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Morphological and molecular characterization of *Apatemon* sp. infecting killifish in Mozambique

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ABSTRACT

Strigeid trematodes of the genus *Apatemon* Szidat, 1928 are intestinal parasites of fish-eating birds, utilizing various fish species as second intermediate hosts. In this study, we report morphometrical and molecular characterization of *Apatemon* sp. metacercariae parasitizing killifish *Nothobranchius furzeri* (Cyprinodontiformes: Nothobranchiidae) in south-east Mozambique. Metacercariae obtained from the cerebral cavity of killifish and two adult individuals isolated from experimentally infected ducklings were used for detailed morphological and molecular description, both resulting in generic affiliation to *Apatemon*. This is the first molecularly confirmed record of this trematode genus in Africa. Considering the morphological variability and wide host range of individual *Apatemon* species, the combination of both morphological and molecular analyses is indispensable for valid identification of this parasite. The results of our molecular analysis together with phylogenetic reconstruction indicated the presence of a new African lineage, reflecting potentially high diversity within the genus *Apatemon* comparable with other digenean genera.

1. Introduction

Adults of the genus *Apatemon* Szidat, 1928 (Strigeidae) are trematodes of fish-eating birds that utilize fish as second intermediate hosts. The complex taxonomic history of this genus has been considered at length [2,7]. Within the genus *Apatemon*, the cosmopolitan distribution, morphological variability and wide host range of the type species *Apatemon gracilis* (Rudolphi, 1819) has contributed to uncertainties in species classification in last decades. Several early records of *A. gracilis* were made under synonyms (see [2]), while other studies refer to other named species [30]. However, the results of some recent molecular studies [7,13] indicate that *A. gracilis* is a cosmopolitan species with a wide host range and polytypic morphology as proposed by Beverley-Burton in 1961 [4]. Fish hosts of *A. gracilis* include members of several families [6,31] and the location of metacercariae within the host can vary according to host family; Nemacheilidae, Cyprinidae and Gobiidae harbour cysts predominantly in the body cavity, Salmonidae in the pericardial cavity and the Gasterosteidae in the eyes [3,6].

In contrast to frequent records of *Apatemon* species from Europe, either in their adult of larval stage, knowledge on this trematode genus in Africa is poorer. Presence of adult *A. gracilis* has been documented in the northern regions (Egypt, [17]) as well as in the south-east Africa (Madagascar, [1]). Records of metacercarial stages, infecting the fish intermediate hosts, supplement the species list with *Apatemon tilapiae* Zhokhov, Miretskaya, Pugacheva & Tessema 2008 [40] recently described from *Oreochromis niloticus* (L.) and *Apatemon barbusi* Zhokhov, Miretskaya, Pugacheva & Tessema 2008 [40] collected from various species of *Barbus* Cuvier & Cloquet, 1816 in Ethiopian Lake Tana [40]. Nevertheless, description of both species mentioned was based only on morphology of larval (metacercarial) stage, making comparative genetic or morphometrical (adults) analyses impossible.

Killifish of the genus *Nothobranchius* Peters, 1868 (Cyprinodontiformes, Nothobranchiidae) are small teleost fishes living in extreme conditions of annually desiccating savannah pools throughout east Africa [38]. Adult fish occur only during the rainy season when savannah depressions are filled with rain water with populations surviving the dry season as diapaused embryos buried in the dry sediment. Annual desiccation limits their lifespan to several weeks to months [8]. Trematodes of the genus *Apatemon* were found infecting a single killifish species *Nothobranchius furzeri* Jubb at two out of fourteen localities in the region of south-east Mozambique, with the overall prevalence 9% and mean abundance 0.53 [22]. Therefore, the

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aim of this study was to describe larval stages (metacercariae) collected from *N. furzeri* in south-east Mozambique and experimentally matured adult specimens of *Apatemon* species based on the morphological and genetic characteristics using the ITS1–5.8S-ITS2 ribosomal gene cluster and mitochondrial cytochrome *c* oxidase subunit I gene.

2. Materials and methods

2.1. Specimen collection

Nothobranchius furzeri used in this study were sampled as part of a wider investigation on killifish parasites in Mozambique (for details see [22]). Sampling was conducted in south-east Mozambique in March 2013. Fish were collected from two localities (Med2 22°06′61.6″S, 33°46′47.9″E, Med4 24°20′93.6″S, 32°59′55.8″E) where infection with *Apatemon* sp. was previously recognized, using a dip net with a triangular metal frame (45 × 42 cm, mesh size 5 mm) on a 1.5 m long wooden pole. Both sites were relatively large (estimate 1000–1200 square meters), with a mud substrate and medium turbidity. The shores and water surface were largely covered with littoral vegetation and water lilies. After species identification, ten fish individuals from each locality were transported to the field laboratory where they were held in containers with aerated water and examined for parasites within 3 days of capture [20]. Fish were euthanized by spinal severance, measured for standard body length (SL), dissected and examined for parasites with special focus on metacercariae of strigeid flukes. Metacercariae were extracted from the brain cavity and preserved in 70% EtOH for molecular determination (PCR, sequencing and BLAST; see below) or in a mixture of glycerine and ammonium picrate (GAP) and 4% formaldehyde for morphological study. An additional fifty fish (25 from Med2 and 25 from Med4) were transported live to the Czech Republic for further analyses and for experimental infection of ducklings.

2.2. Experimental in vivo infection

Six individuals of *N. furzeri* were found to be infected with metacercariae of *Apatemon* sp. (site Med 2) and were used for experimental infection of two ducklings (*Anas platyrhynchos* forma domestica, 2 weeks of age). Domestic ducklings, raised under controlled conditions, were infected perorally with the entire head of three *N. furzeri.* After 5 days, both ducklings were sacrificed, immediately dissected and their gut examined (decantation) for the presence of adult trematodes. Of the two parasite individuals found, a small piece of tissue was removed and stored in 96% EtOH for molecular analysis. The eggs, together with two adult flukes, were preserved in hot formaldehyde and used for morphological description. Preserved adults were stained using iron acetocarmine, dehydrated in ethanol of increasing concentrations, and mounted in Canada balsam for permanent mounting on slides [11].

2.3. Morphometrical analysis

The encysted metacercariae, originally preserved in GAP in the field, were excysted by using antiformin solution according to Sudarikov et al. [32]. Antiformin was prepared as a 50/50 mixture of chlorinated lime solution (100 g of chlorinated lime in 170 ml of water) and baking soda solution (70 g of baking soda in 170 ml of water). The cysts were placed in a watch glass in a drop of water with 1–2 drops of antiformin dripped on them. After a few seconds, the excysted metacercariae were briefly washed in water and immediately preserved in 70% ethanol. Preserved metacercariae were stained using iron acetocarmine, dehydrated in ethanol of increasing concentrations, and mounted in Canada balsam for permanent mounting on slides [11].

Microscopic observations were made with an Olympus BX51 light microscope equipped with differential interference contrast (DIC) optics, a digital image analysis system (Motion Stream, Olympus), equipped with a drawing tube attachment. Morphometric measurements were completed for metacercariae isolated from six *N. furzeri* – 9 metacercariae prior encystation, 11 encysted metacercariae, and 7 chemically excysted metacercariae. The same analysis was applied to the 2 adult trematodes obtained from experimental infection of ducklings (see above), taking into account body size, including the forebody and hindbody; size of the oral sucker, pharynx, ventral sucker and holdfast of metacercariae and the internal organs of the hindbody of the adults. Sizes are given in micrometres (μm).

Adults from laboratory ducklings (Cat. No. D-728) and metacercariae (Cat. No. D-729) are deposited in the Helminthological Collection of the Institute of Parasitology, ASCR, České Budějovice, Czech Republic.

2.4. Molecular analysis

For the generic affiliation of the metacercariae collected from *N. furzeri*, universal primers designed for ITS2 region were used. Genomic DNA from 10 single ethanol-preserved strigeid metacercariae obtained from the tissue of 5 randomly selected *N. furzeri* from each locality (Med2 and Med4), as well as DNA from two parasite individuals obtained after experimental infection of ducklings (Med2), were isolated using the DNeasy Blood & Tissue Kit (Qiagen, DNeasy 96 Protocol) and stored at −20 °C.

The concentration of extracted DNA was measured (NanoDrop 1000, Thermo Scientific). Each 25 μl of PCR reaction contained 12.5 μl 2× concentrated EmeraldAmp GT PCR Master Mix (Takara), 5.5 μl H_2O, 1 μl of 10 μM forward 5′- GCATCGATGAAGAACGCAGC-3′ and reverse 5'-TCCTCCGCTTATTGATATGC-3’ ITS2 universal primers (195–510 bp, the sequences of universal primers for PCR amplification of ITS2 sequences of animals are described in [37,39]) and 5 μl of DNA template (25 ng). Amplification proceeded in a My Cycler (Bio-Rad) following the protocol: 94 °C, 5 min; 35 times 94 °C, 30 s; 50 °C, 30 s; 72 °C, 45 s and a final 10 min extension at 72 °C. Twenty five μl of the PCR products were separated in 1% agarose during the electrophoresis and purified (MinElute® Gel Extraction Kit, Qiagen).

After the molecular identification of trematode species (see above), the genomic DNA originating from a single ethanol-preserved strigeid metacercaria from each locality (Med2 and Med4) was used for amplification of ITS1–5.8S-ITS2 ribosomal gene cluster and mitochondrial cytochrome *c* oxidase subunit I gene (COI). The partial sequences of ITS1 and COI, typical representative markers used in phylogenetic analysis, were subsequently used (see below). DNA was eluted in 50 μL in deionised water. Forward primer D1(F), 5′-AGGAATTCCTGGTAAG TGCAAG-3′, and reverse primer D2(R), 5′- CGTTACTGAGGGAATCC TGG −3′ were used for the ITS1-5.8S-ITS2 ribosomal gene cluster [15], and forward primer Dice1F, 5′-ATTAACCCTCACTAAATTWCNTTRGATCATAAG-3′, and reverse primer Dice11R, 5′-TAATACGACTCACTATAGCWGWACHAAATTTHCGATC-3′ were used for COI [34]. Each 25 μl of PCR reaction contained the following: 12.5 μl 2× concentrated Combi PPP “Master Mix” (Top-Bio), 7.5 μl H_2O, 1 μl of 10 μM each primer (D1/D2 or Dice1F/Dice11R) and 3 μl of DNA template (15 ng). Thermocycling conditions used for amplification of the DNA regions follow Blasco-Costa et al. [7]. PCR amplicons were purified prior to sequencing using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Amplicons were cycle-sequenced from both strands using PCR primers for the ITS1 and COI regions.

The obtained PCR products were sequenced (3130xl Genetic Analyzer, Applied Biosystems), sequences were adjusted (Genious™ v.10.0 software tool) and compared to NCBI database (Basic Local Alignment Search Tool – BLAST) and submitted to GenBank.

2.5. Phylogenetic analysis

Newly generated and published sequences for ITS1 and COI region

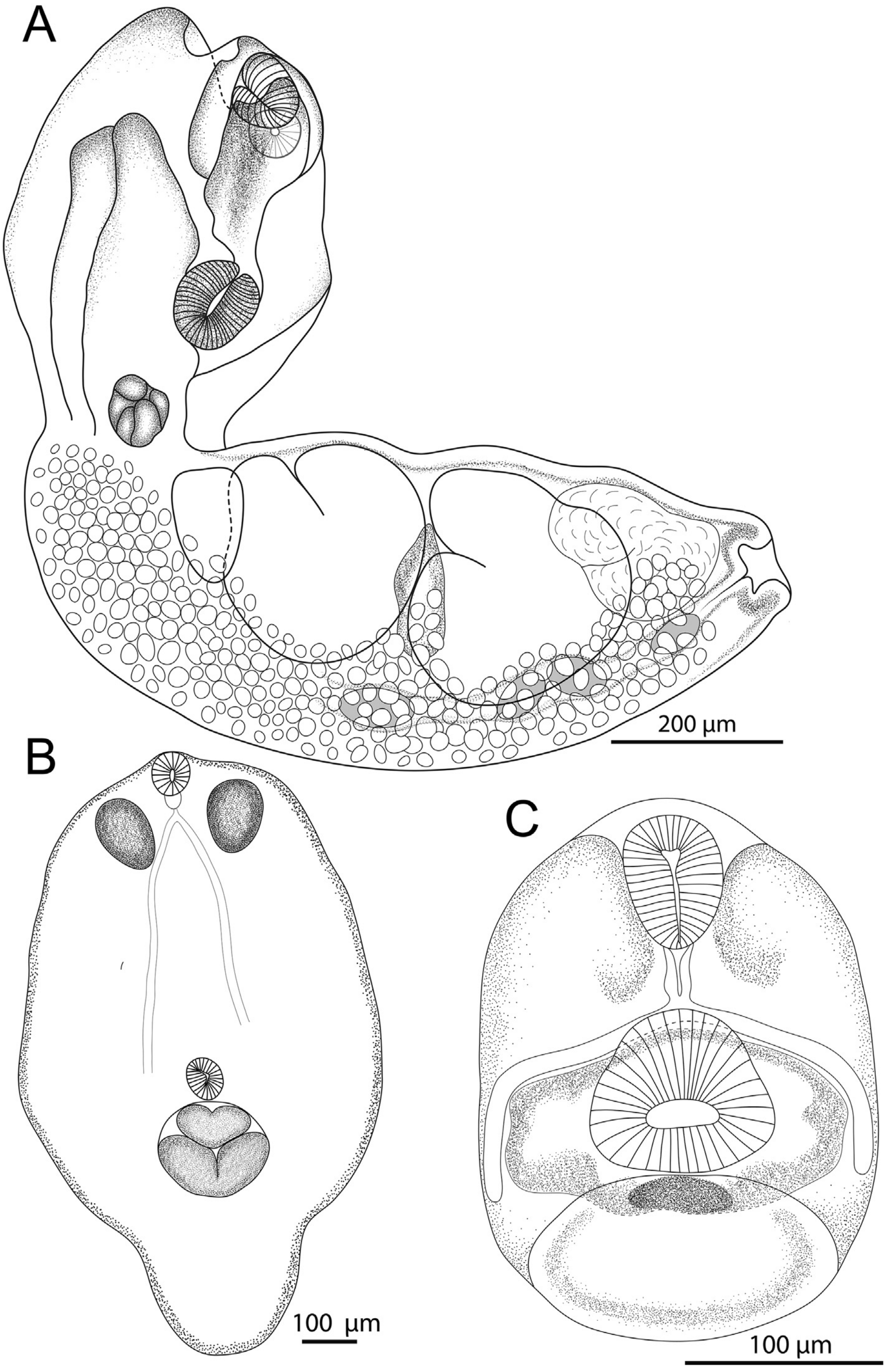

Fig. 1. *Apatemon* sp. A adult in lateral view; B pre-encystment metacercarial stage; C excysted metacercaria.

(selected representative parts) were aligned using MAFFT [18]. The ITS1 dataset (containing 351 bp of the ITS1) included: two sequences of *Apatemon gracilis* (GenBank a.n. AJ314760, AJ314759), two of *Apatemon* sp. 1 (HM064919, HM064918), two of *Apatemon* sp. 3 (HM064921, HM064920), two of *Apatemon* sp. 'jamiesoni' Blasco-Costa, Poulin & Presswell, 2016 [7] (KT334171, KT334170), two of *Australapatemon niewiadomski* Blasco-Costa, Poulin & Presswell, 2016 [7] (KT334175, KT334174), two of *Australapatemon burti* (Miller, 1923) (JX977785, JX977786) and two of newly generated sequences (*Apatemon* sp. ITS1 – locality Med2 [MH305558] and Med4 [MH305559]). COI dataset (394 bp long) included representative sequences: three sequences of *Apatemon gracilis* (GenBank a.n. KY513228, KY513218, KY513220), two of *Apatemon* sp. (KM212029, KY513229), two of *Apatemon* sp. 1 (FJ477183, HM64626), two of *Apatemon* sp. 3 (FJ477185, HM064639), two of *Apatemon* sp. 4 (FJ477186, HM064647), two of *Apatemon* sp. 'jamiesoni' (KT334182, KT334181), two of *Australapatemon niewiadomski* (KT334180, KT334186), two of *Australapatemon burti* (KY207548, JX977727) and two newly generated sequences (*Apatemon* sp. COI – locality Med2 [MH298324] and Med4 [MH298325]). *Apharyngostrigea cornu (Zeder, 1800)* was used as an outgroup in the analyses of the ITS (GenBank accession number HM064969) and COI (GenBank a.n. HM064901) datasets. Extremes of the alignments were trimmed to match the shortest sequence prior to phylogenetic analyses. Phylogenetic analyses were run on the two datasets individually under the maximum likelihood (ML) and Bayesian inference (BI) criteria. A suitable model of molecular evolution GTR + Γ + I (ITS and COI) was selected using jMODELTEST 0.1.1. [24]. The model with the best likelihood was chosen using AIC criteria and phylogenetic trees were reconstructed using Bayesian inference (BI) in the program MrBAYES v. 3.2.2. [16]. MrBAYES analyses were run for 20 million MCMC generations, with four chains and four independent runs and sampling tree topologies every 3000 generation. Burn-in periods were set to 2000 generations. The coherence of each run was checked using TRACER v1.5 [26]. ML analyses were conducted using the PhyML 3.0 software [14]. All model parameters and bootstrap nodal support values were estimated using 1000 repetitions. All phylogenetic topologies (BI and ML) were rooted using outgroup sequences. Genetic divergences among species were estimated using uncorrected p-distances with PAUP* [33].

2.6. *Data analysis*

Ecological descriptors of parasite populations (prevalence, mean abundance and intensity of infection) were calculated for both host population according to Bush et al. [9]. The relationship between fish size and metacercariae abundance was analysed using Spearman correlation. Analyses were performed using Statistica 12.0.

3. Results

3.1. *Natural infection*

The metacercariae of *Apatemon* sp. were found in the cranial cavity in a single killifish species *Nothobranchius furzeri*. Both pre-encysted and encysted metacercariae were located between the brain tissue and the skull bones. Prevalence and abundance of infection varied between sites. At locality Med2, prevalence reached 60% and mean abundance was 3.9. There was a significant positive correlation between the fish size and intensity of infection (Spearman correlation: $n = 6$, $r_s = 0.94$, $P < 0.05$). At locality Med4, only a single fish individual was infected; the overall prevalence being 10% and abundance 0.3. The average standard length of the fish was 43.6 (32–53) mm at locality Med2 and 39.2 (34–46) mm at locality Med4.

3.2. *Parasite description*

3.2.1. *Strigeidae Railliet, 1919; Strigeinae Railliet, 1919; Apatemon Szidat, 1928; Apatemon sp.*

3.2.1.1. Adult. Two adult worms (Fig. 1A) and 15 eggs were isolated from the small intestine of one experimentally infected duckling. Widths of organs correspond to their dorso-ventral diameter since the specimens were mounted laterally. Morphometrical description (Table 1) is based on two adults (one complete and one incomplete without the posterior part of the hindbody, with only one testis).

Total length 1661; Body bipartite, strongly flexed dorsally at junction of forebody and hindbody, tegument smooth. Forebody oval; 645–651 long, 434–478 wide. Hindbody subcylindrical, widest in the middle, 1010 long, 402–484 wide. Ratio of forebody to hindbody length 1:1.6. Oral and ventral suckers well developed, ventral larger than oral. Oral sucker subterminal 107–114 × 101–102. Ventral sucker 135–156 × 120–142. Oral to ventral sucker length ratio 1:1.2–1.5. Pharynx 73–75 × 69–72. Holdfast organ composed of two lobes. Proteolytic gland at base of forebody, 83–84 × 90–100. Reproductive organs confined to hindbody. Testes bilobed, situated in tandem posterior to ovary. Anterior testis 212–274 × 264–343, posterior testis 319 × 326. Ovary ovoid, transversely elongate, 95–101 × 145–156, situated immediately anterior to the testes. Vitelline follicles in hindbody, confined to ventral and subventral fields. Vitelline reservoir median, inter-testicular. Uterus ventral to gonads, with immature eggs. Mature eggs found in duckling's intestine content 92–107 × 60–81 (5 eggs measured). Seminal vesicle highly convoluted, situated dorsally to posterior testis. Copulatory bursa small. Genital cone poorly delimited from surrounding parenchyma. Laurer's canal, Mehlis' gland and excretory vesicle not observed.

Table 1
Comparative metrical data for *Apatemon* sp. adult specimen from experimentally infected *Anas platyrhynchos* and for *Apatemon* species known to infect cranial cavity of the intermediate host obtained from the literature.

	Apatemon sp.	*A.* sp. 'jamiesoni'[a]	*A. gracilis* (Rudolphi, 1819)[b]
Total body length	1661[*]	1300	< 2500
Forebody length	645–651	545	400–720
Forebody width	434–478	333	340–540
Hindbody length	1010[*]	803	930–1800
Hindbody width	402–484	333	430–600
Oral sucker length	107–114	88	110–180
Oral sucker width	101–102	91	80–140
Pharynx length	73–75	58	60–80
Pharynx width	69–72	48	52–80
Ventral sucker length	135–156	148	180–255
Ventral sucker width	120–142	139	110–245
Ovary length	95–101	82	110–130
Ovary width	145–156	130	150–180
Anterior testis length	212–274	167	210–380
Anterior testis width	264–343	152	270–360
Posterior testis length	319[*]	176	270–435
Posterior testis width	326[*]	136	250–340
Forebody to hindbody length ratio	1:1.6[*]	1:1.5	1:2.8
Oral to ventral sucker length ratio	1:1.2–1:1.5	1:1.9	1:2.0

[*] One individual measured.
[a] Data from reference [7].
[b] Data from reference [10].

3.2.1.2. Pre-encysted metacercaria. Tetracotyle-type metacercariae (Fig. 1B; $n = 6$) found in the brain cavity of fish at a stage prior to encystment. Flat, leaf-like body 1311–1891 × 729–1227 when flattened under a coverglass. Hindbody small, poorly differentiated. Oral sucker ventro-terminal, 76–111 × 77–105. Pharynx oval. Paired glandular pseudosuckers on both sides of oral sucker, approximately twice as large as oral sucker. Intestine ending weakly delimited. Ventral sucker 70–114 × 87–100, situated anterior to holdfast. Holdfast protuberant with two lobes, 193–298 × 236–324.

3.2.1.3. Encysted metacercaria. Tetracotyle-type metacercariae enclosed in egg-shaped translucent cyst (Fig. 2), thicker at the narrow pole ($n = 11$). Outer cyst 578–700 × 450–534. Encysted metacercaria 322–490 × 278–360; cyst wall thickest at poles, thickness varies from 75 to 115 at sides to 100–177 at narrow pole.

3.2.1.4. Excysted metacercaria. Seven antiformin excysted metacercariae measured (Fig. 1C, Table 2). Body 326–381 × 248–278 divided into two distinct regions, hindbody adhered to forebody as a result of excystation of formaldehyde fixed cysts. Oral and ventral suckers, pharynx and holdfast organ well-developed in the forebody, but genital primordia barely discernible in the rudimentary hindbody. Oral sucker sub-terminal, 66–112 × 58–76. Ventral sucker larger than oral sucker, circular to transversely elongate, 79–113 × 93–126. Pharynx conical. Holdfast (129–191 × 194–244) dorsal to ventral sucker. Proteolytic gland situated at base of holdfast. Excretory system not observed.

3.3. *Remarks*

Adults of *Apatemon* sp. possess characters typical for *Apatemon* Szidat, 1928, namely a small terminal copulatory bursa with genital

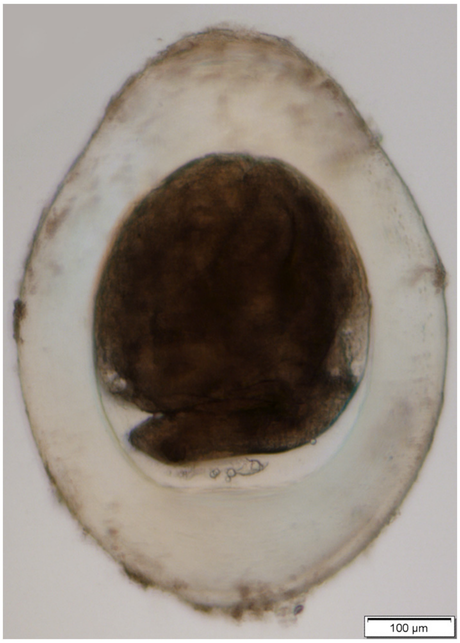

Fig. 2. Microphotograph of encysted metacercariae of *Apatemon* sp.

cone poorly delimited from surrounding parenchyma (see Fig. 1A). Two *Apatemon* species with adult stage described morphology are known to infect cranial cavity of the intermediate host, i. e. *A. gracilis* and *Apatemon* sp. 'jamiesoni' Blasco-Costa, Poulin & Presswell, 2016 [7,10]. Out of them, *A. gracilis* has been reported also from definitive hosts, *A. platyrhynchos* [1] and *Bulbucus ibis* (L., 1758) [17] in Africa. Morphological dimensions of *Apatemon* sp. obtained from experimentally infected *A. platyrhynchos* lie inside the ranges of *A. gracilis* (Rudolphi, 1819) (as re-described by Dubois [10], Table 1), being larger in body size and most other features than *Apatemon* sp. 'jamiesoni' [7], except for ventral sucker length which is smaller than in *A. gracilis* and comparable to *A.* sp. 'jamiesoni'. Oral to ventral sucker length ratio is smaller than that in both species. In addition, forebody to hindbody length ratios in *Apatemon* sp. correspond to *A.* sp. 'jamiesoni', being larger compared to *A. gracilis*. Dimensions of reproductive organs are close or below the lower limit of *A. gracilis*, possibly due to the early stage of the individual measured (Table 1).

The metacercariae of *Apatemon* sp. most closely resemble those of *Apatemon* sp. 'jamiesoni'. (see Table 2). However, although the morphological dimensions of these two species are similar, *Apatemon* sp. 'jamiesoni' metacercarial cysts appear slightly wider [7] compared to typically egg-shaped cysts of *Apatemon* sp. (Fig. 2). Metacercariae of other species known to infect fish cranial cavity, i. e. *A. gracilis*, *A. tilapiae* and *A. barbusi*, have relatively larger body size than those of *Apatemon* sp., although the dimensions of both oral and ventral sucker are similar for all species (Table 2).

3.4. Molecular analysis

The DNA sequences of ITS2 fragment (440 bp) obtained from 10 metacercariae exhibited a 98% identity to ITS2 of *Apatemon gracilis* metacercariae obtained from *Oncorhynchus mykiss*, UK (accession number: AJ301893.1). Genetic divergence between specimens from sites Med2 and Med4 was recognized as negligible, while no intraspecific variability was detected within localities. The ITS2 fragments (440 bp) of two adult worms isolated from one duckling experimentally infected with metacercariae originating from fish caught at site Med2 were 100% identical to the sequences related to metacercariae from the same locality (alignment online tool, http://xylian.igh.cnrs.fr/, [23]). The consensual nucleotide ITS2 sequence of Mozambique isolates – metacercariae and adults – *Apatemon* sp. Med2/4 was deposited in Gene Bank with the accession number (MH305557).

3.5. Phylogenetic analysis

The set of sequences used for phylogenetic analyses contained 15 unambiguously aligned ITS1 sequences (each 351 bp) and 20 COI sequences (each 394 bp). Phylogenetic reconstructions run on the two datasets (ITS1, COI) under the maximum likelihood (ML) and Bayesian inference (BI) criteria depicted highly congruent topology and showed a sister position of the genera *Apatemon* and *Australapatemon.* The sequences of African specimens identified as a species of the genus *Apatemon* clustered in one distinct well-supported clade (Fig. 3).

Newly generated COI sequences from African metacercariae (*Apatemon* sp. COI – locality Med2 and Med4, GenBank a.n. MH298324, MH298325) formed one well supported lineage corresponding to *Apatemon* spp. (including *Apatemon* sp. 1, sp. 3 and sp. 4 sensu Locke et al. 2010 [21] from the North America, and *Apatemon* sp. from Norway sensu Kuhn et al. 2015 [19] and Soldánová et al. 2017 [29]) compared to *Apatemon gracilis* sensu Soldánová et al. 2017 [29]. Sequences of *Apatemon* sp. 'jamiesoni' from New Zealand formed a monophyletic clade, sister to *Apatemon* spp. and *Apatemon gracilis* (Fig. 3B). The divergence between *Apatemon* sp. and other *Apatemon* species varied between 9.4% (*Apatemon* sp. 1 and sp. 3) to 10.4% (*Apatemon* sp. 4 sensu Locke et al. 2010 [21]).

In contrast, using phylogenetic analysis based on the ITS1 region (Fig. 3A) the separation of *Apatemon* clades were not well supported; e.g. clade *Apatemon gracilis* and *Apatemon* sp. 1 and 3. Nevertheless, analyses of the ITS1 dataset also provided support for the remaining COI-derived major clades (i.e. *Apatemon* sp. 'jamiesoni' and *Apatemon* spp. including *Apatemon gracilis*, North-American and African specimens). The sequence of *A. gracilis* identified on the basis of metacercarial morphology by Bell and Sommerville [2] and the sequence of

Table 2
Comparative metrical data for *Apatemon* sp. metacercariae and for *Apatemon* species known to infect cranial cavity obtained from the literature.

	Apatemon sp.	*A. tilapiae*[a]	*A. barbusi*[a]	*Apatemon* sp. 'jamiesoni'[b]	*A. gracilis* (Rudolphi, 1819)[c]
Metacercaria length	326–381	1440–1980	653–963	397–398	550–665
Metacercaria width	248–278	168–900	548–720	271–306	300–327
Oral sucker length	66–112	120–279	82–116	85–90	76–80
Oral sucker width	58–76	84–207	94–131	80–90	76–84
Ventral sucker length	79–113	108–168	98–154	*	84–99
Ventral sucker width	93–126	99–144	122–166	121–137	95
Holdfast length	129–191	153–405	158–222	171–221	34–46
Holdfast width	194–244	180–252	170–284	166–196	46

[a] Data from reference [40].
[b] Data from reference [7]
[c] Data from reference [10].
* Only data for width mentioned; ventral sucker circular to transversely elongate.

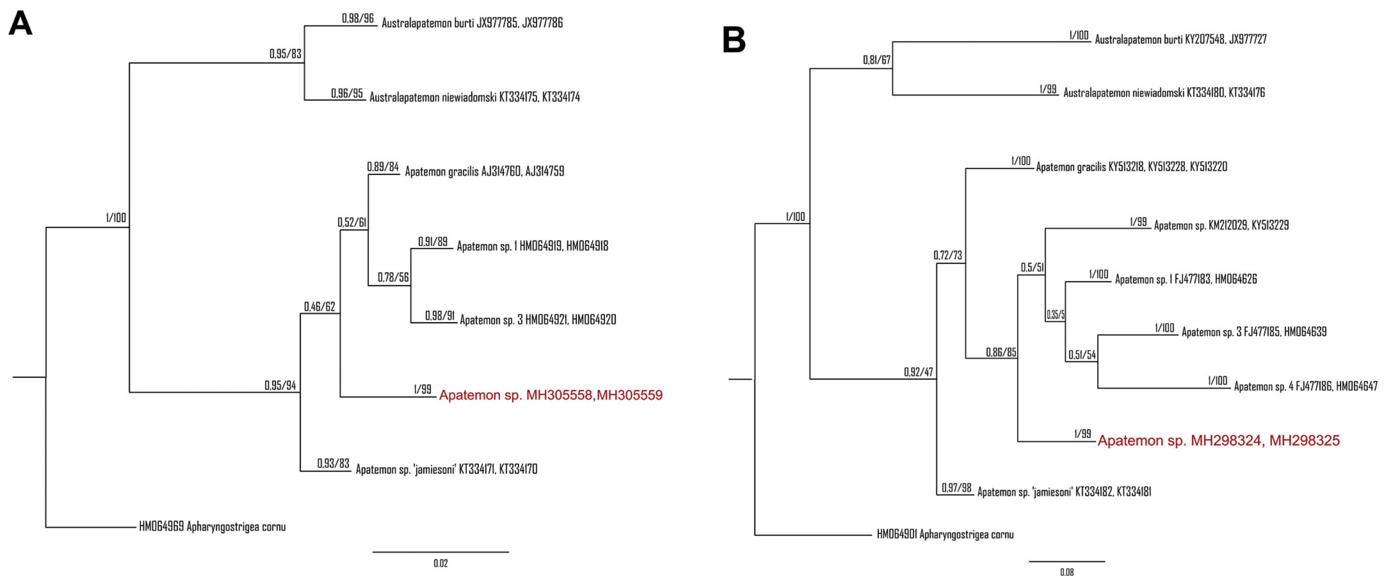

Fig. 3. Phylogenetic tree derived from (A) ITS1 and (B) COI gene sequences showing posterior probability values derived using Bayesian inference with bootstrap percentages resulting from Maximum likelihood analyses above the branches. Scale bars indicate the number of substitutions per site. Taxa in red represent a new lineage of *Apatemon* sp. from Africa from locality Med2 and Med4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

North-American *Apatemon* spp. sensu Locke et al. [21] clustered together, while two newly generated sequences for *Apatemon* sp. (*Apatemon* sp. ITS – locality Med2 and Med4, GenBank a.n. MH305558, MH305559) from Africa exhibited a close relationship with this clade. Genetic divergence between *Apatemon* sp. and other *Apatemon* species varied between 0.9% (*A. gracilis* sensu Soldánová et al. 2017 [29]) to 1.4% (*Apatemon* sp. 1 and sp. 3 sensu Locke et al. 2010 [21]).

4. Discussion

Our study provides the first confirmed record of *Apatemon* sp. metacercariae from the African continent supported by molecular data. Previous records based on morphological descriptions of metacercariae include *Apatemon tilapiae* [40] from *Oreochromis niloticus* (L.) and *Apatemon barbusi* [40] from various species of *Barbus* collected in Ethiopian Lake Tana [40]. Consistent with our results, Zhokhov et al. [40] found cysts of both previously mentioned trematodes in the cranial cavity of infected fish, although only in cases of heavy infection in small individuals. In large fishes, or when the infection rates were low, the metacercariae were found in the abdominal cavity and vitreous humour of the eyes [40]. However, metacercariae of both above mentioned species are considerably larger than the specimens of *Apatemon* parasitising killifish in the present study. The cranial cavity as a site of infection is also mentioned for *Apatemon* sp. 'jamiesoni' from Australia [7]. Smith and Hickman [28] found metacercariae of *A. gracilis* in the abdominal cavity as well as behind the eyeball next to the optic nerve of infected *Galaxias auratus* Johnston, 1883. In infected killifish, the cranial cavity was the exclusive site of infection, independently of the intensity of infection. The location of *Apatemon* cysts within the fish host appears to vary according to host family [3,6], and in the case of sticklebacks and perch the eyes are preferred by the metacercariae of *A. gracilis* [3,6]. To the best of our knowledge, *Apatemon* sp. observed in our study is the first trematode of this genus reported to infect fishes of the family Nothobranchiidae, which extends the second intermediate host range known for *Apatemon* species.

Out of the four killifish species examined by Nezhybová et al. [22] for metazoan parasites in a range of localities in Mozambique, only one species, *Nothobranchius furzeri*, was found to be infected though another congeneric species, *N. orthonotus*, was present at locality Med 4. Both localities sampled were relatively large (estimate 1000–1200 square meters) with dense vegetation, which may serve as suitable habitat for the first intermediate hosts of trematodes. During the sampling survey, the grey heron (*Ardea cinerea* Linnaeus, 1758) was seen hunting at the site, indicating predation of killifish by fish-eating birds as a possible route of transmission of the parasite. Birds from the family Anatidae are among the most frequently mentioned definitive hosts for *Apatemon* flukes but the Phalacrocoracidae were also found to host *Apatemon* flukes [7]. Successful experimental infections resulting in fertile adult flukes have also included birds belonging to the Laridae and Phasianidae [36].

Compared to the two *Apatemon* species recently described from Africa [40], the parasite abundance in killifish was relatively low, reaching a maximum of 9 metacercariae per fish. The intensity of infection positively correlated with killifish length, but the results are based on a small sample size, and, in fact, the largest individuals were not infected. An increase in infection intensity with the host age/size is often explained as a result of exposure time and niche size [25]. As killifish hatch synchronously when their habitat is filled with water, the age of all individuals is, therefore, more-or-less the same and their length similar [27]. Thus, any difference in intensity of parasite infection may represent variation in microhabitat selection by host fish or chance.

Experimental in vivo infection showed that fertile adult trematodes as well as their eggs were found 5 days post infection in the small intestine of a duckling. This relatively short infection period is typical for *A. gracilis* from domestic ducklings and even from other hosts such as herring gulls, eider ducks or domestic chickens, with just 3 to 6 days needed to obtain a fertile trematode [28,36]. Although the killifish were infected by other trematode metacercariae (see [22]), only the adult flukes of the genus *Apatemon* were found to infect ducklings and they were found to be genetically identical to *Apatemon* metacercariae removed from killifish.

The metrics of our adult specimens correspond with, or are slightly larger than, *A. gracilis* from experimental infections in domestic ducklings [4,28,30,35] and are larger than *Apatemon* sp. 'jamiesoni' from Australia [7], [Table 1]. However, the morphology of adult *A. gracilis* is known to differ according to the host family and there is a great morphological variability even within the same host [4,30,35,36]. Metacercariae of *A. gracilis* from stone loach *Barbatula barbatula* (Linnaeus, 1958) described by Vojtek [35] were of similar size to our *Apatemon* sp., while Bell et al. [3] recorded larger specimens from various fish hosts in the Salmonidae, Percidae, Nemacheilidae and Ictaluridae. Of the *Apatemon* species known to infect the cranial cavity of fishes, Australian *Apatemon* sp. 'jamiesoni' parasitizing Eleotrinae and Galaxiinae fishes [7] were of comparable size to our samples while *A. tilapiae* and *A. barbusi* from Ethiopia were distinctly larger [40, Table 2].

In the cranial cavity of killifish large unencysted metacercariae were present, in addition to encysted metacercariae. Blair [5] noted that metacercariae of *Apatemon gracilis* can be found unencysted in fish intermediate hosts for several weeks and their dimensions vary according to the time of encystment, being bigger at the start [35]. Thus, the large size of pre-encysted metaceracariea is in accordance with Stunkard et al. [30], who reported that the metacercariae decrease in size prior to cyst formation and the cyst itself decreases in size as its walls become thicker, the metacercaria becoming tightly compressed within the cyst.

The results of our molecular analysis together with phylogenetic reconstruction based on the newly-generated ITS (*Apatemon* sp. Med2 and Med4, MH305558, MH305559) and COI (*Apatemon* sp. Med2 and Med4, MH298324, MH298325) sequences, combined with sequence data available in GenBank for European, Australian and American *Apatemon* spp., indicated the presence of one new African lineage. This finding reflects the potentially high diversity of the genus *Apatemon* [7,29]. This high degree of diversity in *Apatemon* is comparable with other digeneans such as *Diplostomum* genus [12]. Blasco-Costa et al. [7] noted high variation in COI (6.3–13.1%) within four isolates of *Apatemon* spp. and predicted that further analyses may reveal new species among this group, and among other strigeid genera. Our results support this prediction, revealing one strongly supported lineage of *Apatemon* spp. including new *Apatemon* specimens from Africa that are separated from *A. niewiadomski* isolates of New Zealand origin [7] and *A. gracilis* of Norwegian origin [28]. Two new African ITS sequences (*Apatemon* sp. Med2 and Med4, MH305558, MH305559) exhibited a close relationship with the *Apatemon* spp. clade including North-American *Apatemon* lineages [21] and *A. gracilis* [29], as was previously reported for the representative sequence of *Apatemon* sp. 'jamiesoni' [7].

The single lineage status of our sequences related to the African species (*Apatemon* sp. Med2 and Med4, MH298324, MH298325) was better supported by results from COI sequence data. The sequences for *Apatemon* sp. MH298324 and MH298325 exhibited a closer relationship with *Apatemon* spp. (North America) than to *Apatemon gracilis* (Europe), although support values were not high. COI sequences for *Apatemon* sp. 'jamiesoni' from New Zealand formed a basal lineage, sister to *Apatemon* spp. and *Apatemon gracilis*, as is shown in Blasco-Costa et al. [7] and Soldánová et al. [29]. According to the results of our analyses, with the addition of new African samples, the topology of the COI tree was different – *Apatemon* sp. 'jamiesoni' from New Zealand clustered with greater probability with *Apatemon* spp., *Apatemon* sp. from Africa and *Apatemon gracilis*.

Although we used molecular data to distinguish these *Apatemon* spp. metacercarial specimens from Africa, the formal description of such isolates would require additional collection of cercarial and adult specimens from autochthonous hosts. Molecular determination of further new *Apatemon* spp. isolates and the corresponding phylogenetic analysis could potentially lead to the description of a new *Apatemon* species.

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Declarations of interest

None.

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