***Ascocotyle longa* (Digenea: Heterophyidae) infecting dolphins from the Atlantic Ocean**

**Mariana Bertholdi Ebert1\*, Mercedes Fernández2, Ana Luisa Schifino Valente3, Marta Jussara Cremer4, Pedro Volkmer de Castilho5 and Reinaldo José da Silva1**

1 Laboratory of Parasitology of Wild Animals, Division of Parasitology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil

2 Cavanilles Institute of Biodiversity and Evolutionary Biology, Marine Zoology Unit, Science Park University of Valencia (UV), Paterna, Spain

3 Department of Morphology, Institute of Biology, Federal University of Pelotas (UFPel), Rio Grande do Sul, Brazil

4 Laboratory of Ecology and Conservation of Coastal and Marine Tetrapods, University of the Region of Joinville (UNIVILLE), São Francisco do Sul, Santa Catarina, Brazil

5Laboratory of Zoology, Department of Fishery and Biology Engineering, Santa Catarina State University (UDESC), Laguna, Santa Catarina, Brazil

\*Corresponding author: [mbe.bio@gmail.com](mailto:mbe.bio@gmail.com)

OrcID numbers:

Mariana Bertholdi Ebert: 0000-0001-8200-6411

Mercedes Fernández: 0000-0002-0875-956X

Ana Luísa Schifino Valente: 0000-0002-7854-8768

Marta Jussara Cremer: 0000-0003-3521-1409

Pedro Volkmer de Castilho: 0000-0002-9939-7807

Reinaldo José da Silva: 0000-0002-3426-6873

**ABSTRACT**

The heterophyid *Ascocotyle longa* is a cosmopolitan species that infects a variety of hosts. In this study, we report for the first time the infection of dolphins with *A.* *longa*. We examined the intestines of three dolphin species, *Sotalia guianensis*, *Steno bredanensi,* and *Tursiops truncatus gephyreus*, which were found already dead along the Southeastern and the Southern Brazilian coast. Specimens of *A*. *longa* from the pinniped *Otaria* *flavescens* were also analyzed for comparisons. The worms were identified based on morphological and molecular data using the ribosomal DNA 28S and the mitochondrial DNA COI genes. The phylogenetic analyses recovered *Ascocotyle* as a paraphyletic group, indicating the revision of the species currently comprised within it. Our new data also suggested that *A. longa* might be a cryptic species in need of further investigation. *Ascocotyle* *longa* was probably transmitted to the cetaceans by the consumption of mullets, which are common intermediate hosts for this heterophyid metacercariae. The encounter of this potentially zoonotic heterophyid species on cetaceans and pinnipeds suggests attention to human consumption of mullets at the studied area.

**Keywords** Trematoda, marine mammals, new host*,* 28S rDNA gene, COI mtDNA gene, cryptic species

**Declaration**

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**Authors' contributions**Conceived or designed the study MBE, RJS; Performed research MBE;Collected data MBE, ALSV, MJC, PVC; Analyzed data MBE, MF; Wrote the manuscript MBE, MF; Approved the final manuscript MBE, MF, ALSV, MJC, PVC, RJS

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**INTRODUCTION**

Heterophyids of the genus *Ascocotyle* Looss, 1899 are small flukes commonly found in the intestine of fish-eating birds and mammals (Scholz 1999; Pearson 2008). Among the many species currently included in the genus, *Ascocotyle longa* Ransom, 1920 is one of the most cosmopolitan and widespread, being reported from North to South America, Europe, North Africa, and Middle East Asia (Scholz 1999 and references therein).

*Ascocotyle longa* has a complex three-host life cycle; the only known natural first intermediate host is the cochliopid snail *Heleobia australis* (d’Orbigny, 1835), the metacercariae are frequently found encysted in the muscles and internal organs of mugilid fish (Simões et al. 2010) and the adult parasites are found in the intestine of a wide spectrum of definitive hosts (Scholz 1999 and references therein, 2001; Simões et al. 2010; Martorelli et al. 2012). The species was first described from the intestine of the Alaskan fox *Vulpes lagopus* (Ransom, 1920). Since then, it has been reported not only from mammals, such as cats, dogs, wolves, otters, fur seals, and sea lions but also from naturally infected fish-eating birds such as herons, cormorants, spoonbills and pelicans (Scholz 1999); besides, several birds and mammals, such as chickens, egrets, pelicans, night herons, opossums, white rats, minks, and hamsters, have served as experimental hosts for the species (Scholz 1999 and references therein). Despite the bulk of definitive hosts in which *A. longa* is found, it has never been registered infecting cetaceans so far.

In Brazil, *A.* *longa* natural infections in wild definitive hosts are known only in the cocoi heron *Ardea cocoi* Linnaeus, 1766 from the Southeastern coast (Barros et al. 2002), the Magellanic penguin *Spheniscus magellanicus* (Forster, 1781) (Brandão et al. 2013), and the South American sea lion *Otaria flavescens* Shaw, 1800, both from South Brazilian waters (Pereira et al. 2013). However, the metacercariae of the species have been described from mugilids associated with estuaries and coastal lagoons from São Paulo, Rio de Janeiro, and Espírito Santo States, on the Southeastern coast of Brazil (Almeida-Dias and Woiciechovski 1994; Knoff et al. 1997; Oliveira et al. 2007).

The morphological identification of *Ascocotyle* spp. is challenging due to their small size and the fact that the internal organs and structures as the circumoral spines, which are crucial structures in the taxonomy and the differentiation between species, can be lost or are difficult to observe (Scholz et al. 1997, 1999). As a consequence, *A. longa* has been through many taxonomic rearrangements, including several synonymies with closely related species, i.e., *Ascocotyle arnaldoi* Travassos, 1929, *Ascocotyle byrdi* (Robinson, 1956), and *Ascocotyle longicollis* (Kuntz & Chandler, 1956).

Adding to the difficulty in morphological identifications, molecular information on the genus is still scarce and few sequences are currently available. For the 28S rDNA gene, the sequences deposited in databank are from *A. longa* and *Ascocotyle pindoramensis* (Travassos, 1928) collected from experimentally infected golden hamsters *Mesocricetus auratus* Waterhouse, 1839 from Brazil, which remain unpublished, and therefore, no complementary morphological information is available until date; and a sequence from an unidentified *Ascocotyle* metacercaria collected from the grey *mullet Chelon labrosus* (Risso, 1827) from Italy. Recently, Hernández-Orts et al. (2019) contributed with novel 28S rDNA sequences from *Ascocotyle patagoniensis* Hernández-Orts et al.. 2019 and the newly described species *Ascocotyle cameliae* Hernández-Orts et al., 2019, both species collected from Magellanic penguins *S. magellanicus* from Argentina. In their works, Hernández-Orts et al. (2019) also presented the first phylogenetic positioning of *Ascocotyle*, reporting the genus as paraphyletic. As for the COI mtDNA gene, the sequences available are from the same unpublished material of *A. longa* and *A. pindoramensis* collected from experimentally infected mice *M. auratus* from Brazil, a sequence of *A. longa* collected from the double-crested cormorant *Phalacrocorax auritus* from the USA (O’Hear et al. 2014), and sequences from unidentified *Ascocotyle* metacercaria collected from the pumpkinseed *Lepomis gibbosus* (Maes, 1898) from USA (Ferguson et al. 2012) and the mummichog *Fundulus heteroclitus* (Linnaeus, 1766) from Canada (Van Steenkiste et al. 2015); however, none of these sequences have been used to specifically discuss the phylogenetic position of *Ascocotyle*.

As a part of an ongoing survey for the helminth fauna of cetaceans on the South and Southeastern Brazilian coast, several specimens of *A. longa* were collected from the intestines of dolphins. The aims of the present study are: a) to confirm the species identity based on the examination of morphological characters and phylogenetic analyses of the 28S rDNA and the COI mDNA genes; and b) to report dolphins as new hosts for the identified species. As a consequence of the achieved results, here we contribute to the revision of the phylogenetic position of *Ascocotyle* by using new molecular data and briefly discuss the species boundaries in *A. longa*.

**MATERIAL AND METHODS**

**Collection of sampling**

The worms were collected from the intestines of six dolphins belonging to three different species: one Guiana dolphin *Sotalia guianensis* Van Bénéden, 1864 collected from Cananéia municipality, São Paulo State, Southeastern Brazil (25°00'57.2"S, 47°55'31.0"W); two rough-toothed dolphins *Steno bredanensis* Lesson, 1828, collected from São Francisco do Sul municipality, Santa Catarina State, Southern Brazil (26°09'43.0"S, 48°34'01.6"W); and three Lahille’s bottlenose dolphins *Tursiops truncatus* *gephyreus* Lahille, 1908 (Committee on Taxonomy 2018) collected from Laguna municipality, Santa Catarina State, Southern Brazil (28°27'57.7"S, 48°45'36.6"W). The dolphins were collected between August 2016 and February 2018. The dolphins were found already dead, washed ashore at the coastline, and in decomposition code between 3 and 4 according to Geraci and Lounsbury (2005). During necropsy, the small intestines of the hosts were removed and stored at -20 °C until further examination. The intestines were washed over a sieve of 150 µm mesh. The content was transferred to Petri dishes, diluted in distilled water, and then examined for parasites under a stereomicroscope. Hundreds of digeneans were found in the intestine of each host. Due to the small size of the worms, they were collected with a pipet along with distilled water and placed into a falcon tube, which was further completed with 96% ethanol.

We also examined specimens of *A. longa* identified by Pereira et al. (2013), which were collected from the small intestine of a South American sea lion *O. flavescens* found dead along the coast of Rio Grande do Sul State, Southern Brazil (31°21'38"S, 33°44'35"W), between June 2010 and September 2011.

**Morphological analyses**

A total of 93 worms (29 from *S. bredanensis*, 15 from *T. t. gephyreus*, 13 from S*. guianensis*, and 36 from *O. flavescens*) was stained with Gomori’s trichome, differentiated in tap water, dehydrated in 96% ethanol, cleared with creosote and mounted as permanent slides. Morphological measurements of the specimens were made in a computerized system for image analysis with differential interface contrast (Qwin Lite 3.1, Leica Microsystems, Wetzlar, Germany). The specific identification of the worms was based on the analyses of morphological characters and the comparison with given data on *A. longa* from different hosts by previous studies (Scholz 1999; Barros et al. 2002; Simões et al. 2010; Pereira et al. 2013; Santos et al. 2013).

**DNA extraction and amplification**

Genomic DNA was extracted from seven worms (three specimens from a single *S. bredanensis*, two specimens from a single *T. t. gephyreus* and two specimens from the *O. flavescens)* using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer’s instructions, and adjusted to a final volume of 30 μl. Amplification and sequencing of fragments of the 28S rDNA and the COI mtDNA genes were performed with primers and cycling conditions as shown in Table 1. PCR reactions were performed in a final volume of 25 μl, which consisted of 3 μl DNA extract, 1 μl of each primer, 20 μl of ultrapure water (Sigma‐Aldrich, United Kingdom) and Ready‐to‐Go polymerase chain reaction (PCR) beads (Pure Taq Ready‐to‐Go PCR beads, GE Healthcare, Chicago, IL, USA). PCR products (3 μl) were run on an agarose gel (1%) using gel red and loading buffer to confirm amplicon size and yield. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions with PCR primers using the BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA, USA). Sequences were read with an Applied Biosystems ABI 3.500 DNA genetic analyzer.

**Sequence alignment and phylogenetic analyses**

The newly generated sequences of the 28S rDNA and COI mtDNA genes were assembled and edited using Sequencher v.5.2.4 (Gene Codes, Ann Arbor, MI, USA) and then aligned separately with other Heterophyidae Leiper, 1909, Opisthorchiidae Looss, 1889 and Cryptogonimidae Ward, 1917 sequences retrieved from GenBank (Table 2). Alignments were performed with default parameters of the algorithm MUSCLE (Edgar 2004) implemented on Geneious 7.1.3 (Kearse et al. 2012). The presence of stop codons and indels for the COI mtDNA alignment was verified with the amino acid translation using the echinoderm mitochondrial code table on Geneious 7.1.3 (Kearse et al. 2012).

The best‐fitting models for nucleotide substitution were determined using the Akaike information criterion using the jModelTest software (Posada 2008) as GTR+I+G for both datasets. Phylogenies were reconstructed under Bayesian Inference (BI) and Maximum-likelihood (ML) criteria. The BI analyses were run on the MrBayes v.3.2 program implemented on the CIPRES web portal (Miller et al. 2015). For BI, the Markov chain Monte Carlo (MCMC) chains were run with 106 generations and sampling tree topologies every 100th generations, with the burn‐in set for the first 25% of generations and the consensus tree estimated using the remaining topologies. Nodes with posterior probabilities (pp) greater than 0.90 were considered well supported. The ML analyses were estimated on the RAxML v.8 (Stamatakis 2014) implemented on the CIPRES web portal (Miller et al. 2015) using random starting trees with the best-fitting models selected for each dataset and 1,000 bootstrap replicates to investigate the support of each node in the most likely topologies. Nodes with bootstrap values (bv) greater than 70% were considered well supported. The trees were visualized and edited using FigTree v.1.3.1 (Rambaut 2009) and CorelDraw v6.

The number of base substitutions per site within and among the aligned sequences of each gene was calculated using the Kimura-2-parameter distance model (Kimura 1980) using MEGA7 software (Kumar et al. 2016).

**RESULTS**

**Morphological analyses**

Through morphology and morphometry, we were able to identify the analyzed specimens as *A.* *longa* (Figure 1, Table 3): body pyriform to fusiform, with maximum width at level of ovary; tegumental spines not seen, which were probably lost due to decomposition; oral sucker subterminal, strongly muscular, presenting a preoral lobe and a conical posterior appendage; oral sucker surrounded by a single row of maximum 16 circumoral spines (observed only in few specimens); pre-pharynx long and strongly muscular; oesophagus short; intestinal caeca long, dividing anteriorly to ventral sucker and reaching up to pretesticular level; spherical ventral sucker; genital pore opening immediately anterior to ventral sucker. ovary round to oval, pre-testicular, dextral to seminal receptacle; uterus at the acetabular-testicular area forming numerous loops; seminal receptacle voluminous, widely oval, in the middle of the ovarian-testicular area; Laurer's canal not observed; ventrogenital sac containing the ventral sucker and a large bipartite gonotyl with two pad-like lobes; oval testes situated close to posterolateral margin of the body; vitellaria formed by follicles grouped into two bands, in the latero-posterior region of body, between the ovary and the testes, joining almost horizontal at the region of the seminal receptacle; excretory vesicle x-shaped; and eggs operculated.

Vouchers of *A. longa* used in this study are deposited in the Helminthological Collection of the Institute of Bioscience of Botucatu (CHIBB), at the São Paulo State University - UNESP, São Paulo, Brazil (*S. bredanensis* 581L to 583L, 8851; *S. guianensis* 584L to 586L; *T. t. gephyreus* 587L; *O. flavescens* 588L to 591L, 8850).

**Phylogenetic analyses**

We generated a total of seven new sequences; one of the 28S rDNA gene from a worm collected from *S. bredanensis* (see table 1), with 1,237 bp in length, and six of the COI mtDNA gene from worms collected from *S. bredanensis*, *T. t. gephyreus*, and *O. flavescens* (see table 1), which varied from 361 and 447 bp in length.

The final alignment of the 28S rDNA gene dataset included 33 sequences and 1,111 positions. Both the BI and the ML analyses yielded similar topologies, with most clades well-supported. The 28S rDNA representative phylogenetic reconstruction (Figure 2) recovered the genus *Ascocotyle* as a paraphyletic group, forming a well-supported clade together with *Phocitrema fusiforme* Goto & Ozaki, 1930, *Pholeter gastrophilus* (Kossack, 1910) Odhner, 1914 and *Pygidiopsis macrostomum* Travassos, 1928. Our newly generated sequence of *A.* *longa* clustered in a highly supported monophyletic clade with the other *A*. *longa* sequence recovered from experimentally infected golden hamsters *M. auratus* (MF980611), being the sequence of *A. pindoramensis* (MF980609) their sister taxa. The metacercaria identified only at generic level as *Ascocotyle* sp. collected from *C. labrosus* (KU559561) assumed the position of sister to the clade formed by *P. fusiforme* and *P. gastrophilus,* with *P. macrostomum* as their sister taxa. The sequences of *A. patagoniensis* (MK359081) and *A. cameliae* (MK359080), both collected from the *S. magellanicus* clustered together in a highly supported clade, which was placed as sister to the clade formed by *A*. *longa*, *A.* *pindoramensis*, *Ascocotyle* sp., *P. macrostomum, P. fusiforme*, and *P. gastrophilus*.

The 28S rDNA gene pairwise genetic distance calculated between the two sequencesof *A*. *longa* was 0.6%. The genetic distances among our new sequence and its congeners ranged from 8.1% (*A. pindoramensis*) to 11.3% (*A. patagoniensis*)andamong congeneric species of *Ascocotyle*, the distances ranged from 7.2% (*A. pindoramensis* x *A. longa*) to 12.4% (*A. pindoramensis* x *A. patagoniensis*). The distance between *A*. *longa* and the three genera apparently most closely related, i.e., *P. fusiforme*, *P. gastrophilus* and *P. macrostomum* was 7.1%, 7.9%, and 10.3%, respectively. Between *Ascocotyle* spp., *P. fusiforme*, *P. gastrophilus,* and *P. macrostomum,* the distance ranged from 5.3 to 18.1%.

The COI mtDNA final dataset consisted of 22 sequences and yield a final alignment of 323 positions. Both the BI and the ML phylogenetic reconstructions using the COI mtDNA gene (Figure 3) also recovered *Ascocotyle* as a paraphyletic group, with *A. longa*, *A. pindoramensis*, and *P. macrostomum* clustered together in a well-supported clade. The newly generated sequences of *A.* *longa* from cetaceans and pinnipeds grouped together in a monophyletic clade. However, this clade was positioned as sister to the clade formed by the other *A*. *longa* sequences obtained from experimentally infected mice recovered from GenBank. *Ascocotyle* *pindoramensis* was positioned in a well-supported clade together with *P. macrostomum*, which by its turn, was placed as sister to the *A*. *longa* clade.

The pairwise genetic distances within our newly generated COI mtDNA sequences of *A*. *longa* collected from the cetaceans and the pinniped ranged from 0.3 to 0.6%. The genetic divergence among the newly generated sequences and the sequences of *A*. *longa* collected from the experimentally infected mice ranged from 9.5 to 10.8%. Between our new sequencesand *A. pindoramensis,* the distance varied from 17.3 to 18.6%, and between our new sequencesand *P. macrostomum,* the range was from 18.8 to 20.0%.

**DISCUSSION**

Here we report for the first time the infection of three delphinid species, *S. guianensis, S. bredanensis,* and *T. truncatus gephyreus,* with *Ascocotyle longa*, which now represents new definitive hosts for this trematode species.

Our specimens were collected from stranded and already decomposing carcasses of marine mammals (decomposition codes 4 and 5) inducing a relatively poor condition of the parasites. As a consequence, we could only determine the precise number of circumoral spines in few worms since in most specimens they were not present or visible, probably lost due to decomposition. The same is valid regarding the tegumental spines, which could not be observed in our specimens. Despite that, the combination of morphological characteristics along with morphometrical data contributed to the correct morphological identification of *A.* *longa* in our samples. However, despite the general morphology of our specimens fitted the descriptions of *A. longa* (Scholz 1999, Barros et al. 2002; Simões et al. 2010; Pereira et al. 2013; Santos et al. 2013), we found morphometrical variation among our specimens collected from all three dolphins, the pinniped and the ones from *V. lagopus*, *L. reponda* and *Pelecanus occidentalis carolinensis* Gmelin, 1789 (Scholz 1999), *A. cocoi* (Barros et al. 2002) and *M. auratus* (Simões et al. 2010; Santos et al. 2013) (see Table 3), mainly regarding size of body, oral sucker, ovary, and seminal receptacle. We considered those differences either as intraspecific or due to poor morphological conditions of the samples, which is a similar result reported by Scholz (1999). Nevertheless, we incetivet further comparative analyses to corroborate this statement.

Our analyses using both the 28S rDNA and the COI mtDNA genes recovered paraphyly in *Ascocotyle*, which is in agreement with the results reported by Hernández-Orts et al. (2019). The 28S rDNA genetic distances between congeneric species of *Ascocotyle* (7.2% to 12.4%) was much similar or higher to the recovered values of genetic distances between *Ascocotyle* species and their supposedly closest related genera, i.e., *P. fusiforme*, *P. gastrophilus*, and *P. macrostomum* (5.3% to 18.1%). The same result was obtained using the COI mtDNA gene since the genetic distances between our *A.* *longa* sequences and its congener *A. pindoramensis* (17.3 to 18.6%) was much similar to the recovered distance between *A.* *longa* and the representing species of its closest related genera, *P. macrostomum* (18.8 to 20.0%). Thus, as currently comprised, the genus *Ascocotyle* includes species that are paraphyletic and present high genetic distances among then. These results suggest the need of further evaluation of the present structure of *Ascocotyle* and the taxonomic status of the species currently included within it.

Our newly 28S rDNA sequence clustered together with the other *A. longa* sequence recovered from the experimentally infected *M. auratus*. The genetic divergence found among those two sequences was 0.6%. If one compares this value with the ones found between *A. longa* and their supposed congeneric species (7.2% to 12.4%), then one might consider that the divergence between the two *A. longa* sequences probably represents an intraspecific value. However, besides being one of the most frequently applied markers among studies dedicated to infer phylogenetic relationships of marine trematodes (Nolan and Cribb 2005), the rDNA 28S gene is sometimes considered unresolutive for species determination (Locke et al. 2010). Furthermore, since *Ascocotyle* appears as a paraphyletic group, the identities of the species currently included in it are still in need of further evaluation. Therefore, it is not clear if the divergence found between the two 28S rDNA *A. longa* sequences should be enough to consider them as conspecific.

The four newly COI mtDNA sequences of *A.* *longa* from the delphinid hosts clustered together in a monophyletic clade along with the two new sequences of *A.* *longa* from the pinniped host, revealing that these two groups of marine mammals are infected by the same trematode species. The low COI mtDNA intraspecific divergence found within the *A.* *longa* sequences from dolphins and pinnipeds (0.3 to 0.6%) also confirmed this statement. However, the two clades obtained, one comprising our newly generated sequences and the other formed by the *A. longa* sequences recovered from experimentally infected mice, are reciprocally monophyletic, suggesting that they might not be considered conspecific. The COI mtDNA distance between those two groups of *A*. *longa* (9.5 to 10.8%) was also higher than the expected for COI mtDNA intraspecific divergence rates in trematodes. It has already been suggested that high levels of divergence in trematodes are unlikely among conspecific individuals (Pérez-Ponce de León et al. 2016). According to Vilas et al. (2005), the maximum intraspecific variation in platyhelminths ranges between 0.3% and 2.2% and, individuals considered as the same species which diverge by more than 5% should be investigated as separate species.

Based on our results combining morphological and molecular analyses from ribosomal and mitochondrial genes, we suggest that *A. longa* might be interpreted as a cryptic species. *Ascocotyle longa* is a cosmopolitan species worldwide distributed which infects a variety of hosts and consequently transits between several ecological and evolutionary interactions. These many interactions may influence the gene flow of the species, which could proceed with speciation processes. Therefore, to unravel the species identity and boundaries, we suggest further taxonomic and phylogenetic investigations with more *A. longa* sequences and especially of specimenscollected from different groups of hosts, along with new genes and methodologies.

As already mentioned, the metacercariae of the species have been found infecting mugilids associated with estuaries and coastal lagoons along with the southeastern Brazilian coast (Almeida-Dias and Woiciechovski, 1994; Knoff et al. 1997; Oliveira et al. 2007). Not surprisingly, the three delphinid species serving as hosts for *A.* *longa* present coastal and/or estuarine habits on the Brazilian coast (Silva and Best 1996; Jefferson et al. 2015; Costa et al. 2016) and feed, among others, on mugilid fish (Lodi and Hetzel 1999; Rosas et al. 2010; Laporta et al. 2016), which probably facilitated their infection with the parasite. *Ascocotyle* *longa* is considered one of the causative agents of the emergent fish-borne disease of human heterophyiasis (Muller 2001; Fried et al. 2004), with several cases already reported in Brazil (Chieffi et al. 1990, 1992; Antunes and Almeida-Dias 1994). Because of its zoonotic potential, their encounter infecting the Brazilian dolphins and sea lions highlights the need for attention on human consumption of uncooked or partially cooked mugilid fish, especially caught from the studied area.

This is the first time a species of *Ascocotyle* is found in cetaceans. The encounter of a new parasite in the intestine of three different cetacean species reinforces the importance of systematized routine parasitological surveys in marine mammals. In the case of *Ascocotyle* spp., considering their minute size, they might be readily overlooked during a parasitological examination, unless done by a specialist. The data presented here contributes for future diversity surveys and encourages researchers towards a greater awareness about the importance of the helminth fauna of cetaceans since our understanding of the interactions between parasites and their cetacean hosts require a detailed and updated account of its biodiversity.

**REFERENCES**

Almeida-Dias ER, Woiciechovski E (1994) Ocorrência da *Phagicola longa* (Trematoda: Heterophyidae) em mugilídeos e no homem, em Registro e Cananéia, SP. Hig Alim 8:43-46

Antunes SA, Almeida-Dias ER (1994) *Phagicola longa* (Trematoda: Heterophyidae) em mugilídeos estocados resfriados e seu consumo cru em São Paulo, SP. Hig Alim 8:41-42

Barros LA, Arruda VS, Gomes DC, Pinto RM (2002) First natural infection by *Ascocotyle* (*Phagicola*) *longa* Ransom (Digenea, Heterophyidae) in an avian host, *Ardea cocoi* Linnaeus (Aves, Ciconiiformes, Ardeidae) in Brazil. Rev Bras Zool 19(1):151-155. https://doi.org/10.1590/S0101-81752002000100013

Borges JN, Costa VS, Mantovani C, Barros E, Santos EGN, Mafra CL, Santos CP (2017). Molecular characterization and confocal laser scanning microscopic study of *Pygidiopsis macrostomum* (Trematoda: Heterophyidae) parasites of guppies *Poecilia vivipara*. J Fish Dis 40:191-203. https://doi.org/10.1111/jfd.12504

Brandão M, Luque JL, Scholz T, Kostadinova A (2013). New records and descriptions of digeneans in the Magellanic penguin, *Spheniscus magellanicus* (Aves: Sphenisciformes), from Brazilian coasts. Syst Parasitol 85:79-98. https://doi.org/10.1007/s11230-013-9410-2

Bray RA, Waeschenbach A, Cribb TH, Weedall GD, Dyal P, Littlewood DTJ (2009) The phylogeny of the Lepocreadiidae (Platyhelminthes: Digenea) inferred from nuclear and mitochondrial genes: implications for their systematics and evolution. Acta Parasitol 54:310-329. https://doi.org/10.2478/s11686-009-0045-z

Chieffi PP, Gorla MCO, Torres DMAGV, Dias RMDS, Mangini AC, Monteiro AV, Wolciechovski E (1992) Human infection by *Phagicola* sp. (Trematoda, Heterophyidae) in the municipality of Registro, São Paulo State, Brazil. J Trop Med Hyg 95:346-348

Chieffi PP, Leite OH, Souza-Dias RMD, Torres DMAV, Mangini ACS (1990) Human parasitism by *Phagicola* sp. (Trematoda, Heterophyidae) in Cananéia, São Paulo State, Brazil. Rev Inst Med Trop São Paulo 32:285-288. <https://doi.org/10.1590/s0036-46651990000400008>

Committee on Taxonomy (2018) List of marine mammal species and subspecies. Society for Marine Mammalogy, www.marinemammalscience.org, consulted on 30 september 2019

Costa APB, Rosel PE, Daura-Jorge FG, Simões-Lopes PC (2016) Offshore and coastal common dolphins of the western South Atlantic face-to-face: What the skull and spine can tell us. Marine Mammal Science 32(4):1433-1457. https://doi.org/10.1111/mms.12342

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5):1792-7. https://doi.org/10.1093/nar/gkh340

Ferguson JA, Locke SA, Font WF, Steinauer ML, Marcogliese DJ, Cojocaru CD, Kent ML (2012) *Apophallus microsoma* n. sp. from chicks infected with metacercariae from coho salmon (*Oncorhynchus kisutch*) and review of the taxonomy and pathology of the genus *Apophallus* (Heterophyidae). J Parasitol 98(6):1122-1132. <http://doi.org/10.1645/GE-3044.1>

Fraija-Fernández N, Olson PD, Crespo EA, Raga JA, Aznar FJ, Fernández M (2015) Independent host switching events by digenean parasites of cetaceans inferred from ribosomal DNA. Int J Parasitol 45:167-173. https://doi.org/10.1016/j.ijpara.2014.10.004

Fried B, Graczyk TK, Tamang L (2004) Food-borne intestinal trematodiases in humans. Parasitol. Res 93:159-170. <https://doi.org/10.1007/s00436-004-1112-x>

Geraci JR, Lounsbury VJ (2005) Marine mammals ashore: a field guide for strandings. National Aquarium, Baltimore

Hernández-Orts JS, Georgieva S, Landete DN, Scholz T (2019) Heterophyid trematodes (Digenea) from penguins: A new species of *Ascocotyle* Looss, 1899, first description of metacercaria of *Ascocotyle* (*A.*) *patagoniensis* Hernández-Orts, Montero, Crespo, García, Raga and Aznar, 2012, and first molecular data. Int J Parasitol Parasites Wildl 8:94-105. https://doi.org/10.1016/j.ijppaw.2018.12.008.

Hostettler R, Cutmore SC, Cribb TH (2018) Two new species of Haplorchoides Chen, 1949 (Digenea:Heterophyidae) infecting an Australian siluriform fish, Neoarius graeffei Kner & Steindachner. Syst Parasitol 95:201-211. https://doi.org/10.1007/s11230-018-9775-3

Huston DC, Cutmore SC, Cribb TH (2018) Molecular systematics of the digenean community parasitising the cerithiid gastropod *Clypeomorus batillariaeformis* Habe & Kusage on the Great Barrier Reef. Parasitol Int 67:722-735. <https://doi.org/10.1016/j.parint.2018.07.008>

Jefferson T, Webber M, Pitman R (2015) Marine Mammals of the World. Academic Press, San Diego

Kearse M, Moir R, Wilson A et al (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647-1649. https://doi.org/10.1093/bioinformatics/bts199

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111-120. https://doi.org/10.1007/BF01731581

Knoff M, Luque JL, Amato JFR (1997) Community ecology of the metazoan parasites of grey mullets, *Mugil platanus* (Osteichthyes: Mugilidae) from the Littoral of the State of Rio de Janeiro, Brazil. Rev Bras Biol 57:441-454

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870-1874. https://doi.org/10.1093/molbev/msw054

Kuzmina TA, Tkach VV, Spraker TR, Lyons ET, Kudlai O (2018) Digeneans of northern Fur seals *Callorhinus ursinus* (Pinnipedia: Otariidae) from five subpopulations on St. Paul Island, Alaska. Parasitol Res 117:1079-1086. https://doi.org/10.1007/s00436-018-5784-z

Laporta P, Fruet PF, Siciliano S, Flores PAC, Loureiro JD (2016) Report of the Working Group on the Biology and Ecology of *Tursiops truncatus* in the Southwest Atlantic Ocean. Lat Am J Aquat Mamm 11: 62-70. http://doi.org/10.5597/lajam00216

Le TH, Nguyen KT, Nguyen NTB, Doan TTH, Dung DT, Blair D (2017) The ribosomal transcription units of *Haplorchis pumilio* and *H. taichui* and the use of rDNA 28S sequences for phylogenetic identification of common heterophyids in Vietnam. Parasit Vectors 10: 17. http://doi.org/10.1186/s13071-017-1968-0

Lodi L, Hetzel B (1999) Rough-toothed dolphin, *Steno bredanensis*, feeding behaviors in Ilha Grande Bay, Brazil. Biociencias 7:29-42

Martorelli SR, Lino A, Marcotegui P, Montes MM, Alda P, Panei CJ (2012) Morphological and molecular identification of the fish-borne metacercaria of *Ascocotyle* (*Phagicola*) *longa* Ransom, 1920 in *Mugil* *liza* from Argentina. Vet Parasitol 190:599-603. http://dx.doi.org/10.1016/j.vetpar.2012.07.002

Masala S, Piras MC, Sanna D, Chai JY, Jung BK, Sohn WM, Garippa G, Merella P (2016) Epidemiological and molecular data on heterophyid trematode metacercariae found in the muscle of grey mullets (Osteichthyes: Mugilidae) from Sardinia (western Mediterranean Sea). Parasitol Res 115(9):3409-17. http://doi.org/10.1007/s00436-016-5101-7.

Miller MA, Schwartz T, Pickett BE et al (2015) A RESTful API for Access to Phylogenetic Tools via the CIPRES Science Gateway. Evol Bioinform 16(11):43-48. http://doi.org/10.4137/EBO.S21501

Muller R (2001) Worms and Human Diseases. CABI Publishing, Wallingford

O'Hear M, Pote L, Yost M, Doffitt C, King T, Panuska C (2014) Morphologic and molecular identifications of digenetic trematodes in double-crested cormorants (*Phalacrocorax auritus*) from the Mississippi Delta, USA. J Wildl Dis 50(1):42-49. http://doi.org/10.7589/2012-10-249

Oliveira SA, Blazquez FJH, Antunes SA, Maia AAM (2007) Metacercária de *Ascocotyle* (*Phagicola*) *longa* Ransom, 1920 (Digenea: Heterophyidae), em *Mugil platanus*, no estuário de Cananéia, SP, Brasil. Ciên Rural 37:1057-1059. http://doi.org/10.1590/S0103-84782007000400022

Olson PD, Cribb TH, Tkach VV, Bray RA, Littlewood DTJ (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int J Parasitol 33(7):733-755. http://doi.org/10.1016/S0020-7519(03)00049-3.

Pearson JC (2008) Family Heterophyidae Leiper, 1909. In: Bray RA, Gibson DI, Jones A (Eds) Key to the Trematoda Volume 3. CAB International and Natural History Museum, Wallingford, UK, pp 113-141

Pereira EM,  Müller G,  Secchi E, Pereira J, Valente ALS (2013) Digenetic Trematodes in South American Sea Lions from Southern Brazilian Waters. J Parasitol 99:910-913. http://doi.org/10.1645/GE-3216.1

Pérez-Ponce de León G, Pinacho-Pinacho CD, Mendoza-Garfias B, Choudhury A, García-Varela M (2016) Phylogenetic analysis using the rDNA 28S rRNA gene reveals that the genus Paracreptotrema (Digenea: Allocreadiidae) is not monophyletic; description of two new genera and one new species. J Parasitol 102:131-142. http://doi.org/10.1645/15-815

Pornruseetairatn S, Kino H, Shimazu T, Nawa Y, Scholz T, Ruangsittichai J, Saralamba NT Thaenkham U (2015) A molecular phylogeny of Asian species of the genus *Metagonimus* (Digenea) small intestinal flukes based on representative Japanese populations. Parasitol Res 115:1123-30. http://doi.org/ 10.1007/s00436-015-4843-y

Posada D (2008) jModelTest: Phylogenetic model averaging. Mol Biol Evol 25:1253-1256. http://doi.org/10.1093/molbev/msn083

Rambaut A (2009) Molecular Evolution, phylogenetics and epidemiology: Fig-Tree. Available at http//tree.bio.ed.ac.uk/software/figtree/

Razo-Mendivil U, Rosas-Valdez R, Perez-Ponce de Leon G (2008) A new cryptogonimid (Digenea) from the Mayan cichlid, *Cichlasom urophthalmus* (Osteichthyes: Cichlidae), in several localities of the Yucatan Peninsula, Mexico. J Parasitol 94:1371-1378. <https://doi.org/10.1645/GE-1546.1>

Rosas FCW, Marigo J, Laeta M, Rossi-Santos MR (2010) Natural history of dolphins of the genus *Sotalia*. Latin American Journal of Aquatic Mammals 8(1-2):57-68. http://dx.doi.org/10.5597/lajam00154

Santos CP, Lopes KC, Costa VS, Santos EGN (2013) Fish-borne trematodosis: Potential risk of infection by *Ascocotyle* (*Phagicola*) *longa* (Heterophyidae). Vet Parasitol 193:302-306. https://doi.org/10.1016/j.vetpar.2012.12.011

Sato H, Ihara S, Inaba O, Une Y (2010) Identification of *Euryhelmis costaricensis* metacercariae in the skin of Tohoku hynobiid salamanders (*Hynobius lichenatus*), northeastern Honshu, Japan. J Wildl Dis 46:832-842. http://doi.org/10.7589/0090-3558-46.3.832

Scholz T (1999) Taxonomic study of *Phagicola longa* (Ransom, 1920) (Digenea: Heterophyidae) and related taxa. Syst Parasitol 43:147-158. http://doi.org/10.1023/A:100612050

Scholz T, Aguirre-Macedo ML, Salgado-Maldonado G (2001). Trematodes of the family Heterophyidae (Digenea) in Mexico: a review of species and new host and geographical records. J Nat Hist 35:1733-1772. http://doi.org/10.1080/00222930152667087

Scholz T, Vargas-Vázquez J, Aguirre-Macedo ML, Vidal-Martínez VM (1997) Species of *Ascocotyle* Looss, 1899 (Digenea: Heterophyidae) from the Yucatan Peninsula, Mexico. Syst Parasitol 36:161-181. http://doi.org/10.1023/A:1005757929710

Shumenko PG, Tatonova YV, Besprozvannykh VV (2017) *Metagonimus suifunensis* sp. n. (Trematoda: Heterophyidae) from the Russian Southern Far East: morphology, life cycle, and molecular data. Parasitol Int 66:982-991. http://doi.org/10.1016/j.parint.2016.11.002

Silva VME, Best RC (1996) *Sotalia fluviatilis*. Mamm Spec 5271-5277

Simões SBE, Barbosa HS, Santos CP (2010) The life cycle of *Ascocotyle* (*Phagicola*) *longa* (Digenea: Heterophyidae), a causative agent of fish-borne trematodosis. Acta Tropica 113:226-233. http://doi.org/10.1016/j.actatropica.2009.10.020

Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9):1312-1313. http://doi.org/10.1093/bioinformatics/btu033

Thaenkham U, Blair D, Nawa Y, Waikagul J (2012) Families Opisthorchiidae and Heterophyidae: are they distinct? Parasitol Int 61:90-93. http://doi.org/[10.1016/j.parint.2011.06.004](https://doi.org/10.1016/j.parint.2011.06.004)

Thaenkham U, Dekumyoy P, Komalamisra C, Sato M, Dung DT, Waikagul J (2010) Systematics of the subfamily Haplorchiinae (Trematoda: Heterphyidae), based on nuclear ribosomal DNA genes and ITS2 region. Parasitol Int 59(3):460-465. <http://doi.org/10.1016/j.parint.2010.06.009>

Thaenkham U, Nawa Y, Blair D, Waikagul J (2011) Confirmation of the paraphyletic relationship between Opisthorchiidae and Heterophyidae using a small and large subunit ribosomal DNA sequences as DNA markers. Parasitol Int 60:521-523. http://doi.org/[10.1016/j.parint.2011.07.015](https://doi.org/10.1016/j.parint.2011.07.015)

Van Steenkiste N, Locke SA, Castelin M, Marcogliese DJ, Abbott CL (2015) New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes). Mol Ecol Resour 15(4):945-952. http://doi.org/10.1111/1755-0998.12358

Vilas R, Criscione CD, Blouin MS (2005) A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. Parasitology 131:839-846. http://doi.org/10.1017/S0031182005008437

Wongsawad C, Nantarat N, Wongsawad P (2017) Phylogenetic analysis reveals cryptic species diversity within minute intestinal fluke, *Stellantchasmus falcatus* Onji and Nishio, 1916 (Trematoda, Heterophyidae). Asian Pac J Trop Med 10:165-170. http://doi.org/[10.1016/j.apjtm.2017.01.016](https://doi.org/10.1016/j.apjtm.2017.01.016)

**TABLES AND FIGURES CAPTIONS**

**Table 1** Primers, cycling conditions used during PCR and respective references

**Table 2** Parasite species used in the phylogenetic analyses with data on their host species followed by host common name in parenthesis, the geographical locality where they were found, the GenBank accession numbers (28S rDNA and COI mtDNA) and respective references. The letters EI in parenthesis after host species means that the digenean specimen was recovered from an experimentally infected host

**Table 3** Comparative measurements (in µm) of *Ascocotyle longa* infecting different hosts species. The measurements are presented as the range (min - max) followed by the mean in parentheses. (l) = length; (w) = width; \* = experimentally infected host, † = The author did not specified left/right.

**Fig 1** Adult of *Ascocotyle longa* collected in the intestine of a *Steno bredanensis* from the South Brazilian coast. Scale bar = 200 μm

**Fig 2** Bayesian phylogenetic topology of Heterophyidae and close related families constructed with 28S rDNA dataset. The support values at the branching points are shown as the Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses (Bayesian posterior probability values < 0.90; Maximum likelihood bootstrap values < 70). The branch length scale bar indicates the mean number of substitutions per site. New sequences obtained by this study are highlighted in bold

**Fig 3** Bayesian phylogenetic tree based on partial COI mtDNA sequences of Heterophydae. The support values at the branching points are shown as the Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses (Bayesian posterior probability values < 0.90; Maximum likelihood bootstrap values < 70). The branch length scale bar indicates the mean number of substitutions per site. New sequences obtained by this study are highlighted in bold