Heterophyid trematodes (Digenea) from penguins: A new species of *Ascocotyle* Looss, 1899, first description of metacercaria of *Ascocotyle* (*A.*) *patagoniensis* Hernández-Orts et al. (2012), and first molecular data

Jesús S. Hernández-Orts[a], Simona Georgieva[b,c], Dennis N. Landete[d], Tomáš Scholz[b,*]

[a] *Centro de Investigación Aplicada y Transferencia Tecnológica en Recursos Marinos Almirante Storni (CIMAS – CCT CONICET – CENPAT), Güemes 1030, 8520, San Antonio Oeste, Río Negro, Argentina*
[b] *Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branišovská 31, 370 05, České Budějovice, Czech Republic*
[c] *Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Parque Científico, Universidad de Valencia, Catedrático José Beltrán 2, 46980, Paterna, Valencia, Spain*
[d] *Escuela Superior de Ciencias Marinas, Universidad Nacional del Comahue, San Martín 247, 8520, San Antonio Oeste, Río Negro, Argentina*

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ABSTRACT

Two species of heterophyid trematodes were found in the Magellanic penguin, *Spheniscus magellanicus* (Forster), from Patagonia, Argentina. *Ascocotyle* (*Ascocotyle*) *patagoniensis* Hernández-Orts et al. (2012) is re-described based on new, properly fixed specimens (original material from South American sea lion, *Otaria flavescens* Shaw, was from frozen hosts). Metacercariae of this species are reported and described for the first time from the heart of the silversides, *Odontesthes argentinensis* (Valenciennes) and *O. smitti* (Lahille), from Patagonia. *Ascocotyle* (*Phagicola*) *cameliae* n. sp. is described from the intestine of *S. magellanicus*. The new species is placed into the subgenus *Phagicola* Faust, 1920 because of the presence of a single row of circumoral spines and uterine loops and vitelline follicles being confined posterior to the ventral sucker. However, it differs distinctly from other members of this subgenus by the number (19–24) and length (23–31 μm) of massive circumoral spines and by the morphology of the ventrogenital sac with a large, simple gonotyl devoid of refractile bodies. Molecular data (partial 28S rDNA sequences) for both species are also provided. Matching sequences from metacercarial and adult stages helped elucidate partially the life-cycle of *A.* (*A.*) *patagoniensis*. The interspecific relationships and phylogenetic position of *Ascocotyle* were further assessed on a broad phylogeny on the Opisthorchioidea Looss, 1899. *Ascocotyle* (*P.*) *ornamentata* Shalaby et al. (1993) described from decomposed worms (all circumoral spines were detached) found in a dog in Egypt, with no type-specimens of this species deposited in a repository collection, is considered to be *species inquirenda*.

1. Introduction

Heterophyid trematodes of the genus *Ascocotyle* Looss, 1899 are common parasites of fish-eating birds and mammals and their metacercariae are encysted in fresh-, brackishwater and marine fishes (Scholz et al., 2001; Pearson, 2008). The brackishwater species *Ascocotyle* (*Phagicola*) *longa* Ransom, 1920, whose metacercariae are encysted in mullets throughout the world (see Scholz, 1999a), is considered as a causative agent of human fish-borne disease (Chai and Jung, 2017). Brandão et al. (2013) found this species in the Magellanic penguin, *Spheniscus magellanicus* (Forster), stranded in Brazil. These authors also found another species of the subgenus *Phagicola* Ransom, 1920, which could not be identified to the species level because of poor quality of specimens available. In Patagonia, Argentina, Hernández-

Orts et al. (2012) described *Ascocotyle* (*Ascocotyle*) *patagoniensis* Hernández-Orts et al. (2012) from the South American sea lion *Otaria flavescens* Shaw as the first species of the nominotypical subgenus *Ascocotyle* found in a marine mammal (another three species of other subgenera of *Ascocotyle* were also reported from pinnipeds).

During parasitological examination of a recently dead Magellanic penguin near the type-locality of *A.* (*A.*) *patagoniensis* in Patagonia, Argentina, adults of two heterophyid trematodes of the genus *Ascocotyle* were found. One species belongs to the recently described *A.* (*A.*) *patagoniensis* for which the Magellanic penguin represents its new definitive host, whereas the other represents a new species, which is described in this paper. In addition, metacercariae of *A.* (*A.*) *patagoniensis* were found for the first time in the second intermediate host, silversides, *Odontesthes argentinensis* (Valenciennes) and *Odontesthes smitti*

(Lahille). First molecular data for both species of *Ascocotyle* are also provided.

2. Materials and methods

2.1. Specimen collection

One dead Magellanic penguin (female, about one year old) was collected in the beach near San Matias beacon (40°49′29″S, 64°42′57″W), San Matias Gulf, Río Negro, Argentina in September 2017. Its intestine was removed from the carcass and its content was washed with tap water through a sieve of 0.2 mm mesh. Live heterophyids were gently washed in saline and most of the worms were fixed immediately with almost boiling 4% formaldehyde solution (= formalin) and subsequently stored in 70% ethanol. Selected specimens were also fixed in molecular-grade ethanol for DNA isolation and subsequent molecular analyses.

Heterophyid metacercariae were found encysted in the wall of the conus arteriosus of the heart of 31 *O. argentinensis* (total length = 12.5–39.5 cm) and 31 *O. smitti* (total length = 14.5–33.1 cm) collected from several localities near the San Antonio Oeste Bay (40°43′42″–41°05′00″S; 64°19′11″–65°09′51″W), San Matias Gulf, Argentina between March and September 2017 (see Supplementary Tables 1 and 2 for collection details). Cysts were removed from the heart of three silversides, *O. argentinensis* (total length = 26.0–31.1 cm), and placed in Petri dishes with saline solution. Metacercariae were excysted using fine needles and fixed with almost boiling 4% formaldehyde solution or in a molecular-grade ethanol.

2.2. Morphological data

Adults and metacercariae prepared as whole mounts were stained with Mayer's hydrochloric carmine solution, dehydrated through a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam. Mounted specimens were examined with an Olympus BX51 microscope. Measurements were taken using the Olympus Quick-Photo Image-Program. Measurements are expressed in micrometres (μm) and are presented as the range, with the mean followed by standard deviation (SD) and the number of measured specimens or structures in parentheses. Detailed line drawings were made using a drawing tube attached to an Olympus BX51 microscope.

For scanning electron microscopical (SEM) observations, six entire adult worms of the new species were post-fixed in 2% osmium tetroxide for 2 h, washed in 0.1 M phosphate buffer, dehydrated through an acetone series, critical point-dried and mounted on aluminium stubs on double-sided adhesive carbon tape. Specimens were sputter-coated with gold and examined using a JEOL JSM 7401-F scanning electron microscope at an accelerating voltage of 4 kV at the Laboratory of Electron Microscopy, Institute of Parasitology, Biology Centre ASCR, České Budějovice, Czech Republic.

Specimens of the type-series and vouchers are deposited in the Helminthological Collection of the Museo de La Plata (HCMLP-He), Buenos Aires, Argentina, the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic (IPCAS), the Natural History Museum, Geneva, Switzerland (MHNG-PLAT), and the National Museum of Natural History of the Smithsonian Institution, Washington D.C., USA (NMNH-USNM).

2.3. Molecular data

Total genomic DNA was extracted from either posterior portion (adult worms) or entire specimens (metacercariae) using Chelex extraction protocol as described in Dallarés et al. (2013). The anterior part of adult worms was kept as voucher material (hologenophores *sensu* Pleijel et al., 2008; see Supplementary Fig. 1). PCR amplification

reactions were performed using either (i) Ready-To-Go-PCR Beads (GE Healthcare, Chicago, USA) in a total volume of 25 μl and containing ∼2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP and stabilisers including BSA, 10 mM of each PCR primer and *c*.50 ng of gDNA, or (ii) 2 × MyFi™ DNA Polymerase mix (Bioline Inc., Taunton, USA) in a total volume of 20 μl (containing 10 μl of × MyFi™ DNA Polymerase mix, 8 pmol of each primer and *c*.50 ng of gDNA). The primer pair used was LSU5' (forward; 5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′; Littlewood et al., 2000) and 1500R (reverse; 5′-GCT ATC CTG AGG GAA ACT TCG-3′; Tkach et al., 1999). Amplification products were generated under the following cycling conditions: initial denaturation step of 5 min at 95 °C, followed by 40 cycles of amplification at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min and a final extension step at 72 °C for 7 min. PCR products were purified using QIAquick™ Purification Kit (Qiagen Ltd., Hilden, Germany). Sanger sequencing of both strands was carried out on an ABI BigDye™ chemistry (ABI Perkin-Elmer, London, UK), alcohol-precipitated and run on an Applied Biosystems 3730xl DNA Analyser. Consensus sequences were assembled using Geneious v. 8 (http://www.geneious.com/; Kearse et al., 2012), quality checked and compared with those available in the GenBank database through BLASTn search tool. Contiguous sequences were deposited in the GenBank database under the accession numbers MK359080-MK359083.

2.4. Phylogenetic analyses

The 28S rDNA alignments were constructed using MAFFT v.7 (Katoh and Standley, 2013) under default gap parameters on EMBL-EBL bioinformatics web platform (http://www.ebi.ac.uk/Tools/msa/mafft/). The phylogenetic relationships were assessed on two separate datasets: (i) a dataset comprising currently available data only for *Ascocotyle* spp. which included sequences for three species of the genus; and (ii) a dataset including sequence data for the superfamily Opisthorchioidea Looss, 1899 in order to assess the phylogenetic relationships of the genus at family level. The dataset for the Opisthorchioidea comprised sequences for a total of 47 species of the Opisthorchiidae Looss, 1899, Heterophyidae Leiper, 1909 and Cryptogonimidae Ward, 1917 as currently available in the GenBank database (see Table 1 for details on the included taxa). A newly-generated sequence for a metacercaria of a cryptogonimid species ex *Paralichthys patagonicus* Jordan (total length = 29 cm) collected from the beach near San Matias beacon, San Matias Gulf, Río Negro, Argentina (40°49′29″S, 64°42′57″W) in June 2017 was also added into the analysed large dataset. The outgroup choice was informed from previous analyses on the phylogeny of the Digenea (Olson et al., 2003) and comprised sequences for *Homalometron synagris* (Yamaguti, 1953) (AY222243; Aporeadiidae Skrjabin, 1942) and *Preptetos trulla* (Linton, 1907) (AY222237; Lepocreadiidae Odhner, 1905). The alignment was trimmed and subjected to Gblocks (Castresana, 2000) as implemented in SeaView v.4 (Gouy et al., 2010) where the highly variable parts of the alignment were excluded prior to analysis using less stringent parameters. The phylogenetic affinities were assessed through Bayesian inference analysis using MrBayes v.3.2.6 (Ronquist et al., 2012). Two simultaneous runs were performed for 10,000,000 generations and sampled every 1000 generations. The 'burn-in' period was set for the first 25% of the sampled trees. Prior to analysis the best-fitting models of nucleotide substitution were estimated with jModelTest 2.1.4 (Guindon and Gascuel, 2003; Darriba et al., 2012) under the Akaike Information Criterion (AIC). These were the GTR+Γ for the *Ascocotyle* spp. dataset and GTR + I+Γ in the case of the Opisthorchioidea. Bayesian inference analyses were run on the Cipres Science Gateway v.3.1 (http://www.phylo.org/sub_sections/portal/; Miller et al., 2010). using the MrBayes (3.2.6) on XSEDE tool.

Table 1
Species of the Opisthorchioidea Looss, 1899 included in the phylogenetic analyses with data on the life-cycle stage, host species used, locality and GenBank accession number (28S rDNA). Abbreviations: A, adult; C, cercaria; M, metacercariae.

Species	Life-cycle stage	Host	GenBank accession no.	Locality	Reference
Ingroup					
Heterophyidae Leiper, 1909					
Ascocotyle (Ascocotyle) patagoniensis Hernández-Orts et al. (2012)	A	Spheniscus magellanicus (Forster)	MK359081	San Matias Gulf, Argentina	Present study
	M	Odontesthes argentinensis (Valenciennes)	MK359082	San Matias Gulf, Argentina	Present study
Ascocotyle (Phagicola) cameliae n. sp.	A	S. magellanicus	MK359080	San Matias Gulf, Argentina	Present study
Ascocotyle (Phagicola) longa Ransom, 1920	A	Mesocricetus auratus Waterhouse	MF980611	Brazil	Santos & Borges (Unpublished data)
Ascocotyle (Phagicola) pindoramensis (Travassos, 1928)	A	M. auratus	MF980609	Brazil	Santos & Borges (Unpublished data)
Ascocotyle (Phagicola) sp.	M	Chelon labrosus (Risso)	KU559561	Mistras Lagoon, Sardinia, Italy	Masala et al. (2016)
Amphimerus ovalis Barker, 1911	A	Trionyx muticus (Lesueur)	AY116876	Mississippi, USA	Olson et al. (2003)
Apophallus zalophi Price, 1932	A	Callorhinus ursinus (Linnaeus)	MG806918	St. Paul Island, Alaska, USA	Kuzmina et al. (2018)
Centrocestus formosanus Nishigori, 1924	A	M. auratus	MG874609	Thailand	Thaenkham et al. (Unpublished data)
Cryptocotyle lingua (Creplin, 1825)	C	Littorina littorea (Linnaeus)	AY222228	Germany	Olson et al. (2003)
Euryhelmis costaricensis Brenes, Arroyo & Jiménez-Quirós, 1960	M	Hynobius lichenatus Boulenger	AB521799	Fukushima, Japan	Sato (2010)
Galactosomum bearupi Pearson, 1973	C	Clypeomorus batillariaeformis Habe & Kosuge	MH257773	Heron Island, Australia	Huston et al. (2018)
Galactosomum lacteum (Jägerskiöld, 1896)	A	Phalacrocorax carbo (Linnaeus)	AY222227	Ukraine	Olson et al. (2003)
Galactosomum sp.	C	C. batillariaeformis	MH257775	Heron Island, Australia	Huston et al. (2018)
Galactosomum sp.	C	C. batillariaeformis	MH257774	Heron Island, Australia	Huston et al. (2018)
Galactosomum ubelakeri (Dailey, 1969)	A	C. ursinus	MG806920	St. Paul Island, Alaska, USA	Kuzmina et al. (2018)
Haplorchis popelkae Snyder and Tkach (2009)	A	Elseya dentata (Gray)	EU883584	Victoria River, Australia	Snyder and Tkach (2009)
Haplorchis pumilio Looss, 1896	A	Trichogaster trichopterus (Pallas)	HM004191	Thailand	Thaenkham et al. (2010)
Haplorchis taichui (Nishigori, 1924)	A	Puntius brevis (Bleeker)	HM004181	Thailand	Thaenkham et al. (2010)
Haplorchis yokogawai (Katsuta, 1932)	A	Mystus singaringan (Bleeker)	HM004177	Thailand	Thaenkham et al. (2010)
Haplorchoides daguilarensis Hostettler et al. (2018)	A	Neoarius graeffei (Kner & Steindachner)	MG747499	Kipper Creek, Queensland, Australia	Hostettler et al. (2018)
Haplorchoides maiwariensis Hostettler et al. (2018)	A	N. graeffei	MG747501	Kipper Creek, Queensland, Australia	Hostettler et al. (2018)
Haplorchoides sp.	A	Arius graeffei Kner & Steindachner	AY222226	Australia	Olson et al. (2003)
Heterophyes heterophyes (von Siebold, 1852)	M	Mugil cephalus Linnaeus	KU559558	Italy	Masala et al. (2016)
Heterophyes sp.	M	C. labrosus	KU559560	Italy	Masala et al. (2016)
Metagonimoides oregonensis Price, 1931	C	Pleurocera próxima (Say)	JQ995473	North Carolina, USA	Belden et al. (2012)
Metagonimus hakubaensis Shimazu, 1999	A	Lethenteron reissneri (Dybowski)[c]	KM061388	Japan	Pornruseetairatn et al. (2016)
Metagonimus katsuradai Izumi, 1935	A	Tanakia limbata (Temminck & Schlegel)	KM061391	Japan	Pornruseetairatn et al. (2016)
Metagonimus miyatai Saito, Chai, Kim, Lee & Rim, 1997	A	Plecoglossus altivelis (Temminck & Schlegel)	HQ832633	Miyakoda River, Japan	Pornruseetairatn et al. (2016)
Metagonimus otsurui Saito & Hori, 1962	A	Rhinogobius flumineus (Mizuno)[c]	KM061394	Japan	Pornruseetairatn et al. (2016)
Metagonimus takahashii Takahashi, 1929	A	Carassius auratus langsdorfii Temminck & Schlegel	HQ832636	Kiso River, Japan	Pornruseetairatn et al. (2016)
Metagonimus suifunensis Shumenko et al. (2017)	A	Juga tegulata (Martens)[a,c]	KX387460	Russia: Komarovka	Shumenko et al. (2017)
Metagonimus yokogawai (Katsurada, 1912)	A	P. altivelis	HQ832639	Tenryu River, Japan	Pornruseetairatn et al. (2016)
Phocitrema fusiforme Goto & Ozaki, 1930	A	C. ursinus	MG806921	St. Paul Island, Alaska, USA	Kuzmina et al. (2018)
Procerovum cheni Hsu, 1950	A	Anabas testudineus (Bloch)[c]	HM004193	Thailand	Thaenkham et al. (2010)
Procerovum varium Onji & Nishio, 1916	A	A. testudineus	HM004182	Thailand	Thaenkham et al. (2010)
Pygidiopsis macrostomum Travassos, 1928	A	M. auratus[b]	MF972527	Brazil	Borges et al. (Unpublished data)
Stellantchasmus falcatus Onji & Nishio, 1916	A	Homo sapiens Linnaeus	HM004174	Vietnam	Thaenkham et al. (2010)
Stictodora sp.	M	Chelon saliens (Risso)[d]	KU559563	Off Italy	Masala et al. (2016)
Opisthorchiidae Looss, 1899					
Clonorchis sinensis Looss, 1907	A	H. sapiens	JF823989	Vietnam	Thaenkham et al. (Unpublished data)
Opisthorchis noverca Braun, 1902	A	Sus scrofa domestica Erxleben	KC295443	Meghalaya, India	Tandon et al. (Unpublished data)
Opisthorchis viverrini Poirier, 1886	A	Puntius brevis (Bleeker)	HM004188	Thailand	Thaenkham et al. (Unpublished data)
Opisthorchis sp.	A	"duck"	MF110001	Binh Dinh, Vietnam	Dao et al. (2017)
Cryptogonimidae Ward, 1917					
Cryptogonimidae gen. sp.	M	Paralichthys patagonicus Jordan	#####	San Matias Gulf, Argentina	Present study
Adlardia novaecaledoniae Miller, Bray, Goiran, Justine & Cribb, 2009	A	Nemipterus furcosus (Valenciennes)	FJ788496	New Caledonia	Bray (2009)
Acanthostomum burminis (Bhalerao, 1926)	A	Xenochropis piscator (Schneider)	KC489791	Kanchanaburi Province, Thailand	Jayawardena et al. (2013)

Table 1 (*continued*)

Species	Life-cycle stage	Host	Locality	GenBank accession no.	Reference
Acanthostomum sp.	A	*Mieniplota scabra* (Müller)[e]	Peradeniya, Sri Lanka	KC489792	Jayawardena et al. (2013)
Caecincola parvulus Marshall & Gilbert, 1905	A	*Micropterus salmoides* (Lacépède)	USA	AY222231	Olson et al. (2003)
Mitotrema anthostomatum Manter, 1963	A	*Cromileptes altivelis* (Valenciennes)	Australia	AY222229	Olson et al. (2003)
Siphodera vinaledwardsii (Linton, 1901)	A	*Sciaenops ocellatus* (Linnaeus)	USA	AY222230	Olson et al. (2003)
Pseudosellacotyla lutzi (Freitas, 1941)	A	*Hoplias malabaricus* (Bloch)	Brazil	MH368357	Pantoja et al. (2018)
Outgroup					
Apocreadiidae Skrjabin, 1942					
Homalometron synagris (Yamaguti, 1953)	A	*Scolopsis monogramma* (Cuvier)	Australia	AY222243	Olson et al. (2003)
Lepocreadiidae Odhner, 1905					
Prepetos trulla (Linton, 1907)	A	*Ocyurus chrysurus* (Bloch)	Jamaica	AY222237	Olson et al. (2003)

[a] As *Parajuga subtegulata* Prozorova & Starobogatov, 2004 in GenBank.
[b] Laboratory host.
[c] Adult worms obtained from rodents experimentally infected with metacercariae.
[d] As *Lisa saliens* (Risso) in GenBank.
[e] As *Thiara scabra* (Müller) in GenBank.

3. Results

3.1. Taxonomic summary of Ascocotyle (Phagicola) cameliae n. sp. (Figs. 1–4)

3.1.1. Type-host
Magellanic penguin, *Spheniscus magellanicus* (Forster) (Sphenisciformes: Spheniscidae).

3.1.2. Type-locality
Beach near San Matias beacon, San Matias Gulf, Río Negro, Argentina (40°49′29″S, 64°42′57″W).

3.1.3. Site of infection
Small intestine.

3.1.4. Deposition of material
Holotype (MLP-He 7501), 4 paratypes (MLE-He 7502), 10 paratypes (IPCAS D-805), 3 paratypes (MHNG-PLAT 121254), 2 paratypes (NMNH-USNM) and 5 vouchers (IPCAS D-805).

3.1.5. Zoobank
The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:6AA338D8-65C9-4370-9FB2-73AC8DBB4B9B. The LSID for the new name *Ascocotyle* (*Phagicola*) *cameliae* n. sp. is urn:lsid:zoobank.org:act:######.

3.1.6. Molecular data
A fragment of 1252 bp of the 28S rRNA gene is available in the GenBank database (Acc. No. MK359080). Representative photomicrographs of the sequenced specimens are provided in Supplementary Fig. 1.

3.1.7. Etymology
This species is named in honour of the senior author's sister, Camelia Cristina Hernández Orts.

3.1.8. Description (Figs. 1–4)
Based on 29 mounted specimens and 6 worms studied using SEM: Body pyriform to fusiform (Fig. 1A and B), 567–838 (689 ± 74; n = 26) long and 174–312 (254 ± 32; n = 24) wide, with maximum width at level of ovary (Fig. 1A and B). Body covered with tegumental spines except for region posterior to circumoral spines and around mouth of ventrogenital sac (Fig. 2A and B; 3A-D). Spines flat, pectinate, with 2–5 tooth-like projections (digits) in anterior and middle part of body, single-toothed in posterior end of body (Fig. 3E-H). Preoral lobe usually weakly developed (Fig. 1A and B; 4C), exceptionally widely conical (Fig. 4D).

Oral sucker subterminal, 62–122 (89 ± 12; n = 26) wide, with conical, short posterior appendage, 62–79 (69 ± 6 n = 12) long, usually not reaching to pharynx (Fig. 1A and B). Oral sucker surrounded by single row of 19–24 (22, 22, 19, 24, 20, 20, 22, 20, 21, 20, 22, 20, 21; mean 21; n = 13) massive circumoral spines (Fig. 2C–E; 4A–D); spines 23–31 (26 ± 2; n = 45) long and 6–9 (8 ± 1; n = 47) wide (in frontal view). Prepharynx straight or slightly sinuous, 79–138 (100 ± 21; n = 12) long; pharynx oval, strongly muscular, 45–69 (57 ± 6; n = 24) long and 31–65 (42 ± 7; n = 22) wide. Oesophagus sinuous, shorter than prepharynx; intestinal caeca narrow, long, reaching to ovarian level, bent inwards (medially) in their terminal part (Fig. 1B). Ventral sucker well-developed, embedded, spherical, 48–74 (61 ± 7; n = 22) long and 54–81 (70 ± 6; n = 23) wide, slightly dextral and nearly equatorial, situated at 41–55% (49% ± 4%; n = 23) of body length. Mouth of ventral sucker small, transversely oval; ratio of width of suckers 1: 1.04–1.53 (1.28 ± 0.15; n = 23).

Testes double, unlobed, widely oval, symmetrical, situated close to posterolateral margin of body (Fig. 1A,B), 66–138 (96 ± 15; n = 34)
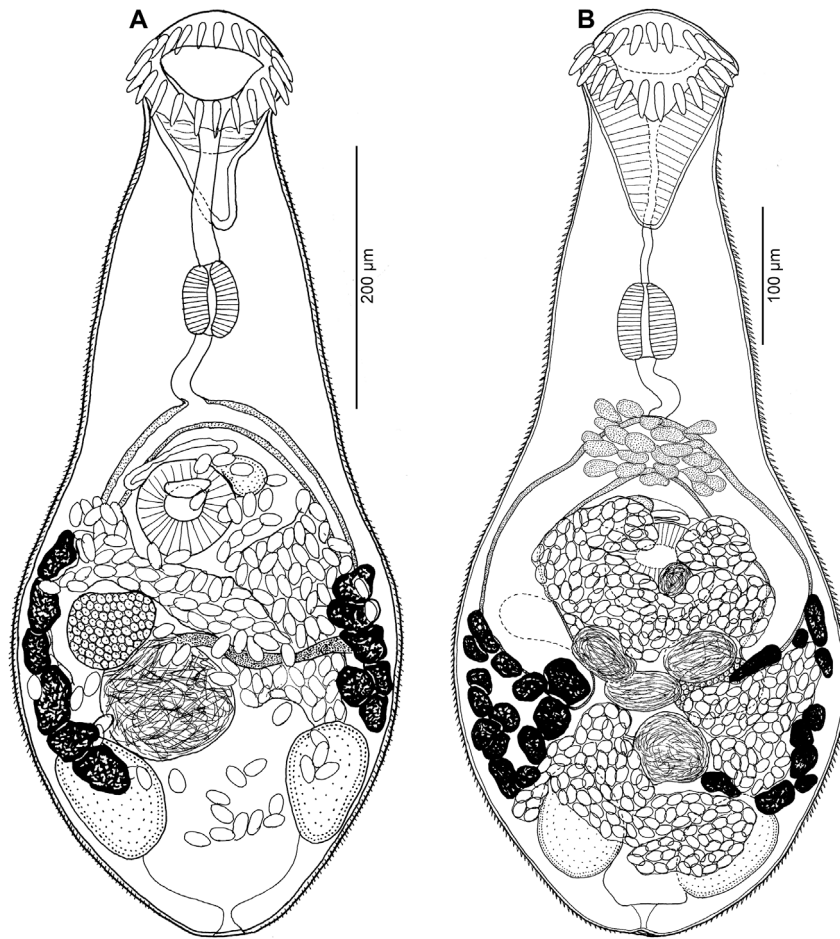
Fig. 1. *Ascocotyle* (*Phagicola*) *cameliae* n. sp. from the intestine of *Spheniscus magellanicus* collected in Patagonia, Argentina. (A) Total, ventral view of holotype (MLP-He 7501). (B) Total, dorsal view of paratype (IPCAS D-805).

long and 45–82 (65 ± 9 n = 29) wide. Seminal vesicle sinuous and tubular unless full of sperms; then its posterior part expands laterally, looking like separated dorsal chamber; this chamber may be subdivided to 2 or 3 parts (Fig. 1B). Ejaculatory duct thick-walled, curved proximally, sinistral. Ventrogenital sac formed, containing relatively small pad-like gonotyl, composed of 1 lobe of vesicular tissue (Fig. 4E). Mouth of ventrogenital sac narrow, transverse, slit-like. Genital pore (mouth of ventrogenital sac) widely oval, transverse, slightly anterosinistral to ventral sucker.

Ovary triangular to widely oval, dextral, posterolateral to ventral sucker, 40–79 (57 ± 9; n = 21) long and 48–102 (70 ± 13; n = 20) wide. Seminal receptacle voluminous, widely oval, submedian, posterior and mediodorsal to ovary (Fig. 1A and B), 56–167 (95 ± 28; n = 19) long and 36–176 (101 ± 31; n = 19) wide. Laurer's canal not observed. Vitellarium formed by follicles grouped into 2 lateral bands, situated from ventral sucker to testes level. Common vitelline ducts slightly sinuous, almost horizontal, ventrally passing posterior part of ovary and anterior part of seminal receptacle, medially joined and widened to form vitelline reservoir. Uterus tubular, forming numerous loops between anterior margin of ventral sucker and posterior margin of testes (Fig. 1A and B). Metraterm opening sinistrally into ventrogenital sinus. Eggs operculate, 19–22 (20 ± 1; n = 60) long and 9–11 (10 ± 1; n = 60) wide. Excretory vesicle Y-shaped.

3.1.9. Remarks

Pearson (2008) did not recognise individual subgenera of *Ascocotyle* as valid and considered them as synonyms of this genus. In the present paper, we recognise the subgenera *Ascocotyle* Looss, 1899, *Leighia*

Sogandares-Bernal and Lumsden, 1963 and *Phagicola* because their separation is quite straightforward and also corresponds to cercarial morphology.

The new species is placed in the subgenus *Phagicola* because uterine loops and vitelline follicles are confined posterior to the ventral sucker and a single row of circumoral spines is present. *Ascocotyle* (*P.*) *cameliae* n. sp. differs from other members of this subgenus by the number of circumoral spines (one complete row of 19–24), their size (length 23–31 μm) and by the morphology of the ventrogenital sac with a small pad-like gonotyl, composed of a single lobe of vesicular tissue without refractile bodies (see Supplementary Table 3).

Ascocotyle (*P.*) *ascolonga* (Witenberg, 1929), *A.* (*P.*) *bulbosa* Ukoli (1968) and *A.* (*P.*) *longa* have 16 spines in a single row, whereas *A.* (*P.*) *macrostoma* (Robinson, 1956) has 18 spines in a single row and *A.* (*P.*) *pindoramensis* (Travassos, 1928) does not possess circumoral spines (Witenberg, 1929; Robinson, 1956; Ukoli, 1968; Scholz, 1999a; Simões et al., 2006). The new species can be distinguished from *A.* (*P.*) *angrense* Travassos, 1916, *A.* (*P.*) *diminuta* (Stunkard & Haviland, 1924), *A.* (*P.*) *lageniformis* Chandler (1941), *A.* (*P.*) *nana* Ransom, 1920 and *A.* (*P.*) *pithecophagicola* Faust, 1920 by the different number of circumoral spines and absence of accessory spines on the dorsal side, i.e. 17–19 spines +2 accessory spines in *A.* (*P.*) *angrense*, 15–18 + 0–2 in *A.* (*P.*) *diminuta*, 16 + 2 in *A.* (*P.*) *lageniformis*, 16 + 6 in *A.* (*P.*) *nana* and 16 + 4 in *A.* (*P.*) *pithecophagicola* (see Chandler, 1941; Font et al., 1984; Ostrowski de Núñez, 1993; Scholz, 1999b).

Three other species included in the subgenus, *A.* (*P.*) *ampullacea* Miller and Harkema (1962), *A.* (*P.*) *angeloi* Travassos, 1928 and *A.* (*P.*) *intermedius* Srivastava, 1935, can be distinguished from *A.* (*P.*) *cameliae*
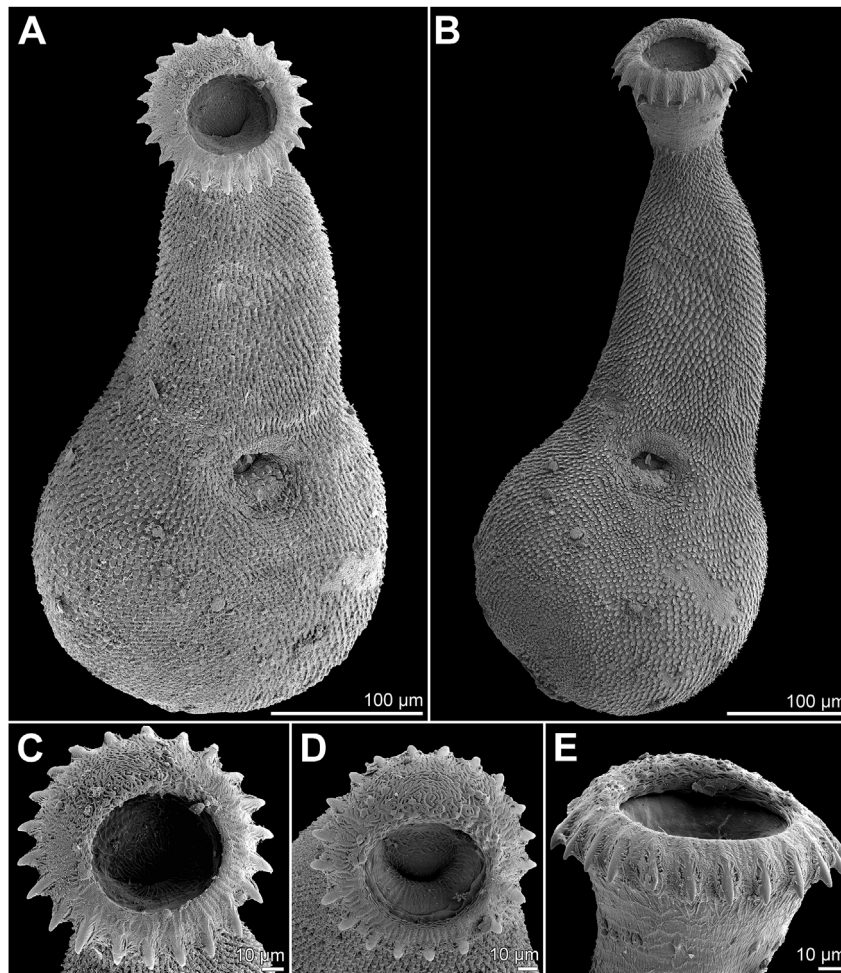
Fig. 2. *Ascocotyle* (*Phagicola*) *cameliae* n. sp. from the intestine *Spheniscus magellanicus* collected in Patagonia, Argentina. Scanning electron micrographs. (A and B) Total, ventral view. (C and D). Anterior end, apical view. (E). Anterior end, ventral view.

by possessing 2 complete rows of circumoral spines (Srivastava, 1935; Miller and Harkema, 1962; Ostrowski de Núñez, 1998).
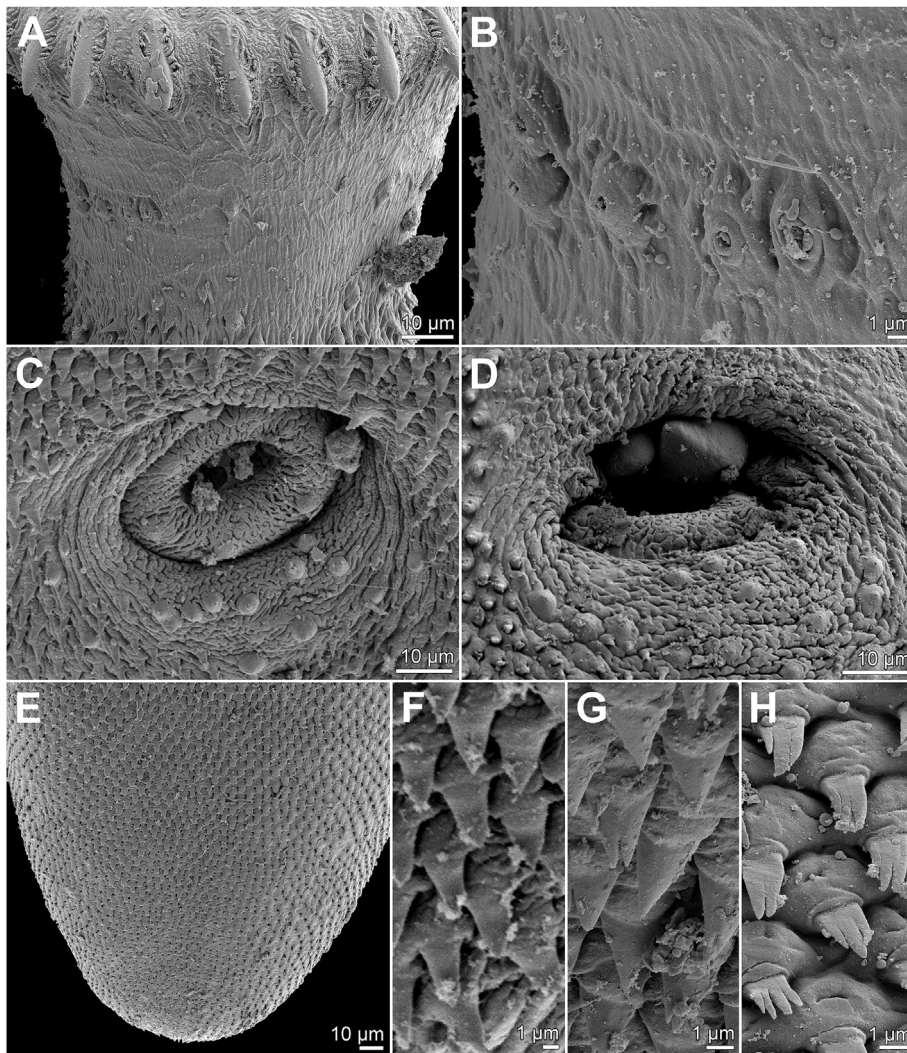
The number of circumoral spines of *A.* (*P.*) *cameliae* overlaps with that of *A.* (*P.*) *inglei* Hutton and Sogandares-Bernal (1959), *A.* (*P.*) *micracantha* Coil and Kuntz (1960) and *A.* (*P.*) *septentrionalis* Van den Broek (1967) (19–24 vs 19, 20 and 16–20 spines, respectively). However, the new species can be distinguished from them by the length of circumoral spines (23–31 μm in *Ascocotye* (*P.*) *cameliae* vs 14.5–15 μm in *A.* (*P.*) *inglei*, 10.4–11.2 μm in *A.* (*P.*) *micracantha* and 10–18 μm in *A.* (*P.*) *septentrionalis*) and the shape of the gonotyl (i.e. single lobed without refractile bodies in *A.* (*P.*) *cameliae* vs 4 separate groups of refractile bodies in *A.* (*P.*) *inglei*, 5 or 6 "chitinous bars" or "gland cells" in *A.* (*P.*) *micracantha*, and several "chitinous bars of spines" in *A.* (*P.*) *septentrionalis*) (Hutton and Sogandares-Bernal, 1959; Coil and Kuntz, 1960; Van den Broek, 1967; Scholz, 1999a).

Ascocotyle (*P.*) *cameliae* most closely resembles *A.* (*P.*) *sinoecum* Ciurea (1933) in gross morphology and the number of circumoral spines (19–24 vs 19–22). *Ascocotyle* (*P.*) *cameliae* differs from *A.* (*P.*) *sinoecum* by the shape of the gonotyl (single lobed vs two pad-like lobes), the length of the circumoral spines (23–31 μm vs 19 μm), the length of the posterior oral appendage (41–92 μm vs 90–121 μm), the extent of the caeca (ovary vs anterior border of testes), the width of the testes (45–82 μm vs 85–132 μm), the anterior extent of vitelline follicles (ventral sucker vs ovary) and by the size of the eggs (19–22 × 9–11 μm vs 22–24 × 13–15 μm) (Ciurea, 1933). Moreover, *A.* (*P.*) *sinoecum* has been reported from terrestrial and aquatic mammals from the Black Sea and Far East (Ciurea, 1933; Dalimi and Mobedi, 1992; Raga, 1992;

Demidenko and Korolev, 2004), whereas *Ascocotyle* (*P.*) *cameliae* occurs in marine birds from the Atlantic coast of Patagonia.

Ascocotyle (*P.*) *italica* Alessandrini (1906) and *A.* (*P.*) *minuta* Looss (1899) were poorly described based on a very few specimens collected from dogs in Italy and Egypt, respectively (Looss, 1899; Alessandrini, l906). The number of circumoral spines of *A.* (*P.*) *cameliae* overlaps with that of *A.* (*P.*) *italica* and *A.* (*P.*) *minuta* (19–24 vs 18–20) (Looss, 1899; Deiana, 1961). However, the new species differs from *A.* (*P.*) *italica* in the width of the body (174–312 μm vs 330–360 μm), the size of circumoral spines (23–31 × 6–9 μm wide vs 18–20 × 4–5 μm) and the anterior extent of vitelline follicles (ventral sucker vs ovary to testes) (Deiana, 1961). Moreover, the gonotyl of *A.* (*P.*) *italica* apparently possesses refractile bodies (see Fig. 2 in Deiana, 1961), whereas these structures were not observed in the new species. *Ascocotyle* (*P.*) *minuta* differs from *A.* (*P.*) *cameliae* in the length of the body (500 μm vs 597–838 μm), the size of the circumoral spines (12 × 4 μm vs 23–31 × 6–9 μm), the length of the pharynx (41 μm vs 45–69 μm), the extent of the caeca (ventral sucker vs ovary), the size of the testes (23 × 9 μm vs 66–138 × 45–82 μm), the size of the eggs (23–24 × 14 μm vs 19–22 × 9–11 μm), and the anterior extent of the vitelline follicles (posterior border of the ovary vs ventral sucker).
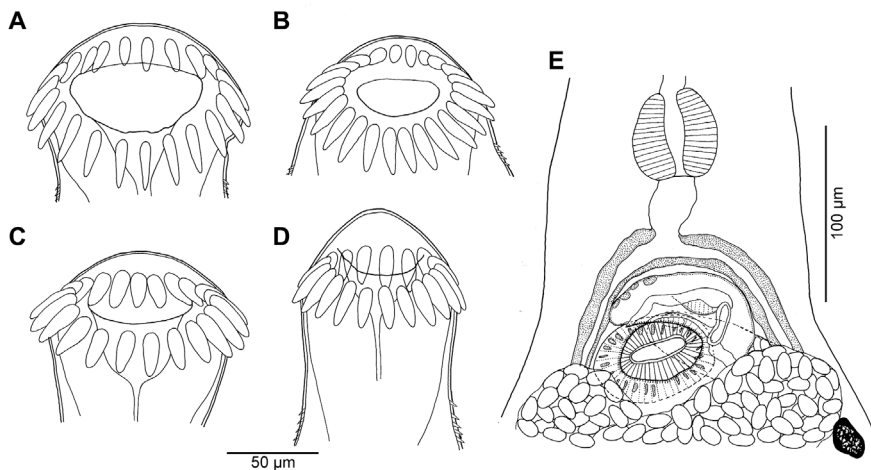
Brandão et al. (2013) reported trematodes placed in the subgenus *Phagicola* from Magellanic penguins stranded on the coast of the state of Rio de Janeiro, Brazil. These trematodes were not identified to species level because their circumoral spines were completely or partly lost, and their precise number could not be counted. The specimens from penguins of Brazil, reported as *Ascocotyle* (*P.*) sp., showed similar

Fig. 3. *Ascocotyle* (*Phagicola*) *cameliae* n. sp. from the intestine of *Spheniscus magellanicus* collected in Patagonia, Argentina. (A–D) Anterior end with circumoral spines; note variation in spine number, 22 spines (A and B), 19 spines (C) and 24 spines (D). (E) Terminal genitalia, ventral view of paratype (IPCAS ####).

morphology to that of *A.* (*P.*) *cameliae*, such as the shape and size of the body and other internal structures (including similar size of circumoral spines), and the posterior extension of uterine loops, reaching to the posterior extremity. However, *Ascocotyle* (*P.*) sp. differs from the new species in having a simple gonotyl with 4–8 refractile pockets (vs small pad-like gonotyl without refractile bodies) and caeca extending to the level of testes (vs ovary level).

Fig. 4. *Ascocotyle* (*Phagicola*) *cameliae* n. sp. from the intestine *Spheniscus magellanicus* collected in Patagonia, Argentina. Scanning electron micrographs. (A) Detail of circumoral spines; note absence of tegumental spines. (B). Detail of papillae in region devoid of tegumental spines. (C and D) Detail of the mouth of the ventrogenital sac; note gonotyl in (D). (E) Posterior end, ventral view; note simple tegumental spines reaching up to the posterior extremity. (F) Pectinate (with 4–5 digit-like processes – teeth) tegumental spines on the anterior part of the body. (G) Pectinate (with 2–3 digit-like processes) tegumental spines on the middle part of the body. (H) Simple or 2-toothed tegumental spines on the posterior part of the body.

3.2. Taxonomic summary of ascocotyle (ascocotyle) patagoniensis Hernández-Orts et al. (2012) (Fig. 5)

3.2.1. New definitive host

Magellanic penguin, *Spheniscus magellanicus* (Forster) (Sphenisciformes: Spheniscidae).

3.2.2. New locality

Beach near San Matias beacon, San Matias Gulf, Río Negro, Argentina (40°49′29″S, 64°42′57″W).

3.2.3. Site of infection

Small intestine.

3.2.4. Second intermediate hosts

Silversides *Odontesthes argentinensis* (Valenciennes) and *O. smitti* (Lahille) (Atheriniformes: Atherinopsidae).

3.2.5. Site of infection in second intermediate hosts

Wall of the conus arteriosus.

3.2.6. Locality for second intermediate hosts

San Antonio Oeste Bay, San Matias Gulf, Argentina (40°43′42″–41°05′00″S; 64°19′11″–65°09′51″W).

3.2.7. Infection parameters in second intermediate host

Prevalence: 100% (n = 31) in *O. argentinensis* and 100% (n = 31) in *O. smitti*.

3.2.7. Deposition of material: Adults: 1 voucher specimen (MLP-He 7503) and 1 voucher specimen (IPCAS D-786/1). Metacercariae: 2 voucher specimens (MLP-He 7504), 1 voucher specimen (IPCAS D-787/1) and 1 voucher specimen (NMNH-USNM).

3.2.8. Molecular data

A fragment of 1252 bp of the 28S rRNA gene is available in the GenBank database (Acc. Nos. MK359081-MK359082). Representative photomicrographs of the sequenced specimens are provided in Supplementary Fig. 1.

3.2.9. Description of adult (Fig. 5A and B)

Based on 2 heat-fixed specimens from *P. magellanicus:* Body pyriform, 419–572 long and 170–178 wide, covered with slender, simple spines (Fig. 5A). Preoral lobe well developed, 20 long (Fig. 5A and B); oral sucker subterminal, 56–62 wide, with conical, long appendage, 156 long. Oral sucker surrounded by 36–41 massive circumoral spines arranged in 2 complete rows of 18–21 spines each (Fig. 5B). Spines in anterior row 16–18 long and 6–7 wide (in frontal view; n = 6). Spines in posterior row 16–17 long and 6–7 wide (in frontal view; n = 4). Prepharynx 137 long; pharynx strongly muscular, oval to spherical, 48–54 long and 31–42 wide. Oesophagus shorter than prepharynx; caeca sacciform, short and very wide, completely preacetabular (Fig. 5A). Ventral sucker spherical, deeply embedded, measuring 40–43 long and 50–55 wide, slightly dextral and postequatorial, situated at 61–63% of body length. Mouth of ventral sucker small, spherical; ratio of width of suckers 1: 1.12–1.13.

Testes double, unlobed, widely oval, symmetrical, situated near posterior extremity, 43–58 long and 40–56 wide. Seminal vesicle tubular, voluminous. Ventrogenital sac formed, containing large, pad-like gonotyl, without refractile bodies (Fig. 5A). Mouth of ventrogenital sac narrow, slit-like. Genital pore oval, slightly anterosinistral to ventral sucker.

Ovary triangular to widely oval, dextral, 40–46 long and 41–44 wide; seminal receptacle voluminous, dorsomedial, 89–97 long and 114–120 wide. Laurer's canal not observed. Vitellarium formed by follicles grouped into 2 lateral bands, situated laterally from ventral sucker to post-testicular level. Common vitelline ducts straight,

ventrally passing posterior to ovary and midlevel of seminal receptacle, dextrally joined to form vitelline reservoir. Uterus tubular, forming numerous loops reaching posterior level of intestinal caeca and posterior extremity of body. Eggs operculate, 18–21 (20 ± 1; n = 11) long and 10–12 (11 ± 1; n = 11) wide. Excretory vesicle subterminal, subdorsal, Y-shaped.

3.2.10. Description of metacercaria (Fig. 5C–E)

Based on 4 excysted metacercariae from heart of *Odontesthes argentinensis:* Cyst spherical, thick-walled. Body of excysted metacercariae pyriform, 402–482 long and 111–156 wide, covered with flat tegumental spines (Fig. 5C and D). Eyespot pigment absent.

Preoral lobe well developed 13–28, conical (Fig. 5C, E); oral sucker subterminal, 34–57 wide, with long, conical appendage, 94–150 long (Fig. 5C). Oral sucker surrounded by 36–41 massive circumoral spines arranged in 2 complete rows of 18–21 spines each (18 + 18 = 36 spines in 2 specimens; 19 + 19 = 38 spines, n = 1; 21 + 20 = 41 spines, n = 1) (Fig. 5E). Spines in anterior row 12–14 long and 3–4 wide (in frontal view). Spines in posterior row 12–15 long and 3–4 wide (in frontal view).

Prepharynx 111–131 long; pharynx oval, strongly muscular, 37–43 long and 29–38 wide; oesophagus absent. Caeca sacciform, short and very wide, completely preacetabular, filled with large, spherical refractile corpuscles (Fig. 5C and D). Ventral sucker well developed, deeply embedded, spherical, markedly postequatorial (situated at 64–74% of body length), 40–43 and 45–49 wide; sucker-width ratio 1: 0.74–1.19.

Testes symmetrical, situated near posterior extremity, 36–47 long and 29–43 wide. Ventrogenital sac well developed, gonotyl large, anterosinistral to ventral sucker, without refractile bodies, but with several folds on posterior margin (Fig. 5C,D); mouth of ventrogenital sac large, median, slightly anterior to opening of ventral sucker. Ovary widely oval, dextral, posterolateral to ventral sucker. Anlagen of vitelline follicles forming single longitudinal bands near lateral margin between posterior extremity and mid-acetabular level. Excretory vesicle wide, subterminal, Y-shaped.

3.2.11. Remarks

This species was described by Hernández-Orts et al. (2012) from South American sea lions, *Otaria flavescens*, in Patagonia, Argentina. It was distinguished from the 10 remaining species of the nominotypical subgenus *Ascocotyle* by the number of circumoral spines (in 2 rows of 18–23 spines each), a gonotyl without papillae and a very wide seminal receptacle (Hernández-Orts et al., 2012). The original description was based on decomposed specimens that were obtained from frozen hosts. As a result, the specimens from *O. flavescens* were unnaturally relaxed, more elongate and their tegument was detached; many of them were also devoid of circumoral spines.

Most of the present specimens from Magellanic penguins were alive when fixed with hot fixative, which kept their natural shape and circumoral spines. The present material differs from those described by Hernández-Orts et al. (2012) from South American sea lions in a smaller size of the body (pyriform vs elongate), the length of the posterior oral appendage and the shape of the caeca (sacciform vs tubular). Despite these differences, the specimens from Magellanic penguins are considered conspecific with those from *O. flavescens*, all found in Patagonia, because they are indistinguishable in most taxonomically important characteristics, such as the number of circumoral spines arranged in 2 complete rows of 17–23 spines each in specimens from sea lions and 19–24 spines each in the present material, shape of the preoral lobe, sucker ratio, shape and length of the posterior appendage, the absence of refractile bodies in the gonotyl (compare Fig. 5A and B in the present paper with Figs. 1–8 in Hernández-Orts et al., 2012). The Magellanic penguin, *S. magellanicus*, represents a new definitive host of this parasite, which is reported from a bird for the first time.

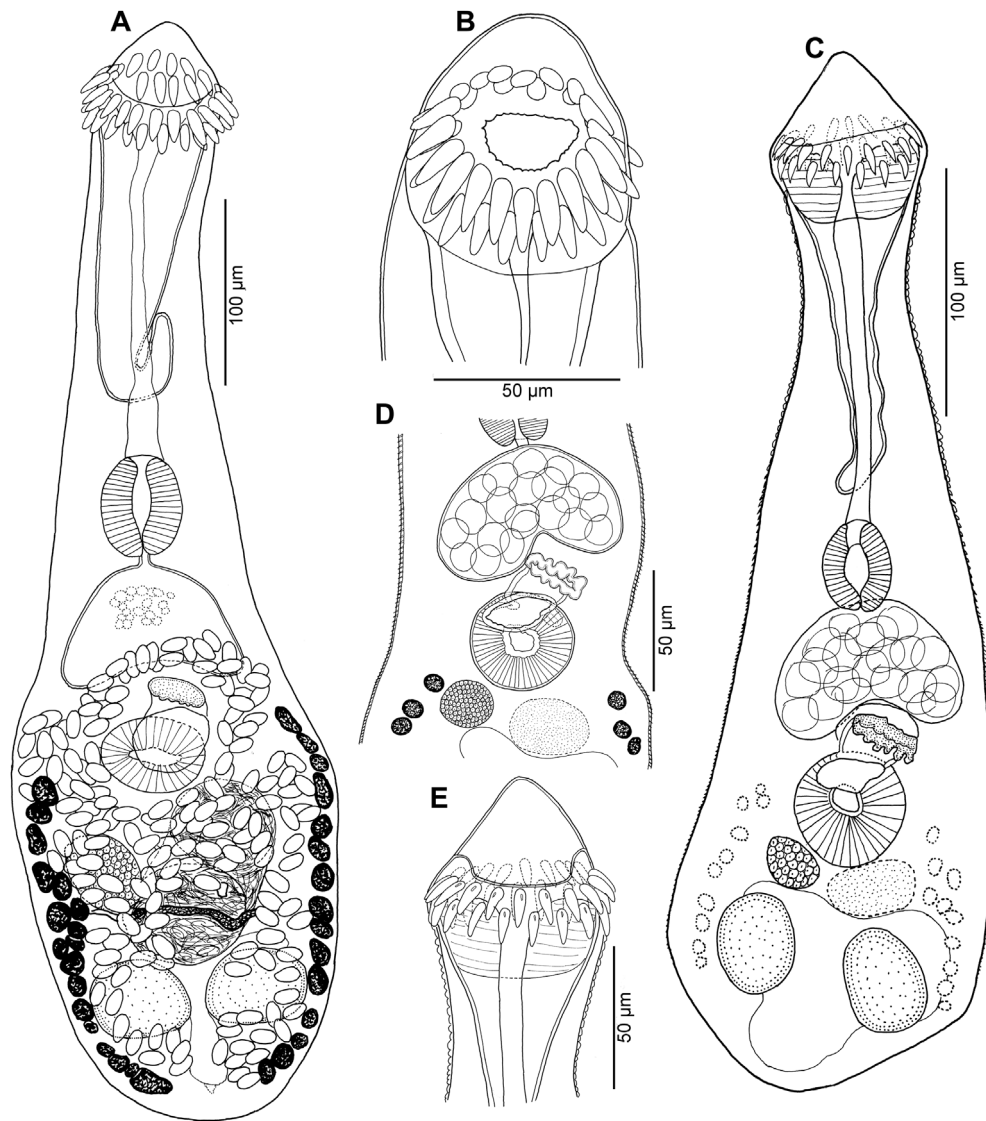Hernández-Orts et al. (2012) collected about 4500 specimens of *A.*

Fig. 5. *Ascocotyle* (*Ascocotyle*) *patagoniensis* Hernández-Orts et al. (2012). Adults from the intestine of *Spheniscus magellanicus* (A and B) and metacercariae from the heart of *Odontesthes argentinensis*, Patagonia, Argentina. (C–E). (A) Total, ventral view, voucher (MLP-He 7503). (B) Anterior end with circumoral spines, voucher (IPCAS D-786). (C) Total, ventral view. (D) Middle part of body with ventrogenital complex, ventral view. (E) Anterior end with circumoral spines.

(*A.*) *patagoniensis* from 2 out of 56 South American sea lions from Patagonia. The latter authors reported the type-locality of *A.* (*A.*) *patagoniensis* as "North Patagonia (40°43′–43°20′S, 63°04′–65°07′W)". Most of the specimens of *A.* (*A.*) *patagoniensis* (including type-series) were collected from a 13-year-old sea lion male stranded in San Antonio Oeste (40°45′38″S; 64°58′39″W), San Matias Gulf, Río Negro, Argentina, which should be considered as the type-locality of the species. Few specimens (45 worms) were also collected from a 12-year-old sea lion female found stranded in Promontorio Belén (40°09′06″S; 63°48′23″W), San Matias Gulf, Río Negro, Argentina. The penguin examined in this study was collected in the same area where the sea lions were found stranded, i.e. in the San Matias Gulf in Argentina.

Hernández-Orts et al. (2012) considered the life-cycle of *A.* (*A.*) *patagoniensis* to remain elusive, because they did not find any heterophyid metacercariae in the 542 marine teleosts from 20 species collected from the Patagonian shelf in Argentina. However, the authors did not examine the silversides, *O. argentinensis* and *O. smitti*, which in fact serve as the second intermediate host of this trematode as evidenced by the present study. The silversides are demersal, marine fish living from southern Brazil to Uruguay and Argentina (Froese and Pauly, 2018). Our results thus showed that the life-cycle of *A.* (*A.*) *patagoniensis* occurs in the coastal waters of the San Matias Gulf where both penguins and sea lions are commonly observed feeding on silversides (Hernández-Orts J.S., unpublished data).

3.3. Phylogenetic relationships and genetic divergence

The newly-generated sequences were 1252 bp (*Ascocotyle* spp.) and 1250 bp (Cryptogonimidae gen. sp.) long. The adult specimen of *A.* (*A*) *patagoniensis* shared identical sequence with a metacercaria recovered from *O. argentinensis* and confirming their conspecificity. The sequence for the isolate of the new species, *A.* (*P.*) *cameliae* n. sp., differed substantially from those of *A.* (*A.*) *patagoniensis*, i.e. by a total of 84 bp. On the other hand, the metacercaria from *Paralichthys patagonicus* differed by 179 and 146 bp, respectively from both species of *Ascocotyle* from the South-West Atlantic.

The analysed 28S dataset for *Ascocotyle* spp. comprised 1208 bp. *Phocitrema fusiforme* Goto & Ozaki, 1930 (MG806921) from the northern fur seal, *Callorhinus ursinus* Linnaeus was the outgroup choice. The tree topology resulting from the Bayesian inference analysis is presented in Fig. 6. The newly-sequenced specimens from the Southwest Atlantic, *A.* (*A.*) *patagoniensis* and *A.* (*P.*) *cameliae* n. sp., clustered

Fig. 6. Bayesian inference (BI) phylogram based on the partial (D1–D3 domains) sequences of 28S rDNA for the species of *Ascocotyle*. Posterior probability values are given above the branches. Support values with < 0.95 posterior probability are omitted. The branch length scale-bar indicates the expected number of substitutions per site. The newly-generated sequences are colour indicated (in blue) and highlighted in bold. The outgroup is represented in grey colour. Host origins of the specimens sequenced are symbol indicated on the tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

as sister species in a strongly-supported clade apart from the currently available specimens for *Ascocotyle* spp., all members of subgenus *Phagicola*, i.e. *A.* (*P.*) *longa*, *A.* (*P.*) *pindoramensis* and an otherwise unidentified metacercaria of *Ascocotyle* (*P.*) sp. from the thicklip grey mullet, *Chelon labrosus* (Risso). The latter species was recovered as earliest diverging in respect to the remaining representatives of the genus. Unexpectedly, under the current taxon sampling the newly-described species *A.* (*P.*) *cameliae,* a member of the subgenus *Phagicola*, did not cluster in a monophyletic clade with the other members of the subgenus and was recovered as a closer relative to members of the subgenus *Ascocotyle*.

The analysed 28S dataset for the Opisthorchioidea included a total of 50 species and was comprised of 1271 characters of which 69 were excluded prior to analysis. The topology of the phylogenetic phylogram resulting from the Bayesian majority rule consensus tree is present in Fig. 7. Relationships within and between the opisthorchioids were to a large extent consistent with previous phylogenetic assessments of the Opisthorchiidae + Heterophyidae (Kuzmina et al., 2018). The Heterophyidae and Opisthorchiidae fell within a major multitaxon clade sister to the earliest diverging clade of the Cryptogonimidae, although with poor support. The resulted phylogeny recovered species of the Opisthorchiidae and Heterophyidae clustered together in two major clades (denoted here as A and B, respectively), albeit with poor support (i.e. 0.68 and 0.93, respectively). *Centrocestus formosanus* (Nishigori, 1924) was placed as the earliest diverging taxon to the Opisthorchiidae + Heterophyidae. The position of the Opisthorchiidae was not well defined as the five currently available sequenced species were nested within a strongly-supported major subclade within Clade A, together with the heterophyids *Apophallus zalophi* Price, 1932, *Cryptocotyle lingua* (Creplin, 1825) and *Euryhelmis costaricensis* (Rudolphi, 1819). *Metagonimus* spp. and *Metagonimoides* spp. were placed in an unresolved earlier diverging position within Clade A. The second major clade (Clade B, Fig. 7) comprised sequence data for the genera *Procerovum* Onji & Nishio, 1916, *Haplorchis* Looss, 1899, *Haplorchoides* Chen, 1949, *Stictodora* Looss, 1899, *Galactosomum* Looss, 1899, *Stellantchasmus* Onji & Nishio, 1916, *Heterophyes* Cobbold, 1866, *Phocitrema* Goto & Ozaki, 1930, *Pygidiopsis* Looss, 1907 and *Ascocotyle*. Under the current taxon sampling *Ascocotyle* was resolved as paraphyletic forming a strongly supported major subclade together with *Phocitrema fusiforme* and *Pygidiopsis macrostomum* Travassos, 1928. The clustering pattern was consistent with that inferred from the small dataset including only *Ascocotyle* spp. The paraphyly of *Ascocotyle* was revealed not only at subgeneric, but also at generic level, i.e. the newly-sequenced *A.* (*P.*) *cameliae* n. sp. and *A.* (*A.*) *patagoniensis* clustered within a subclade with strong support and sister to the remaining taxa, namely *Ascocotyle* spp. + *Phocitrema fusiforme* + *Pygidiopsis macrostomum*.

The Cryptogonimidae was recovered as a basal to the Heterophyidae + Opisthorchiidae. The newly-sequenced metacercaria from the caecal wall of *Paralichthys patagonicus* was placed as sister to the freshwater cryptogonimid *Caecincola parvulus* Marshall & Gilbert, 1905.
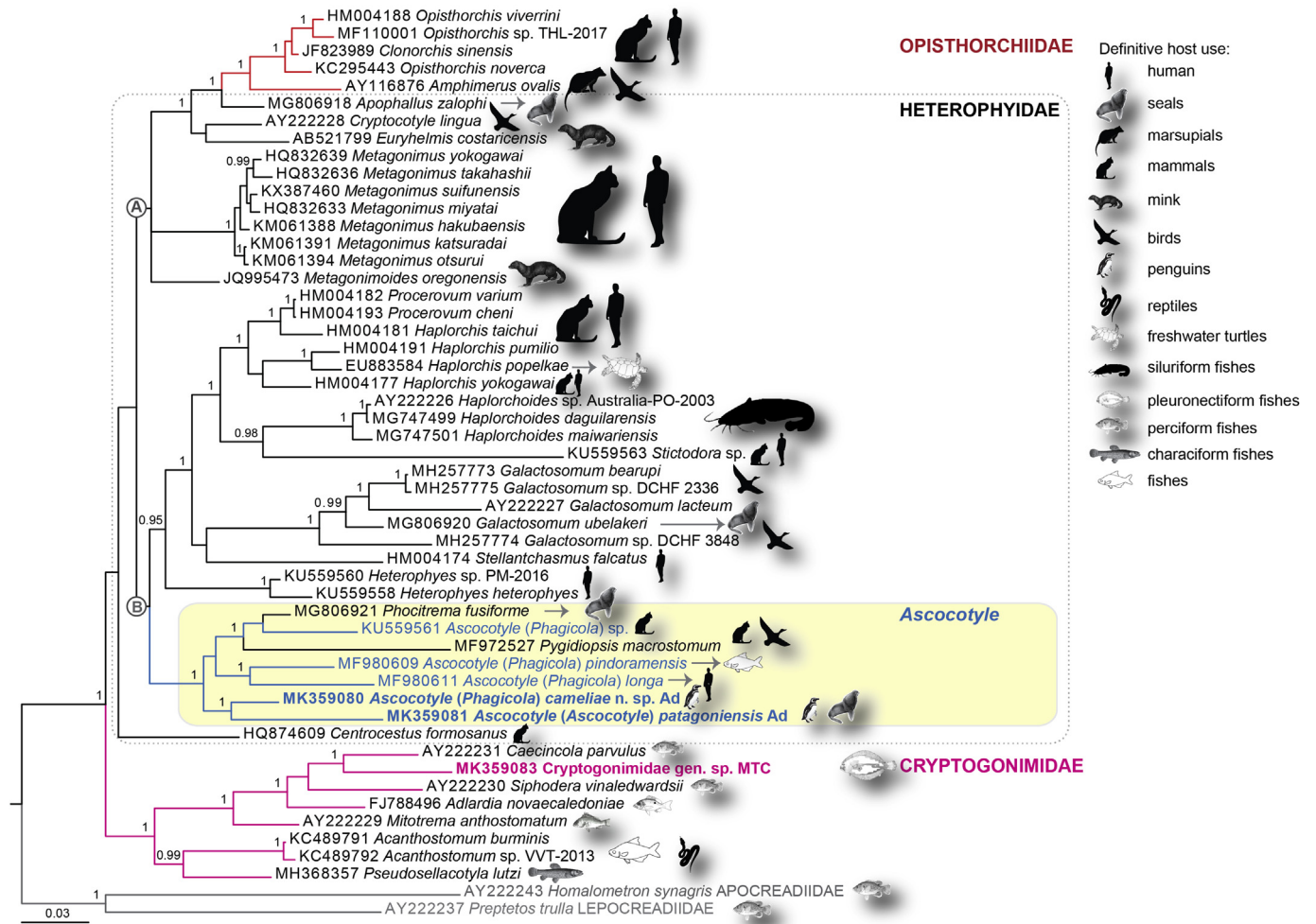
4. Discussion

Three species of the heterophyid genus *Ascocotyle* were found in Magellanic penguins in the South Atlantic off South America (Brazil and Argentina), i.e. *A.* (*A.*) *patagoniensis*, *A.* (*P.*) *cameliae* n. sp. and *A.* (*P.*) *longa*. For *A.* (*A.*) *patagoniensis*, which was originally described from South American sea lions, penguins represent new definitive hosts. The other species, *A.* (*P.*) *cameliae*, is new for science and may be specific to its definitive host, whereas the third species, *A.* (*P.*) *longa*, has a very wide spectrum of definitive hosts and large distribution area (Scholz, 1999a).

The number of circumoral spines and their arrangement are one of the most important characters used in the taxonomy of species of *Ascocotyle*, as their number is usually stable, species-specific. Both species dealt with in the present paper possess relatively many circumoral spines, but their number is subjected to some, apparently intraspecific (individual) variability. Metacercariae of *A.* (*A.*) *patagoniensis* possess 18–21 spines in a single row, i.e. between 36 and 41 spines in total.

The systematics of subgenus *Phagicola* has been dealt with mainly in the Americas (e.g. Ostrowski de Núñez, 1993, 1998; Scholz, 1999a,b; Scholz et al., 2001; Simões et al., 2006). In contrast, insufficient information exists about the morphology for the species described from the Mediterranean area in the Palaearctic region, namely *A.* (*P.*) *italica*, *A.* (*P.*) *minuta*, *A.* (*P.*) *sinoecum* and *A.* (*P.*) *ornamentata* Shalaby et al. (1993). These species were described from very few specimens or even a single specimen (see Looss, 1896), which does not allow to assess intraspecific variability of these taxa. Moreover, morphological descriptions were superficial and incomplete, with some important distinguishing characteristics (e.g. shape of the gonotyl) undescribed (Looss, 1896; Alessandrini, 1906; Ciurea, 1933; Shalaby et al., 1993). Moreover, type material of none of these problematic species is available.

The most recently proposed species, *A.* (*P.*) *ornamentata*, was described based on decomposed worms without circumoral spines and the morphological description was very poor including numerous basic errors in the English (Shalaby et al., 1993). In addition, type-specimens of this species were not deposited in a repository collection. Therefore, this taxon is considered *species inquirenda*.

The present study is the first exploring the diversity of *Ascocotyle* spp. in the respective definitive (penguin) and intermediate (fish) hosts from the South-West Atlantic, using both morphological and molecular data. Despite the very few currently available sequences for members of the genus, the topological structure of the present phylogeny rejected monophyly of *Ascocotyle*. Furthermore, despite the very few currently available sequences for members of the genus, the subgeneric clustering was not recovered by our analysis, This implies that the present structure of the genus considered by the present authors, in contrast to the concept of Pearson (2008), is unsatisfactory and calls for further thorough re-evaluation of its concept. Thus, future studies including wider taxon sampling of *Ascocotyle* spp., their morphological examination and new molecular data are needed. Including greater representation of sequence data of all life-cycle stages would provide

Fig. 7. Bayesian inference (BI) phylogram based on the partial (D1–D3 domains) sequences of 28S rDNA for the Opisthorchioidea. Posterior probability values are given above the branches. Support values with < 0.95 posterior probability are omitted. The branch length scale-bar indicates the expected number of substitutions per site. The newly-generated sequences are highlighted in bold. Species of *Ascocotyle* are indicated in blue and the respective clade is demarcated with yellow rectangular. Doted rectangular outline the members of the family Heterophyidae. Outgroup taxa are represented in grey colour. The respective definitive hosts are symbol indicated on the tree. *Abbreviations*: Ad, Adult; MTC, metacercaria. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

better assessment and subsequent bounds of both the generic and subgeneric concepts.

The present phylogenetic analysis is mostly consistent with those of Thaenkham et al. (2011, 2012) and Kuzmina et al. (2018). In both cases the Opisthorchiidae was recovered as nested within the Heterophyidae. This calls for reconsideration of the concepts of the Opisthorchiidae and Heterophyidae with reference to their morphology and respective synapomorphies. Despite the lack of well-established position of the Opisthorchiidae, the Cryptogonimidae was recovered as an earlier diverging within the Opisthorchioidea. In this case the topology is constrained by the host use, where the members of the family are parasites of fish in contrast with the heterophyids and opisthorchiids, where the preferred definitive hosts are birds and mammals.

Compliance with ethical standards

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2018.12.008.

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