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ORIGINAL PAPER

Species of *Apatemon* Szidat, 1928 and *Australapatemon* Sudarikov, 1959 (Trematoda: Strigeidae) from New Zealand: linking and characterising life cycle stages with morphology and molecules

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Abstract Species of *Apatemon* Szidat, 1928 and *Australapatemon* Sudarikov, 1959 are reported from New Zealand for the first time, and their life cycles are resolved using molecular sequence data (28S and ITS rDNA regions and mitochondrial COI). The metacercaria of *Apatemon* sp. 'jamiesoni' ex *Gobiomorphus cotidianus* and its cercaria ex *Potamopyrgus antipodarum* are described in detail. Its adult, found in *Anas platyrhynchos* and *Phalacrocorax punctatus*, is identified by molecular sequence data. *Apatemon* sp. 'jamiesoni' uses a different species of snail host, exhibits consistent differences in the genetic markers examined and its single described adult differs from known species so as to be considered distinct, but its formal description awaits additional adult specimens. *Australapatemon niewiadomski* n. sp. is described from *Anas platyrhynchos*. It is distinguished morphologically by the absence of a ringnapf and its overall smaller size compared to most other *Australapatemon* spp. except *Au. magnacetabulum* and *Au. minor*, which are smaller in nearly all features than the new species. *Au. niewiadomski* n. sp. metacercaria and its intermediate host (*Barbronia weberi*) are identified via matching of molecular sequence data. The status of *Apatemon* and *Australapatemon* as distinct genera is confirmed based on their respective monophyly, and genetic divergence between them is comparable to other well-established genera in the Strigeidae. The diagnosis of *Australapatemon* is emended. Life history data, accurate patterns of host specialisation and distribution, alongside concurrent molecular and morphological evidence would be useful for an integrative taxonomical approach towards the elucidation of species diversity in this group.

Keywords *Apatemon* · *Australapatemon* · Strigeidae · Taxonomy · Phylogenetics · Genetic divergence

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Introduction

The Strigeidae Railliet, 1919 is a species-rich family comprising mainly parasites of birds. The criterion of host specificity has been utilised as the basis for the systematics of members of this group on several occasions (Dubois 1938; Sudarikov 1959). For instance, two subfamilies are recognised according to their host groups (birds or mammals). At a lower taxonomic level, Sudarikov (1959) recognised two genera based on differences observed in the life cycles of several species of *Apatemon* Szidat, 1928: *Apatemon*, with metacercariae encysting in fish and *Australapatemon* Sudarikov, 1959, with metacercariae encysting in leeches. Their taxonomic status has been questioned several times: Dubois and Pearson (1965) reduced *Australapatemon* to the level of subgenus, but Yamaguti (1971) restored it to generic level. In the most recent review of the family they were proposed as distinct genera (Niewiadomska 2002), but different authors still seem to hold different opinions (Bell and Sommerville 2002; Bell et al. 2002). Independent evidence that confirms the validity of their status is still lacking.

The taxonomic history of species within these genera is also complex. For example, the morphological variability of the type species *Apatemon gracilis* (Rudolphi, 1819) has been noted repeatedly (Dubois 1938; Stunkard et al. 1941; Dubois and Rausch 1948; Dubois 1968). Some workers suggested

that it be divided into numerous subspecies (Dubois 1953; Dubois 1968), and yet others, noting that variability can occur within the same host, preferred to consider *A. gracilis* as a cosmopolitan species with a wide host range and polytypic morphology (Beverley-Burton 1961). Elucidating the range of variability within this species, or species complex, is confounded by the fact that many studies claiming to refer to *A. gracilis* have been found to be referring to other named, or unnamed species (e.g. Szidat 1929; Stunkard et al. 1941; Dubois and Rausch 1948). The distinction between other species of *Apatemon* may be questionable, as exemplified by the morphometric and molecular synonymisation of *A. gracilis* and *A. annuligerum* (von Nordmann, 1832) (Bell and Sommerville 2002; Bell et al. 2002). Likewise, the problem extends to other strigeid genera and species. *Australapatemon burti* (Miller, 1923), described originally from North America (now distributed in the Holarctic and Neotropical regions), has been reported several times as *A. gracilis* (e.g. Stunkard et al. 1941; Dubois and Rausch 1948, 1950; Dubois 1951; Sudarikov 1959; Dubois and Rausch 1960) with a host range that includes at least ten different anatid species (see Dubois 1968; Drago et al. 2007; Hinojosa-Saez et al. 2009; Hernández-Mena et al. 2014). Despite the efforts of multiple researchers over the last century, it seems obvious that our understanding of the diversity in this trematode group has been severely hampered by the intraspecific plasticity and interspecific/intergeneric homogeneity in morphological features. The use of molecular species diagnostics in combination with detailed morphological examination of specimens (e.g. Blasco-Costa et al. 2010; Barcak et al. 2014; McNamara et al. 2014) represents the best approach to elucidate the species diversity and assess the extent of morphological plasticity within these strigeid taxa.

Within the Strigeidae, the extent of morphological variability appears to differ between groups, and molecular studies designed to examine species delineation have produced a range of results: some molecular studies have upheld the morphologically diagnosed species (Bell et al. 2001), others have found new species (Hernández-Mena et al. 2014), and still others have found separately described species to be genetically identical (Bell and Sommerville 2002). It is therefore imperative that morphological studies are supported by molecular evidence when possible. At this stage, although larval or adult specimens of *Apatemon* (Bell et al. 2001; Bell and Sommerville 2002; Moszczynska et al. 2009; Locke et al. 2010) and *Australapatemon* (Hernández-Mena et al. 2014) have been used in molecular phylogenies at various taxonomic levels, these specimens were seldom identified to species. Furthermore, sequence data exist for the 28S ribosomal DNA (rDNA) region, the internal transcribed spacers (ITS) of the ribosomal gene or the mitochondrial cytochrome *c* oxidase subunit I (COI) of merely a handful of species from 7 out of 13 genera considered valid within the family. There is, therefore, a need to establish a phylogenetic context in which to explore the relationships amongst genera and species, and to aid species delineation for newly collected specimens belonging to these genera and more generally to the Strigeidae.

In this study, we describe morphologically and characterise genetically a new species of *Australapatemon* based on adults and larval stages, and the larval stages and one adult specimen of an unnamed *Apatemon* species. Life cycle stages of these species are matched using sequence data for three molecular markers, 28S and ITS rDNA, and mitochondrial COI. Their sequences are analysed together with available sequences of strigeid species from GenBank and the phylogenetic analyses confirm the validity and distinct status of the two genera, *Australapatemon* and *Apatemon*. Furthermore, we present an amended diagnosis of *Australapatemon* and explore the patterns of intraspecific and interspecific genetic divergence in a number of strigeid genera.

Materials and methods

Specimen collection

Potamopyrgus antipodarum (Gray) (Gastropoda: Tateidae) were sampled from Lake Waihola and Tomahawk Lagoon in Otago, South Island, New Zealand, using dip nets, at various times of year between 2011 and 2013. Infected *P. antipodarum* (freshwater mudsnail) were screened for cercariae after incubating them in lake water at 25 °C under intense light for 24 h. Cercariae were collected for live observation and digital microphotography and fixed with 96 % ethanol for subsequent DNA extraction. *Barbronia weberi* (Blanchard) (Hirudinea: Salifidae) were collected by hand from beneath rocks at Lake Hayes in Otago. In the lab, leech specimens were pressed between glass slides to confirm infection and were killed in hot water before dissection and removal of metacercarial cysts. Metacercarial cysts and host tissue were fixed in 96 % ethanol for molecular identification.

Gobiomorphus cotidianus McDowall (Actinopterygii: Eleotridae; common bully) were collected from Lakes Waihola and Waipori (Otago) as part of other studies during 2009–2010. Fish were euthanized by spinal severance and, in some cases, frozen for later dissection. Metacercariae extracted from the body cavity and mesenteries were fixed in either 96 % ethanol for molecular analyses or 70 % ethanol for morphological study.

Adult mallard ducks (*Anas platyrhynchos* L.) were donated by duck hunters from Manuka Island in Clutha River, Mount Watkin and Karitane Estuary, South Island (New Zealand), during the official hunting season (May to July 2013), shot under licence in accordance with the Fish and Game New Zealand regulations governing the region of Otago, or were found as fresh roadkill. A spotted shag (*Phalacrocorax*

punctatus (Sparrman), following the taxonomy of (Kennedy and Spencer 2014)) was also found as roadkill on the Portobello Road, Otago Peninsula. Viscera were dissected and intestinal worms were preserved in either 96 % ethanol for molecular analyses or 70 % ethanol for morphological study.

Morphological data

Adults and metacercariae were stained using iron acetocarmine, dehydrated through a graded ethanol series, cleared in clove oil and mounted in Canada balsam. Histological sections of adults were pre-stained with eosin and post-stained with haematoxylin and mounted in Entallan® (Merck, Germany). Measurements of adults were taken from drawings at ×400 magnification. Live cercariae were stained with Neutral Red and examined as wet mounts under a light compound microscope at a magnification of ×1000. Visualising flame cells was facilitated with the addition of urea. Measurements of cercariae were taken from digital photographs using ImageJ software (Wayne Rasband, NIH, USA). All measurements in the text are in micrometres unless otherwise stated and are given as the range followed by the mean±standard deviation in parentheses.

The type and voucher material are deposited in the Platyhelminthes collection of the Natural History Museum of Geneva (MHNG), Switzerland. Comparative material examined comprised type-specimens of *Apatemon hypseleotris* Negm-Eldin & Davies, 2001 [Museum of Victoria, Melbourne; accession numbers F84195 – F84213] and voucher specimens of *Apatemon vitelliresiduus* Dubois & Angel, 1972 [South Australian Museum, Adelaide; accession numbers AHC22032 and 22033].

Molecular data

We characterised molecularly specimens of *Australapatemon niewiadomski* n. sp. (four adult worms and one metacercaria) and *Apatemon* sp. 'jamiesoni' (two adult, two metacercariae and two cercariae) from New Zealand (see Table 1). A tip of the holdfast of the single specimen of *Apatemon* sp. 'jamiesoni' ex *A. platyrhynchos* was used for the DNA extraction (DNA voucher of the morphological voucher described below). Genomic DNA was extracted from ethanol-fixed isolates in 200 μL of a 5 % suspension of Chelex® in deionised water and containing 0.1 mg/mL proteinase K, followed by incubation at 56 °C for 5 h, 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) [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 [900 bp; primers D1, 5′-AGG AAT TCC TGG TAA GTG CAA G-3′, and D2, 5′-CGT TAC TGA GGG AAT CCT GGT-3′ (Galazzo et al. 2002)] and partial fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) [500 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 dNTP, 0.5 mM each primer and 0.7 unit BIOTAQ™ DNA polymerase (Bioline Ltd.). 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 ITS1-5.8S-ITS2 gene cluster. Thermocycling conditions for the COI 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 PCR primers for the 28S and COI regions, as well as 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 ITS1-5.8S-ITS2 gene cluster [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 on an ABI 3730XL Analyser (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Contiguous sequences were assembled and edited using Sequencher™ (GeneCodes Corp. v. 5) and submitted to GenBank (see accession numbers in Table 1).

Molecular analyses

Newly generated sequences for the 28S rDNA, the ITS1-5.8S-ITS2 gene cluster and the COI fragment were aligned in three independent datasets together with published sequences of strigeids identified at least to generic level from GenBank, using MUSCLE implemented in MEGA v. 5 (Tamura et al. 2011). Sequences for the COI were aligned with reference to the amino acid translation using the echinoderm and flatworm mitochondrial genetic code (Telford et al. 2000) but were analysed as nucleotides exclusively (all codon positions included). We selected the two longest available sequences for each distinct species from GenBank. The 28S dataset (968 bp long) included two representative sequences of species of *Apharyngostrigea* Ciurea, 1927 and one of each *Cardiocephaloides* Sudarikov, 1959 and *Ichthyocotylurus* Odening, 1969 retrieved from GenBank (see accession

Table 1 Summary data for the taxa used as ingroup in the phylogenetic analyses

Species	Isolate code	Life cycle stage	Host species	Locality	GenBank accession number		
					COI	ITS	28S
Apatemon gracilis						AJ301888	
Apatemon gracilis						AJ314760	
Apatemon sp. 'jamiesoni'	ApaGco1	M	*Gobiomorphus cotidianus*	Lake Waipori, New Zealand	**KT334182**	**KT334170**	
Apatemon sp. 'jamiesoni'	ApaGco2	M	*Gobiomorphus cotidianus*	Lake Waihola, New Zealand		**KT334171**	
Apatemon sp. 'jamiesoni'	ApaPan1	C	*Potamopyrgus antipodarum*	Tomahawk lagoon, New Zealand			**KT334166**
Apatemon sp. 'jamiesoni'	ApaPan2	C	*Potamopyrgus antipodarum*	Lake Waihola, New Zealand	**KT334181**	**KT334172**	**KT334167**
Apatemon sp. 'jamiesoni'	ApaApl	A	*Anas platyrhynchos*	Balclutha, New Zealand			**KT334168**
Apatemon sp. 'jamiesoni'	ApaPpu	A	*Phalacrocorax punctatus*	Otago Harbour, New Zealand			**KT334169**
Apatemon sp. 1		M	*Etheostoma nigrum*	Quebec, Canada	FJ477183		
Apatemon sp. 1		M	*Etheostoma nigrum*	Lake Saint François, Canada	HM064633	HM064916	
Apatemon sp. 1x		M	*Etheostoma nigrum*	Lake Saint François, Canada	HM064635		
Apatemon sp. 1x		M	*Etheostoma nigrum*	Lake Saint François, Canada	HM064636		
Apatemon sp. 2		M	*Galaxiella pusilla*	Australia		JX051357	
Apatemon sp. 3		M	*Ambloplites rupestris*	Lake Saint Pierre, Canada	FJ477185		
Apatemon sp. 3		M	*Ambloplites rupestris*	Lake Saint Pierre, Canada	HM064645	HM064920	
Apatemon sp. 3		M	*Galaxiella pusilla*	Australia		JX051358	
Apatemon sp. 4		M	*Ambloplites rupestris*	Lake Saint François, Canada	FJ477186		
Apatemon sp. 4		M	*Ambloplites rupestris*	Lake Saint Pierre, Canada	HM064647		
Apharyngostrigea pipientis		M	*Rana pipiens*	Boucherville, Etang Saulaie, Canada	HM064884		
Apharyngostrigea pipientis		M	*Rana pipiens*	Boucherville, Etang Saulaie, Canada	HM064885	HM064966	
Apharyngostrigea pipientis		A	*Nycticorax nycticorax*	Nelson Co., North Dakota, USA			JF820597
Apharyngostrigea cornu		A	*Ardea herodias*	Lake Saint Louis, Canada	JF769451		
Apharyngostrigea cornu		A	*Ardea alba*	Pánuco, Veracruz, México	JX977777	JX977837	
Apharyngostrigea cornu		A	*Ardea cinerea*	Kherson Region, Ukraine			AF184264
Australapatemon burti		A	*Anas diazi*	Estado de México, México	JX977727	JX977787	
Australapatemon niewiadomski n. sp.	AusApl1	A	*Anas platyrhynchos*	Balclutha, New Zealand	**KT334177**	**KT334173**	
Australapatemon niewiadomski n. sp.	AusApl2	A	*Anas platyrhynchos*	Balclutha, New Zealand	**KT334178**	**KT334174**	
Australapatemon niewiadomski n. sp.	AusApl3	A	*Anas platyrhynchos*	Balclutha, New Zealand	**KT334179**		
Australapatemon niewiadomski n. sp.	AusApl4	A	*Anas platyrhynchos*	Balclutha, New Zealand	**KT334180**	**KT334175**	**KT334165**
Australapatemon niewiadomski n. sp.	AusBwe	M	*Barbronia weberi*	Lake Hayes, New Zealand	**KT334176**		**KT334164**
Cardiocephaloides medioconiger		A	*Larus sp.*	Laguna de Términos, México	JX977782		
Cardiocephaloides medioconiger		A	*Larus sp.*	Laguna de Términos, México	JX977783	JX977843	
Cardiocephaloides sp.[*]		A	*Larus occidentalis*	Guerrero Negro, Baja California Sur, México	JX977784	JX977844	
Cardiocephaloides longicollis		A	*Larus ridibundus*	Kherson Region, Ukraine			AY222171
Cotylurus gallinulae		A	Aythya affinis	La Esperanza, Sonora, México	JX977781	JX977841	
Ichthyocotylurus erraticus		A	*Coregonus autumnalis*	Lough Neagh, Northern Ireland, United Kingdom			AY222172
Ichthyocotylurus pileatus		M	*Perca flavescens*	Lake Saint Louis, Canada	HM064721	HM064931	

Table 1 (continued)

Species	Isolate code	Life cycle stage	Host species	Locality	GenBank accession number		
					COI	ITS	28S
Ichthyocotylurus sp. 2		M	*Perca flavescens*	Lake Saint Louis, Canada	HM064728		
Ichthyocotylurus sp. 3		M	*Notropis hudsonius*	Lake Saint François, Canada	HM064729		
Parastrigea cincta		A	*Eudocimus albus*	Caimanero, Sinaloa, México	JX977757	JX977817	
Parastrigea diovadena		A	*Eudocimus albus*	Caimanero, Sinaloa, México	JX977729	JX977789	
Parastrigea plataleae		A	*Platalea ajaja*	Topolobampo, Sinaloa, México	JX977761	JX977821	
Uncultured organism[a]			*Radix* sp.	Germany			EF417284

Accession numbers in bold represent new sequences obtained for this study

A adult, *M* metacercaria, *C* cercaria

[a] Name as in GenBank

numbers in Table 1). Further, we included a sequence of an unidentified uncultured organism from *Radix ovata* (Draparnaud, 1805), which showed high similarity to our sequences in BLAST®. The ITS dataset (containing 643 bp of the ITS1-5.8S-ITS2 gene cluster) included representative sequences for five species of *Apatemon*, three species of *Parastrigea* Szidat, 1928, two species each of *Apharyngostrigea* and *Cardiocephaloides*, and one species each of *Cotylurus* Szidat, 1928 and *Ichthyocotylurus*, retrieved from GenBank (see Table 1). The COI dataset (413 bp long) included two sequences per species and preferably from two distinct studies when possible. It contained representative sequences for four species of *Apatemon*, three species each of *Ichthyocotylurus* and *Parastrigea*, two species each of *Apharyngostrigea* and *Cardiocephaloides* and one of *Cotylurus* from GenBank. Three species of *Tylodelphys* Diesing, 1850 (Diplostomidae) were used as outgroups in the analyses of the ITS and COI datasets. Sequences for the 28S rDNA region of *Tylodelphys* spp. were unavailable, instead we used two sequences of species of *Diplostomum* von Nordmann, 1832 (Diplostomidae) as outgroups for the analyses. Extremes of the alignments were trimmed to match the shortest sequence prior to phylogenetic analyses. Phylogenetic analyses were run on the three datasets individually under the maximum likelihood (ML) and Bayesian inference (BI) criteria, employing the models of nucleotide evolution GTR+Γ (28S dataset) and GTR+Γ+I (ITS and COI) all estimated using jModelTest 2.1.1 (Guindon and Gascuel 2003; Darriba et al. 2012). ML analyses were conducted using the programme RAxML v. 7.3 (Stamatakis 2006; Stamatakis et al. 2008). All model parameters and bootstrap nodal support values (1000 repetitions) 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 10^7 generations and sampling tree topologies every 10^3 generation. Burn-in periods were set to 10^6 generations according to the standard deviation of split frequency values (<0.01). A consensus topology and nodal support estimated as posterior probability values (Huelsenbeck et al. 2001) were calculated from the remaining trees. All analyses were performed on the computational resource CIPRES (Miller et al. 2010). Genetic divergence between sequences was calculated as *p*-distances in MEGA (gaps/ambiguities excluded in pairwise comparisons). Genetic divergences in the COI fragment were estimated from an additional alignment containing our new sequences and sequences of all unique haplotypes available in GenBank for the species included in the phylogenetic analyses. Histograms of intraspecific and interspecific genetic divergence between congeneric species were built in R v. 3.1 (R Development Core Team 2010) using the library *ggplot2* (Wickham 2009).

Results

The distinctive cysts of an unnamed species of *Apatemon* are seen in virtually all dissections of the common bully fish. A furcocercaria resembling that of *Apatemon* is also known from the freshwater mudsnail that has been intensively studied in our lab. Since species of *Apatemon* are most commonly found infecting anatid birds, we sought the adults in mallard ducks and a roadkill spotted shag. While we found one incidence of infection by the same species of *Apatemon*, we also discovered a hitherto unreported species of *Australapatemon* in the mallards. Subsequently, we examined freshwater leeches, in order to find the metacercarial stage of this species. Life cycle stages of each species were confirmed by genetic concurrence and described morphologically below.

BI and ML phylogenetic reconstructions derived from each dataset depicted congruent sister relationships among sequences of the genera represented in our study (Fig. 1). In all analyses, sequences of specimens identified as either *Apatemon* or *Australapatemon* clustered in two distinct well-supported monophyletic clades. *Apatemon* and

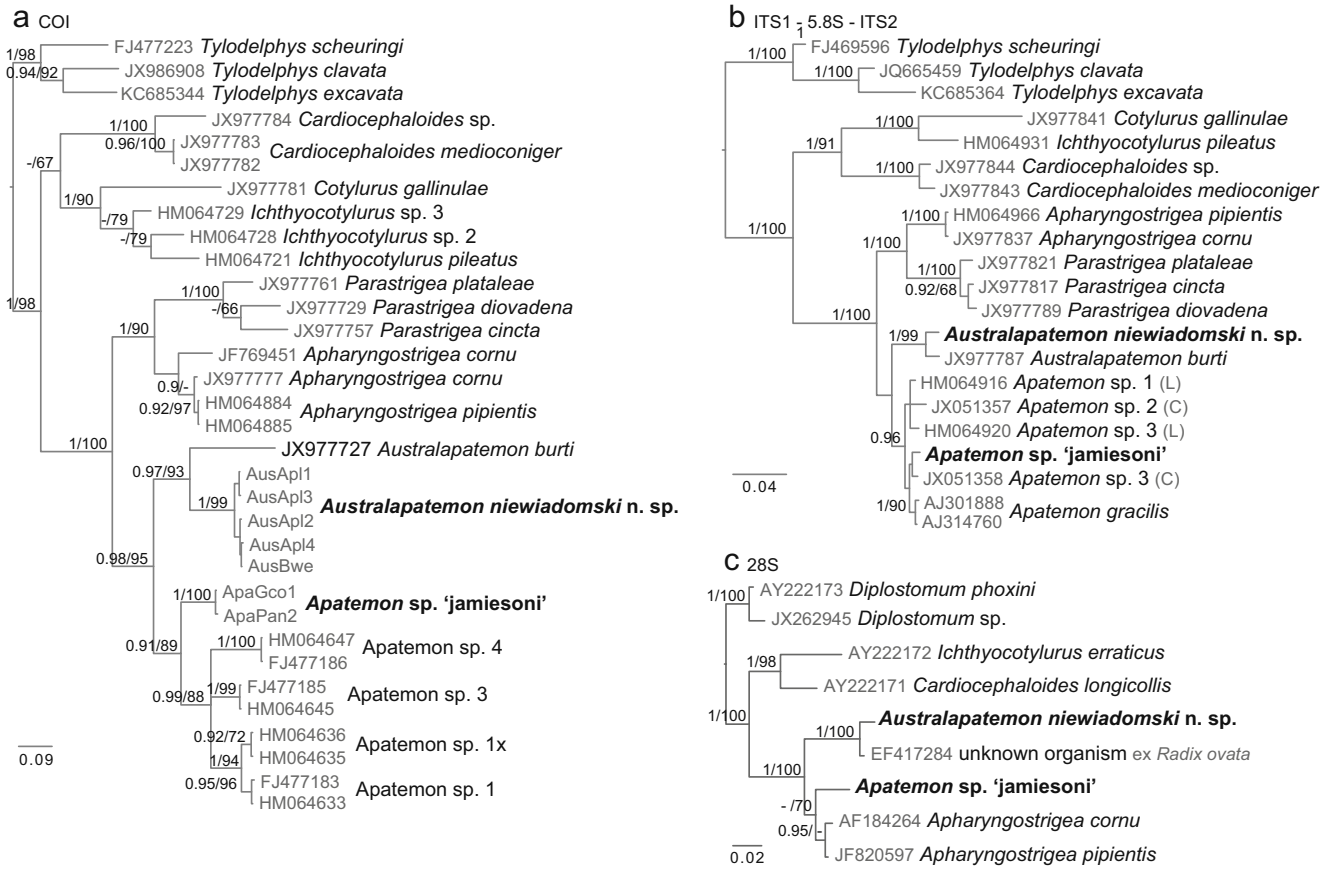

Fig. 1 Bayesian inference phylograms derived from **a** COI, **b** ITS1-5.8S-ITS2 and **c** 28S rRNA gene sequences with posterior probability values followed by bootstrap percentages above the branches (posterior probabilities <0.90 and bootstrap values <60 not reported). *Scale bars* indicate the number of substitutions per site. Taxa in bold were newly sequenced in this study. Abbreviations in brackets: L, Locke et al. 2010; C, Coleman, R. A. unpublished

Australapatemon appeared as sister taxa (strong support only in the COI tree, Fig. 1a), closely related to the clade formed by *Apharyngostrigea* and *Parastrigea* (when included, Fig. 1a, b). The clade including *Cardiocephaloides*, *Ichthyocotylurus* and *Cotylurus* (Fig. 1a–c) was depicted as the earliest divergent in all phylogenetic hypotheses. Newly generated COI sequences from New Zealand fall into two distinct well-supported reciprocally monophyletic lineages corresponding to *Apatemon* sp. 'jamiesoni' ex *P. antipodarum* and *G. cotidianus*, and *Au. niewiadomski* n. sp. ex *Barbronia weberi* and *A. platyrhynchos* (Fig. 1a). Sequences of unidentified *Apatemon* spp. from North America formed a monophyletic clade, sister to *Apatemon* sp. 'jamiesoni'. *Au. niewiadomski* n. sp. was sister to *Au. burti* from North America (Fig. 1a). The sequence of *Apharyngostrigea cornu* (Zeder, 1800) from Mexico appeared more closely related to *Apharyngostrigea pipientis* (Faust, 1918) than to *Ap. cornu* from Canada, although with low support. Newly generated ITS and 28S rDNA sequences corroborated the distinct status of the two COI lineages, *Apatemon* sp. 'jamiesoni' and *Au. niewiadomski* n. sp. The ITS tree depicted all genera, including *Apatemon* and *Australapatemon*, as well-supported monophyletic groups. However, most relationships among species of *Apatemon* were unresolved (Fig. 1b). *A. gracilis* isolates from Europe formed a supported reciprocally monophyletic lineage, the representative sequence of *Apatemon* sp. 'jamiesoni' ex *A. platyrhynchos*, *G. cotidianus* and *P. antipodarum* was sister to *Apatemon* sp. 3 (Coleman, unpublished) ex *Galaxiella pusilla* (Mack) from Australia but unsupported. As for the COI reconstruction, *Au. niewiadomski* n. sp. was found to be sister to *Au. burti* from North America (Fig. 1b). The 28S phylogenetic tree included few species (seven) due to the lack of comparative sequence material in GenBank, but the results were mostly congruent with those above (Fig. 1c). The sequence of an unidentified organism ex *R. ovata* retrieved from GenBank was sister to *Au. niewiadomski* n. sp.

Overall, intraspecific genetic divergence in COI sequences of the strigeid species (unique haplotypes sequences of each species available from GenBank plus the newly sequenced isolates; a total of 116 sequences and 411 nt) ranged from 0.1 to 2.7 % (Fig. 2), with few exceptions. Within this range, *Au. niewiadomski* n. sp. had the widest intraspecific genetic divergence range (0.2–2.7 %). Genetic divergence values

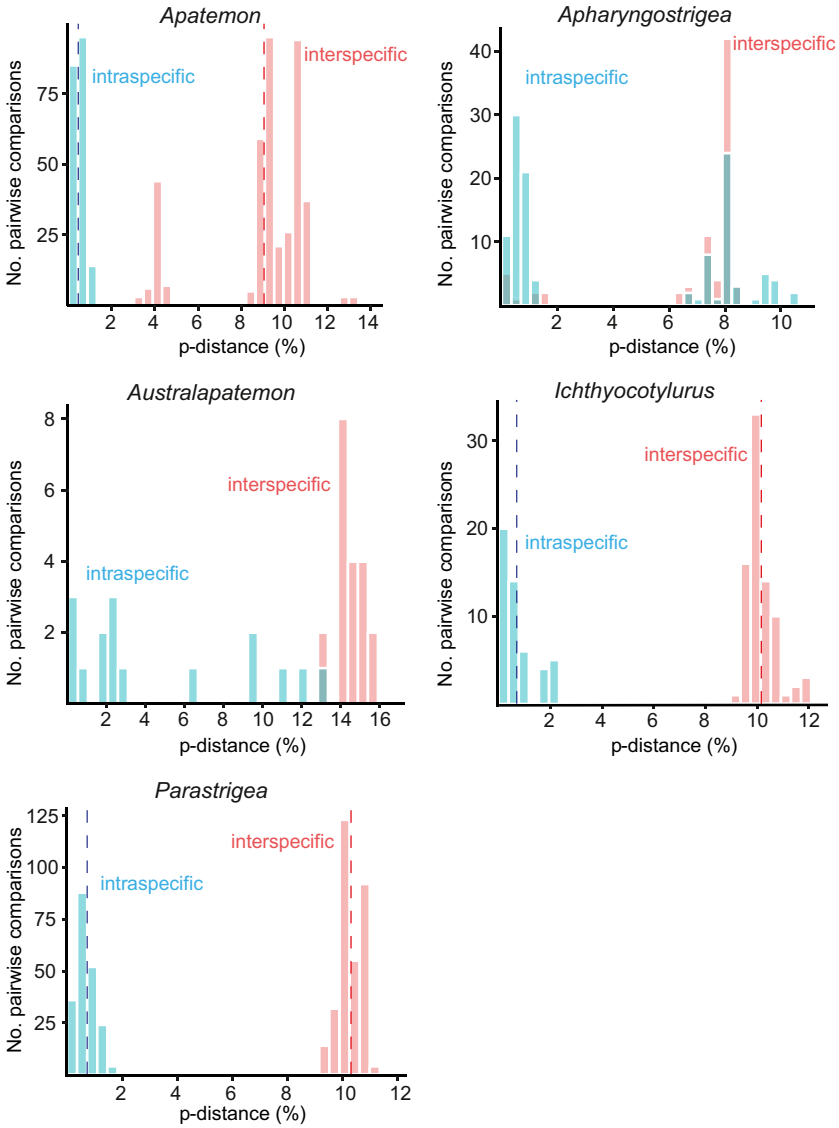

Fig. 2 Histograms of intraspecific and interspecific genetic divergence (calculated as uncorrected *p*-distance) for the barcode region of the COI gene in five strigeid genera. *Bars in blue colour* represent intraspecific divergence; in *red*, interspecific divergence between congeneric species and intermediate colour indicates intraspecific and interspecific divergence values overlap. *Dashed lines* indicate mean genetic divergence (estimated only for genera with non-overlapping intraspecific and interspecific genetic divergence values) (colour figure online)

between *Au. burti* sequences from Mexico were the highest (6.3–13.1 %) at intraspecific level, well above the range for the other strigeid species. *Apharyngostrigea cornu* showed 0.2–0.6 % intraspecific genetic divergences within Canada, but it diverged 7.5–10.3 % from *Ap. cornu* sequences from Mexico. A representative sequence of *Ap. cornu* ex *Nyctanassa violacea* L. (GenBank ID JX977780) from Mexico diverged 6.8–7.1 % from other sequences of *Ap. cornu* from the same geographical origin. The large intraspecific genetic divergences between *Au. burti* and *Ap. cornu* sequences respectively are comparable to interspecies divergence values (see below, Fig. 2).

Genetic divergence between congeneric species varied between 7.5 and 15.6 % in the COI (Fig. 2), except within *Apatemon*, if *Apatemon* sp. 1 and 1x (Locke et al. 2010) are considered as distinct species (3.4–4.6 % divergence between them). Three genera (*Apatemon*, *Ichthyocotylurus*, *Parastrigea*), in which intraspecific and interspecific divergence ranges did not overlap, showed an average interspecific divergence 14–19 times higher than the average intraspecific divergence (Fig. 2). Mean interspecific genetic divergence between the closest *Apatemon* lineages (*Apatemon* sp. 1 and *Apatemon* sp. 1x) was just over 8× higher than the mean intraspecific divergence. Two sequences labelled as *Ap. cornu* from Mexico (GenBank IDs JX977777 and JX977779) diverged only 0.2–1.2 % from representative sequences of *Ap. pipientis* from Canada, leading to an overlap between intraspecific and interspecific distances (Fig. 2). Newly sequenced specimens of *Apatemon* and *Australapatemon* did not show intraspecific differences in their ITS and 28S sequences. Interspecific genetic divergence in the ITS region (including the ITS1-5.8S-ITS2 gene cluster) was 0.9–2.1 % in *Apatemon*, the lowest between sequences of *Apatemon* sp. 1 ex *Etheostoma nigrum* Rafinesque from Canada and

A. gracilis ex *Salmo salar* L. from UK; 1.7 % in *Apharyngostrigea*; 1.9 % in *Australapatemon* and 0.8–1.7 % in *Parastrigea*. Genetic divergence in the 28S region between *Australapatemon niewiadomski* n. sp. and the sequence of the unidentified larval stage from *R. ovata* deposited in GenBank was 1.2 %. Average genetic divergence between *Apatemon* and *Australapatemon* was 15.2±1.4 % for the COI and 4.7±0.6 % for the 28S, which fell within the range of variation of other strigeid genera (14.5–21.4 % in COI; 3.1–8.8 % in 28S). For the ITS regions *Apatemon* and *Australapatemon* showed the lowest mean genetic divergence (4.4±0.7 %) of all strigeids considered (4.4–18.4 %).

Family Strigeidae Railliet, 1919

Subfamily Strigeinae Railliet, 1919

Genus *Australapatemon* Sudarikov, 1959

***Australapatemon niewiadomski n.* sp.**

Type - host: *Anas platyrhynchos* L. (definitive host).

Second intermediate host: *Barbronia weberi* (Blanchard) (Hirudinea: Salifidae).

Site of infection: Intestine (definitive host); body parenchyma (second intermediate host).

Prevalence: In 2 out of 2 birds (Manuka Island); in 1 out of 4 birds (Karitane estuary); in 3 out of 4 leeches.

Intensity: In definitive host: 5–137 worms per bird, mean intensity 48.3; in second intermediate host: range 4–6, mean intensity 5.0.

Type - locality: Manuka Island, Clutha River, Otago, New Zealand (46° 11′ 56″ S, 169° 42′ 07″ E, elev. 15 m)

Other localities: Karitane Estuary, Otago, New Zealand (45° 37′ 28″ S, 170° 38′ 10″ E, brackish, sea level) (mallard). Lake Hayes (44° 58′ 30″ S, 168° 49′ 01″ E, freshwater, elev. 315 m) (leech).

Type - material: Holotype MHNG-PLAT-91860; paratypes MHNG-PLAT-91861 (27 specimens).

Representative DNA sequences: 28S rDNA, KT334164-KT334165; ITS1-5.8S-ITS2, KT334173-KT334175; COI, KT334176-KT334180.

Etymology: This species is named after Professor Katarzyna Niewiadomska who is internationally recognised as the authority on members of the digenean superfamily Diplostomoidea.

Description of adult (Fig. 3a–d; Table 2)

[Measurements based on 27 specimens ex *Anas platyrhynchos* L. Measurements all in micrometres. Widths of organs in the forebody and hindbody correspond to their dorso-ventral diameter since the specimens are mounted laterally.]

Total length 1345–1997 (1714±177); body distinctly bipartite; maximum width at level of ventral sucker in forebody. Tegument smooth. Forebody cup-shaped; 452–712 (577±62) long, 361–536 (443±41) wide. Hindbody subcylindrical; widest at level of anterior testis; 888–1412 (1137±135) long, 348–545 (420±44) wide. Ratio of forebody to hindbody length 1:1.6–2.4 (2.0±0.2). Oral sucker subterminal, 103–145 (125±11)×97–145 (122±13). Ventral sucker situated in posterior mid-forebody, 130–217 (181±21)×142–193 (171±15). Sucker length ratio 1:1.1–1.7 (1:1.5±0.2). Holdfast organ composed of two lobes; associated proteolytic gland at base of forebody, level with, or slightly anterior to junction with hindbody, 36–85 (62±12)×79–139 (106±16). Prepharynx absent. Pharynx small, feebly muscular, not easily observed, 55–76 (62±7)×49–72 (55±6). Testes in tandem, large; anterior testis asymmetrical, bilobed, smaller lobe ventral; anterior margin positioned at 18–33 (26±4)% of hindbody; 191–361 (265±41)×169–333 (246±35) at widest point. Posterior testis asymmetrical, bilobed; smaller lobe ventral; posterior margin positioned at 43–82 (70±9)% of hindbody; 193–327 (267±30)×200–385 (240±42) at widest point. Seminal vesicle highly convoluted; dorsal in post-testicular region. Ovary ovoid, transversely elongate, dorsal; positioned 7–21 (16±4)% of hindbody; 91–148 (115±12)×118–194 (157±21). Laurer's canal long, broad and convoluted. Mehli's gland dorsal; anterior to ovary. Vitellaria follicular, confined to hindbody, densely distributed occupying pre-ovarian region and extending posteriorly in two ventro-lateral fields to level of copulatory bursa. Vitelline reservoir intertesticular; median. Uterus extends anterior to ovary, ventral to gonads, with 0–27 (9±6) large eggs, 94–103 (98±3)×55–72 (59±4). Copulatory bursa large with terminal opening. Genital cone well delimited from surrounding parenchyma, one fifth to one ninth of hindbody length; 129–314 (228±46)×131–213 (164±19). Ejaculatory duct joins distal part of uterus at apex of cone. Hermaphroditic duct with internal rugae, runs along central axis of genital cone (see Fig. 3b, c). Ringnapf absent. Excretory vesicle and pore not observed.

Description of encysted metacercaria (Fig. 3d)

Based on two live specimens ex *Barbronia weberi*. Tetracotyle-type metacercariae enclosed in egg-shaped, translucent, laminated cyst. Outer cyst 361–366×255–288; encysted metacercaria 284–306×208–245. Cyst wall thickness varies from 25 to 37, thickest at poles.

Remarks

Of the nine species currently included in the genus *Australapatemon*, only the type species, *A. intermedius* (Johnston, 1904) has been reported from the Australasian region. This species was described parasitising *Cygnus atratus* (Lath.) in New South Wales, Australia (Johnston 1904). There are no records of the genus from New Zealand. The following

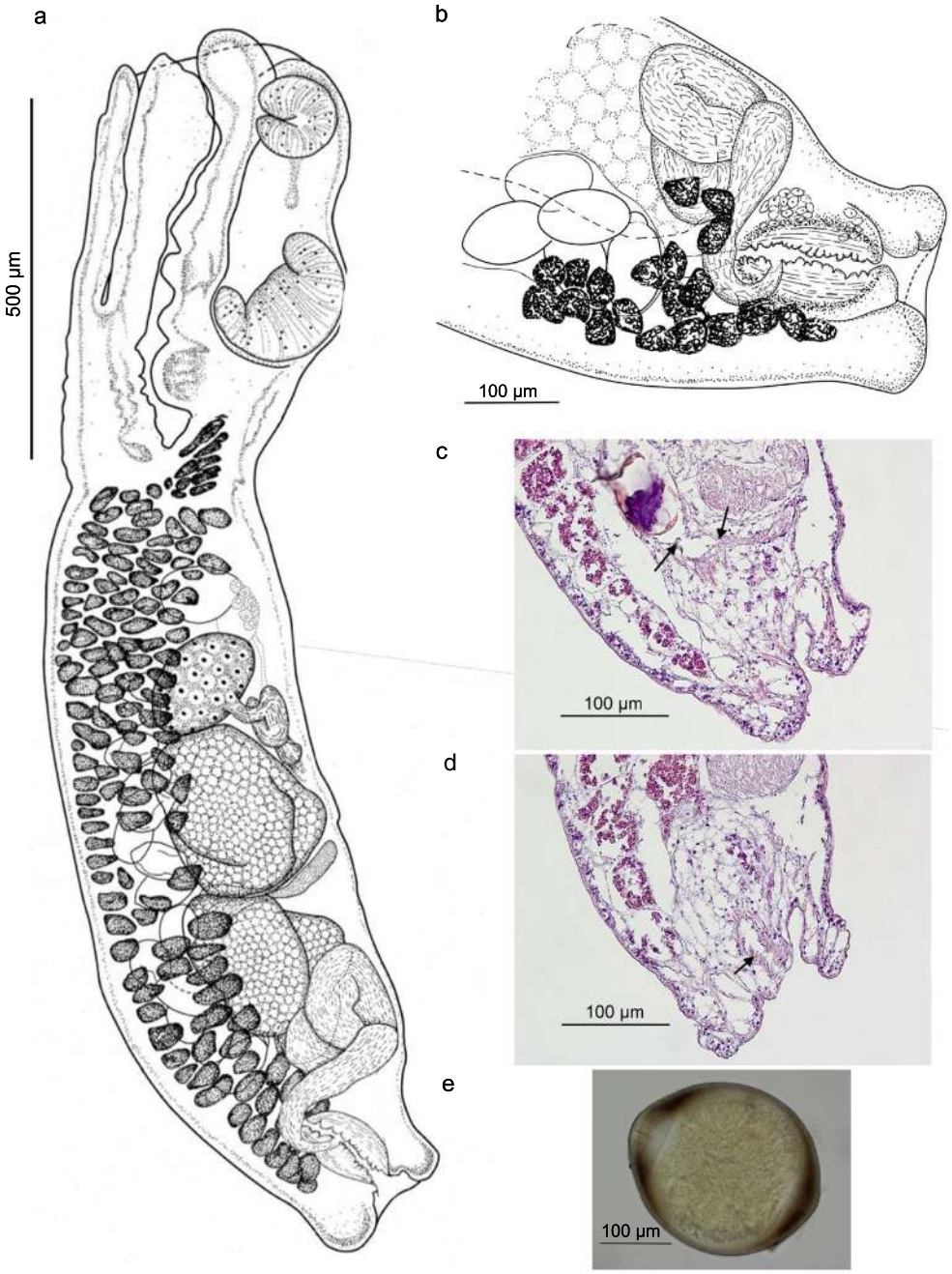

Fig. 3 *Australapatemon niewiadomski* n. sp. **a** Adult (holotype) lateral view. **b** Detail of the terminal genitalia. **c** Histological section of the posterior end of an adult; *left arrow* indicates the entrance of the uterus into the genital cone; *right arrow* points at the connection of the ejaculatory duct to the uterus. **d** Histological section of the posterior end of an adult; an *arrow* points to the muscular fibres of the hermaphroditic duct. **e** Metacercaria photograph

comparative diagnoses are based on original descriptions, or re-descriptions where noted.

The absence of a muscular ring (ringnapf) at the genital atrium distinguishes the adults of *Australapatemon niewiadomski* n. sp. from *Au. canadensis* Dubois & Rausch, 1950, *Au. fuhrmanni* Dubois, 1937, *Au. congolensis* Dubois & Fain, 1956 and *Au. anseris* Dubois, 1967, all of which possess this character. Of the remaining five species (see Table 2), *Au. bdellocystis* (Lutz 1921) and *Au. intermedius* (adult re-described by Dubois and Pearson (1965)) are much larger in size (up to 2.5 and 3.6 mm, respectively) and in most morphometric features. The latter two species have larger forebody, hindbody, oral sucker, pharynx, ovary and testes than *Australapatemon niewiadomski* n. sp. In addition, *Au. bdellocystis* has a distinctive spherical forebody and *Au. intermedius* has a very large genital cone and multilobed testes. *Australapatemon burti* (Miller, 1923) (adult described by Stunkard et al. (1941)) is distinguishable from *Au. niewiadomski* n. sp. morphometrically by having a shorter pharynx (36–45 vs 55–76 in *Au. niewiadomski* n. sp.) and a narrower oral sucker (65–90 vs 97–145 in *Au. niewiadomski* n. sp.) besides a higher upper limit for body length range and lower limits for the range of most other features (forebody, hindbody, suckers and egg length, and ovary).

Table 2 Comparative metrical data for species of *Australapatemon* (adult and metacercarial stages)

	Australapatemon niewiadomski n. sp.	*Au. bdellocystis* (Lutz, 1921)[c]	*Au. burti* (Miller, 1923)[d]	*Au. intermedius* (Johnston, 1904)[e]	*Au. magnacetabulum* (Dubois, 1988)	*Au. minor* (Yamaguti, 1933)
Adult						
Total body length (mm)	1.35–1.99	2.50	1.80–2.50	3.24–3.60	1.08–1.40	0.80–1.20
Forebody length	452–712	800	300–600	1120–1160	420–450	250–460
Forebody width	361–536	800	300–450	860–1000	360–370	340–440
Hindbody length	888–1412	c.1600	700–1300	2240–2530	660–950	540–750
Hindbody width	348–545	c.800	350–500	850–970	270–310	300–400
Oral sucker length	103–145	150 d	90–125	200–250	92–95	80–100
Oral sucker width	97–145	–	65–90	145–170	70–80	60–90
Pharynx length	44–64	100 d	36–45d	85–90	70–73	40–65[c]
Pharynx width	41–60	–	–	65–85	55–68	33–65[c]
Ventral sucker length	130–217	–	–	300–320	130–200	100–120
Ventral sucker width	142–193	200	90–140	270–310	105–170	80–110
Proteolytic gland length	36–85	–	–	65–70	90–95	–
Proteolytic gland width	79–139	–	–	160–180	120–190	–
Ovary shape	Oval	Spherical	Ovoid	Reniform[c]	Reniform	Oval
Ovary length	91–148	–	70–120[c]	210	63–105	90–110
Ovary width	118–194	200	90–165[c]	330	90–115	100–140
Anterior testis length	191–361	400		460–490	75–165	130–180
Anterior testis width	169–333	450	200–300	490–650	105–175	140–210
Posterior testis length	193–327	–	–	400–490	75–190	–
Posterior testis width	200–385	–	–	480–650	120–235	–
Genital cone length	154–224	–	–	640–850	115–165	–
Genital cone width	115–126	–	–	420–500	115–150	–
Egg length	94–103	–	90–100	89–103	100–105	100
Egg width	55–72	–	62–70	62–69	60–63	60
Ovary to body length ratio[a] (%)	7–21	18[b]	12[b]	11	30[b]	6[b]
Anterior testis to body length ratio[a] (%)	18–33	31[b]	24[b]	19[b]	42[b]	19[b]
Posterior testis to body length ratio[a] (%)	43–82	86[b]	70[b]	65[b]	73[b]	73[b]
Forebody to hindbody length ratio	1:1.6–2.4	1:2.5[b]	1:1.1–2.4[c]	1:2.0–2.5[c]	1:2.3[b]	1:1.4–2.6[c]
Oral to ventral sucker width ratio	1:1.2–1.5	1:1.3	1:1.08[b]	1:1.4[b]	1:2.2[b]	1:1.5[b]
Metacercaria						
Cyst outer length	361–366	–	450–607	295–393[f]	–	386–461[c]
Cyst outer width	255–288	–	295–451	246–328[f]	–	318–386[c]
Encysted metacercaria length	284–306	–	–	–	157–221[g]	–
Encysted metacercaria width	208–245	–	–	–	137–189[g]	–
Cyst wall thickness	25–37	–	38–78	–	9–49[g]	–

Body length is given in millimetres; all other measurements are in micrometres

[a] Pre-ovarian, pre-anterior testis or pre-posterior testis field length as a percentage of hindbody length (as in Dubois 1968)

[b] Measured or calculated from published drawings in source literature

[c] Data from Dubois (1968)

[d] Data from Stunkard et al. (1941)

[e] Data in Dubois and Pearson (1965) taken from Johnston's specimens

[f] Data from Johnston and Beckwith (1947)

[g] Data from Davies and Ostrowski de Núñez (2012)

Australapatemon magnacetabulum (Dubois, 1988) and *Au. minor* (Yamaguti 1933) are smaller in size (1.1–1.4 and 0.8–1.2 mm, respectively, vs 1.4–2.0 in *Au. niewiadomski* n. sp.) and ranges for most features (forebody length, hindbody, suckers, proteolytic gland and ovary width and testes length) do not overlap for either species with those for the new

species. Furthermore, *Au. magnacetabulum* has the ovary positioned more anteriorly in the hindbody than *Au. niewiadomski* n. sp. (pre-ovarian field length 6 % of the hindbody length vs 7–21 %), and has been found only in raptors.

The few records of metacercarial cyst measurements in the literature appear to show that cyst size bears little relation to adult worm size. *Australapatemon burti* and *Au. minor*, generally smaller adult worms than *Au. niewiadomski* n. sp., develop in much larger metacercarial cysts (outer cysts 450–607×295–451 and 386–461×318–386 respectively vs 361–366×255–288 in *Au. niewiadomski* n. sp.) (Stunkard et al. 1941; Dubois 1968), whereas the cysts of *Au. intermedius* (the species with the largest adult) measure 295–393×246–328 (as *Cercaria lessoni* in Johnston and Beckwith 1947) a range similar to that of *Au. niewiadomski* n. sp., and the size of the encysted metacercaria in *A. magnacetabulum* is smaller than that in *Au. niewiadomski* n. sp. (157–221×137–189 vs 284–306×208–245). In addition, *Au. burti* has a thicker cyst wall than *Au. niewiadomski* n. sp. (38–78 vs 25–37). The above comparisons of the adults and metacercarial cysts together support the distinct status of *Au. niewiadomski* n. sp.

Family Strigeidae Railliet, 1919

Subfamily Strigeinae Railliet, 1919

Genus *Apatemon* Szidat, 1928

***Apatemon* sp. 'jamiesoni'**

Other names in the literature: *Apatemon* sp. in Blasco-Costa et al. (2013; 2015), Hammond-Tooke et al. (2012), Hechinger (2012), Herrmann et al. (Herrmann and Poulin 2011, Herrmann and Poulin 2012a, 2012b), Hock and Poulin (2012), Kelly et al. (2009), Poulin (2013), Cercaria F1 of Winterbourn (1974).

Hosts: *Anas platyrhynchos* L. and *Phalacrocorax punctatus* (Sparrman) (definitive hosts).

Second intermediate host: *Gobiomorphus cotidianus* McDowall, *Gobiomorphus breviceps* (Stokell) and *Galaxias anomalus* (Stokell).

First intermediate host: *Potamopyrgus antipodarum* (Gray)

Site of infection: Intestine (definitive hosts); body cavity, mesenteries, muscles, liver, gonads, cranial cavity (second intermediate hosts); gonads (first intermediate host).

Prevalence: In one spotted shag out of nine; in one out of one mallard; 100 % out of 77 common bully from Lake Waihola; in 2 out of 3232 *P. antipodarum* from Lake Waihola (0.06 %).

Intensity: In definitive hosts: one in mallard, 40 individuals in spotted shag; in second intermediate host: 9–800 individuals, mean intensity 204 in common bully from Lake Waihola.

Localities: Mount Watkin, Otago, New Zealand (45° 33′ S, 170° 34′ E, elev. 365 m) (mallard); Otago Harbour, New Zealand (45° 79′ S, 170° 71′ E, marine/brackish, sea level) (spotted shag); Tomahawk Lagoon, Otago New Zealand (45° 54′ 02″ S, 170° 32′ 35″ E, freshwater/ brackish, sea level) (snail and bully); Lake Waihola and Waipori, Otago, New Zealand (46° 01′ 12″ S, 170° 05′ 42″ E and 45° 58′ 09″ S, 170° 06′ 49″ E, respectively, brackish, sea level) (snail and bully); plus many other localities throughout New Zealand's North and South Islands.

Voucher material: adult MHNG-PLAT-91644.

Representative DNA sequences: 28S rDNA, KT334166-KT334169; ITS1-5.8S-ITS2, KT334170-KT334172; COI, KT334181-KT334182.

Etymology for the epithet: We distinguish *Apatemon* sp. from other unknown species of *Apatemon* with the epithet 'jamiesoni' in memory of the late Professor Ian Jamieson, our friend and colleague who through his research contributed enormously to bird conservation in New Zealand. Disclaimer: this does not intend to be a nomenclatural act and this name should not be interpreted as a species name.

Description of adult (Fig. 4a; Table 3)

[Based on a single specimen from *Anas platyrhynchos* L. Measurements all in micrometres. Widths of organs in the hindbody correspond to their dorso-ventral diameter since the specimen is mounted laterally.]

Total length 1348; body distinctly bipartite; maximum width at level of ventral sucker. Tegument smooth. Forebody cup-shaped; 545×333. Hindbody subcylindrical; widest at level of first testis; 803×333. Forebody to hindbody length ratio 1:1.5. Oral sucker subterminal (88×91); ventral sucker in posterior mid-forebody (148×139). Oral to ventral sucker length ratio 1:1.7. Holdfast with two lobes; associated proteolytic gland at base of forebody, level with, or slightly anterior to constriction (45×94). Prepharynx absent. Pharynx 58×48. Testes tandem, large; anterior testis round; anterior margin positioned 38 % length of hindbody; 167×152. Posterior testis asymmetrical, bilobed; posterior margin positioned 74 % length of hindbody; 176×136. Seminal vesicle highly convoluted; located dorsally in post-testicular region. Ovary ovoid, transversely elongate, median; positioned 23 % length of hindbody; 130×182. Laurer's canal long, broad and convoluted. Mehli's gland dorsal; anterior to ovary. Vitellaria follicular, densely distributed in hindbody, occupying pre-ovarian region, extending posteriorly in two ventro-lateral fields to level of copulatory bursa. Vitelline reservoir inter-testicular; median. Uterus extends anterior to ovary, ventral to gonads, with three eggs (immature, therefore measurements not given). Copulatory bursa small. Genital cone not delimited from surrounding parenchyma. Hermaphroditic duct rugose, opens close to apex of genital cone.

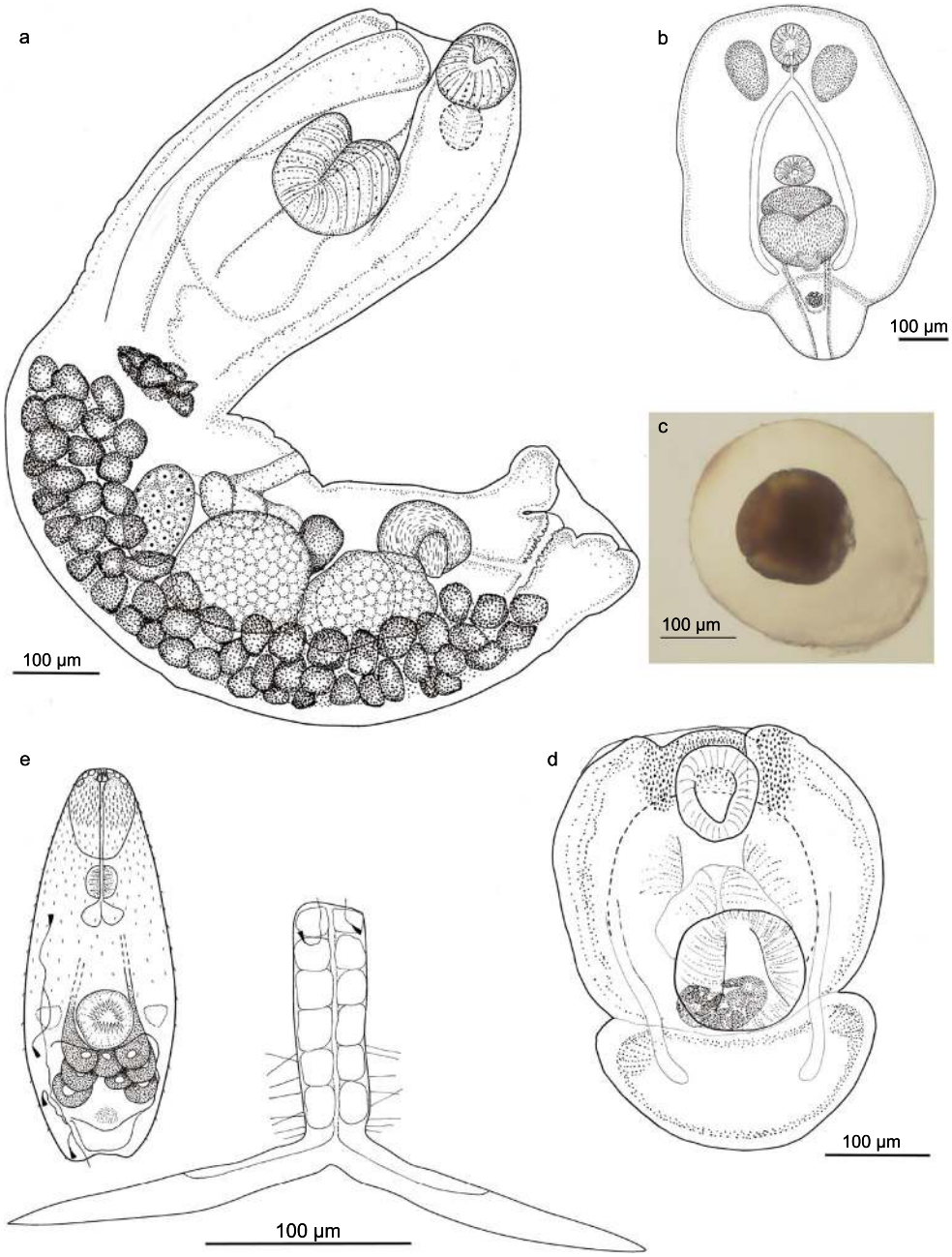

Fig. 4 *Apatemon* sp. 'jamiesoni'. **a** Adult in lateral view; **b** pre-encystment metacercarial stage; **c** encysted metacercaria (photo); **d** excysted metacercaria; **e** cercaria, ventral view

Description of metacercaria (Fig. 4b–d)

Pre-encystment metacercaria: [Based on 25 large stained and mounted specimens in ventral view]. Tetracotyle-type metacercariae found in the body cavity of fish at all stages of growth prior to encystment. Smallest (95×56 μm), are flat, round to oval, newly invaded cercariae without tails and largest (225×135 μm), are thicker, leaf-like, often recurved ventrally; all sizes between these two extremes represented. Largest pre-encystment metacercariae have a small, poorly differentiated hindbody. Paired, glandular pseudosuckers either side of oral sucker, approximately the same width and twice the length of oral sucker. Oral sucker ventro-terminal. Pharynx oval, weakly delimited. Oesophagus short, caeca ending slightly anterior to hindbody. Ventral sucker circular or transverse oval, situated slightly anterior to level of holdfast. Holdfast ventrally protuberant with two lobes. Paired excretory bladders thin V-shape, elongate, opening into single, terminal excretory pore. Genital primordia in median hindbody, visible in older specimens.

Encysted metacercaria: [Based on 38 specimens]. Tetracotyle-type metacercariae in tough egg-shaped, translucent, laminated cyst, thicker at the narrow pole. Outer cyst 601–852×512–647 (719±67.7×573±34.3). Encysted

Table 3 Comparative metrical data for *Apatemon* sp. 'jamiesoni' adult specimen from *Anas platyrhynchos* together with morphologically similar *Apatemon* species taken from the literature

	Apatemon sp. 'jamiesoni'	*A. buteonis* (Yamaguti, 1933)	*A. fuligulae* Yamaguti, 1933	*A. gracilis* (Rudolphi, 1819)[a]	*A. hypseleotris* Negm-Eldin & Davies, 2001	*A. jamesi* Palmieri, Krishnasamy, Sullivan, 1979	*A. somateriae fischeri* Dubois, 1968	*A. somateriae somateriae* (Dubois, 1948)	*A. vitellires-iduus* Dubois & Angel, 1972
Total body length (mm)	1.3	2.5–3.6	1.5-3.0	<2.5	1.6–2.5	1.1–1.5	<2.8	<3.3	2.2–2.6
Forebody length	545	900–1000	500–1000	400–720	600–1000	180–400	630–900	510–1200	710–900
Forebody width	333	700	400–700	340–540	400–600		560–840	510–750	510–640
Hindbody length	803	1600–2400	1000–2000	930–1800	1000–1600	800–1040	1120–1920	960–2100	1480–1700
Hindbody width	333	500–600	400–700	430–600	400–650		530–800	570–840	500–560
Oral sucker length	88			110–180		78–87	160–220	135–200	115–160
Oral sucker width	91	100–110	90–150	80–140	120–140	37–51	140–200	110–170	110–127
Pharynx length	58	110	50–62	60–80	45–68		100–150	80–130	85–93
Pharynx width	48	88	57–88	52–80	51–60		90–140	75–120	70–75
Ventral sucker length	148	140	140–200	180–255	140–170	74–147	230–280	170–300	190–210
Ventral sucker width	139	160	150–175	110–245	130–180	92–129	190–250	190–315	165–180
Proteolytic glands length	45				100	41–55			65–80
Proteolytic glands width	94				100	46–83			60–70
Ovary	Oval	Reniform	Oval	Oval	Reniform/bilobed	Round/subround	Oval	Oval	Oval
Ovary length	82	150–250	100–150	110–130	100–130	87–110	130–150	120–180	140–150
Ovary width	130	200–250	140–250	150–180	130–200	60–101	190–210	140–235	180–190
Anterior testis length	167	200–400	250–380	210–380	300–450	129–221	350–460	270–470	260–330
Anterior testis width	152	250–400	200–440	270–360	350–450	115–212	270–490	330–470	240–300
Posterior test length	176	no data	350	270–435	320–450	120–239	360–570	390–570	300–370
Posterior testis width	136	no data	230	250–340	320–400	83–193	270–480	340–530	260–300
Ovary to body length ratio[b] (%)	23	31[c]	23[c]	38[c]	16[c]	24[c]	15[c]	19–26	27–36
Anterior testis to body length ratio[b] (%)	38	43[c]	30[c]	51[c]	21[c]	37[c]	26[c]	31[c]	39[c]
Posterior testis to body length ratio[b] (%)	74	77[c]	78[c]	81[c]	80[c]	73[c]	77[c]	79[c]	81[c]
Forebody to hindbody length ratio	1:1.5	1:1.8[c]	1:2.4[c]	1:2.8[c]	1:1.8[c]	1:1.9[c]	1:2.4[c]	1:1.5–2.7	1:1.9–2.1[c]
Oral to ventral suckerlength ratio	1:1.9	1:1.7[c]	1:1.6[c]	1:2.0[c]	1:1.0[c]	1:1.3[c]	1:1.3[c]	1:1.2[c]	1:1.4[c]
Pharynx to oral sucker length ratio	1:1.4	1:1.1[c]	1:1.8[c]	1:2.0[c]	1:2.3[c]	1:2.5[c]	1:1.3[c]	1:1.6[c]	1:1.56[c]
Proteolytic gland width to length ratio	1:0.5	1:0.7[c]	1:0.9[c]	1:0.6[c]	1:0.6[c]	1:0.7	1:0.4[c]	1:0.4[c]	1:0.6[c]

Body length is given in millimetres; all other measurements are in micrometres

[a] Data from Dubois (1968)

[b] Pre-ovarian, pre-anterior testis or pre-posterior testis field length as a percentage of body length

[c] Ratios inferred from measuring drawings in source literature

metacercaria 291–380×293–355 (340±20.5×318±15.5). Thickness of cyst wall 28–40 (34±3.4) at sides, 31–51 (41±5.0) at rounded pole and 50–100 (71±12.7) at narrow pole.

Excysted metacercaria: [Based on two stained and mounted excysted specimens]. Body (total length 397–398) divided into two distinct regions. Forebody cup-shaped; 289–290×271–306. Hindbody arises from posteroventral part of the forebody and is bluntly rounded at the posterior extremity; 108–110×208–255. When extended, body elongated scoop-shaped, but when contracted the forebody adopts a cup-like shape. Pseudosuckers anterodorsal or lateral to oral sucker depending on state of contraction of specimen. Oral sucker (85–90×80–90) sub-terminal; pharynx conical, weakly-muscular. Intestinal caeca extend almost to extremity of hindbody. Ventral sucker at the base of forebody; circular to transversely elongate, larger than oral sucker; 121–137 wide. Holdfast posterior to the ventral sucker, consisting of two large lobes, which can be more or less protruded; 171–221×166–196. Proteolytic gland situated at base of holdfast. Genital primordia observed as strongly-stained cells in centre of hindbody. Excretory system not observed.

Description of cercaria (Fig. 4e)

[Based on photographs of 14 live-stained specimens]. Typical strigeid furcocercaria. Body of the same length or slightly longer than tail stem, 85–195×33–70 (121±33.8×55±12.4); tail shorter than furcae, with six pairs of caudal bodies in tail stem; tail 61–134×19–47 (104±22.0×32±7.5); furca 73–157 (129±25.1). Spines on entire body, sparser towards posterior extremity; 5–10 pre-oral spines forming apical tuft; post-oral spines arranged in ca. 9 rows reaching to the mid-length of anterior organ, larger than body spines; long (ca. 9 μm) tail spines on posterior one third of tail stem. Mouth subterminal. Terminal anterior organ 21–53×16–39 (37±9.1×27±6.6), larger than ventral sucker. Ventral sucker post-equatorial, 10–23×11–24 (17±3.6×18±3.5), with three or four rows of 10–16 spines. Penetration gland-cells four pairs, posterior to ventral sucker. Colourless eye-spots, oval to sub-triangular (ca. 13×9), lateral and level with anterior margin of ventral sucker. Prepharynx 27–48 (35±7.3) long, pharynx round to oval, 8–19×8–16 (12±2.4×11±1.3); oesophagus very short; caeca bilobed, sacculate, terminating some distance from anterior margin of ventral sucker. Genital primordia between penetration gland-cells and excretory bladder, indistinct. Excretory bladder bilobed, V- or U-shaped. Flame cell formula 2[(1+1)+(1+1+[1])]=10; excretory system with transverse commissure posterior to ventral sucker; caudal flame cells at level of first pair of caudal bodies.

Remarks

Of the species currently included in the genus *Apatemon*, three have been reported from Australia. *Apatemon hypseleotris* was described from the western carp dudgeon *Hypseleotris klunzingeri* Ogilby, an eleotrid fish closely related to *Gobiomorphus* Gill (Thacker and Hardman 2005) and *A. vitelliresiduus* was described parasitising the Musk duck *Biziura lobata* (Shaw) (Johnston 1904). In addition *A. gracilis* was reported from the Black duck *Anas superciliosa* Gm. (Smith and Hickman 1983). There are no records of the genus from New Zealand.

As a basis for comparison, we take the species of *Apatemon* as designated by Dubois in his work of 1968, and accept the synonyms therein. Consequently we recognise the four species designated by Dubois (1968) (*A. buteonis* (Yamaguti, 1933), *A. fuligulae* Yamaguti, 1933, *A. somateriae* Dubois, 1948, *A gracilis*). In addition, we recognise three further species described after 1968 (*A. jamesi* Palmieri, Krishnasamy & Sullivan 1979, *A. vitelliresiduus* and *A. hypseleotris*). *A. annuligerum* was convincingly shown to be synonymous with *A. gracilis* from the UK (Bell and Sommerville 2002). *Apatemon indicus* Vidyarthi, 1937 (synonymised with *A. casarcus* Vidyarthi, 1937 by Dubois (1968)), *A. japonicus* Ishii, 1932, *A. graciliformis* Szidat, 1928 and *A. parvitestis* Ishii, 1935 were all designated *species inquirendae* by Dubois (1953; 1968) and we have not used them for comparison herein. The following comparative diagnoses are based on original descriptions, or re-descriptions where noted.

The formal description of *Apatemon* sp. 'jamiesoni' requires further collection of adult specimens in a reasonable state of preservation to document the morphological variability of the species since the specimens found in the accidentally dead spotted shag were too degraded. Nonetheless, we distinguish this first identified adult specimen of *Apatemon* in mallards from New Zealand as follows. *Apatemon* sp. 'jamiesoni' is characterised by its relatively small overall size and the comparatively small size of its ovary and testes. This *Apatemon* species is smaller in body size and in most other features than *Apatemon gracilis* (as re-described by Dubois (1968)), *A. hypseleotris*, *A. somateriae somateriae*, *A. s. fischeri*, *A. vitelliresiduus*, *A. buteonis* and *A. fuligulae* (see Table 3). Notably, the length of the ovary and size of the testes of *Apatemon* sp. 'jamiesoni' lie outside the range of all the above species. Likewise, *Apatemon* sp. 'jamiesoni' measures lie outside of those for the suckers and pharynx of *A. s. somateriae*, *A. s. fischeri* and *A. vitelliresiduus*. In addition, *A. vitelliresiduus* has vitellaria that extend into the forebody and *A. buteonis* has post-equatorial testes and a ringnapf. Moreover, *A. gracilis* has metacercarial cysts that are lemon-shaped as opposed to the egg-shaped cysts of *Apatemon* sp. 'jamiesoni' and *A. hypseleotris* and *A. fuligulae* have notably

smaller cysts (430–570×320–460 and 385×200, respectively). The species that most closely resembles *Apatemon* sp. 'jamiesoni' is *A. jamesi*. However, although the body size of these two species is similar, *Apatemon* sp. 'jamiesoni' has a larger forebody compared to the hindbody and round suckers (see Table 3) instead of elongate oval as in *A. jamesi*. Furthermore, *A. jamesi* is unusual in having a leech second intermediate host.

***Apatemon hypseleotris* Negm-Eldin & Davies, 2002**

Host: ex *Columba livia* Gm. (exp.).

Locality: Victoria, Australia.

Material re-examined: Museum of Victoria, Melbourne; accession numbers F84195 (holotype, metacercaria) and F84213 (paratype, experimentally grown adult) and other material deposited by the authors (F84196-F84212).

Remarks

On our examination of the original material, we observed that the ovary on one specimen appears kidney shaped as described by Negm-Eldin and Davies (2002), but this does not apply to all specimens, which instead are oval. In addition, we observed the seminal vesicle forming three-dimensional convolutions that we believe the authors interpreted equivocally as constrictions when viewed on a single plane. It is feasible that the seminal vesicle has been misinterpreted, as the mounted specimens are not at all clear and detail is difficult to see.

Discussion

This study successfully resolves the life cycles of two strigeid species by using molecular tools and provides morphological descriptions of the adults and larval stages of *Australapatemon niewiadomski* n. sp. and *Apatemon* sp. 'jamiesoni'. *Australapatemon niewiadomski* n. sp. is distinguished morphologically by the absence of a ringnapf and its overall smaller size than most other *Australapatemon* spp., except *Au. magnacetabulum* and *Au. minor*, which are smaller and whose ranges for most features do not overlap with the new species. In addition, *Au. niewiadomski* n. sp. is also molecularly distinguished from *Au. burti* and its metacercariae and intermediate host identified via matching of molecular sequence data. Developmental stages of the metacercariae of *Apatemon* sp. 'jamiesoni' and its cercariae are described in detail. Its adult, present in both *A. platyrhynchos* and *P. punctatus*, is identified by linking molecularly sequence data with the other life cycle stages. *Apatemon* sp. 'jamiesoni' uses a different species of first intermediate snail host to other *Apatemon* spp. and exhibits consistent molecular differences in the genetic markers examined. Notwithstanding that the formal description of *Apatemon* sp. 'jamiesoni' awaits collection of additional adult specimens; altogether, this evidence confirms the distinct species status of these two strigeid species from New Zealand.

Furthermore, we confirm the status of *Apatemon* and *Australapatemon* as distinct genera based on their respective monophyly for the three molecular markers considered and genetic divergence between them that is comparable to other well-established genera in the family. We noted an error in the terminology employed for the duct that runs along the genital cone in the generic diagnosis of *Australapatemon* (Niewiadomska 2002). We observed in our specimens that the ejaculatory duct joins the uterus at the apex of the genital cone (Fig. 3b); thus, the duct running along the genital cone should be the hermaphroditic duct as it also carries both the eggs and the sperm. Our observation is in agreement with the diagnosis presented in Dubois (1968) for the subgenus *Australapatemon*. We proposed an amended diagnosis as follows:

Genus *Australapatemon*

Body bipartite; forebody globular, cup- or bell-shaped, with holdfast organ composed of two lobes; hindbody elongate saccular, with neck region absent. Oral and ventral suckers well developed; ventral sucker larger than oral. Testes tandem, irregular in shape, in middle or anterior half of hindbody. Ovary round, oval or reniform. Vitellarium confined to hindbody. Copulatory bursa large with terminal opening. Genital cone large (one third to one fifth of hindbody length), encloses long hermaphroditic duct with internal rugae formed by union of distal part of uterus and ejaculatory duct, opens close to apex of cone. In anseriform birds. Cosmopolitan. Metacercariae of 'tetracotyle' type, in leeches. Cercariae with flame cell formula 2[(1+1)+(2+2+[1])]=14; excretory commissures anterior and posterior to ventral sucker, may be incomplete; penetration glands in two groups of four, posterior to ventral sucker; alimentary system well developed.

Despite molecular differences between *Apatemon* and *Australapatemon*, the morphological distinction is not always clear-cut, the most notable example being that of *A. hypseleotris*. *A. hypseleotris* was distinguished from its morphologically closest congener (*A. gracilis*) by a kidney shaped ovary and bipartite, or strongly constricted seminal vesicle (Negm-Eldin and Davies 2002). The authors also use the distinction that *A. hypseleotris* can develop in both leeches and fish as metacercariae. On our examination of the original material, it is clear that, while the ovary on one specimen appears kidney shaped, this does not apply to all specimens. The apparently bipartite seminal vesicle we believe is an error of interpretation, where the three-dimensional convolutions of the seminal vesicle can appear to be constricted when viewed in a single plane. The apparent ability of *A. hypseleotris* to develop in both leeches and fish is possibly unique among

species of the *Apatemon* group. Indeed, experimental infections using various taxa have only ever been able to successfully produce metacercariae in either one or the other host group. Thus, cercariae of *Australapatemon* have only been developed experimentally in leeches (Stunkard et al. 1941; Johnston and Angel 1951; McCarthy 1990), and those of *Apatemon* only develop in one or more species of fish (Vojtek 1964; Blair 1976). Negm-Eldin and Davies (2002) stated that encysted cercariae were found naturally in fish, but not in leeches, and it seems reasonable to assume (notwithstanding the experimental results) that the fish is their natural second intermediate host, all of which supports the placement of *A. hypseleotris* in *Apatemon*. However, all the above being said, the cercariae described in the same paper display all the characteristic features of *Australapatemon*, having seven caudal bodies, long caeca reaching to below the ventral sucker and a total of 14 flame cells, and the authors compare their cercariae closely to species now placed in *Australapatemon*. Further collection and molecular characterisation of this apparently aberrant species may elucidate its position once and for all.

We also found that Coleman's unpublished sequences of *Apatemon* spp. ex *Galaxiella pusilla* from Australia were distinct to all other *Apatemon* spp. (or lineages) sequenced to date, including their geographically closest counterpart from New Zealand. Although none of the three *Apatemon* spp. reported from Australia (see above) has been found in a galaxiid host before, A. *hypseleotris* is able to infect distantly related fish host (eleotrids, salmonids, poeciliidae) at least experimentally (Negm-Eldin and Davies 2002) and the intermediate host of *A. vitelliresiduus* is still unknown. Thus, the Australian sequences reported here could correspond to the two *Apatemon* spp. (*A. vitelliresiduus*, *A. hypseleotris*) described on the basis of morphology alone from Australia. However, comparative morphological and molecular data from these species will be necessary to confirm it.

The number of genetically distinct COI lineages (putative species) of *Apatemon* almost equals the number of morphospecies regarded as valid to date (see remarks section above) even though only one named species (*A. gracilis*) has been molecularly characterised. The global distribution of *Apatemon* spp. and the broad host range of some known species (according to records based on morphology alone) lead us to think that a lot more species may exist than are currently recognised. Likewise, *Australapatemon* species, as well as a number of other strigeids, may have been underestimated. For instance, based on morphology *Au. burti* seems to have a wide geographical distribution (Holarctic and Neotropic) and low host specificity (infects at least nine species of *Anas* L., *Aythya affinis* Eyton and *Oxyura jamaicensis* Gmelin) (e.g. Dubois 1968; Drago et al. 2007; Hinojosa-Saez et al. 2009; Hernández-Mena et al. 2014). Originally described in North America, *Au. burti* has been reported among the most common cercariae occurring in pulmonate snails in Europe but often as *A. gracilis* (see Faltýnková et al. 2007 and references within). Nonetheless, *Au. burti* adults have never been found in the Palaearctic and all records in this region are based on identification of the cercarial larval stage (Faltýnková and Haas 2006; Faltýnková et al. 2007; Soldánova et al. 2010; Tolstenkov et al. 2012). It seems plausible that future molecular studies of *Au. burti* as currently recognised on the basis of its morphology will uncover a number of cryptic species. Based on genetic divergence, adult specimens identified as *Apharyngostrigea cornu* from US and Mexico (Locke et al. 2011; Hernández-Mena et al. 2014) could also represent two distinct cryptic species. The use of multiple genetic markers to corroborate genetic differences in COI and the deposition of voucher material in appropriate collections would help clarifying the taxonomy of the group. Overall, our results emphasise the need for further analyses of patterns of intraspecific and interspecific variation based on the implementation of an integrative taxonomy (Dayrat 2005; Will et al. 2005) to enhance the re-evaluation of strigeid species and advance our understanding of their relationships, distribution and host specialisation of this cosmopolitan trematode group.

Linking life cycles of strigeids

The utilisation of molecular data for inferring complete life cycles of trematodes (Criscione et al. 2005; Pérez-Ponce de León and Nadler 2010) has proved successful and is rapidly increasing (e.g. Cribb et al. 1998; Cribb et al. 2011; Locke et al. 2011; Galaktionov et al. 2012; Georgieva et al. 2012; Georgieva et al. 2013; Presswell et al. 2014; Chibwana et al. 2015). The life cycle stages of *Apatemon* sp. 'jamiesoni' and *Au. niewiadomski* n. sp. are here confirmed by genetic concurrence of three molecular markers. Most trematodes are highly specific to their first intermediate host; thus, it is striking to find in the literature that a single species such as *A. gracilis* is able to infect snail species belonging to at least six different families (Acroloxidae, Bithyniidae, Lymnaeidae, Physidae, Planorbidae and Viviparidae; see Yamaguti (1975)). This equals the number of different host families infected by all other strigeids put together (approximate estimation based on data from Yamaguti (1975)). To the best of our knowledge *Apatemon* sp. 'jamiesoni' is the first strigeid reported to infect tateiid snails, which extends the first intermediate host range known for *Apatemon* and the Strigeidae. We envisage the discovery of a number of cryptic species when, for instance, molecular characterisation of *A. gracilis* cercariae from different snail intermediate hosts are carried out. Furthermore, species belonging to genera studied here represent an example of the difficulty of delimiting species that exhibit high intraspecific morphological plasticity and limited interspecific, even intergeneric morphological differentiation. In such scenarios, data on multiple life history stages and accurate patterns of

host specialisation and distribution besides concurrent molecular and morphological evidence will be highly useful for an integrative taxonomical approach towards the elucidation of species diversity and a meaningful classification of this group.

While we searched for adults of *Apatemon* sp. 'jamiesoni' in mallards, we also discovered a hitherto unreported species of *Australapatemon* which metacercaria was subsequently discovered in non-native freshwater leeches, *Barbronia weberi*. Can we elucidate the biogeographical history of this *Australapatemon* species from what we know of its life cycle? Mallard ducks became established in New Zealand after the 1940s, largely from descendants of UK-sourced introductions and some US-sourced mallards that possibly originated from game-farm mallards originally imported from Europe (Guay et al. 2015). The fact that *Au. niewiadomski* n. sp. uses two non-native hosts, the leech and the mallard, poses an interesting question. Is the species native to New Zealand or was it introduced from either Europe or US? *Au. niewiadomski* n. sp. could have been already present in native grey ducks (*Anas superciliosa* Gmelin; that started to hybridise with mallards soon after their introduction and are currently on their way to extinction (Guay and Tracey 2009)), or it may have been introduced with mallards from either Europe or USA. Whatever the truth is, our study provides the first record of any *Australapatemon* and *Apatemon* spp. in New Zealand birds (see McKenna (2010)). Unfortunately, nowadays it would be very difficult to obtain permits to examine native duck species. In addition to the definitive host, evaluation of intermediate host specificity towards other native freshwater leeches as well as the identity of the first intermediate host (native or introduced gastropod) could shed some light on the validity of these hypotheses. Establishing the phylogenetic relationships among *Australapatemon* species and prospecting for new species in neighbouring countries such as Australia and the Pacific islands in the future will also be useful since the current molecular dataset is very limited and does not allow biogeographical inferences. Undoubtedly, unravelling life cycle stages of parasites will improve our understanding of their roles in ecosystems, their transmission pathways (Poulin et al. 2014) and perhaps as in this case, the origin of these species interactions.

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