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

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## Abstract

We report for the first time the infection of dolphins with *Ascocotyle longa* found in the intestines of three different species, *Sotalia guianensis*, *Steno bredanensis*, and *Tursiops truncatus gephyreus*, which were found washed ashore along the south-eastern and southern Brazilian coast. The worms were identified based on morphological and molecular data using the 28S rDNA gene and the COI gene. Specimens of *A. longa* from the pinniped *Otaria flavescens* were also analyzed. As the first isolation of *A. longa* from cetaceans, the present study increases the distribution area and range of definitive hosts of this trematode, and provides new molecular data to complement the phylogeny of the group in future studies, thus contributing to the scientific knowledge of this potentially zoonotic parasite.

**Keywords** Trematoda · Marine mammals · New host · 28S rDNA gene · COI gene

## Introduction

Heterophyids of the genus *Ascocotyle* Looss, 1899 are small flukes, often cosmopolitan and potentially zoonotic, which are commonly found in the intestine of fish-eating birds and

mammals (Pearson 2008). Despite the wide range of definitive hosts in which *Ascocotyle* spp. have been reported, they have never been found infecting cetaceans.

The morphological identification of *Ascocotyle* spp. is challenging due to their small size and the difficulty in observing internal and external structures used for taxonomy and differentiation among species (Scholz et al. 1997; Scholz 1999; Scholz et al. 2001). Adding to that, molecular data are still scarce and a few sequences are currently available. Hernández-Orts et al. (2019) recently presented the first 28S rDNA phylogenetic positioning of *Ascocotyle* spp.; however, for other genes such as COI, the available sequences have never been used to specifically discuss the phylogenetic position of the genus.

As a part of an ongoing survey of the helminth fauna of cetaceans on the southeastern and southern Brazilian coast, several specimens of *Ascocotyle* were collected from the intestines of dolphins. The present study aimed to confirm the digenean species identity based on the examination of morphological characters and phylogenetic analyses of the 28S rDNA and the COI genes and to report dolphins as new hosts for the identified species. The present study increases the distribution patterns and host biodiversity of *Ascocotyle*, and provides new molecular data to be used as complement for

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discussions regarding the phylogeny of the group in future studies, thus contributing to scientific knowledge of the genus as a benefit.

## Material and methods

The intestines of 57 dolphins were screened for parasites (33 Guiana dolphins *Sotalia guianensis* (Van Bénédén, 1864), 22 Lahille's bottlenose dolphins *Tursiops truncatus gephyreus* Lahille, 1908 (Committee on Taxonomy 2018), and two rough-toothed dolphins *Steno bredanensis* Lesson, 1828) which were found dead and washed ashore at the southeastern and southern Brazilian coastline from August 2016 to February 2018. The worms were collected from the intestines of six of the analyzed dolphins: one *S. guianensis* (identification number 75085, adult, 1.95 m long, female) from the municipality of Cananéia, São Paulo State, 25° 00' 57.2" S, 47° 55' 31.0" W; two *S. bredanensis* (identification number 19643, 2.60 m long, adult, male; identification number 44958, 2.46 m long, adult, sex not determined), from the municipality of São Francisco do Sul, Santa Catarina State (26° 09' 43.0" S, 48° 34' 01.6" W); and three *T. t. gephyreus* (identification number 47602, total length not determined, adult, male; identification number 47897, total length not determined, adult, male; identification number 1915401, total length not determined, adult, sex not determined) from the municipality of Laguna, Santa Catarina State (28° 27' 57.7" S, 48° 45' 36.6" W). During dissection, the small intestines of the dolphins were removed, washed over a sieve, and their content examined under a stereomicroscope. The worms were preserved in 70% ethanol until further morphological and molecular analyses. Specimens of *Ascocotyle longa* Ransom, 1920 from the small intestine of a South American sea lion *Otaria flavescens* Shaw, 1800 collected by Pereira et al. (2013) were also examined for comparison purposes.

The worms were stained with Gomori's trichrome or carmine, cleared with creosote, and mounted as permanent slides. Morphological analyses were made in a computerized system for image analysis with differential interface contrast (Qwin Lite 3.1, Leica Microsystems). The specific identification of the worms was made based on morphological characters and comparison with data on *A. longa* from different hosts stated in previous studies (Scholz 1999; Barros et al. 2002; Simões et al. 2010; Pereira et al. 2013; Santos et al. 2013).

Genomic DNA of the worms was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Fragments of the 28S rDNA and the COI genes were amplified using Pure Taq™ Ready-to-Go™ PCR beads (GE Healthcare). The primers used were 28S rDNA, LSU5 (5'-TAGG TCGACCCGCTGAAYTTAAGCA-3') and 1500R (5'-GCTATCCTGAGGGAACTTCG-3') (Olson et al. 2003); COI, JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3')

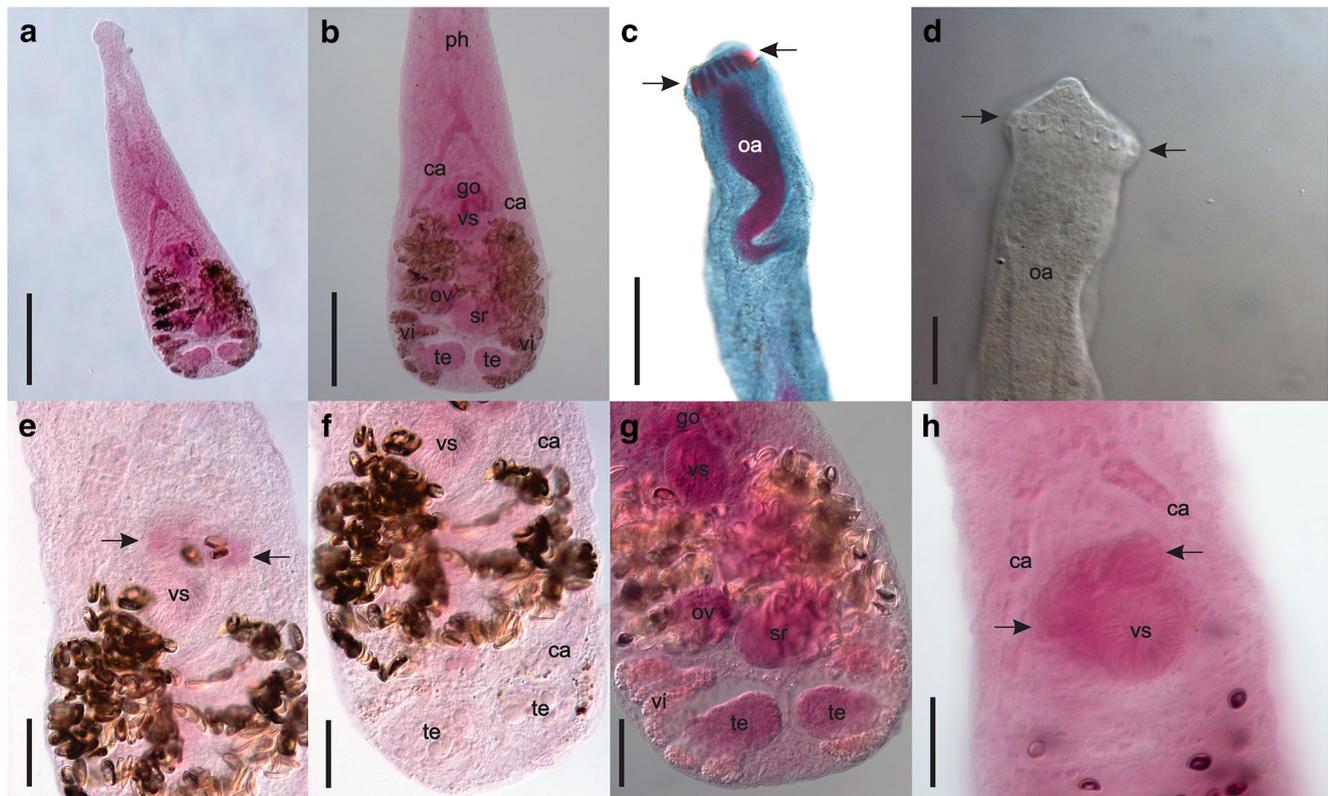
(Morgan and Blair 1998) and JB4.5 (5'-TAAA GAAAGAACATAATGAAAATG-3') (Razo-Mendivil et al. 2008). The thermocycling profile for the 28S rDNA gene amplification included initial denaturation at 95 °C for 7 min, followed by 40 cycles of amplification at 95 °C for 40 s, 57 °C for 45 s, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. For the COI gene amplification, the initial denaturation was at 94 °C for 5 min, followed by 40 cycles of amplification at 92 °C for 30 s, 47 °C for 45 s, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced with PCR primers using the BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequences were read with an Applied Biosystems ABI 3.500 DNA genetic analyzer.

The 28S rDNA and COI newly generated sequences were assembled and edited using Sequencher v.5.2.4, and aligned separately with other species of the families Heterophyidae Leiper, 1909, Opisthorchiidae Looss, 1899, and Cryptogonimidae Ward, 1917 retrieved from GenBank, as shown in the table provided as Electronic Supplementary Material 1. Alignments were performed using MUSCLE implemented on Geneious 7.1.3. The presence of stop codons and indels for the COI alignment was verified on Geneious 7.1.3.

Prior to the phylogenetic analyses, the best-fitting model for nucleotide substitution was determined using the Akaike information criterion on jModelTest program 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 Cipres Science Gateway using MrBayes v.3.2.7a on XSEDE tool. For BI, the Markov chain Monte Carlo (MCMC) chains were run with 10<sup>6</sup> generations and sampling tree topologies every 100th generations. The burn-in was set for the first 25% of generations. Nodes with posterior probabilities greater than 0.90 were considered well supported. The ML analyses were estimated on the Cipres Science Gateway using RAXML-HPC v.8 on XSEDE tool, with 1000 bootstrap replicates. Nodes with bootstrap values greater than 70% were considered well supported.

## Results and discussion

We found a total of 5563 specimens of *Ascocotyle* in the intestines of the dolphins. Only one out of 33 *S. guianensis* was infected with 15 digenean specimens; both *S. bredanensis* were infected with 1498 and 1005 specimens, respectively; and three out of 22 *T. t. gephyreus* were infected with 1323, 1482, and 230 specimens, respectively. A total of 93 digeneans (29 from *S. bredanensis* identification number



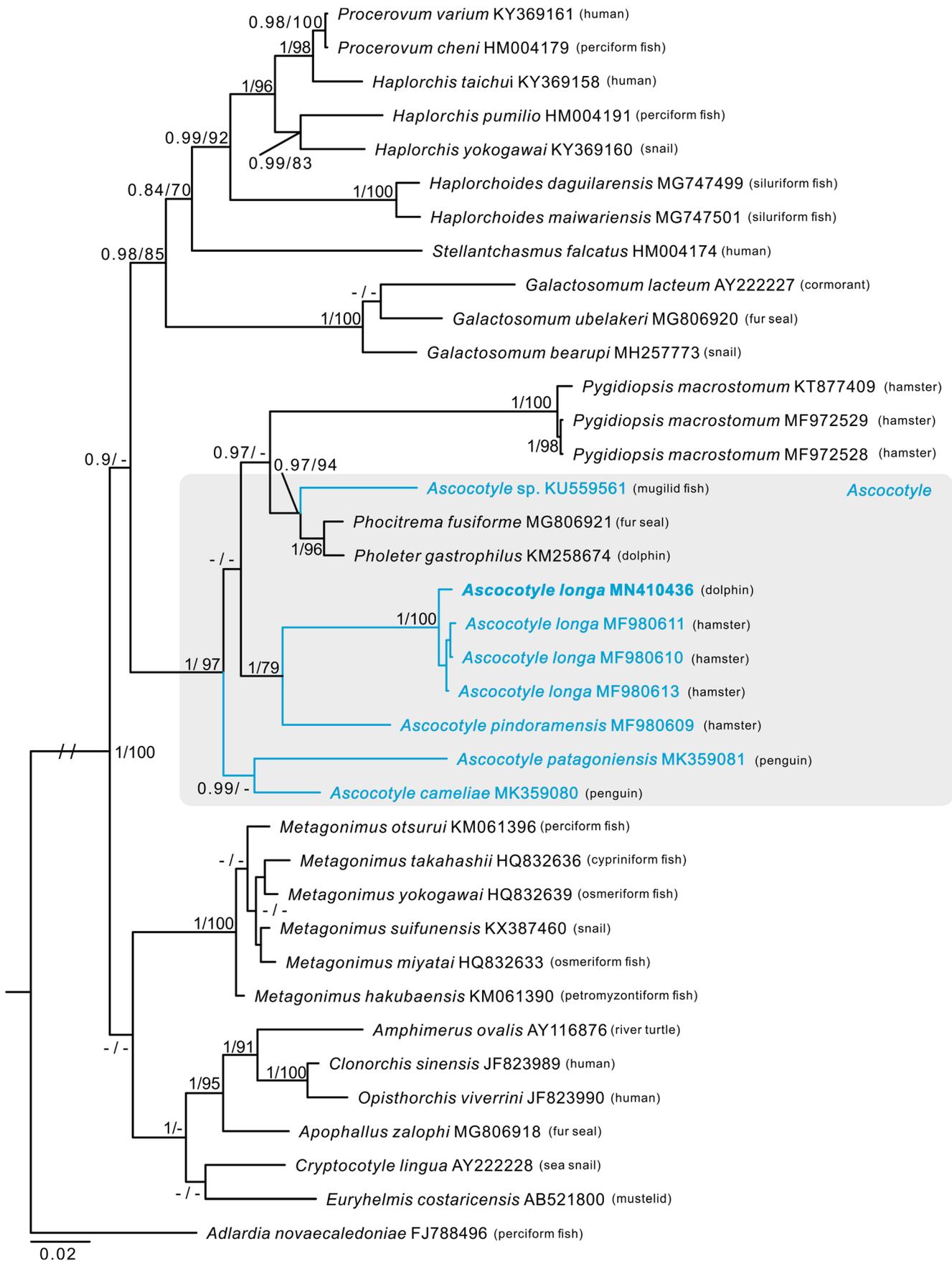
**Fig. 1** Adult *Ascocotyle longa* collected from *Otaria flavescens* (a–h) and *Steno bredanensis* (c) from the southern Brazilian coast. **a** Whole worm, scale bar = 200  $\mu$ m. **b** Posterior extremity of the worm, scale bar = 100  $\mu$ m. **c** Anterior extremity of the worm; arrow indicates the circumoral spines stained with Gomori's trichrome, scale bar = 50  $\mu$ m. **d** Anterior extremity of the worm; arrow indicates the circumoral spines; Hoyer medium, scale bar = 50  $\mu$ m. **e** Detail of the bipartite gonotyl;

arrows indicate pad-like lobes, scale bar = 50  $\mu$ m. **f** Posterior extremity of the worm showing the end of caeca reaching up the pretesticular area, scale bar = 50  $\mu$ m. **g** Posterior extremity of the worm, scale bar = 50  $\mu$ m. **h** Detail of the bipartite gonotyl; arrows indicate pad-like lobes, scale bar = 200  $\mu$ m. ca = caeca; go = gonotyl; ov = ovary; oa = oral appendage; ph = pharynx; sr = seminal receptacle; te = teste; vi = vitellarium; vs = ventral sucker

19643, 15 from *T. t. gephyreus* identification number 1915401, 13 from *S. guianensis* identification number 75085, and 36 from *O. flavescens*) were morphologically analyzed. Morphological and morphometric analyses of the parasites enabled us to assign the specimens found in this study as *A. longa* (Fig. 1; voucher specimens at the CHIBB São Paulo State University - UNESP under numbers 581L-591L and 8850-8851). The main morphological characteristics considered to identify the species were the presence of a subterminal oral sucker surrounded by a single row of 16 circumoral spines (due to decomposition, most or all spines were detached and a complete circle with spines could be observed in only few specimens), with a preoral lobe and a conical posterior appendage; the presence of a ventrogenital sac containing a spherical ventral sucker and a bipartite gonotyl with two pad-like lobes; long intestinal caeca reaching up to the pretesticular level; oval testes situated close to the posterolateral margin of the body; and vitellarium formed by seven to nine follicles grouped into two bands in the latero-posterior region of the body.

Despite the combined morphological characteristics and morphometrical data of our specimens fitting the descriptions

of *A. longa* provided by Scholz (1999), Barros et al. (2002), Simões et al. (2010), Pereira et al. (2013), and Santos et al. (2013), we found morphometrical variation among *A. longa* collected from the dolphins, the pinniped and the ones from *Vulpes lagopus* Linnaeus, 1758, *Lutra reponda*, *Pelecanus occidentalis carolinensis* Gmelin, 1789 (Scholz 1999), *Ardea cocoi* Linnaeus, 1766 (Barros et al. 2002), and *Mesocricetus auratus* Waterhouse, 1839 (Simões et al. 2010; Santos et al. 2013), mainly regarding the size of the body, oral sucker, ovary, and seminal receptacle. Scholz (1999) synonymized several species of *Ascocotyle* as *A. longa* after observing slight morphological differences among the specimens analyzed by him, concluding that those variations should not be considered enough to maintain different species. We consider that our results might be also either an effect of intraspecific variation and/or due to the relatively poor morphological conditions of our samples, which were collected from stranded and decomposed carcasses of marine mammals. Nevertheless, we encourage further robust comparative analyses to investigate the morphological intraspecific boundaries within *A. longa* to corroborate our findings.



◀ **Fig. 2** Bayesian phylogenetic topology of Heterophyidae and closely related families constructed with the 28S rDNA gene dataset. Support values at branching points are shown as Bayesian posterior probabilities followed by maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses. Branch length scale bar indicates mean number of substitutions per site. New sequences are highlighted in bold

We successfully extracted genomic DNA of seven worms (three specimens from *S. bredanensis* identification number 19643, two specimens from *T. t. gephyreus* identification number 1915401, and two specimens from the *O. flavescens*). Despite several attempts, we were not able to obtain good quality DNA from the worms collected from *S. guianensis*, probably because of the poor preservation of the specimens. A total of seven new sequences were obtained; one of the 28S rDNA gene from a worm collected from the *S. bredanensis* (MN410436), and six of the COI gene from worms collected from *S. bredanensis* (MN419235, MN419239), *T. t. gephyreus* (MN419238, MN419240), and *O. flavescens* (MN419236, MN419237). The 28S rDNA and COI final alignments included 37 sequences and 1111 positions and 27 sequences and 323 positions, respectively.

Both the BI and the ML analyses of the 28S rDNA alignment yielded similar topologies, with most clades well supported. The 28S rDNA phylogenetic reconstruction (Fig. 2) recovered the genus *Ascocotyle* as paraphyletic, forming a well-supported clade together with *Phocitrema fusiforme* Goto and Ozaki, 1930, *Pholeter gastrophilus* (Kossack, 1910) Odhner, 1914, and *Pygidiopsis macrostomum* Travassos, 1928. Our newly generated sequence clustered in a highly supported monophyletic clade with the other *A. longa* sequences recovered from experimentally infected golden hamsters *M. auratus* (MF980610, MF980611, MF980613), thus confirming its identity as *A. longa*, being the sequence of *Ascocotyle pindoramensis* (Travassos, 1928) (MF980609) their sister taxa. The metacercaria identified only at generic level as *Ascocotyle* sp. collected from the gray mullet *Chelon labrosus* (Risso, 1827) (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 *Ascocotyle cameliae* Hernández-Orts et al., 2019 (MK359080) and *Ascocotyle patagoniensis* Hernández-Orts et al., 2019 (MK359081), both collected from Magellanic penguins *Spheniscus magellanicus* (Forster, 1781), clustered together in a highly supported clade, which was placed as sister to the clade formed by *A. longa*, *A. pindoramensis*, *Ascocotyle* sp., *P. fusiforme*, *P. gastrophilus*, and *P. macrostomum*.

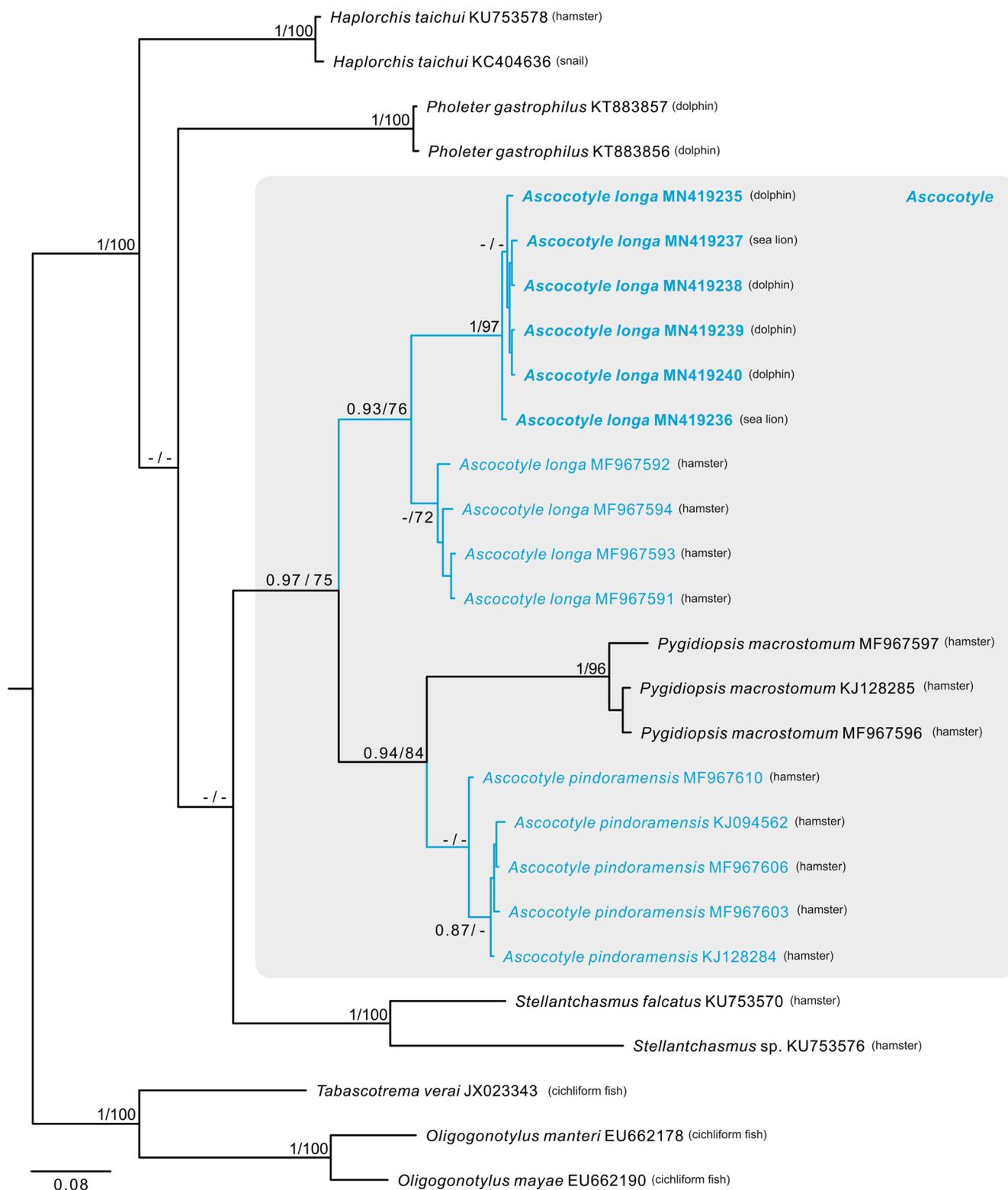
For the COI alignment, both the BI and the ML phylogenetic reconstructions (Fig. 3) also recovered *Ascocotyle* as paraphyletic, with *A. longa*, *P. macrostomum*, and *A. pindoramensis* clustered together in a well-supported clade.

The newly generated sequences of *A. longa* from dolphins and the pinniped (MN419235-40) grouped together in a monophyletic clade, revealing that these two groups of marine mammals are infected by the same lineage of *A. longa*. However, this clade was positioned as sister to another clade formed by *A. longa* sequences (MF967591-94) obtained from experimentally infected mice recovered from GenBank. These reciprocally monophyletic clades formed by sequences of *A. longa* suggest the need of further investigation into intra-specific boundaries of the species and, in this case, the possibility of the existence of a complex of sibling species. New samples of *A. longa* from different hosts should be collected to test this hypothesis.

Paraphyly of *Ascocotyle* revealed by both the 28S rDNA and the COI analyses is in agreement with the results already reported by Hernández-Orts et al. (2019). Our new data reinforces that, as currently comprised, *Ascocotyle* might not be a natural group and needs further evaluation of its taxonomic status and the identity of the species included in this genus. *Ascocotyle longa* is a cosmopolitan species distributed worldwide which infects a variety of hosts and consequently takes part in several ecological and evolutionary interactions. These interactions may influence the gene flow of the species, which could proceed with speciation processes. Our study serves as preliminary data that should be accessed in future studies to unravel species boundaries and identity in *Ascocotyle*, thus contributing to scientific knowledge of the group. Therefore, we suggest further morphological, taxonomic, and phylogenetic investigations using more robust data on *A. longa*.

Metacercariae of *A. longa* have been found infecting mugilids associated with estuaries and coastal lagoons along the southeastern Brazilian coast (Oliveira et al. 2007). Not surprisingly, the three delphinid species serving as hosts for *A. longa* in this study present coastal and/or estuarine ecological and feeding habits on the Brazilian coast (Jefferson et al. 2015) with mugilid fish being one of the most required items of their diet (Lodi and Hetzel 1999; Rosas et al. 2010; Laporta et al. 2016), which could potentially harbor metacercariae of *A. longa* that infected the dolphins. *Ascocotyle longa* is considered one of the causative agents of human heterophyiasis (Simões et al. 2010), with several cases already reported in Brazil (Antunes and Almeida-Dias 1994). The occurrence of *A. longa* in Brazilian dolphins and sea lion implies the presence of its metacercariae in fish at the studied areas. Because of the zoonotic potential of *A. longa*, we suggest attention to human consumption of uncooked or partially cooked mugilid fish caught in this region.

This study reports for the first time the infection with *A. longa* in delphinid species, *S. guianensis*, *S. bredanensis*, and *T. truncatus gephyreus*, which now represent new definitive hosts for this trematode species. Even with a low prevalence, we found a considerable intensity of infection; therefore, the possibility of delphinids serving as postcyclic or



**Fig. 3** Bayesian phylogenetic topology based on partial COI sequences of Heterophyidae. Support values at branching points are shown as Bayesian posterior probabilities followed by maximum-likelihood

bootstraps. Dashes are shown for branches not supported by the analyses. Branch length scale bar indicates mean number of substitutions per site. New sequences are highlighted in bold

accidental hosts for *A. longa* might be discarded. Therefore, here we expand the biodiversity of cetacean parasites and

reinforce the importance of systematized routine parasitological surveys in marine mammals. Moreover, in the case of

*Ascocotyle* spp., considering their minute size, they might be readily overlooked during parasitological examinations, unless done by careful specialists.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00436-020-06956-1>.

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**Authors' contributions** MBE and RJS designed the study; MBE, ALSV, MJC, and PVC collected the data; MBE performed the research; MBE and MF analyzed the data; MBE and MF wrote the manuscript; MBE, MF, ALSV, MJC, and PVC revised and approved the final manuscript.

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**Data availability** Permission to collect and transport deceased stranded dolphins was given by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) under registration 11980-1 (SISBIO) and 5820094 (ABIO).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Code availability** Not applicable.

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