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ORIGINAL PAPER

Molecular and morphological evidence for nine species in North American *Australapatemon* (Sudarikov, 1959): a phylogeny expansion with description of the zygocercous *Australapatemon mclaughlini* n. sp.

Michelle A. Gordy[1] · **Sean A. Locke**[2] · **Timothy A. Rawlings**[3] · **Angela R. Lapierre**[4] · **Patrick C. Hanington**[1]

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Abstract Zygocercous (aggregating) cercarial larvae were recently discovered emerging from a physid snail during a molecular survey of cercariae from molluscs in lakes in central Alberta, Canada. This manuscript delves into the characterization of these cercariae through morphological and molecular techniques and provides the first genetic information for a zygocercous larval trematode. Analyses of cytochrome *c* oxidase I of mitochondrial DNA and two partial regions of nuclear ribosomal DNA sequences revealed the zygocercous cercariae to belong to the genus *Australapatemon* Sudarikov, 1959. Further analyses of sequences of *Australapatemon burti* (Miller, 1923), from cercariae and adults collected from across North America, indicate a complex of nine genetically-distinct lineages within this species, a surprising level of diversity. The zygocercous cercariae, along with adult worms collected from ducks in Manitoba, Canada, and from Mexico, represent one of these lineages, and are herein described as *Australapatemon mclaughlini* n. sp. Seven lineages cannot yet be identified, but one is tentatively identified as *Australapatemon burti*.

Keywords *Australapatemon* · Strigeidae · Anatidae · Pulmonata · Phylogenetics · Zygocercous

Introduction

Reports of digenetic trematodes with zygocercous cercariae, often referred to as "Rat King", "Rattenkönig" or "Aggregacercaria", are rare. In the past century (since 1888), there have been only 11 descriptions of aggregating cercariae. Aggregation is a unique behavioural adaptation that results in the joining together of several to hundreds of individual cercariae into bundles or nets (Beuret and Pearson 1994; Cable 1956, 1963; Cable and McLean 1943; Dronen 1973; Hendrickson and Kingston 1974; Komiya 1941; Martin 1968; Martin and Gregory 1951; Miller 1929, 1930; Pintner 1891; Ward 1916; Wardle 1988). Zygocercous cercariae join by the tails to form rosettes, like the "Rat King" phenomenon from which they were originally described, or pine cone-like structures (reviewed in Suppl. Table 1). This adaptation is believed to assist cercariae in being consumed by their next host, thought to be a fish, based on a few experimental studies (Dronen 1973). Little information regarding life cycle progression or host use is available for zygocercous specimens described to date. Moreover, there are no available genetic resources for any of these cercariae, resulting in limited understanding of their phylogenetic affinity, beyond tentative identifications at the family level.

The present study began with a morphological and molecular characterization of zygocercous cercariae emerging from the snail, *Physella gyrina* (Say, 1821), collected from a lake in

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✉ Patrick C. Hanington
pch1@ualberta.ca

[1] University of Alberta School of Public Health, 357F South Academic Building, Edmonton, AB T6G 2G7, Canada

[2] Department of Biology, University of Puerto Rico at Mayagüez, Box 9000, Mayagüez 00681–9000, Puerto Rico

[3] Department of Biology, Cape Breton University, 1250 Grand Lake Road, PO Box 5300, Sydney, NS B1P 6L2, Canada

[4] Department of Biology, Concordia University, 7141 Sherbrooke West, Montreal, QC H4B 1R6, Canada

central Alberta. The morphology of these cercarial larvae was compared to that of other zygocercous larval trematodes in the literature, and to close relatives identified through mitochondrial cytochrome *c* oxidase 1 (*cox1*) DNA sequences. Sequence comparisons suggested the zygocercous cercariae belonged to *Australapatemon burti* (Miller, 1923), based on an adult trematode sequenced by Hernández-Mena et al. (2014), although the zygocercous forms we collected displayed morphological differences to cercariae of this species (Miller 1923, 1926; Cort and Brooks 1928; Stunkard et al. 1941). An expanded sampling effort provided additional *cox1* sequences from cercariae collected during a large-scale survey of digeneans in central Alberta (Gordy et al. 2016) and from cercariae and adult worms collected across North America. These results placed the zygocercous cercariae in one of nine genetically distinct lineages of *Australapatemon*, matching sequence from adult *A. burti* from ducks sampled by Hernández-Mena et al. (2014) in Mexico. The morphological differences between the zygocercous and non-zygocercous cercariae, along with morphology and host-use of adults in Manitoba and Mexico, further corroborated the distinctions among lineages identified in *cox1* comparisons. These findings indicate hidden species diversity within *Australapatemon*. Among the nine lineages distinguished herein, one is tentatively identified as *A. burti* (Miller, 1923), and one is a new species of *Australapatemon*.

Methods

Specimen collection

Most data reported here are based on cercariae from snails collected as part of a parasitological survey of several lakes in central Alberta (see methods within Gordy et al. 2016), from June 2013 to September 2015. On July 13th and August 10th, 2015, collections from Rochon Sands Provincial Park at Buffalo Lake (52.4638361 N, −112.8843833 W) yielded two *P. gyrina* snails infected with a trematode with zygocercous type cercariae. These snails were placed in small plastic containers with artificial spring water (ASW) (Ulmer 1970), fed red leaf lettuce ad libitum, and monitored over several days to count and capture emerging cercariae. One individual cercaria of an aggregate was separated in a dilute solution of tricaine mesylate, used to relax the aggregate, before using fine forceps to pull it apart. The aggregates were otherwise impossible to separate. The individual cercaria was wet mounted and photographed using the Zeiss Axio Imager.A2 compound microscope and mounted Zeiss AxioCam MRc camera. The number of zygocercous aggregates per day was recorded, and finally, both cercariae and snail were preserved in 100% ethanol for later analyses. A permanent mount was prepared for the zygocercous cercariae aggregates using Grenacher's Borax-Carmine stain and mounted in Canada balsam. Drawings were made from photographs of wet-mounted specimens.

Additional material was obtained from gastropod and avian hosts elsewhere in North America. The latter included cercariae from planorbid snails at two localities in California (Santa Clara area, sampled in August 2009 and Pleasanton area, June 2009) and cercariae from *Helisoma campanulatum* (Say, 1821) from a lake in Cape Breton, May 2012. In addition, we included data from adult worms from Anatidae (Anserinae: *Anas acuta* (Linnaeus, 1758) (n = 2), *Aythya collaris* (Donovan, 1809) (n = 1), *Bucephala albeola* (Linnaeus, 1758) (n = 1), and Anatinae: *Oxyura jamaicensis* (Gmelin, 1789) (n = 1)) collected from the southern end of Lake Manitoba, Manitoba, Canada in 2008 and 2009. In the latter collections, live adults and cercariae from freshly killed hosts were placed directly into 70–95% ethanol.

Voucher samples of permanent mount slides for the zygocercous cercariae and for several representative adult worms, including type material, were donated to the Royal Alberta Museum, in Edmonton, Alberta, Canada.

Molecular analyses

Cercariae collected in Alberta were initially identified by partial sequencing of the mitochondrial *cox1* gene, using primers Dice1F and Dice11R for amplification, and a shortened version of these primers for Sanger sequencing (Van Steenkiste et al. 2014), as previously described (Gordy et al. 2016). Additionally, partial large subunit (28S) and internal transcribed spacer regions (ITS1–5.8S–ITS2) of ribosomal DNA (rDNA) sequences were generated for select specimens to include in phylogenetic analyses. Universal primers BD1 and BD2 were used to amplify ITS1–5.8S–ITS2 sequences as previously described for *Clonorchis sinensis* (Looss, 1907) (Tatonova et al. 2012). Sequences for 28S rDNA were amplified as previously described for other trematodes (Gordy et al. 2016). *Cox1* fragments from cercariae and adults obtained outside Alberta were amplified and sequenced as described by Moszczynska et al. (2009). Sequences of rDNA from non-Albertan specimens were generated using primers and protocols in Littlewood and Olson (2001) (for 28S) and Galazzo et al. (2002) for ITS1–5.8S–ITS2. All newly generated sequences were submitted to GenBank (accession numbers: HM385485–HM385486, HM385534–HM385538, KY207548–KY207628, KY570946–KY570948, KY587394–KY587403, KY587405–KY587406, MF124269–MF124270).

Our molecular analysis built on a recent phylogeny of *Australapatemon* Sudarikov, 1959 and *Apatemon* Szidat, 1928 (Blasco-Costa et al. 2016). The *cox1,* 28S, and ITS strigeid sequences used and generated by Blasco-Costa et al. (2016) were separately aligned with newly obtained

sequences using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar 2004) in Geneious v.10.0.5 (http://www.geneious.com, Kearse et al. 2012). Alignments were trimmed to the shortest available sequence prior to phylogenetic analyses (*cox1*: 408 nt; 28S: 809 nt; ITS: 525 nt). MEGA7 was used for model testing, initial maximum likelihood (ML) analyses, and *p* distance calculations (Kumar et al. 2016). Model selection for each dataset was based on ML fits of 24 different nucleotide substitution models, with the best model of evolution being that with the lowest BIC score (Bayesian information criterion). The models of nucleotide evolution used for ML trees in MEGA7 were HKY + G + I (*cox1*) and K2 + G (28S and ITS) and tree nodes were assessed with 1000 bootstrap replicates. All ML analyses in MEGA7 employed four discrete gamma categories, used complete deletion if there were gaps/missing data and inferred trees using nearest-neighbour-interchange as the heuristic method, while initial trees were generated automatically with the neighbour-joining method. Tree options in Geneious were slightly different; thus, the second-best models were used, namely HKY85 + G (*cox1*) and JC69 + G (28S and ITS). The PhyML plugin (Guindon et al. 2010) in Geneious was used to test the robustness of initial ML trees generated in MEGA7. The following options were selected to run these analyses: bootstrap branch support with 1000 replicates, transition/transversion ratio estimated, proportion of invariable sites estimated, number of substitution categories was four, gamma distribution parameter estimated, optimized for topology/length/rate, and using an SPR topology search. The MrBayes plugin v. 3.2.6 (Huelsenbeck and Ronquist 2001) in Geneious was used for Bayesian inference (BI) analyses. All BI trees were constructed from two independent MCMC runs of four chains (temp 0.2) for 10^7 generations, sub-sample frequencies of 10^4 generations, and burn-in of 10^5 generations, per the standard deviation of split frequency values (<0.01). A consensus topology and nodal support, estimated as posterior probability values, were generated from trees remaining beyond the burn-in period (Huelsenbeck and Ronquist 2001). The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.652, as estimated by PhyML).

The web app for Automatic Barcode Gap Discovery (ABGD; wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) was used to test hypotheses of lineage separation (Puillandre et al. 2012), coupled with a priori assumptions of species differentiation, using a 5% cut-off value for *cox1* (Vilas et al. 2005). Default values were applied to a *p* distance matrix input into the ABGD program. The ABGD method has been used previously as a supportive tool for delimiting species among trematodes of the families Clinostomidae (Locke et al. 2015b; de León Pérez-Ponce et al. 2016), Diplostomidae (Locke et al. 2015a) and Opecoelidae (López et al. 2015; Oliva et al. 2015).

Morphological analyses of cercariae

The zygocercous cercariae and other cercariae (representative samples from each following intermediate host: *Stagnicola elodes* (Say, 1821), *Helisoma trivolvis* (previously named *Planorbella trivolvis* (Say, 1817)) and *Pysella gyrina*) with high nucleotide identity (>95%) to *A. burti* sequences, matched through BLAST (tblastn), were selected for morphological analyses. Samples of cercariae stored in 95–100% ethanol were imaged using a scanning electron microscope (SEM) (Model XL30 by FEI Company North America NanoPort, 5350 NE Dawson Creek Drive Hillsboro, Oregon 97,124 USA). Samples were prepared by first transferring to a 0.2-μm GTTP membrane atop a 25-mm swinnex filter holder with o-ring (Millipore). While the membrane was still wet, the top of the filter holder was tightened into place. Then, using a 1-ml luer lock syringe, the samples were taken to 100% hexamethyldisilazane (HMDS) through the following series: 1 ml of 100% ethanol (twice), 1 ml of 75% ethanol:25% HMDS, 1 ml of 50% ethanol:50% HMDS, 1 ml of 25% ethanol:75% HMDS, and 1 ml of 100% HMDS (twice). After the fluid was run through the syringe, there was a five-minute waiting period before the next fluid was run through. After the samples were in 100% HMDS, all remaining fluid was pushed through by filling the syringe with air and pressing through until no further liquid came out the other end. Finally, the membranes were dried completely for several hours before being cut and placed onto the SEM stud for sputtering and subsequent imaging.

Measurements were taken from SEM images using Scandium 5.0 (Olympus Soft Imaging Systems) to capture the following major external morphological features for comparison between specimens and those in the literature: cercarial body length and width, tail stem length and width, furcal length and width, oral sucker to ventral sucker length, ventral sucker to tail stem length, ventral sucker length and width, length and width of spines found on the body, tail stem and furcae. Additionally, for the zygocercous cercariae, measurements were taken for the papules found on the tail stem. Because the dehydration step during the SEM processing appeared to cause some collapsing of the tissues, creating wrinkles, measurements were also taken from a small number of cercariae preserved for permanent mounting, to test for artefacts or distortion due to different methods of specimen preparation. Because resolution of the permanent-mount material was not as great as in that prepared for SEM, the only measurements taken were of body length and width and tail stem length and width. Light microscope images were taken using a compound Zeiss Axio Scope.A1 and PictureFrame v. 2.3 (Optronics) software, and measurements were made using ZEN (Zeiss) software. Sizes of morphological features were compared using an independent samples Kruskal-Wallis analysis of variance, and post hoc multiple comparisons test, with

Bonferroni correction for multiple tests, and an alpha level of 0.05, using IBM SPSS Statistics v. 24.0.

Morphological analyses of adults

In the lineages genetically distinguished herein for which adult specimens were available, DNA was often extracted from a subsample of a worm, and the remainder was stained in acetocarmine and mounted laterally on a slide in Canada balsam (i.e. as hologenophores, sensu Pleijel et al. 2008). In one lineage (LIN8), DNA was extracted from the entire worm, and the voucher is an intact worm that appeared indistinguishable from the sequenced specimen when it was taken from the same host (i.e. a paragenophore, sensu Pleijel et al. 2008). The adult vouchers were compared to published descriptions, with emphasis on accounts originating geographically close or from the same host as in the original description. Measurements were made with both an ocular micrometre and using imaging software, and one specimen was drawn using a camera lucida. Measurements were taken from uncollapsed, laterally oriented eggs along the entire length of the uterus, unless eggs were clearly different in size or shape due to differences in maturation. Unless otherwise stated, measurements (in μm) are reported as a range followed in parentheses by mean ± standard deviation, and number of specimens in which the structure was measured.

Results

Molecular phylogenetics

The *cox1* sequence derived from the zygocercous cercariae showed a 99.56% nucleotide similarity to *A. burti* (JX977725) from *Anas americana* in Mexico (isolate 180 from Hernández-Mena et al. 2014). Other matches to other *A. burti* isolates from Hernández-Mena et al. (2014) were attained with *cox1* sequences from non-zygocercous cercariae derived from a previous study (Gordy et al. 2016). Therefore, nucleotide alignments and phylogenetic analyses of these combined specimens were used to assess the relationships among these samples to clarify these extreme differences in cercarial behaviour.

Confirming initial tblastn results, both ML and BI trees in all three data sets (*cox1*, 28S, ITS) strongly supported the placement of the zygocercous cercaria (MGC1935) in *Australapatemon*, nested among other *A. burti* samples from Mexico, and separate from *Australapatemon niewiadomski* Blasco-Costa, Poulin, and Presswell, 2016 and, in separate clades, other strigeid genera (Blasco-Costa et al. 2016) (Fig. 1 and Suppl. Fig. 1). Both 28S and ITS markers, though generally supportive, and confirming genus-level monophyly seen in Blasco-Costa et al. (2016), were less informative for discriminating between species than *cox1,* possibly because sequences were available from fewer samples and had relatively low intrageneric divergence (mean intrageneric divergence across the Strigeidae: 28S ≤1.2%, ITS ≤6%) (Suppl. Table 2). Thus, initial species-level delineation among samples within the genus *Australapatemon* was achieved by analysis of *cox1*.

The *cox1* phylogeny showed nine lineages (LIN1–LIN9) within *A. burti*. Members within each lineage differed by less than 6.8% and by at least 6.7% from those in other lineages. The lineages corresponded to identical clusters of sequences in ABGD (prior maximal distance $P = 2.15e\text{-}02$, MinSlope = 1.5) (Puillandre et al. 2012) (evolutionary divergences as *p* distances given in Suppl. Table 2). Lineages 2, 3 and 5 were each represented by a single sequence. In the other six lineages, maximum intraspecific divergence ranged from 0.5–6.8% (LIN1 (*A. burti*): 6.5%, LIN4: 5.0%, LIN6: 3.3%, LIN7 (*Australapatemon mclaughlini* n. sp.): 0.5%, LIN8: 1.0%, LIN9: 6.8%), and mean intraspecific divergence from 0.2–3.5% (LIN1 = 1.8%, LIN4 = 3.5%, LIN6 = 2.2%, LIN7 = 0.2%, LIN8 = 0.3%, and LIN9 = 3.0%). Between lineages, the greatest interspecific divergence value was found between LIN5 and LIN8 (14.4%), and the smallest interspecific difference between LIN6 and LIN7 (6.7%). Overall, the range of genetic divergence by gene region among each new lineage and congeneric species was 6.7–14.4% (*cox1*), 0.0–1.2% (28S), and 0.4–1.9% (ITS).

Sequences of *cox1* from the zygocercous cercariae grouped phylogenetically with those from six adult worms from North America to form a distinct lineage, LIN7 (*A. mclaughlini* n. sp.). Five of the adult worms were collected from Northern Pintail (*A. acuta*) in Lake Manitoba, Manitoba, Canada, and one adult worm was from an American widgeon, *Anas americana* (Gmelin, 1789) collected from Baja California Sur, Mexico, i.e., isolate 180, identified as *A. burti* by Hernández-Mena et al. (2014) (JX977725.1). LIN7 was positioned within a monophyletic clade also composed of lineages 4 (LIN4), 5 (LIN5), and 6 (LIN6). With the exception of LIN5, all members of this clade utilize *P. gyrina* as an intermediate host and anatid birds as their definitive host. A single cercarial specimen, representing LIN5, was found emerging from the snail *S. elodes* (Fig. 1).

Host use and parasite morphology

The genetic distinction between clades was supported by differences in intermediate and definitive-host use in several lineages that were sampled more than once (LIN6 and LIN8 in *P. gyrina*, LIN9 in *S. elodes*; LIN7 in *P. gyrina* and *Anas* spp.; LIN8 in *O. jamaicensis*). However, because host specificity may correlate with sampling effort, the support that host-specific distributions imply for putative species is also likely

related to sampling effort. For example, 68/80 sequenced cercarial isolates were from *S. elodes* (Lymnaeidae), and consequently a lineage in our samples might be associated only with *S. elodes* by chance, even if it naturally occurs in other hosts. On the other hand, only 7/80 sequenced cercarial samples were obtained from *P. gyrina* (Physidae), such that lineages found only in this host are more likely truly specific to it. To address this, we did not assess host specificity of three lineages recovered from single individual hosts (LIN2, LIN3, LIN5). In other lineages, we randomized the 80 lineage-snail-host associations 10,000 times. The observed host distribution of LIN9 (nine isolates all from in *S. elodes*) was not different from random ($P = 0.351$), but the likelihoods of recovering LIN6, LIN7 and LIN8 only from *P. gyrina* were very small ($P \leq 0.0099$) if these lineages were equally capable of infecting other snail species sampled. The mixed snail-host associations of LIN1 mirror those of the data as a whole (60/66 LIN1 isolates in *S. elodes*; $P = 0.827$). Neither the wide snail-host spectrum of LIN1, nor the narrow host ranges of the other lineages seem to be purely an artefact of sampling effort, because the number of cercarial isolates sequenced was not related to the number of snail-host species in each lineage (Spearman's rho = 0.616, $P = 0.11$, $n = 8$). Permuting the smaller database of adult-parasite avian-host associations (consisting of seven lineages, LIN1, 2, 4, 6, 7, 8, 9, from nine individual birds in seven host species) 10,000 times showed the probability of two LIN8 occurring only in *O. jamaicensis* (one in Manitoba, one in Durango, Mexico (Hernández-Mena et al. 2014)) to be 0.0278. The only other potential host specificity among the adult worms is in LIN7, which was only recovered from *Anas* spp. The probability of two LIN7 samples occurring in *Anas* was 0.275. Thus, three *cox1* lineages (LIN6, LIN7 and LIN8) are supported by host-specific distributions that are unlikely to be an artefact of sampling effort.

Cercarial morphometrics (14/16) were significantly different across lineages (Suppl. Table 3). There was no significant difference between measurements in SEM and those of permanently mounted, stained samples, nor from wet-mounts. The only lineage within which there were significant morphometric differences among cercariae was LIN1 (Suppl. Table 4). Notably, there were no differences between genetically divergent samples within LIN9 ($P > 0.05$ for all comparisons between MGC1376 and MGC1360).

Within LIN1, cercariae varied morphometrically. For instance, samples MGC1557 (*S. elodes*) and MGC1179 (*H. trivolvis*) had significantly different body and furcal spine dimensions, as well as different furcal lengths (4/16 morphometrics in Table 1; Suppl. Table 4). This lineage was recovered from five different pulmonate species in Western and Eastern Canada and the Southwestern USA (Fig. 1 and Suppl. Table 5). Despite this broad distribution, the only link to an adult was with the isolate from *Anas diazi* (Ridgway, 1886) in Mexico (sample 138 of Hernández-Mena et al. 2014).

Varying degrees of morphological distinction were observed among adults of LIN2, LIN4, LIN7, LIN8 and LIN9 (Tables 2 and 3). Adults of most lineages could be distinguished mainly by their total length, ratio of hindbody to forebody length and egg size. Dense vitelline follicles prevented visualization of the ovary in all specimens, and the genital cone characteristic of *Australapatemon* was observed in two specimens of LIN7, as well as in the single specimen of LIN9. No substantial difference was seen among the adults of LIN4 and LIN8, and cercariae of both lineages emerged from *P. gyrina*.

Because these parasites mature in migratory waterfowl, and some species have been recorded in diverse birds from distant localities (e.g. McDonald 1981; Drago et al. 2007; Drago and Lunaschi 2010), we compared the morphology of the adult vouchers of the nine lineages with all species of *Australapatemon* (Table 2; Suppl. Table 6), although we focus on species known in North America. One lineage is newly described herein (LIN7 = *A. mclaughlini* n. sp.), and most others could not be assigned to described species, nor described.

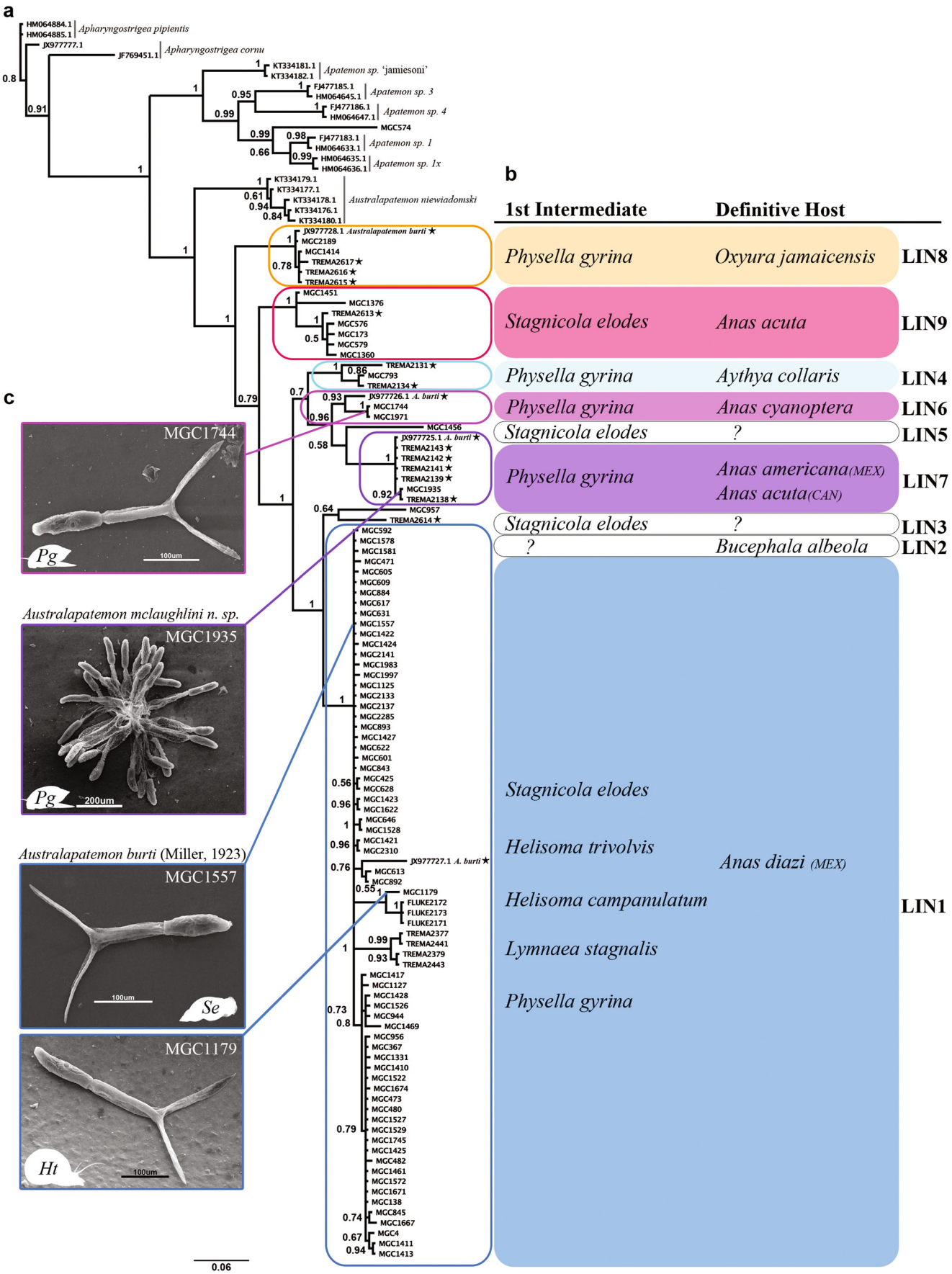
Description

Australapatemon mclaughlini n. sp.
Family Strigeidae Railliet, 1919
Subfamily Strigeinae Railliet, 1919
Genus *Australapatemon* Sudarikov, 1959

Description of adult (Fig. 2a, b; Table 2)

[Measurements from 7 specimens (4 subsampled hologenophores, 2 paragenophores, and holotype), ex *Anas acuta* L. measurements in micrometres; widths dorso-ventral]

Total length 1484–1851 (1671 ± 143, 5); body distinctly bipartite. Maximum width of forebody at level of ventral sucker. Forebody cup-shaped, 347–473 (386 ± 55, 5) long, 475–630 (527 ± 64, 5) wide. Hindbody arcuate, curved dorsally, widest at level of anterior testis, 1137–1378 (1298 ± 92, 6) long, 535–662 (616 ± 48, 6) wide. Ratio of forebody to hindbody length 1:2.9–3.7 (3.4 ± 0.3, 5). Oral sucker terminal, 78–117 (102 ± 14, 5) × 88–113 (102 ± 9, 5). Ventral sucker in median dorsal wall of forebody, 125–160 (142 ± 15, 4) × 173–185 (177 ± 15, 4). Holdfast organ bilobed; proteolytic gland not observed. Pharynx small, difficult to observe, 39–58 (52 ± 11, 3) long. Testes tandem, large; anterior testis asymmetrical, bilobed; anterior margin at 17–27 (23 ± 4, 4) % of hindbody; 341 × 390 (1). Posterior testis asymmetrical, bilobed; posterior margin at 72–79 (77 ± 3, 4) % of hindbody; 293(1) × 240–341 (291 ± 72, 3). Seminal vesicle convoluted in dorsal post-testicular region, often distending adjacent tegument. Ovary not observed. Vitellaria follicular, confined to hindbody, densely distributed, extending posteriorly in two


a
HM064884.1
HM064885.1
Apharyngostrigea pipientis
JX977777.1
JF769451.1
Apharyngostrigea cornu
KT334181.1
KT334182.1
Apatemon sp. 'jamiesoni'
FJ477185.1
HM064645.1
Apatemon sp. 3
FJ477186.1
HM064647.1
Apatemon sp. 4
MGC574
FJ477183.1
HM064633.1
Apatemon sp. 1
HM064635.1
HM064636.1
Apatemon sp. 1x
KT334179.1
KT334177.1
KT334178.1
KT334176.1
KT334180.1
Australapatemon niewiadomski
JX977728.1 Australapatemon burti ★
MGC2189
MGC1414
TREMA2617★
TREMA2616★
TREMA2615★
MGC1451
MGC1376
TREMA2613★
MGC576
MGC173
MGC579
MGC1360
TREMA2131★
MGC793
TREMA2134★
JX977726.1 A. burti★
MGC1744
MGC1971
MGC1456
JX977725.1 A. burti★
TREMA2143★
TREMA2142★
TREMA2141★
TREMA2139★
MGC1935
TREMA2138★
MGC957
TREMA2614★
MGC592
MGC1578
MGC1581
MGC471
MGC605
MGC609
MGC884
MGC617
MGC631
MGC1557
MGC1422
MGC1424
MGC2141
MGC1983
MGC1997
MGC1125
MGC2133
MGC2137
MGC2285
MGC893
MGC1427
MGC622
MGC601
MGC843
MGC425
MGC628
MGC1423
MGC1622
MGC646
MGC1528
MGC1421
MGC2310
JX977727.1 A. burti★
MGC613
MGC892
MGC1179
FLUKE2172
FLUKE2173
FLUKE2171
TREMA2377
TREMA2441
TREMA2379
TREMA2443
MGC1417
MGC1127
MGC1428
MGC1526
MGC944
MGC1469
MGC956
MGC367
MGC1331
MGC1410
MGC1522
MGC1674
MGC473
MGC480
MGC1527
MGC1529
MGC1745
MGC1425
MGC482
MGC1461
MGC1572
MGC1671
MGC138
MGC845
MGC1667
MGC4
MGC1411
MGC1413
0.06
b
1st Intermediate
Definitive Host
Physella gyrina
Oxyura jamaicensis
LIN8
Stagnicola elodes
Anas acuta
LIN9
Physella gyrina
Aythya collaris
LIN4
Physella gyrina
Anas cyanoptera
LIN6
Stagnicola elodes
?
LIN5
Physella gyrina
Anas americana(MEX)
Anas acuta(CAN)
LIN7
Stagnicola elodes
?
LIN3
?
Bucephala albeola
LIN2
Stagnicola elodes
Helisoma trivolvis
Helisoma campanulatum
Lymnaea stagnalis
Physella gyrina
Anas diazi (MEX)
LIN1
c
MGC1744
Pg
100um
Australapatemon mclaughlini n. sp.
MGC1935
Pg
200um
Australapatemon burti (Miller, 1923)
MGC1557
100um
Se
MGC1179
Ht
100um

◀ **Fig. 1** **a** Bayesian inference phylogram generated from partial *cox1* gene sequences with posterior probability values >0.50 reported. GenBank (GB) accession numbers are associated with samples derived from the database, while all other sample names represent new sequences. Sample names correspond to new GB accession numbers provided in Suppl. Table 5. Sequences from adult worms are indicated by a *black star*. Adults collected in Mexico and studied by Hernández-Mena et al. (2014) are labelled *A. burti*. *Scale bar* denotes number of substitutions per site. Lineages are identified by differently coloured rounded rectangles that correspond to same colour-shaded rectangles directly to the right, **b** indicating first intermediate and definitive host use for each lineage. Singletons are not indicated on the tree and are denoted by unshaded rectangles. Each lineage is labelled at the far right of the rectangles as LIN1–LIN9. Question marks denote missing host information. **c** Examples of cercarial morphologies from LIN1, LIN6 and LIN7, in SEM. Coloured outlines correspond to lineage colours, and lines indicate placement within each lineage. First intermediate host use is indicated on each image: *Pg Physella gyrina*, *Se Stagnicola elodes* and *Ht Helisoma trivolvis*

ventro-lateral fields to level of copulatory bursa. Vitelline reservoir intertesticular; median. Eggs 115–131 (120 ± 5, 6) × 83–88 (85 ± 2, 6). Copulatory bursa large with dorsally oriented terminal opening. Genital cone delimited from surrounding parenchyma, one eighth to one sixth of hindbody length; 192–218 (202 ± 14, 3) × 166–188 (177 ± 16, 2). Hermaphroditic duct within genital cone lined with internal rugae.

Description of cercaria (Fig. 2c, d; Table 4)

[Measurements in micrometres, based on three aggregates preserved in 100% ethanol and imaged with SEM. Body and tail lengths/widths are composite measurements of SEM and permanent mount cercariae from a total of six aggregates.] Freshwater, apharyngeate, distomate and zygocercous furcocercaria. Body elliptical, 78.69–149.50 (115.56 ± 18.71, 69) long, 29.82–49.00 (39.08 ± 5.07, 71) wide. Tegument spines dense at oral sucker and becoming sparser towards posterior extremity of body. Body spines 1.41–2.53 (2.00 ± 0.36, 12) long, 0.25–0.55 (0.45 ± 0.09, 12) wide. No apparent eye-spots. Ventral sucker post-equatorial, 4.83–7.70 (6.46 ± 1.27, 6) long, 7.36–13.59 (9.44 ± 2.25,

Table 1 Statistics tests of morphometric comparisons of cercariae in Table 4, using Kruskal-Wallis analysis of variance (H) and post-hoc multiple comparisons test, with Bonferroni correction. Statistical significance ($\alpha = 0.05$) is indicated by *bold text* and an *asterisk*. Only significant comparisons between and within lineages and species are listed

	H	df	*P*	No.	Significant comparisons	Adjusted *P* (Bonferroni)
Body length	17.714	6	**0.007***	99	1935(LIN7)-1179(LIN1)	**0.010***
Body width	10.919	6	0.091	98		
Tail length	42.085	6	**0.000***	78	1557(LIN1)-1935(LIN7)	**0.007***
					1557(LIN1)-*C. laramiensis*	**0.008***
					1744(LIN6)-1935(LIN7)	**0.000***
					1744(LIN6)-*C. laramiensis*	**0.007***
Tail width	40.307	6	**0.000***	66	1557(LIN1)-1935(LIN7)	**0.017***
					1744(LIN6)-1935(LIN7)	**0.000***
Furca width	15.615	5	**0.008***	48		
OS to VS length	2.865	4	0.581	19		
VS length	5.418	3	0.144	16		
VS width	4.984	3	0.173	16		
VS to tail length	6.835	3	0.077	16		
VS spine length	9.705	3	**0.021***	20		
VS spine width	12.497	3	**0.006***	26		
Body spine length	38.495	3	**0.000***	134	1557(LIN1)-1744(LIN6)	**0.033***
					1557(LIN1)-1179(LIN1)	**0.006***
					1557(LIN1)-1935(LIN7)	**0.000***
					1744(LIN6)-1935(LIN7)	**0.009***
Body spine width	25.988	3	**0.000***	99	1179(LIN1)-1935(LIN7)	**0.000***
					1557(LIN1)-1935(LIN7)	**0.003***
					1744(LIN6)-1935(LIN7)	**0.021***
Furca spine length	13.341	3	**0.004***	26	1557(LIN1)-1179(LIN1)	**0.015***
					1744(LIN6)-1179(LIN1)	**0.037***
Furca spine width	28.526	3	**0.000***	39	1557(LIN1)-1179(LIN1)	**0.0010***
					1557(LIN1)-1935(LIN7)	**0.000***

VS ventral sucker, *OS* oral sucker

Table 2 Morphometrics from adults of genetically distinguished lineages of *Australapatemon* and comparison with congeners (μm). Data from the present study are reported as mean (range) and ranges are reported from other studies

	LIN2 (n = 1)	LIN4 (n = 2)	*Australapatemon mclaughlini* n. sp. MGC1935 LIN7	LIN8 (n = 1)	LIN9 (n = 1)	*Australapatemon anseris* (Dubois, 1967)	*Australapatemon bdellocystis* (Lutz, 1921)	*Australapatemon burti* (Miller, 1923)
Total length	1865	1558 (1505–1610)	1689 (1484–1851)	1263	1160	3330-4500b	2500	1800–2500
Forebody length	545	465 (450–479)	396 (347–473)	403	340	600–1260	800	300–600
Forebody width	484	450 (450–450)	540 (475–630)	393	445	640–860	800	300–450
Hindbody length	1320	1093 (1055–1131)	1307 (1137–1378)	860	820	1750–3500	1600	700–1300
Hindbody width	417	555 (550–560)	614 (535–662)	400	384	620–890	800	350–500
Hindbody/forebody length	2.42	2.35 (2.34–2.36)	3.30 (2.91–3.70)	2.13	2.41	1.9–3.0	2	1.2–2.4b
Oral sucker length	152	115 (115–115)	108 (104–117)	113		150–220	150	90–125
Oral sucker width	116	132 (132–132)	105 (101–113)	132		120–170		65–90
Pharynx length			52 (39–58)			70–104	100	36–45
Pharynx width						40–100		36–45
Ventral sucker length	153	184 (184–184)	144 (125–160)	175	139	210–330	200	90–140
Ventral sucker width	146	181 (181–181)	177 (173–185)		131	245–310		90–140
Ovary length						200–210	200	70–120
Ovary width						230–250		70–120
Anterior testis length						380–600	400	200–300
Anterior testis width		280				380–660	450	200–300
Posterior testis length						420–670	400	200–300
Posterior testis width		300	240 (240–240)			420–770	450	200–300
Genital cone length			205 (192–218)		143	440–660		145-200[b]
Genital cone width			188 (188–188)		173	300–450		110-155[b]
Egg length	120–130	91.5 (90–93)	123 (110–131)	95	106–121	90–110		90–100
Egg width	75–80	67 (67–67)	84 (65–86)	57	67–72	65–70		62–70
Other				spines on FB lobes				
Source						Dubois 1968	Dubois 1968	Stunkard et al. 1941

	Australapatemon canadensis (Dubois & Rausch, 1950)	*Australapatemon congolensis* (Dubois & Fain, 1956)	*Australapatemon fuhrmanni* (Dubois, 1937)	*Australapatemon intermedius* (Johnston, 1904)	*Australapatemon magnacetabulum* (Dubois, 1988)	*Australapatemon minor* (Yamaguti, 1933)	*Australapatemon niewiadomski* Blasco-Costa, Poulin, & Presswell, 2016
Total length	2040-3200a	1530-2400a	2250-3300a	3240-5000a	1080–1400	2500	1350–1999
Forebody length	510–960	490–740	590–960	1000–1500	420–450	250–870	452–712

Table 2 (continued)

	LIN2 (n = 1)	LIN4 (n = 2)	*Australapatemon mclaughlini* n. sp. MGC1935 LIN7	LIN8 (n = 1)	LIN9 (n = 1)	*Australapatemon anseris* (Dubois, 1967)	*Australapatemon bdellocystis* (Lutz, 1921)	*Australapatemon burti* (Miller, 1923)
Forebody width	370–770	560–860	540–1000	860–1250	360–370	280–630	361–536	
Hindbody length	870–2270	1004–1630	1260–1960	2200–3000	660–950	540–1730	888–1412	
Hindbody width	420–900	490–640	510–780	850–1270	270–310	250–670	348–545	
Hindbody/forebody length	1.2–2.8	2.1–2.4	1.7–2.9	2.0–2.5		1.4–2.6	1.6–2.4	
Oral sucker length	120–220	145–190	135–200	150–250	92–95	80–145	103–145	
Oral sucker width	105–170	115–140	105–170	145–220	70–80	60–135	97–145	
Pharynx length	60–85	55–67	78–104	80–130	70–73	40–65	44–64	
Pharynx width	60–85	38–42	57–92	50–85	55–68	33–65	41–60	
Ventral sucker length	140–245	215–245	180–260	250–340	130–200	92–198	130–217	
Ventral sucker width	160–235	175–210	160–260	250–340	105–170	80–198	142–193	
Ovary length	105–190	145–155	90–160	210	63–105	66–135	91–148	
Ovary width	125–210	235–240	135–280	330	90–115	105–163	118–194	
Anterior testis length	250–470	210–270	235–390	460–490	75–165	99–306	191–361	
Anterior testis width	235–440	290–310	190–420	490–650	105–175	10–326	169–333	
Posterior testis length	335–640	230–300	270–420	400–490	115–120	130–408	193–327	
Posterior testis width	240–475	300–340	200–470	480–650	70–95	99–367	200–385	
Genital cone length	235–470	260–280	310–400	640–850	115–165	150–280	154–224	
Genital cone width	180–330	210–240	210–320	420–500	115–150	120–190		
Egg length	95–125	87–98	98–122	72–110	100–105	99–132	94–103	
Egg width	65–80	53–65	64–81	62–73	60–63	50–77	55–72	
Other								
Source	Dubois 1968	Dubois 1968	Dubois 1968	Dubois 1968	Dubois 1988	Dubois 1968	Blasco-Costa et al. 2016	

[a] Lower range of length taken from illustration

[b] From Dubois 1968

Table 3 Comparison of adult morphology between lineages. Listed are structures that differ among the lineages

	A. mclaughlini n. sp. LIN7	LIN2	LIN4	LIN8	LIN9
A. mclaughlini n. sp. LIN7					
LIN2	VS, HB:FB				
LIN4	VS, HB:FB, eggs	TL, VS, OS, HB:FB, eggs			
LIN8	TL, FB shape, OS, VS, HB:FB, eggs	TL, VS, OS, HB:FB, eggs	Similar		
LIN9	TL, FB W, HB W, GC, HB:FB	TL, VS, HB:FB, eggs	VS, eggs, FB shape	FB shape, VS, eggs	TL, FB shape, VS, eggs

TL total length, *OS* oral sucker, *PH* pharynx, *VS* ventral sucker, *FB* forebody, *HB* hindbody, *HB:FB* hindbody length/forebody length, *OV* ovary, *AT* anterior testis, *PT* posterior testis, *GC* genital cone

6) wide, and containing 4–5 rows of large spines 1.38–2.26 (1.75 ± 0.46, 3) long, 0.30–0.45 (0.38 ± 0.07, 3) wide. Tail stem thinner or of similar width to body at junction but widens further towards furcae, 101.24–247.40 (179.29 ± 27.37, 50) long, 18.66–82.70 (47.28 ± 11.39, 43) wide, aspinose, and covered in protruding papules 2.36–3.92 (3.0 ± 0.44, 14) long, 1.22–3.03 (1.50 ± 0.19, 14) wide. Furcae 47.33–98.90 (80.30 ± 28.70, 3) long (from base of tail stem to bundle with other furcae), 12.17–34.19 (21.70 ± 8.40, 11) wide, and narrowing to a blunt end. Furcal spines 1.06–1.59 (1.40 ± 0.23, 5) long, 0.62–1.06 (0.80 ± 0.17, 8) wide. Excretory bladder bilobed and roughly triangular in shape, in posterior body region near base of tail stem. Cercarial aggregation by attachment of distal portion of furcae and specialized furcal muscles, in masses of 4–44 individuals. Aggregates non-swimming, resting on substrate in spherical mass, emerging from snail at rate of 20 per day (based on two captive snails observed over 3 days), occurring free and between mantle tissue and shell of snail, suggesting aggregation immediately post-emergence.

Details of type material

Type-host: *Anas acuta* (definitive host)

First intermediate host: *Physella gyrina* Say

Site of infection: intestine (definitive host); ovo-testes and digestive gland (first intermediate host).

Prevalence: in 1 out of 2 birds (Lake Manitoba); in 2 of 127 snails collected (Buffalo Lake)

Type-locality: Delta Marsh, Lake Manitoba, Manitoba, Canada (50.183° N, −98.383° W) (definitive host); Rochon Sands Provincial Park, Buffalo Lake, Alberta, Canada (52.464° N, −112.884° W) (first intermediate host)

Other localities: Guerrero Negro, Baja California Sur (27.959° N, −114.056° W) (definitive host: *Anas americana* JX977725, sample 180 (Hernández-Mena et al. 2014)).

Type-material: holotype (adult worm); holotype (cercariae). Deposited in the Royal Alberta Museum

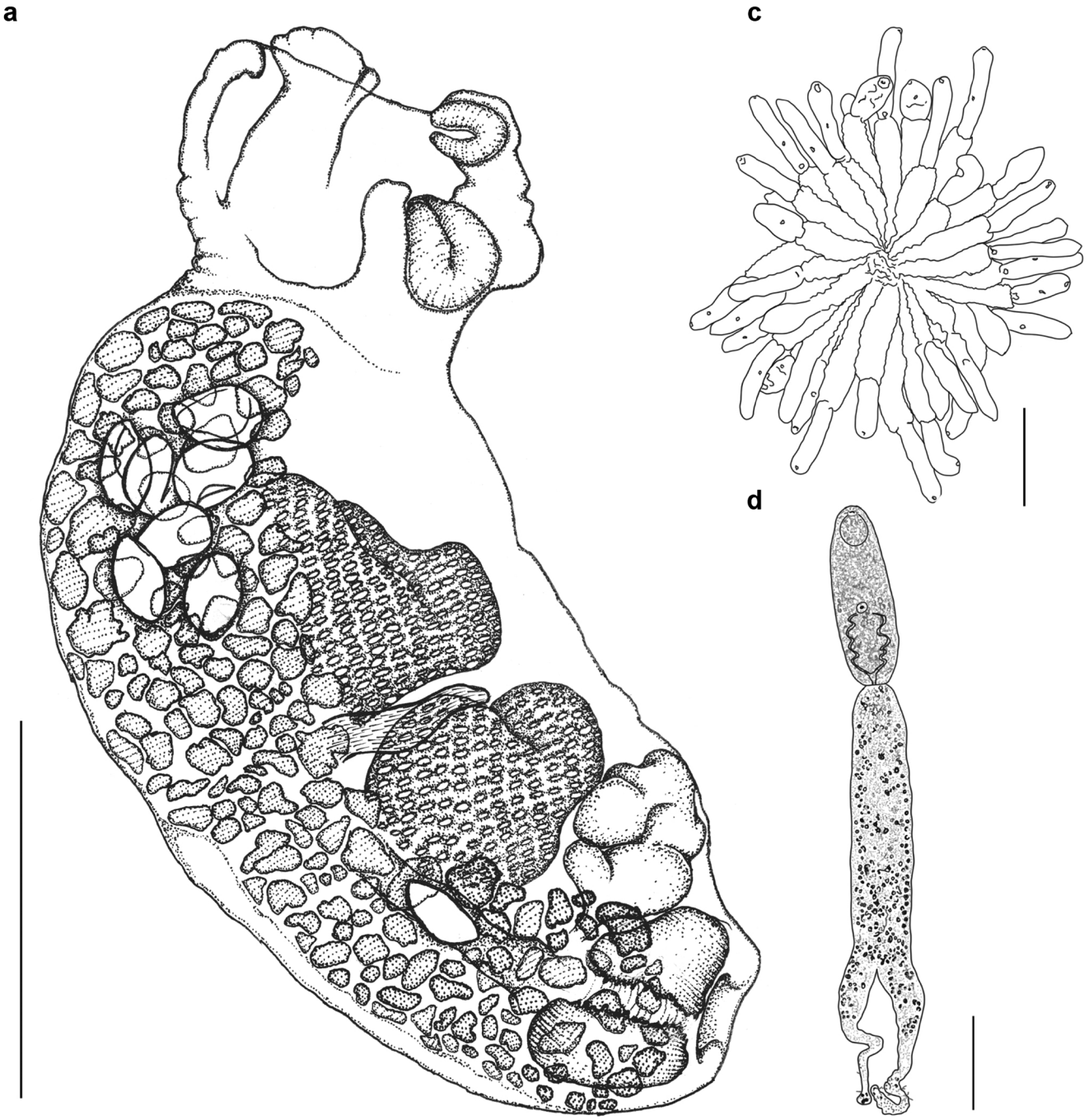
Representative DNA sequences: *cox1*: KY207615, KY587395, KY587402, KY587403, KY587405, KY587406, JX977725; 28S rDNA: KY207627; ITS1–5.8S–ITS2: KY207628

Etymology: This species is named after J. Daniel Mclaughlin, who collected and generously provided adult worms studied herein, and who has made numerous, important contributions to the systematics and ecology of helminths in aquatic systems in North America.

Remarks

Adults of *Australapatemon mclaughlini* n. sp. possess characters typical of *Australapatemon* Sudarikov, 1959, namely, a muscular genital cone delimited from surrounding parenchyma that is traversed by a rugose hermaphroditic duct. The length of the hindbody relative to the forebody (see Fig. 2b) is greater in adults of *A. mclaughlini* n. sp. compared to all species of *Australapatemon*, as well as the lineages characterized genetically herein, but not *Australapatemon anseris* (Dubois, 1967). *Australapatemon mclaughlini* n. sp. is also distinguished from most species and lineages (other than *Australapatemon canadensis* (Dubois and Rausch, 1950), *Australapatemon fuhrmanni* (Dubois, 1937), *Australapatemon minor* (Yamaguti, 1933), and LIN2 and LIN9, by having larger eggs (Suppl. Table 6). Several features distinguish *A. mclaughlini* n. sp. from species of *Australapatemon* reported in North America. Compared with *A. anseris* (a predominantly European species reported in North America by McDonald 1981), *A. mclaughlini* n. sp. is characterized by a smaller total length, oral sucker, pharynx, ventral sucker, posterior testis and genital cone, and greater egg length. *Australapatemon mclaughlini* n. sp. is distinguished from *A. burti* (as described by Stunkard et al. 1941) and *A. canadensis* by having a long hindbody relative to its

Fig. 2 *Australapatemon mclaughlini* n. sp. **a** Adult (holotype); *scale bar = 500* μm. **b** Silhouettes of paratypes (paragenophores and hologenophores, based on photographs taken prior to subsampling and DNA extraction); *scale bar = 500* μm. **c** Outline of cercarial zygocercous aggregate; *scale bar = 200* μm. **d** Individual cercaria, ventral view; *scale bar = 50* μm


a
c
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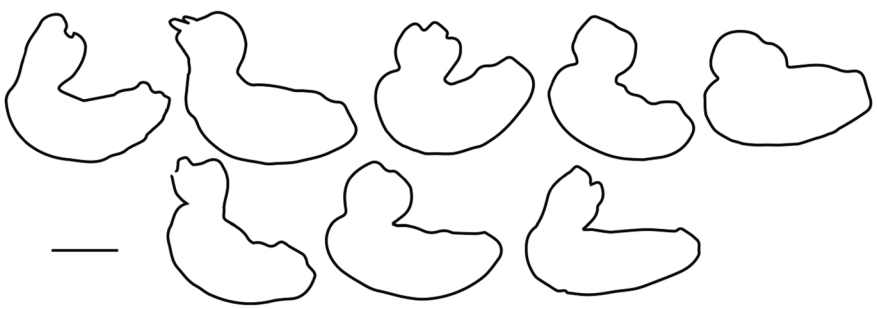
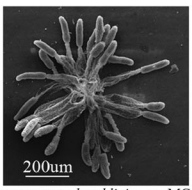
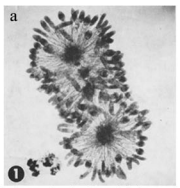

b

forebody, and eggs are longer in *A. mclaughlini* n. sp. than in *A. burti*. *Australapatemon mclaughlini* n. sp. has smaller total length and is more robust (wider relative to length in both fore- and hindbody) than *A. canadensis*. The aggregating habit of cercariae of *A. mclaughlini* n. sp. differs from the behaviour of cercariae in *Australapatemon bdellocystis* (Lutz, 1921), *A. burti*, *Australapatemon intermedius* (Johnston, 1904), *Australapatemon magnacetabulum* (Dubois, 1988) and *A. minor* (Dubois 1968; Davies and Ostrowski de Núñez, 2012) and other genetic lineages herein. The cercariae of *A. mclaughlini* n. sp. are also distinct from cercariae of other lineages genetically characterized herein, as well as from *Cercaria burti* Miller, 1923, in body, tail, and tegumental spine sizes (Tables 1 and 4; Fig. 3). In addition, spines on the body of cercariae of *A. burti* extend only to the ventral sucker (Miller 1923, 1926) while those of *A. mclaughlini* n.

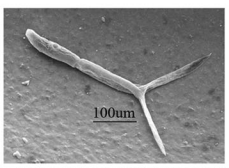
Table 4 Morphometric comparisons among cercariae. Measurements are given as ranges (in micrometres). **a** The zygocercous cercariae of *Australapatemon mclaughlini* n. sp. (LIN7) are compared to two zygocercous diplostomoids, *Cercaria laramiensis* (Hendrickson and Kingston, 1974) and *Cercaria absurda* (Miller, 1927) and **b** representative cercariae from LIN1 and LIN6 (see Fig. 1) are compared to original descriptions of *Australapatemon* (*Cercaria*) *burti* (Miller, 1923)

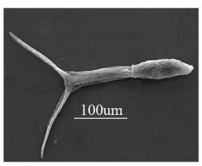


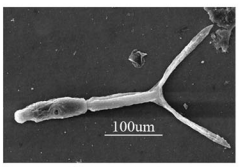


a

	Australapatemon mclaughlini n. sp. MGC1935 LIN7	*Cercaria laramiensis* (Hendrickson & Kingston, 1974)	*Cercaria absurda* (Miller, 1927)
Number of cercariae per aggregate	4–26	4–150	3–4
Number of aggregates per day	20	–	–
Body length (μm)	79–149[b]	91–150	183
Body width (μm)	30–49[b]	46–71	55
Tail Length (μm)	101–247[b]	230–260	285
Tail width (μm)	19–83[b]	41–60	114
Furca length (μm)	47–99	95–171	80
Furca width (μm)	12–34	27–40	30
Oral to ventral sucker length (μm)	43–80	49–81	–
Ventral sucker to tail stem (μm)	31–56	–	–
Ventral sucker length (μm)	4.83–7.70	19–23	–
Ventral sucker width (μm)	7.36–13.59	20–23	–
Ventral sucker spine length (μm)	1.38–2.26	–	–
Ventral sucker spine width (μm)	0.30–0.45	–	–
Body spine length (μm)	1.41–2.53	–	–
Body spine width (μm)	0.25–0.55	–	–
Tail spine length (μm)	–	–	11–16
Tail spine width (μm)	–	–	–
Furca spine length (μm)	1.06–1.59	–	–
Furca spine width (μm)	0.62–1.06	–	–
Tail Papillae	Yes	Yes	No
Tail Papilla length (μm)	2.36–3.92	–	not applicable
Tail Papilla width (μm)	1.22–3.03	–	not applicable







b

	Australapatemon burti MGC1179 LIN1	*Australapatemon burti* MGC1557 LIN1	*Australapatemon sp.* MGC1744 LIN6	*Cercaria burti* (Miller, 1923)
Number of cercariae per aggregate	not applicable	not applicable	not applicable	not applicable
Number of aggregates per day	not applicable	not applicable	not applicable	not applicable
Body length (μm)	138–156	106–131	111–135	88–240
Body width (μm)	42–48	37–44	30–43	34–52
Tail Length (μm)	163–178	118–131	110–145	113–165
Tail width (μm)	26–36	26–27	21–32	26
Furca length (μm)	163–194	120–162	129–162	139–181
Furca width (μm)	15–30	13–19	9–18	–
Oral to ventral sucker length (μm)	56–82	55–64	58–68	–
Ventral sucker to tail stem (μm)	54–62	40–51	41–53	–
Ventral sucker length (μm)	12.44–14.39	4.92–11.29	5.61–7.91	–
Ventral sucker width (μm)	13.65–14.67	6.18–11.38	6.97–11.76	–
Ventral sucker spine length (μm)	1.17–1.25	0.74–1.74	0.69–0.98	–
Ventral sucker spine width (μm)	0.28–0.30	0.19–0.26	0.19–0.24	–
Body spine length (μm)	1.06–2.18	0.75–1.93	1.02–2.15	–
Body spine width (μm)	0.18–0.35	0.19–0.42	0.19–0.42	–
Tail spine length (μm)	–	1.04–2.04	0.85–1.49	–
Tail spine width (μm)	–	0.28–0.56	0.24–0.50	–
Furca spine length (μm)	1.83–2.62	0.80–1.49	0.99–1.55	–
Furca spine width (μm)	0.60–0.81	0.14–0.34	0.24–0.37	–
Tail Papillae	No	No	No	No
Tail Papilla length (μm)	not applicable	not applicable	not applicable	not applicable
Tail Papilla width (μm)	not applicable	not applicable	not applicable	not applicable

NA not available
a Reuse with permission from Allen Press
b Measurements derived from both permanent mount and SEM prep

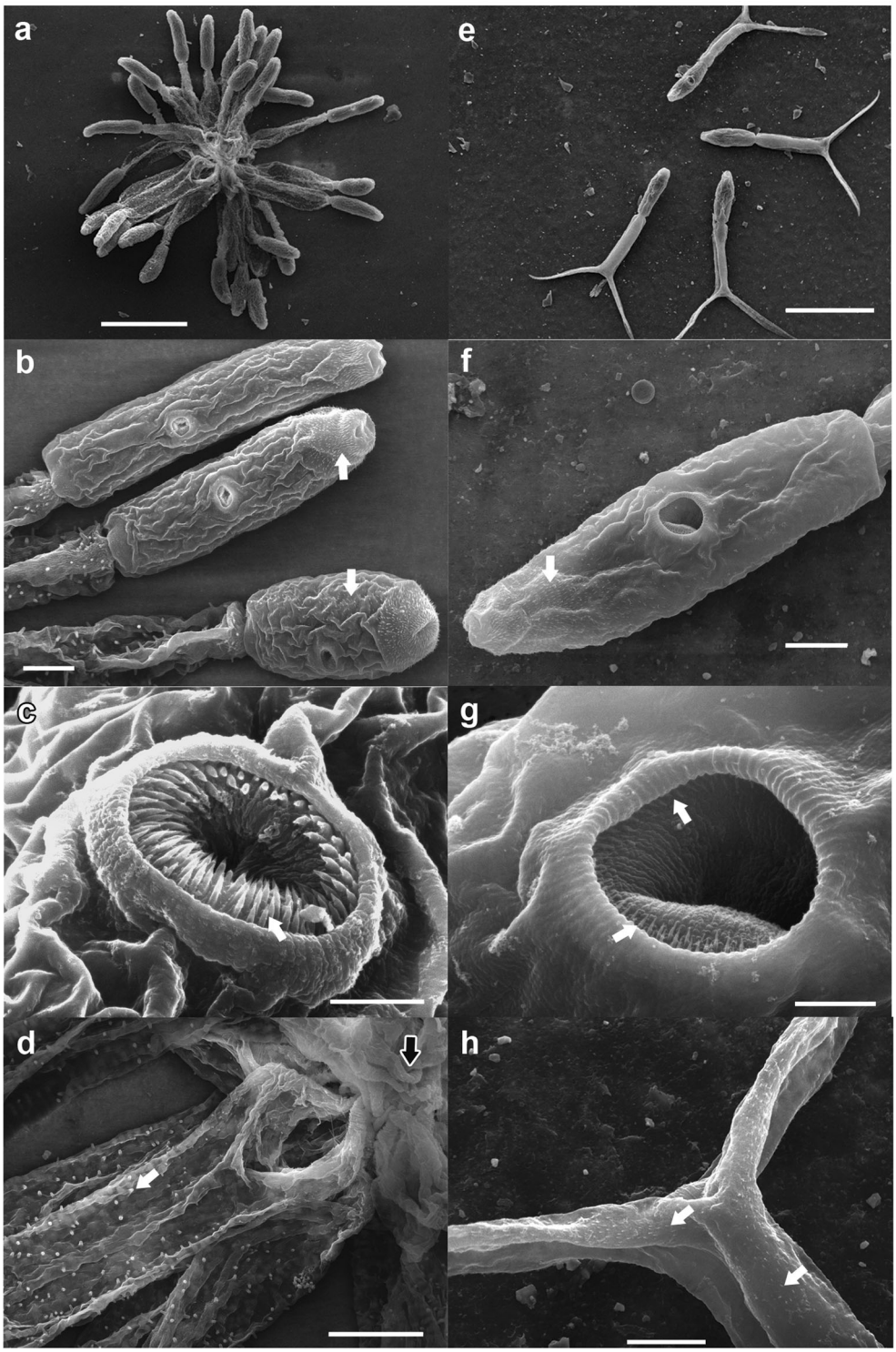


Fig. 3 Scanning electron micrographs (SEM) of zygocercous (LIN7, MGC1935: **a**–**d**) *Australapatemon mclaughlini* n. sp. and non-zygocercous (LIN1, MGC1179: **e**–**h**) *Australapatemon burti* cercariae. **a** Zygocercous aggregate; *scale bar = 100* μm. **b** Ventral view of three individual zygocercous bodies. *White arrows* indicate tegumental spination; *scale bar = 20* μm. **c** Ventral sucker of zygocercous cercaria. *White arrows* indicate sucker spines; *scale bar = 5* μm. **d** Zoomed-in view of cercaria tail among the aggregate tail bundle. *White arrow* indicates papules on tail, *black arrow* indicates narrowing of furcal muscles, specialized for holding on to others; *scale bar = 50* μm. **e** Four individual cercariae; *scale bar = 100* μm. **f** Ventral view of individual cercaria body. *White arrow* indicates body spination; *scale bar = 20* μm. **g** Zoomed-in view of ventral sucker. *White arrows* indicate ventral sucker spines; *scale bar = 5* μm. **h** Zoomed-in view of cercaria furcae. *White arrows* indicate tail and furcal spines used for measurements; *scale bar = 20* μm

sp. span the length of the cercarial body (Fig. 3b). The presence of tegumental spines on cercariae of *A. mclaughlini* n. sp. distinguishes it from *Cercaria laramiensis*, which lacks spines (Hendrickson and Kingston 1974) (Table 4, Suppl. Table 1 and Fig. 3). The only other described aggregating cercariae possibly belonging to the Diplostomoidea, *Cercaria absurda* Miller, 1927, forms chain-like aggregates (Miller 1930) rather than the rosette formation seen in *A. mclaughlini* n. sp.

The other four genetically distinguished lineages of *Australapatemon* in which adults were recovered (LIN2, LIN4, LIN8, LIN9) also displayed varying degrees of morphological distinction from species already described in this

genus (Suppl. Table 3, Suppl. Table 6). The single adult specimen of LIN2 had larger oral and ventral suckers and eggs than *A. burti*, and was smaller in total length, but otherwise similar, to *A. canadensis*. The two adult specimens in LIN4 were smaller in total length and oral sucker width, but with a larger oral sucker, and were otherwise similar to *A. burti*. Compared with *A. canadensis*, adults of LIN4 were smaller in total length and egg size, and had a more spherical forebody. The adult of LIN8 was smaller in total length and oral sucker width, and had a larger pharynx and ventral sucker than *A. burti*, but was otherwise similar to *A. burti*. In comparison to *A. canadensis*, the forebody of LIN8 was more spherical, and the hindbody wider relative to its length. The adult of LIN9 also resembled *A. burti*, although it was smaller in total length, with longer hindbody relative to forebody. It differed from *A. canadensis* in its smaller total length, ventral sucker, genital cone and in its flattened, ovoid forebody. However, because most adults from these four lineages were incomplete specimens (subsampled for DNA extraction), and only 1–2 adults were obtained per lineage (Table 2), assessment of morphological variation was not possible and key features for many comparisons were not observed. For example, adults of LIN4 and LIN8 resemble both *A. burti* and *Australapatemon congolensis* (Dubois and Fain, 1956), two species usually discriminated by dimensions of the genital cone (Dubois 1968), a structure obscured by vitelline fields in vouchers of these lineages. Moreover, high divergence in *cox1* within one lineage (LIN9) may indicate the presence of additional species (see discussion). For these reasons, lineages 2, 4, 8 and 9 were not identified or described as new species based on this material.

Discussion

Historically, the status of *A. burti* (Miller, 1923) has been in a state of flux. When still referred to as *Cercaria burti*, it was believed that these cercariae may be the larval form of *Apatemon gracilis* (Rudolphi, 1819), a worm commonly found in palmate birds across Europe, Japan and North America. It was determined, however, that because *C. burti* had a longer metacercarial developmental period and utilized pulmonate snails in North America, it was likely a different species from *A. gracilis*, known to infect branchiate snails (Stunkard et al. 1941). However, since then, *A. burti* has been one of the most common cercariae found in Europe among pulmonate snails, despite no reports of adult *A. burti* in this region (Faltýnková et al. 2007; Blasco-Costa et al. 2016 and references within).

The distinction between *Apatemon* and *Australapatemon* has also been questioned, with the latter considered a subgenus of *Apatemon* by Dubois (1968). Recent authorities, however, consider *Australapatemon* a valid genus, distinguished from *Apatemon* by a well-defined genital cone in the adult (Niewiadomska 2002). This was supported by the phylogenetic analyses of Blasco-Costa et al. (2016) based on two species of *Australapatemon* and several species of *Apatemon*. Blasco-Costa et al. (2016) noted high variation in *cox1* (6.3–13.1%) within the four isolates of *A. burti* from Mexico (Hernández-Mena et al. 2014) studied here and predicted that further analyses may reveal cryptic species among this group, and among other strigeid genera. Our results support this prediction, revealing nine strongly supported lineages nested within *A. burti* from Mexico, and separate from *A. niewiadomski* from New Zealand, the only two species from which molecular data were available. With data linking larvae to adults across North America, it is apparent that these nine lineages are supported by statistically significant distinctions in host use, morphological characters that do not overlap with other known North American *Australapatemon* species, and by high *cox1* sequence divergence between monophyletic clades. The molecular, morphological and host-use data gathered here suggests four adults identified as *A. burti* (Hernández-Mena et al. 2014) belong to four separate species with ranges extending into the USA and Canada. The prior collections in Mexico include *A. diazi* (JX977727.1–LIN1), *Anas cyanoptera* (Vieillot, 1816) (JX977726.1–LIN6), *A. americana* (JX977725.1–LIN7, *A. mclaughlini* n. sp.) and *O. jamaicensis* (JX977728.1–LIN8) (Hernández-Mena et al. 2014).

Among the samples considered here, LIN1 appears the most likely to contain *A. burti* as described by Miller (1923, 1926) and others (Cort and Brooks 1928; Stunkard et al. 1941). The wide geographic distribution and diversity of snails infected by LIN1 are consistent with this identification. *Australapatemon* (*Cercaria*) *burti* was described from *Helisoma* (*Planorbis*) *trivolvis* in Burt Lake, Michigan (Miller 1923, 1926), and found in *Lymnaea humilis* (*modicella*) (Say, 1822) in the type locality soon after (Cort and Brooks 1928), suggesting early on that this species is a generalist. Similarly, cercariae of LIN1, the only first-intermediate-host generalist in this report, were found emerging from *H. trivolvis* (MGC1179 in Tables 1 and 4 and Figs. 1 and 3), *H. campanulatum*, *Lymnaea stagnalis* (Linnaeus, 1758), *S. elodes* (MGC1557 in Tables 1 and 4 and Fig. 1), and *P. gyrina* (Fig. 1), from localities in Nova Scotia, Alberta and California, which together encompass the type locality. All other lineages reported here infect a single snail species, none of which belong to *Helisoma* or *Lymnaea*, the hosts associated with *A. burti* in the type locality. This line of evidence also suggests members of LIN4, 8 and 9 are not *A. burti*, despite morphological resemblance of adults to those described by Stunkard et al. (1941). Unfortunately, we encountered no adults from LIN1, but the samples from *A. diazi* studied by Hernández-Mena et al. (2014) were also evidently similar to *A. burti*. *Anas diazi* is limited to the lower

Southwestern USA and Mexico (Lepage et al. 2014), implying that another definitive host species must be transmitting LIN1 to snails in Alberta, where this bird has never been reported (Committee ABR 2015). Previous studies have reported *A. burti* from at least ten other anatid species (see Blasco-Costa et al. 2016 and reference within). Further sampling is needed to better understand definitive host specificity. However, if LIN1 does truly represent *A. burti*, then it would not be surprising to find a wide variety of anatids can be infected with members of this lineage.

However, several factors suggest an alternative hypothesis for the high genetic diversity within LIN1, namely, that it is comprised of multiple, recently derived species. For example, within our molecular phylogeny, the cercariae that utilize *Helisoma* species display greater nucleotide substitutions within *cox1* than those from other snail hosts. Also, while the mean intraspecific *cox1* divergence in LIN1 is under the 5% cut-off, the range extends to 6.5%, suggesting more than one species within this lineage by this measure alone, even if no partition was made in ABGD barcode gap analysis. There is also significant variation in cercarial morphometrics within LIN1 (Table 1 and Suppl. Table 4). Different snail hosts could account for this phenotypic variability in LIN1, but it is also possible that multiple, closely related, host-specific species lie within it.

Isolates of LIN9 also display high intra-clade *cox1* divergence (1.0–6.8%) that exceeds the 5% cut-off hypothesis for species delimitation, but again, no further distinction was indicated by ABGD. Sample MGC1376 is 6.5–6.8% different from all other representatives in LIN9, yet there was no significant morphometric difference between this and other isolates in LIN9 (MGC1360). In both LIN1 and LIN9, we predict that further sampling, more detailed morphological analysis, and data from other molecular markers may support separate species within these lineages.

The aggregating habit of cercariae of *A. mclaughlini* n. sp. highlights the importance of pairing classical approaches with molecular analyses. By taking such a combined approach, our results can be shown to support the hypothesis of Cable and McLean (1943) that aggregation has evolved independently as a secondary specialization, in this case, within a member of the genus *Australapatemon.* This has implications for the life cycle of *A. mclaughlini* n. sp. and the taxonomy of the genus *Australapatemon*. One of the characters distinguishing *Australapatemon* from *Apatemon* is that metacercariae of members of the former genus are found in leeches, rather than fish (Blair 1976; Blasco-Costa et al. 2016; Dubois 1968; Johnston and Angel 1951; McCarthy 1990; Negm-Eldin and Davies 2002; Niewiadomska 2002; Stunkard et al. 1941; Vojtek 1964). The spherical structure and loss of the ability to swim en masse of zygocercariae is generally thought to imply ingestion by a fish host, but only Dronen (1973) has successfully infected fish with (echinostomatid) zygocercous cercariae. Notably, Hendrickson and Kingston (1974) were unable to infect fish with zygocercous cercariae closely resembling the isolates we collected. This raises several possibilities for the zygocercous phenotype of *A. mclaughlini* n. sp. and the potential mechanism for infecting its next host, namely, (1) external penetration of leech tegument, a counter-intuitive strategy for aggregating cercariae, (2) ingestion by leeches, which generally lack fine-scale visually acuity (Harley et al. 2011, 2013), (3) ingestion by a fish, rather than a hirudinid second intermediate host or (4) loss of the second intermediate host altogether, with ingestion of the aggregate by the definitive host. The latter is possible because both bird hosts of *A. mclaughlini* n. sp., *A. acuta* and *A. americana,* are dabbling ducks that mainly eat bits of vegetation in water, and other small items like invertebrates. Davies and Ostrowski de Núñez (2012) noted a similar incongruence in the life cycle of *A. magnacetabulum*, in which infection of leeches was verified experimentally, but which was described from birds (*Rupornis magnirostris* (Gmelin, 1788), *Strix rufipes* (King, 1828)) (Dubois 1988) not known to feed on leeches.

The encounter of multiple lineages in a strigeid morphotype may not seem surprising, considering the diversity that has emerged in the sister family Diplostomatidae (Locke et al. 2015a) and the intraspecific plasticity and genetic diversity among the Strigeidae (Blasco-Costa et al. 2016 and references within). However, the diversity encountered here is nonetheless remarkable; we are unaware of a molecular survey revealing nine candidate species within one nominal digenetic trematode. Most other studies report much less cryptic diversity, and the number of cryptic species encountered is driven by sampling effort, both in the Diplostomoidea (Blasco-Costa and Locke 2017) and other parasitic and free-living taxa (Poulin and de León Pérez-Ponce 2016; de León Pérez-Ponce and Poulin 2017). Interestingly, in the only study of digenetic trematodes reporting a comparable number of lineages (8) within a single morphotype, sampling effort was much larger than herein (324 isolates sequenced by Miura et al. 2005, versus 97 sequenced herein). Our material was collected mainly for the purposes of molecular analysis, and one disappointing consequence is that most of the lineages could not be described or identified. However, morphological and ecological distinctions were nonetheless observed among vouchers of these lineages, several of which were considered a single species by researchers with deep expertise in molecular phylogenetics and morphological taxonomy (Hernández-Mena et al. 2014). Further identifications or taxonomic descriptions will require additional collections, but the general finding of (at least) nine species of *Australapatemon* in North America has implications for diversity and distribution of species in this genus. For one, the two-to-three species of *Australapatemon* known in North America (*A. burti*, *A. canadensis* and possibly *A. anseris*) (Dubois 1968; McDonald 1981) clearly do not represent the true diversity of the genus in the Nearctic. Although the migratory ranges of anatids and other definitive hosts make wide

distributions plausible in all species in *Australapatemon*, most are known from a single biogeographic region (Dubois 1968; McDonald 1981; Davies and Ostrowski de Núñez 2012). Moreover, few digeneans that mature in birds have been confirmed from more than one biogeographic region with DNA sequences. There are two noteworthy exceptions: a recent molecular study of the diplostomid, *Austrodiplostomum ostrowskiae*, infecting double-crested cormorants, revealed its range extends to both the Nearctic and Neotropics (García-Varela et al. 2016); and perhaps the most suggestive exception is *Trichobilharzia querquedulae* (McLeod, 1937), a schistosome parasite of anatids that Ebbs et al. (2016) found to be globally distributed. Despite these exceptions, it is more common to find putatively cosmopolitan avian parasites to be made up of geographically isolated, genetically distinct lineages (Caffara et al. 2011; Locke et al. 2015a). This suggests that the lineages reported here likely include undescribed species, rather than new North American records of existing species of *Australapatemon*, and that molecular verification is desirable for species described in North America and reported in South America (Drago et al. 2007; Drago and Lunaschi 2010). Taken together, these results add to our understanding of the life cycles of these parasites—linking larvae to adults and other larval stages—and extend our knowledge of regional trematode distributions and local biodiversity.

To characterize diversity within the *A. burti* group, and the genus *Australapatemon*, molecular data are needed from the other seven species in this genus: *Australapatemon minor* (Yamaguti,1933), *A. bdellocystis* (Lutz, 1921), *A. fuhrmanni* (Dubois, 1937), *A. canadensis* (Dubois and Rausch, 1950), *A. congolensis* (Dubois et Fain, 1956), *A. anseris* (Dubois, 1967), and *A. magnacetabulum* (Dubois, 1988). Further studies may reveal even greater species diversity within the genus *Australapatemon*, and likely within other genera of the Strigeidae.

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