamíferos (1). *Revista de Medicina Veterinaria y Parasitología* **8**, 43–65.

Chakrabarti, K. (1968) On a new strigeid metacercaria, *Diplostomulum cerebralis* n. sp. from an Indian freshwater fish. *Zoologischer Anzeiger* **181**, 303–306.

Chibwana, F.D. & Nkwengulila, G. (2010) Variation in the morphometrics of diplostomid metacercariae (Digenea: Trematoda) infecting the catfish, *Clarias gariepinus* in Tanzania. *Journal of Helminthology* **84**, 61–70.

Chibwana, F.D. & Nkwengulila, G. (2015) The nervous systems of *Tylodelphys* metacercariae (Digenea: Diplostomidae) from the catfish *Clarias gariepinus* (Clariidae) in freshwater habitats of Tanzania. *Journal of Helminthology*. doi:10.1017/S0022149X15001005.

Chibwana, F.D., Blasco-Costa, I., Georgieva, S., Hosea, K.M., Nkvvengulila, G., Scholz, T. & Kostadinova, A. (2013) A first insight into the barcodes for African diplostomids (Digenea: Diplostomidae): Brain parasites in *Clarias gariepinus* (Siluriformes: Clariidae). *Infection, Genetics and Evolution* **17**, 62–70.

Chibwana, F.D., Nkwengulila, G., Locke, S.A., McLaughlin, J.D. & Marcogliese, D.J. (2015) Completion of the life cycle of *Tylodelphys mashonense* (Sudarikov, 1971) (Digenea: Diplostomidae) with DNA barcodes and rDNA sequences. *Parasitology Research* **114**, 3675–3682.

Ciurea, I. (1928) Sur une nouvelle *Proalaria* et sa metacercaire. *Bulletin de la Section Scientifique de l'Academie Roumaine. Bucharest* **11**, 1–14.

Ciurea, I. (1933) Sur quelques larves des vers parasites de l'homme, des mammifères et des oseaux ichtyophages, trouvés chez les poissons des grands lacs de la Bessarabie, du Dniester et de son liman. *Archives roumaines de pathologie experimentale et de microbiologie* **6**, 151–170.

Creplin, F.C.H. (1829) *Novae Observationes de Entozois*. Berlin, Ferdinandum Dummierum.

Creplin, F.C.H. (1846) Nachträge zu Gurlt's Verzeichniss der Thiere, bei welchen Entozoen gefunden worden sind. *Archiv für Naturgeschichte* **12**, 129–160.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**, 772.

Diesing, K.M. (1850) *Systema helminthum*. 679 pp. Vienna, W. Braumüller.

Diesing, K.M. (1858) Revision der Myzhelminthen. Abtheilung: Trematoden. *Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften. Mathematisch-Naturwissenschaftliche Classe, Wien* **32**, 307–390.

Drago, F.B. & Lunaschi, L.I. (2008) Description of a new species of *Tylodelphys* (Digenea, Diplostomidae) in the wood stork, *Mycteria americana* (Aves, Ciconiidae) from Argentina. *Acta Parasitologica* **53**, 263–267.

Drago, F.B., Lunaschi, L.I. & Schenone, M. (2011) Digenean parasites of the Neotropic cormorant, *Phalacrocorax brasilianus* (Gmelin, 1789) (Aves:

Phalacrocoracidae) from Argentina: distribution extension and new host records. *Check List* **7**, 871–875.

Drago, F.B., Lunaschi, L.I. & Draghi, R. (2014) Digenean fauna in raptors from northeastern Argentina, with the description of a new species of *Strigea* (Digenea: Strigeidae). *Zootaxa* **3785**, 258–270.

Dubois, G. (1927) Description de nouveaux Trematodes d'oiseaux du genre *Hemistomum*. *Bulletin de la Société Neuchateloise des Sciences Naturelles NS* **1**, 33–44.

Dubois, G. (1931) Revision des 'Hemistomes' et étude de formes nouvelles. *Bulletin de la Société Neuchateloise des Sciences Naturelles* **56**, 375–412.

Dubois, G. (1936) Nouveaux principes de classification des Trématodes du groupe des Strigeida (note préliminaire). *Revue Suisse de Zoologie* **43**, 507–515.

Dubois, G. (1937a) Sur quelques Strigéidés (notes préliminaires). *Revue Suisse de Zoologie* **44**, 391–396.

Dubois, G. (1937b) Contribution à l'étude des diplostomes d'oiseaux du musée de Vienne. *Bulletin de la Société Neuchateloise des Sciences Naturelles* **62**, 99–128.

Dubois, G. (1938) Monographie des Strigeida (Trematoda). *Memoires de la Société Neuchateloise des Sciences Naturelles* **6**, 1–535.

Dubois, G. (1961) Le genre *Diplostomum* von Nordmann, 1832 (Trematoda: Strigeida). *Bulletin de la Société Neuchateloise des Sciences Naturelles* **84**, 113–124.

Dubois, G. (1964) Du statut de quelques Strigeata La Rue, 1926 (Trematoda). I. *Bulletin de la Société Neuchateloise des Sciences Naturelles* **87**, 27–71.

Dubois, G. (1968) Synopsis des Strigeidae et des Diplostomatidae (Trematoda). Premiere Partie. *Mémoires de la Société Neuchâteloise des Sciences Naturelles* **10**, 1–258.

Dubois, G. (1969) Notes helminthologiques. 2. Diplostomatidae Poirier et Cyathocotylidae Poche (Trematoda). *Revue Suisse de Zoologie* **76**, 3–21.

Dubois, G. (1970) The bases for the taxonomy of Strigeata Trematoda Strigeida. *Revue Suisse de Zoologie* **77**, 663–685.

Dubois, G. & Angel, L.M. (1972) Strigeata (Trematoda) of Australian birds and mammals from the helminthological collection of the University of Adelaide. *Transactions of the Royal Society of South Australia* **96**, 197–215.

Dubois, G. & Fain, A. (1956) Contribution a l'étude des Strigeida du Congo Beige. I. *Bulletin de la Société Neuchateloise des Sciences Naturelles* **79**, 17–38.

Dubois, G. & Rausch, R.L. (1950) A contribution to the study of North American strigeids (Trematoda). *The American Midland Naturalist* **43**, 1–31.

El-Naffar, M., Khalifa, R. & Sakla, A. (1980) Parasitofauna of the Egyptian aquatic birds. II. Trematode parasites of the giant heron (*Ardea goliath*) in Assiut governorate. *Journal of the Egyptian Society of Parasitology* **10**, 107–116.

Faltynkova, A., Nasincova, V. & Kablaskova, L. (2007) Larval trematodes (Digenea) of the great pond snail, *Lymnaea stagnalis* (L.), (Gastropoda, Pulmonata) in Central Europe: a survey of species and key to their identification. *Parasite* **14**, 39–51.

Faltynkova, A., Nasincova, V. & Kablaskova, L. (2008) Larval trematodes (Digenea) of planorbid snails (Gastropoda: Pulmonata) in Central Europe: a survey of species and key to their identification. *Systematic Parasitology* **69**, 155–178.

Flores, V. (1997) Contribution to the morphological description of *Tylodelphys barilochensis* and *Tylodelphys crubensis* (Trematoda, Diplostomidae) encephalon parasites of *Galaxias maculatus* (Teleostei, Galaxiidae). *Boletín Chileno de Parasitología* **52**, 84–88.

Flores, V. & Baccalá, N. (1998) Multivariate analyses in the taxonomy of two species of *Tylodelphys* Diesing, 1850 (Trematoda: Diplostomidae) from *Galaxias maculatus* (Teleostei: Galaxiidae). *Systematic Parasitology* **40**, 221–227.

Fuhrmann, O. (1916) Notes helminthologiques suisses. I. Sur une nouvelle espèce de cercaire à queue fourchue. *Revue Suisse de Zoologie* **24**, 389–396.

Furmaga, S. (1957) The helminth fauna of predatory birds (Accipitres et Striges) of the environment of Lublin. *Acta Parasitologica Polonica* **5**, 215–297.

Galazzo, D.E., Dayanandan, S., Marcogliese, D.J. & McLaughlin, J.D. (2002) Molecular systematics of some North American species of *Diplostomum* (Digenea) based on rDNA-sequence data and comparisons with European congeners. *Canadian Journal of Zoology* **80**, 2207–2217.

García-Varela, M., Sereno-Uribe, A.L., Pinacho-Pinacho, C.D., Hernández-Cruz, E. & Pérez-Ponce de León, G. (2015) An integrative taxonomic study reveals a new species of *Tylodelphys* Diesing, 1950 (Digenea: Diplostomidae) in central and northern Mexico. *Journal of Helminthology*. doi: 10.1017/S0022149X15000917.

Georgieva, S., Soldánová, M., Pérez-del-Olmo, A., Dangel, D.R., Sitko, J., Sures, B. & Kostadinova, A. (2013) Molecular prospecting for European *Diplostomum* (Digenea: Diplostomidae) reveals cryptic diversity. *International Journal for Parasitology* **43**, 57–72.

Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704.

Gupta, R. (1962) Two new species of the genus *Tylodelphys* Diesing, 1850 (Trematoda: Diplostomatidae) from Indian birds. *Proceedings of the National Academy of Sciences, India. Section B, Biological sciences* **32**, 417–420.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R. & Bollback, J.P. (2001) Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **294**, 2310–2314.

Hughes, R.C. (1929) Studies on the trematode family Strigeidae (Holostomidae) no. XIX. *Diplostomulum scheuringi* sp. nov. and *D. vegrandis* (La Rue). *Journal of Parasitology* **15**, 267–271.

Jain, S. & Gupta, A. (1970) On a new species of the genus *Tylodelphys* Diesing, 1850 (Trematoda: Diplostomidae Poirier, 1886) from an Indian bird. *Indian Journal of Helminthology* **22**, 79–83.

Kennedy, C.R. (1995) Richness and diversity of macroparasite communities in tropical eels *Anguilla reinhardtii* in Queensland, Australia. *Parasitology* **111**, 233–245.

King, P.H. & Van As, J.G. (1997) Description of the adult and larval stages of *Tylodelphys xenopi* (Trematoda: Diplostomidae) from southern Africa. *Journal of Parasitology* **83**, 287–295.

Kozicka, J. & Niewiadomska, K. (1960a) Studies on the biology and taxonomy of trematodes of the genus *Tylodelphys* Diesing, 1850 (Diplostomatidae). *Acta Parasitologica Polonica* **8**, 379–401.

Kozicka, J. & Niewiadomska, K. (1960b) *Tylodelphys podicipina* sp. n. (Trematoda, Strigeidae) and its lifecycle. *Acta Parasitologica Polonica* **8**, 25–36.

Lagrue, C. & Poulin, R. (2015) Measuring fish body condition with or without parasites: does it matter? *Journal of Fish Biology* **87**, 836–847.

Littlewood, D.T.J., Curini-Galletti, M. & Herniou, E.A. (2000) The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Molecular Phylogenetics and Evolution* **16**, 449–466.

Locke, S.A., McLaughlin, D.J. & Marcogliese, D.J. (2010) DNA barcodes show cryptic diversity and a potential physiological basis for host specificity among Diplostomoidea (Platyhelminthes: Digenea) parasitizing freshwater fishes in the St. Lawrence River, Canada. *Molecular Ecology* **19**, 2813–2827.

Locke, S.A., Al-Nasiri, F.S., Caffara, M., Drago, F., Kalbe, M., Lapierre, A.R., McLaughlin, J.D., Nie, P., Overstreet, R.M., Souza, G.T.R., Takemoto, R.M. & Marcogliese, D.J. (2015) Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. *International Journal for Parasitology* **45**, 841–855.

Lockyer, A.E., Olson, P.D. & Littlewood, D.T.J. (2003) Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biological Journal of the Linnean Society* **78**, 155–171.

Lunaschi, L.I. & Drago, F.B. (2004) Descripción de una especie nueva de *Tylodelphys* (Digenea: Diplostomidae) parásita de *Podiceps major* (Aves: Podicepediae) de Argentina. *Anales del Instituto de Biología serie Zoología* **75**, 245–252.

Luton, K., Walker, D. & Blair, D. (1992) Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). *Molecular and Biochemical Parasitology* **56**, 323–327.

Macko, J.K. (1961) [Plathelminths and their investigation at most often occuring free-living birds in east Slovakia]. *Sborník Východoslovenského Múzea* II-IIIA, 129–154.

Mashego, S.N. & Saayman, J.E. (1989) Digenetic trematodes and cestodes of *Clarias gariepinus* (Burchell, 1822) in Lebowa, South Africa, with taxonomic notes. *South African Journal of Wildlife Research* **19**, 17–20.

Mehra, H.R. (1962a) Two new species of the genus *Tylodelphys* Diesing, 1850 (Diplostomatidae) from Indian birds. *Proceedings of the National Academy of Sciences, India. Section B, Biological sciences* **32**, 417–420.

Mehra, H.R. (1962b) A new species of the genus *Glossodiplostomum* Dubois, 1932, of the family Diplostomidae Poirier, 1886. *Proceedings of the National Academy of Sciences, India. Section B, Biological sciences* **32**, 125–128.

Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. pp. 1–8 *in Proceedings of the Gateway Computing Environments Workshop (GCE)*, New Orleans, Louisiana, USA, 14 November.

Moema, E.B.E., King, P.H., Rakgole, J.N. & Baker, C. (2013) Descriptions of diplostomid metacercariae (Digenea: Diplostomidae) from freshwater fishes in the Tshwane area. *Onderstepoort Journal of Veterinary Research* **80**, 1–7.

Moszczynska, A., Locke, S.A., McLaughlin, J.D., Marcogliese, D.J. & Crease, T.J. (2009) Development of primers for the mitochondrial cytochrome *c* oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminths. *Molecular Ecology Resources* **9**, 75–82.

Mwita, C.J. (2014) The community of parasites infecting *Clarias gariepinus* in the Tanzanian waters: a case of Lake Victoria. *Open Journal of Ecology* **4**, 873–882.

Mwita, C. & Nkwengulila, G. (2008) Determinants of the parasite community of clariid fishes from Lake Victoria, Tanzania. *Journal of Helminthology* **82**, 7–16.

Niewiadomska, K. (1960) On two cercariae of the genus *Tylodelphys* Dies: *T. excavata* (Rud.) and *T. clavata* (Nord.) Diplostomatidae. *Acta Parasitologica Polonica* **8**, 427–437.

Niewiadomska, K. (1963a) New observations on the morphology of cercariae of *Tylodelphys excavata* (Rud.) and *T. clavata* (Nord.) nec Ciurea (=*C. letifera* Fuhrm.) (Trematoda, Diplostomatidae). *Bulletin de l'Academie Polonaise des Sciences (Biol)* **11**, 383–387.

Niewiadomska, K. (1963b) Remarks on the discussion on certain species of the genus *Tylodelphys* Dies. (Trematoda, Diplostomatidae). *Acta Parasitologica Polonica* **11**, 307–313.

Niewadomska, K. (2002) The Diplostomoidea Poirier, 1886. pp. 150–166 *in* Gibson, D.I., Jones, A. & Bray, R.A. (*Eds*) *Keys to the Trematoda, Vol. 1*. Oxon, UK, CAB International and The Natural History Museum.

Niewiadomska, K. & Laskowski, Z. (2002) Systematic relationships among six species of *Diplostomum* Nordmann, 1832 (Digenea) based on morphological and molecular data. *Acta Parasitologica* **47**, 20–28.

Niewiadomska, K. & Szymanski, S. (1991) Host-induced variability of *Diplostomum paracaudum* (Iles, 1959) metacercariae (Digenea). *Acta Parasitologica Polonica* **36**, 11–17.

Niewiadomska, K. & Szymanski, S. (1992) Host-induced variability of *Diplostomum pseudospathaceum* Niewiadomska, 1984 metacercariae (Digenea). *Acta Parasitologica* **37**, 11–17.

Nigrelli, R.F. & Maraventano, L.W. (1944) Pericarditis in *Xenopus laevis* caused by *Diplostomulum xenopi* sp. nov., a larval strigeid. *Journal of Parasitology* **30**, 184–190.

Odening, K. (1962) Trematoden aus einheimischen Vögeln des Berliner Tierparks und der Umgebung von Berlin. *Biologisches Zentralblatt* **81**, 419–468.

Odening, K. (1970) Neue funde von Diplostomidae (Trematoda) aus vögeln des berliner tierparks. *Mitteilungen aus dem Zoologische Museum in Berlin* **46**, 167–181.

Odening, K. & Bockhardt, I. (1961) Über einige Diplostomatidae (Trematoda, Strigeata) aus Vögeln.

Monatsberichte der Deutschen Akademie der Wissenschaften zu Berlin **3**, 202–213.

Otachi, E.O., Locke, S.A., Jirsa, F., Fellner-Frank, C. & Marcogliese, D.J. (2015) Morphometric and molecular analyses of *Tylodelphys* sp. metacercariae (Digenea: Diplostomidae) from the vitreous humour of four fish species from Lake Naivasha, Kenya. *Journal of Helminthology* **89**, 404–414.

Pandey, K.C. (1968) Studies on metacercaria of fresh water fishes of India III. On two new species of *Diplostomulum* Brandes, 1892. *Proceedings of the National Academy of Sciences, India – Section B, Biological sciences* **1968** (abstract 36), 108.

Pandey, K.C. (1970) Studies on metacercaria of freshwater fishes of India III. On two new species of *Diplostumulum* Brandes, 1892. *Proceedings of the Indian Academy of Sciences – Section B* **72**, 162–170.

Pandey, K. & Tewari, S. (1984) A redescription of *Diplostomum ophthalmi* Pandey, 1968; with a note on validity of certain other Indian species. *Uttar Pradesh Journal of Zoology* **4**, 179–184.

Quaggiotto, E.A. & Valverde, F. (1992) New metacercariae of the genus *Tylodelphys* (Trematoda, Diplostomatidae) in lake populations of *Galaxias maculatus* (Teleostei, Galaxiidae). *Boletín Chileno de Parasitología* **47**, 19–24.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.

Sela, I., Ashkenazy, H., Katoh, K. & Pupko, T. (2015) GUIDANCE2: accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters. *Nucleic Acids Research* **43**, W7–W14.

Singh, K.S. (1956) On some strigeids from India. *Journal of the Zoological Society of India* **8**, 47–56.

Sokolov, S.G., Lebedeva, D.I. & Yadrenkina, E.N. (2013) The first data on the parasite fauna of rotan *Perccottus glenii* Dybowski, 1877 (Actinopterygii: Odontobutidae) in water bodies of forest-steppe zone of the West Siberian Plain. *Parazitologia* **47**, 448–460.

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690.

Stamatakis, A., Hoover, P. & Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology* **57**, 758–771.

Sudarikov, V.E. (1960) [Superfamily Diplostomatoidea Nicoll, 1937]. pp. 157–530 *in* Skrjabin, K.I. (*Ed.*) *[Trematodes of animals and man. Principles of trematodology]*. Vol. 17. Moscow, Nauka (in Russian).

Sudarikov, V.E. (1974) [Order Strigeidida (La Rue 1926) Sudarikov 1959]. pp. 29–244 *in* Skrjabin, K.I. (*Ed.*) *[Trematodes of animals and man. Principles of trematodology]*, Vol. 25. Moscow, Nauka (in Russian).

Szidat, L. (1935) Warum wirft der storch seine junger aus dem nest? Ein beitrag zur brutbiologie und oekologie des weissen storches. (*Ciconia ciconia* L.), sowie zur kenntnis seiner parasitenfauna. *Journal of Ornithology* **83**, 76–87.

Szidat, L. (1969) Structure development and behaviour of new strigeatoid metacercariae from subtropical fishes of South America. *Journal of the Fisheries Research Board of Canada* **26**, 753–786.

Szidat, L. & Nani, A. (1951) Diplostomiasis cerebralis del Pejerrey. Una grave epizootia que afecta a la economia nacional producida por larvas de trematodes que destruzen el cerebro de los pejerreyes. *Revista del Instituto Nacional de Investigacion de las Ciencias Naturales y Museo Argentino de Ciencias Naturales* **1**, 323–384.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.

Tinsley, R.C. & Sweeting, R.A. (1974) Studies on the biology and taxonomy of *Diplostomulum* (*Tylodelphylus*) *xenopodis* from the African clawed toad, *Xenopus laevis*. *Journal of Helminthology* **48**, 247–263.

Van Cleave, H.J. & Mueller, J.F. (1934) Parasites of Oneida Lake fishes. Part. III. A biological and ecological survey of the worm parasites. *Roosevelt Wild Life Annuals* **3**, 161–334.

von Nordmann, A. (1832) *Mikrographische Beiträge zur Naturgeschichte der wirbellosen Thiere*. 118 pp. Berlin, Reimer.

Werle, E., Schneider, C., Renner, M., Volker, M. & Fiehn, W. (1994) Convenient single-step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Research* **22**, 4354–4355.

Yamaguti, S. (1971) *Synopsis of digenetic trematodes of vertebrates*. Tokyo, Japan, Keigaku Publishing.

Zhokhov, A.E., Morozova, D.A. & Tessema, A. (2010) Trematode metacercariae from the cranial cavity of African catfish *Clarias gariepinus* (Burchell, 1822) from Lake Tana, Ethiopia. *Inland Water Biology* **3**, 160–164.