UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS - *CAMPUS* DE BOTUCATU
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (ZOOLOGIA)

TESE DE DOUTORADO

HELMINTOS GASTROINTESTINAIS DE CETÁCEOS: ABORDAGENS MORFOLÓGICAS E MOLECULARES

Mariana Bertholdi Ebert

Botucatu, SP
2021

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Mariana Bertholdi Ebert

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Zoologia) do Instituto de Biociências da Universidade Estadual Paulista – UNESP, Campus de Botucatu, São Paulo, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Zoologia).

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Palavras-chave: cetáceos; helmintofauna; identificação molecular.

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RESUMO

O objetivo deste trabalho foi inventariar a biodiversidade de parasitos gastrointestinais de cetáceos encontrados encalhados ou acidentalmente capturados em redes de pesca ao longo do litoral brasileiro, utilizando analises morfológicas e moleculares. Para tanto, 260 cetáceos hospedeiros pertencentes a 11 espécies identificadas foram investigados. Foi coletado um total de 23.649 espécimes de parasitos. Destes, 23.210 eram trematódeos pertencentes a 12 taxa, seguidos por 290 acantocéfalos pertencentes a 5 *taxa*, 123 nematódeos de dois *taxa*, e 26 cestoides de 4 *taxa* diferentes. *Ascocotyle longa* é registrado pela primeira vez em cetáceos e as analises moleculares indicam a necessidade de investigações mais robustas a respeito da identidade desta espécie. *Ogmogaster antarctica* é registrado em um novo hospedeiro, *Balaenoptera acutorostrata*. *Pholeter gastrophilus* é registrado pela primeira vez no litoral brasileiro. *Synthesium tursionis* é revisado, indicando a necessidade de revalidação de seu sinônimo *Synthesium longissimum* e a descrição de uma nova espécie, *Synthesium* sp. 1. Quatro novas espécies pertencentes ao gênero *Synthesium* deverão ser propriamente descritas no futuro. De maneira geral, foram reconhecidos sete novos hospedeiros e localidades para helmintos já descritos, dentre trematodeos, cestoides, acantocéfalos e nematóides. As análises morfológicas e moleculares dos espécimes encontrados permitiram discussões importantes a respeito da filogenia, evolução e distribuição de parasitos de cetáceos, aumentando assim, o conhecimento acerca das relações dos parasitos com seus hospedeiros e constituindo uma linha de base para futuras pesquisas sobre diversidade parasitaria em cetáceos.

Palavras-chave: helmintofauna, cetáceos, Trematoda, Cestoda, Acanthocephala, Nematoda, identificação molecular

Abstract

ABSTRACT

This study aimed to record the diversity of gastrointestinal parasites of cetaceans that were found stranded or accidentally caught in fishing nets along the Brazilian coast, through morphological and molecular approaches. For that, 260 cetaceans belonging to 11 different species were investigated for their parasite fauna. A total of 23,649 specimens of parasites was recovered. Of this total, 23,210 were trematodes belonging to 12 *taxa,* followed by 290 acanthocephalans belonging to 5 *taxa*, 123 nematodes from two *taxa,* and 26 cestodes belonging to 4 different taxa. Based on the obtained data, it was possible to identify 12 taxa of trematodes belonging to 6 different genera, of which 4 can be considered new species. *Ascocotyle longa* is recorded for the first time in cetaceans and the molecular analyzes indicated the need for further investigations regarding the identity of this species. *Ogmogaster antarctica* is registered in a new host, *Balaenoptera acutorostrata*. *Pholeter gastrophilus* is registered for the first time on the Brazilian coast. *Synthesium tursionis* is revised, indicating the need for the resurrection of its synonymy *Synthesium longissimum* and the erection of a new species. Four new species belonging to the genus *Synthesium* should be properly described in the future. Overall, seven new hosts and locations for helminths already described were recognized, including trematodes, cestodes, acanthocephalans, and nematodes. The morphological and molecular analyzes of the specimens found enabled important discussions regarding the phylogeny, evolution, and distribution of cetacean parasites, thus increasing knowledge about the relationships of the parasites with their hosts and constituting a baseline for future research on parasitic diversity in cetaceans.

Key-words: helminth fauna, cetaceans, Trematoda, Cestoda, Acanthocephala, Nematoda, molecular identification

Introdução geral

INTRODUÇÃO GERAL

Os cetáceos no Brasil

Os cetáceos, popularmente conhecidos como golfinhos, botos e baleias, são mamíferos marinhos completamente adaptados à vida aquática (Di Beneditto et al., 2010). A ordem Certactiodactyla Brisson, 1762 está dividida em três subordens; Archaeoceti Flower, 1833 (fósseis), Mysticeti Cope, 1864 e Odontoceti Flower, 1867. Mysticeti, ou misticetos, são representados pelas baleias com barbatanas e estão divididos em quatro famílias e 14 espécies, sendo oito delas encontradas no litoral brasileiro. Já Odontoceti, ou odontocetos, são representados pelos golfinhos e botos e dividem-se em nove famílias e 69 espécies, das quais 35 ocorrem no Brasil (ICMBio, 2010; Di Beneditto et al., 2010).

A distribuição dos cetáceos ao longo dos 8.000 km da costa brasileira ocorre de forma contínua devido a diversidade de ecossistemas presentes ao longo de nosso litoral (baías, manguezais, recifais etc.), os quais representam habitats favoráveis para reprodução e alimentação destes animais (IBAMA, 2005).

A maioria das espécies listadas no Plano de Ação de Mamíferos Aquáticos do Brasil (IBAMA, 2001) está classificada na categoria DD (*Deficient Data*) da *IUCN Red List of Threatened Species* (http://www.iucnredlist.org), indicando que não existem informações adequadas para avaliação do *status* de conservação, ameaças e outras características das espécies, inclusive dados de diversidade parasitária ou relação parasito-hospedeiro. Muito desta carência se deve a inexistência de estudos e destinação adequada de material biológico e, consequentemente, dados substanciais a serem empregados em projetos que visam à conservação destes animais no Brasil (IBAMA, 2005). Desta forma, é importante o incentivo a estudos parasitológicos de caráter contínuo e padronizado.

Diversidade de parasitos em cetáceos

Os primeiros registros de helmintos em cetáceos datam do início de 1930, época em que a caça comercial era permitida (Baylis, 1932; Price, 1932). Naquela época, no entanto, nenhuma metodologia de coleta ou fixação para fins de análises morfológicas era aplicada (Fraija-Fernández et al., 2016).

Atualmente, a maioria dos estudos parasitológicos de cetáceos ainda utiliza material proveniente de carcaças encalhadas ocasionalmente ou capturadas

acidentalmente em redes de pesca (Raga et al., 2008), o que dificulta a coleta de amostras de parasitos em bom estado de conservação. Como consequência, ainda há uma escassez de informações morfológicas e sobretudo genéticas a respeito destes organismos (Raga et al., 2008).

Cetáceos são parasitados por uma grande diversidade de helmintos, que se distribuem por diversos tecidos, órgãos e cavidades. A helmintofauna de cetáceos inclui atualmente pouco mais de 170 espécies divididas em quatro grandes grupos taxonômicos: os nematoides são o grupo mais diverso, com 62 espécies registradas, seguidos por trematódeos digenéticos com 54 espécies, cestoides com 38 espécies e acantocéfalos com 20 espécies registradas (Fraija-Fernández et al., 2016).

Dentre o filo Nematoda, as famílias Pseudaliidae Railliet & Henry, 1909, Anisakidae Skrjabin & Karokhin, 1945 e Tetrameridae Travassos, 1914 são as mais representativas entre os parasitos de cetáceos. Os cestoides estão representados principalmente pelas famílias Tetrabothriidae Linton, 1891, Diphyllobothriidae Lühe, 1910 e Phyllobothriidae Braun, 1900. Polymorphidae Meyer, 1931 abriga todas as espécies de acantocéfalos infectantes de cetáceos. Já os trematódeos digenéticos estão distribuídos em quatro famílias: Cyathocotylidae Mühling, 1898 (recentemente sinonimizada com Brauninidae Wolf, 1903), Notocotylidae Lühe, 1909, Heterophyidae Leiper, 1909 e Brachycladiidae Odhner, 1905. A família Cyathocotylidae (= Brauninidae) inclui a espécie *Braunina cordiformis* Wolf, 1903; Notocotylidae inclui as espécies do gênero *Ogmogaster* Jägerskiöld, 1891; Heterophyidae é representada por *Pholeter gastrophilus* (Kossack, 1910) Odhner, 1914 e, recentemente, por *Ascocotyle longa* Ransom, 1920 (ver Anexo 2); e Brachycladiidae é a mais representativa, incluindo 10 gêneros e 42 espécies (Gibson, 2005; Fraija-Fernández et al., 2016; Ebert et al., 2017; Shiozaki et al., 2019).

Diversidade de parasitos gastrointestinais de cetáceos no Brasil

Os primeiros registros de parasitos de cetáceos no Brasil foram realizados na década de 70. Apesar do aumento no número de estudos sobre a diversidade de parasitos de cetáceos desde esta época, a fauna parasitária dos cetáceos brasileiros ainda é pouco investigada e a maioria dos estudos não utiliza métodos padronizados, sendo limitados a registros de espécies sem maiores investigações taxonômicas.

Atualmente, há registros de apenas 21 espécies de parasitos gastrointestinais de cetáceos encontrados no Brasil (Luque et al., 2010; Carvalho et al., 2010; Di Azevedo et al. 2015, 2017; Ebert et al., 2013, 2017, 2020a, 2020b, 2021) (Tabela 1). Muitas espécies necessitam de revisões taxonômicas e com o avanço das análises moleculares, é notável o aumento de descobertas de novas espécies e reorganizações filogenéticas desses organismos (Motta et al 2008; Iñiguez et al., 2009; Marigo et al., 2015; Ebert et al., 2017, 2020a, 2020b, 2021).

Tabela 1. Lista de registros de helmintos gastrointestinais e seus respectivos hospedeiros cetáceos encontrados no Brasil.

Parasitos	Hospedeiros	Sitio de Infecção	Localidade	Referências
Trematoda				
Brachycladiidae				
Synthesium neotropicale Ebert et al. 2017	*Tursiops truncatus* (Montagu, 1821)	I	SC	Ebert et al., 2017
Synthesium pontoporiae (Raga, 1984)	*Pontoporia blainvillei* (Gervais & d'Orbigny, 1844)	I	RS, SP, PR, RJ, SC	Andrade et al., 1996; Marigo et al., 2002, 2008; Silva e Cousin, 2004; Alves et al., 2017
*Synthesium tursionis** (Marchi, 1973)	*Delphinus capensis* Cuvier, 1829	I	PR	Andrade et al., 1999
	Sotalia guianensis (Van Beneden, 1864)	I	BA, SP, PR, RJ,SC	Andrade e Pereira, 1998; Marigo et al., 1999, 2010; Ebert et al., 2017; Alves et al., 2017
	T. truncatus	I	PR, SC	Andrade et al., 1999; Marigo et al., 2008
	Stenella frontalis (Cuvier, 1829)	I	PR	Andrade et al., 1999
Cyathocotylidae				
Braunina cordiformis Wolf, 1903	*S. guianensis*	E	RJ, PR	Santos et al., 1996; Marigo et al., 2001, 2010; Di Beneditto e Ramos 2001, 2004; Pinto et al., 2004; Melo et al., 2006
	Steno bredanensis (Lesson, 1828)	E	RJ, RS	Santos et al., 1996; Ott e Danilewicz, 1996; Pinto et al., 2004
	T. truncatus	E	RJ	Santos et al., 1996; Pinto et al., 2004
	Delphinus delphis Linnaeus, 1758	E	-	Travassos et al., 1969; Pinto et al., 2004
	S. frontalis	E	SP	Marigo, 2003
Heminuridae				
Hemiuridae Looss, 1899	*P. blainvillei*	I	RS	Hoss et al. (2017)
Heterophyidae				
Ascocotyle longa Ransom, 1920	*T. truncatus*	I	SC	Ebert et al., 2020b
	S. bredanensis	I	SC	Ebert et al., 2020b
	S. guianensis	I	SP	Ebert et al., 2020b
Pholeter gastrophilus (Kossack, 1910)	*S. bredanensis*	E	SC	Ebert et al., 2021

	Feresa attenuata Gray, 1874	E	SP	Ebert et al., 2021
	T. truncatus	E	SP	Ebert et al., 2021
Notocotylidae				
Ogmogaster antarctica Johnston, 1931	*Balaenoptera borealis* Lesson, 1828	I	RJ	Muniz-Pereira et al., 1999; Pinto et al. 2004
	Balaenoptera physalus (Linnaeus, 1758)	I	RJ	Muniz-Pereira et al., 1999; Pinto et al. 2004
	Balaenoptera acutorostrata Lacépède, 1804	I	SC	Ebert et al., 2020a
Nematoda				
Anisakidae				
Anisakis brevispiculata Dollfus, 1966	*Kogia breviceps* (de Blainville, 1838)	E	CE	Di Azevedo et al., 2017
	Kogia sima (Owen, 1866)	E	CE	Di Azevedo et al., 2017
Anisakis insignis (Diesing, 1851)	*Inia geoffrensis* (de Blainville, 1817)	E		Dailey e Brownell Jr., 1972; Diesing, 1851; Vicente et al., 1997
Anisakis paggiae Mattiucci, Nascetti, Dailey, Webb, Barros, Cianchi & Bullini, 2005	*K. sima*	E	CE	Di Azevedo et al., 2015, 2017
	K. breviceps	E	CE	Di Azevedo et al., 2017
Anisakis physeteris (Baylis, 1923)	*K. breviceps*	E	PE, RJ	Santos e Lodi, 1998; Pinto et al., 2004
	Physeter macrocephalus Linnaeus, 1758	E	RJ	Muniz-Pereira et al., 1999
	B. borealis	E	-	Pinto et al., 2004
	B. physalus	E	-	Pinto et al., 2004
Anisakis nascettii Mattiucci, Paoletti & Webb, 2009	*Mesoplodon europaeus* (Gervais, 1855)	E	CE	Di Azevedo et al. 2014
Anisakis simplex (Rudolphi, 1809)	*F. attenuata*	E	SP	Zerbini e Santos, 1997
	Phocoena dioptrica Lahille, 1912	E	RS	Pinedo et al., 2002
	Pseudorca crassidens (Owen, 1846)	E	RS	Andrade et al., 2001; Pinto et al., 2004
	Stenella clymene (Gray, 1850)	E	BA	Bastos e Marigo, 2002
Anisakis sp.	*Grampus griseus* (Cuvier, 1812*)*	E	BA	Maia-Nogueira, 2000
	Stenella coeruleoalba (Meyen, 1833)	E	SP	Rosas et al., 2002
	S. guianensis	E	SP	Marigo et al., 2010
Anisakis typica (Diesing, 1860) Baylis, 1920	*K. sima*	E	SP	Souza et al., 2002

	P. blainvillei	E	RS	Andrade et al., 1996; Di Beneditto e Ramos, 2001; Marigo et al., 2002; Silva e Cousin, 2004
	S. guianensis	E	RJ, PR	Santos et al., 1996; Iñiguez et al., 2009; Marigo et al., 2001; Di Beneditto e Ramos, 2001, 2004; Melo et al., 2006
	S. coeruleoalba	E	BA	Maia-Nogueira et al., 2001
	S. frontalis	E	CE	Di Azevedo et al., 2017
	Lagenodelphis hosei	E	CE	Di Azevedo et al., 2017
	Peponocephala electra	E	CE	Iñiguez et al., 2011
	S. clymene	E	CE	Iñiguez et al., 2011
	Stenella longirostris (Gray, 1828)	E	PE, CE	Marigo et al., 1998; Iñiguez et al., 2011
	K. breviceps	E	CE	Iñiguez et al., 2011
	S. bredanensis	E	CE	Iñiguez et al., 2011
Anisakis ziphidarum Paggi, Nascetti, Webb, Mattiucci, Cianchi & Bullini, 1988	*K. sima*	E	CE	Di Azevedo et al. 2017
Contracaecum sp. Railliet & Henry, 1912	*P. blainvillei*	E	-	Dailey e Brownell Jr., 1972
	S. guianensis	E	ES	Borobia e Barros, 1989; Di Beneditto e Ramos, 2001
Pseudoterranova sp. Mozgovoi, 1953	*K. breviceps*	E	PE	Santos e Lodi, 1998; Pinto et al., 2004
	B. borealis	E	-	Pinto et al., 2004
	B. physalus	E	-	Pinto et al., 2004
	S. longirostris	E	CE	Di Azevedo et al., 2017
	K. breviceps	E		Santos e Lodi, 1998
Acantocephala				
Polymorphidae				
Bolbosoma capitatum (von Linstow, 1880) Porta, 1908	*Balaenoptera brydei* Olsen, 1913	I	RJ	Pinto et al., 2004
	P. crassidens	I	RS	Andrade et al., 2001
	Globicephala melas (Traill, 1809)	I	-	Machado-Filho, 1964
	S. coeruleoalba	I	SP	Rosas et al., 2002

Bolbosoma turbinella (Diesing, 1851) Porta, 1908	*B. borealis*	I	RJ	Machado-Filho, 1964; Muniz-Pereira et al., 1999
	P. blainvillei	I	RS	Andrade et al., 1996; Marigo et al., 2002; Silva e Cousin, 2004, 2006
	S. coeruleoalba	I	SP	Rosas et al., 2002
Bolbosoma sp. Porta, 1908	*P. blainvillei*	I	RS, SC	Di Beneditto e Ramos, 2001
	S. guianensis	I	PR, SC	Marigo et al., 2010; Alves et al., 2017
	T. truncatus	E/I	CE	Carvalho et al., 2010
	S. longirostris	E/I	CE	Carvalho et al., 2010
Corynosoma australe Johnston, 1937	*P. blainvillei*	I	RS	Andrade et al., 1996
Corynosoma cetaceum Johnston & Best, 1942	*P. blainvillei*	E/I	RS	Andrade et al., 1996; Di Beneditto e Ramos, 2001; Marigo et al., 2002; Silva e Cousin, 2004, 2006; Hoss et al. 2017
Corynosoma sp. Lühe, 1904	*P. blainvillei*	I	-	Dailey e Brownell Jr., 1972
Cestoda				
Tetrabothriidae				
Trigonocotyle sp. Baer, 1932	*S. longirostris*	I	CE	Carvalho et al., 2010
	S. clymene	I	CE	Carvalho et al., 2010
Tetrabothrius forsteri (Krefft, 1871) Fuhrmann, 1904	*S. longirostris*	I	CE	Carvalho et al., 2010
	S. clymene	I	CE	Carvalho et al., 2010
Tetrabothrius sp. Rudolphi, 1819	*S. longirostris*	I	CE	Carvalho et al., 2010
Strobicephalus triangularis (Diesing, 1850)	*S. longirostris*	I	CE	Carvalho et al., 2010
	S. clymene	I	CE	Carvalho et al., 2010
	Peponocephala electra (Gray, 1846)	I	CE	Carvalho et al., 2010
	S. frontalis	I	CE	Carvalho et al., 2010
Diphyllobothriidae				
Diphyllobothrium sp. Cobbold, 1858	*K. breviceps*	I	CE	Carvalho et al., 2010
	Globicephala macrorhynchus Gray, 1846	I	CE	Carvalho et al., 2010

* = necessita revisão taxonômica (ver cap. 1).

Objetivos

OBJETIVOS

Geral

Realizar um levantamento da diversidade dos helmintos parasitas de cetáceos encontrados encalhados ao longo do litoral brasileiro.

Específicos

I. Realizar um estudo taxonômico e molecular dos helmintos gastrointestinais de cetáceos encontrados ao longo do litoral brasileiro;
II. Descrever espécies novas utilizando ferramentas morfológicas e moleculares.

Material e Métodos

MATERIAL E MÉTODOS

Coleta de hospedeiros

Para este estudo foram utilizadas carcaças de cetáceos encontrados encalhados ou acidentalmente capturados em redes de pesca ao longo do litoral brasileiro, desde a costa do estado do Ceará até o estado de Santa Catarina, durante os anos de 1997 a 2018. As carcaças foram obtidas por meio de instituições parceiras as quais mantém programas de monitoramento de fauna marinha e/ou participantes do Programa de Monitoramento de Praia (PMP-BS) da Petrobras (Tabela 2).

Tabela 2. Instituições parceiras através quais foram obtidas as carcaças dos cetáceos utilizadas neste estudo, suas respectivas siglas de identificação e região geográfica onde realizam monitoramento de fauna marinha.

Instituição	Sigla	Região abrangente	Localização geográfica
Associação de Pesquisa e Preservação de Ecossistemas Aquáticos - AQUASIS	AQ	Ceará; litoral central	3°41'09.1"S 38°38'39.5"W
Universidade Estadual do Rio de Janeiro/ Laboratório de Mamíferos Aquáticos	MQ	Rio de Janeiro; litoral central	22°51'27.8"S 43°09'33.8"W
Projeto Biopesca	BP	São Paulo; litoral norte	24°00'53.9"S 46°24'27.6"W
Instituto de Pesquisas Cananéia/Projeto Boto-Cinza/PMP-BS	IPEC	São Paulo; litoral sul	25°00'36.7"S 47°55'17.6"W
Universidade Federal do Paraná/ Centro de Estudos do Mar	CEM	Paraná; litoral norte	25°34'34.5"S 48°20'45.4"W
Universidade da Região de Joinville/ Projeto Toninhas	UNE	Santa Catarina; litoral norte	26°12'57.0"S 48°31'09.1"W
Universidade Estadual de Santa Catarina/ PMP - BS	UDE	Santa Catarina; litoral sul	28°28'38.6"S 48°45'56.2"W

Um total de 260 cetáceos de 11 espécies diferentes foi utilizado para este estudo. Devido ao grau de decomposição, não foi possível a coleta de intestinos e estômagos de todas as carcaças da amostragem. Desta forma, foi analisado um total de 245 intestinos e 85 estômagos (Tabela 3; Figura 1).

Tabela 3. Espécies de cetáceos hospedeiros utilizados neste estudo, amostragem total de carcaças, o total (N) de intestinos e estômagos utilizados para cada espécie de hospedeiro e a Instituição responsável pela coleta das carcaças seguido pela amostragem da instituição por espécie de cetáceo entre parênteses (n).

Hospedeiros	Total	Intestinos (N)	Estômagos (N)	Instituição coletora (N)
MISTICETOS				
Mysticeti	1	1	0	UNE (1)
Balaenopteridae				
Balaenopteridae	1	1	0	UNE (1)
Balaenoptera sp.	2	2	1	IPeC (2)
Balaenoptera acutorostrata (Lacépède, 1804)	2	2	0	UNE (1) IPeC (1)
Balaenoptera brydei	1	1	0	UNE (1)
ODONTOCETOS				
Delphinidae				
Delphinidae	2	2	2	UNE (2)
Delphinus capensis (Gray, 1928)	2	2	0	CEM (2)
Feresa attenuata (Gray, 1875)	1	1	1	IPeC (1)
Pseudorca crassidens (Owen, 1846)	1	1	0	UNE (1)
Sotalia guianensis (van Bénéden, 1864)	111	98	52	CEM (14) IPEC (37) UNE (31) MQ (14) BP (9) AQ (6)
Stenella frontalis (Cuvier, 1829)	8	8	2	CEM (2) IPEC (1) UNE (2) BP (3)
Stenella sp.	9	8	3	UNE (9)
Steno bredanensis (Lesson, 1828)	3	3	3	BP (1) UNE (2)
Tursiops truncatus (Montagu, 1821)	35	35	6	CEM (1) IPEC (2) UNE (23) MQ (1) UDE (8)
Pontoporidae				
Pontoporia blainvillei (Gervais & d'Orbigny, 1844)	77	77	14	UDE (13) IPEC (6) UNE (56) CEM (2)
Physeteridae				
Kogia breviceps (de Blainville, 1838)	4	3	1	UDE (2) UNE (1) IPEC (1)

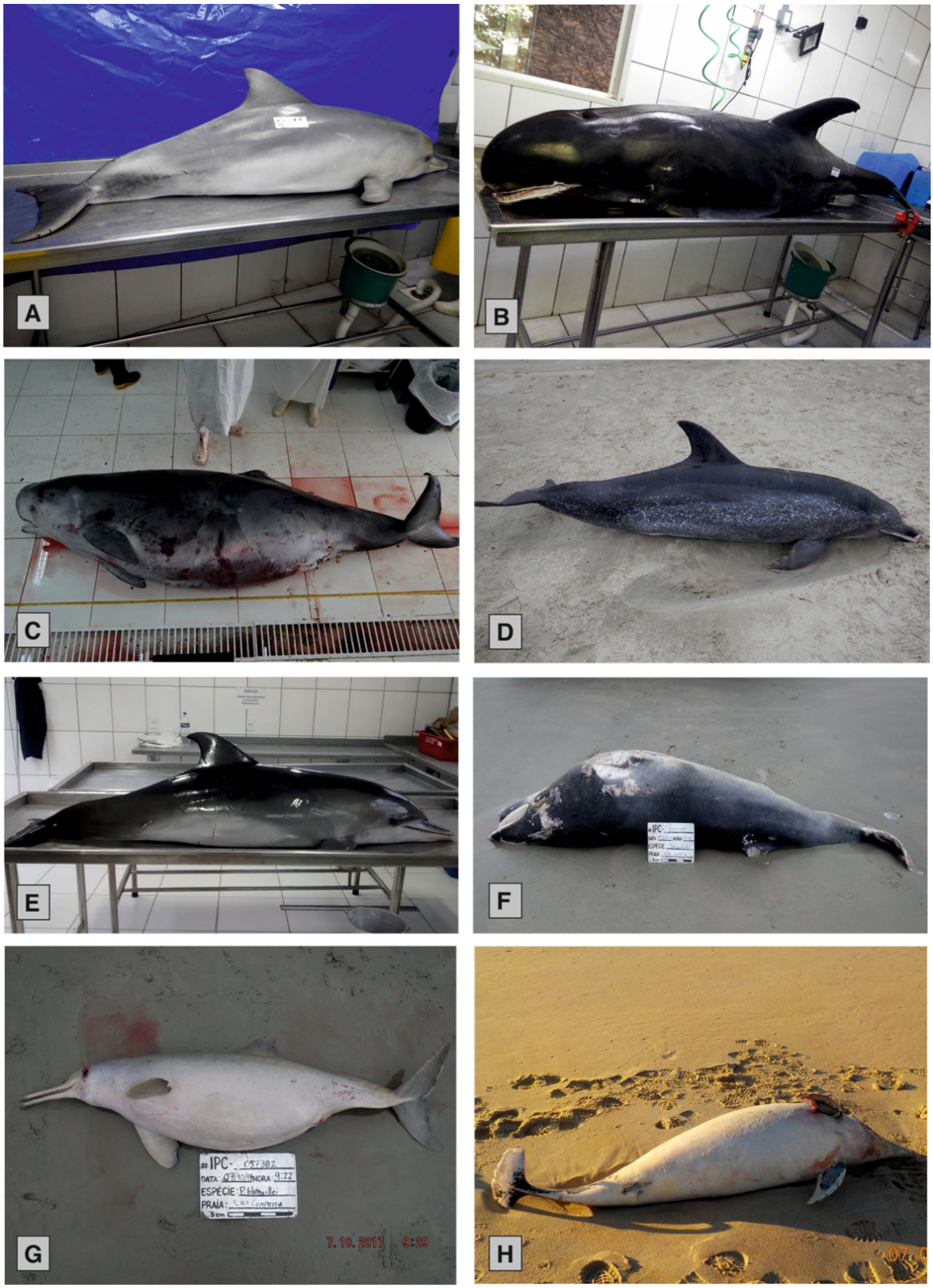


Figura 1. Exemplares de carcaças de cetáceos de diferentes espécies utilizadas neste estudo. A) *Sotalia guianensis*; B) *Feresa attenuata*; C) *Kogia breviceps*; D) *Stenella frontalis*; E) *Stenella* sp. juvenil; F) *Balaenoptera acutorostrata*; G) *Pontoporia blainvillei* prenha; H) *Sotalia guianensis* (fotos: Projeto Monitoramento de Praias – Bacia de Santos).

Coleta dos parasitas

As carcaças de cetáceos obtidas encontravam-se em estado de decomposição inicial (códigos 2 - fresco/morte recente) a avançada (código 4 - decomposição avançada), segundo o estabelecido para mamíferos marinhos por Geraci & Lounsburry (2005). Durante as necropsias dos cetáceos, seus tratos digestórios (estômago e intestino) foram separados, suas extremidades amarradas com barbante, e armazenados em congelador (-20º C) para análise posterior (Figura 2A-B). Quando possível, a abertura dos mesmos foi realizada de imediato, no intuito de minimizar os efeitos do congelamento sobre os parasitas. Os tratos digestórios foram abertos com tesoura sobre uma peneira com abertura de malha de 150 µm a lavados sob água corrente (Figura 2C-D). As mucosas estomacais e intestinais foram analisadas a olho nu para detecção de cistos ou parasitas aderidos as mesmas (Figura 2E-F). Os parasitas encontrados após a lavagem do conteúdo gastrointestinal foram coletados, limpos em soro fisiológico e armazenados em álcool etílico 70% para análises morfológicas e álcool etílico 96% para análises moleculares.

Identificação morfológica dos parasitas

Das amostragens de parasitas coletadas de cada hospedeiro, foram selecionados os espécimes mais íntegros para identificação morfológica. Os espécimes de trematódeos, acantocéfalos e cestoides foram corados com carmim clorídrico alcooólico ou tricrômico de Gomori, desidratados em uma bateria de álcool etílico de diferentes concentrações e diafanizados em Eugenol ou creosoto (o protocolo especifico para cada espécie de parasita é determinado no capitulo ou anexo correspondente, se necessário). Os espécimes de nematoides foram diafanizados diretamente em fenol. Após esse processo, os espécimes foram preparados em montagens temporárias ou permanentes em Bálsamo do Canadá para as análises morfológicas.

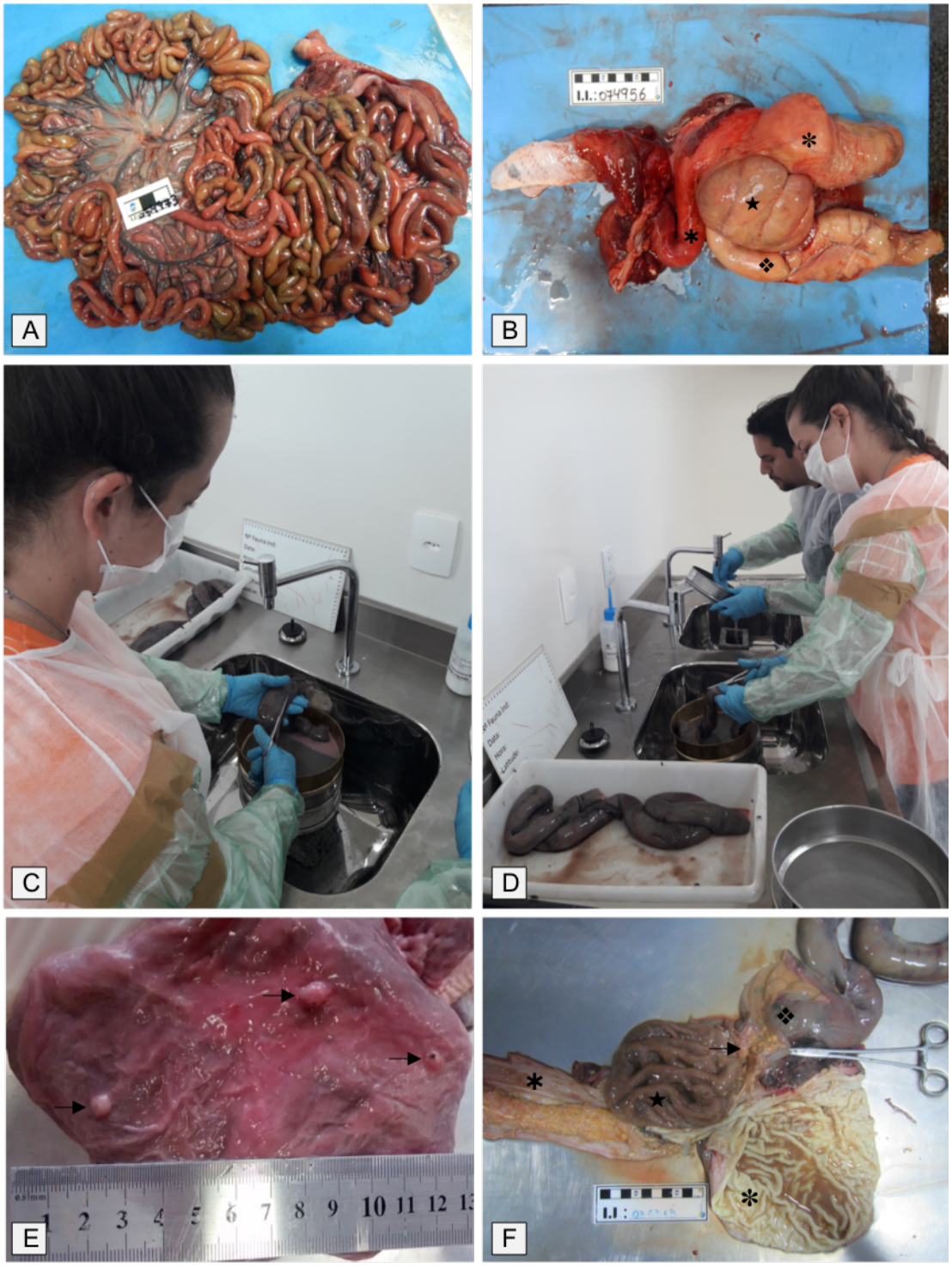


Figura 2. Necropsias e coleta de parasitas gastrointestinais de cetáceos. A) Intestino de *Pontoporia blainvillei* separado para análise. B) Aparelho digestório de *Stenella frontalis* separado para análise. C) Abertura com tesoura do intestino sobre peneira. D) Abertura e lavagem dos intestinos. E) Mucosa da segunda câmara do estômago de *Steno bredanensis* com cistos de *Pholeter gastrophilus* (setas). F) Trato digestório de *Sotalia guianensis* aberto para coleta de parasitas, em destaque *Braunina cordiformis* aderidos a mucosa (seta). ✱=esôfago; ✻=primeira câmara do estômago; ★ = segunda câmara do estômago; ❖ = ampola duodenal. (fotos: Projeto Monitoramento de Praias – Bacia de Santos / Acervo pessoal).

A identificação e classificação dos espécimes foi realizada com auxílio de chaves apropriadas para cada grupo de parasita e artigos de descrição específica para cada espécie (determinados nos capítulos e anexos correspondentes).

Os espécimes foram analisados com auxílio de um sistema computadorizado de análise de imagens com contraste interferencial, utilizando o software LAS V3 (Leica), acoplado em microscópio Leica DMLB 5000. As variáveis morfométricas a serem medidas foram definidas com base em artigos de descrição e análises especificas (determinados nos capítulos correspondentes para cada espécie).

Identificação molecular dos parasitas

Foram realizadas análises moleculares somente para os trematódeos encontrados. As extrações de DNA genômico a partir de fragmentos de parasitas adultos previamente identificados foram realizadas utilizando o kit Qiagen® DNeasy™ Blood & Tissue com volume final de 30 µl. As amostras de DNA foram submetidas à reação em cadeia pela polimerase (PCR) utilizando primers com alvo no DNA ribossomal (subunidade maior do DNA ribossomal - gene 28S - subunidade menor do DNA ribossomal - gene 18S - e região intergênica 1 - ITS1) e DNA mitocondrial (nicotinamida dinucleotídeo dehidrogenase subunidade 3 - gene ND3 - e citocromo oxidase subunidade I - gene COI).

As reações de PCR foram realizadas utilizando o kit comercial Ready-to-Go™ (GE Healthcare) PCR beads, 3 a 5 µl de DNA genômico e 1 a 1,25 µl de cada par de primers, em um volume final de 25 µl. Os primers de amplificação utilizados para cada gene ou região e as condições especificas de ciclo de PCR estão descriminados na Tabela 4 e Figura 3, respectivamente.

Após a verificação de amplificação por eletroforese em gel de agarose 1%, os produtos de PCR foram purificados utilizando o kit comercial QIAquick™ PCR Purification (Qiagen®), e quantificados em espectrofotômetro de moléculas NanoDrop 2000®.

Para as reações de sequenciamento foi utilizado o kit comercial BigDye™ terminator cycle sequencing ready reaction (Applied Biosystems). As amostras foram sequenciadas no sequenciador ABI 3500 Genetic Analyser (Applied Biosystems).

Tabela 4. Descrição detalhada dos primers de amplificação para trematódeos utilizados para cada gene ou região e suas respectivas referências.

Gene ou Região	Primer	Sentido do Primer	Sequência do Primer	Referências
18S / SSU	WormA	F	GCG AAT GGC TCA TTA AAT CAG	Littlewood & Olson, 2001
	WormB	R	CTT GTT ACG ACT TTT ACT TCC	Littlewood & Olson, 2001
	300F	F	AGG GTT CGA TTC CGG GAG	Littlewood & Olson, 2001
	930F	F	GCA TGG AAT AAT GGA ATA GG	Littlewood & Olson, 2001
	1200F	F	CAG GTC TGT GAT GCC C	Littlewood & Olson, 2001
	1270R	R	ACT TAA AGG AAT AGA CGG	Littlewood & Olson, 2001
28S /LSU	LSU5	F	TAG GTC GAC CCG CTG AAY TTA AGC A	Olson et al., 2003
	1200R	R	GCA TAG TTC ACC ATC TTT CGG	Lockyer et al., 2003
	1500R	R	GCT ATC CTG AGG GAA ACT TCG	Olson et al., 2003
ITS1	ITS1F	F	GAC GAC CAA ACT TGA TCA TT	Marigo et al. 2015
	ITS1R	R	TGC GCT CTT CAT CGA CAC ACG A	Marigo et al. 2015
ND3	ND3F	F	GCT TAA TTK KTA AAG CYT TGR ATT CTT ACT	Fernández et al., 1998
	ND3d	R	CTA CTA GTC CCA CTC AAC RTA ACC YT	Fernández et al., 2000
COI	JB3	F	TTT TTT GGG CAT CCT GAG GTT TAT	Morgan & Blair, 1998
	JB4.5	R	TAA AGA AAG AAC ATA ATG AAA ATG	Razo-Mendivil et al., 2008

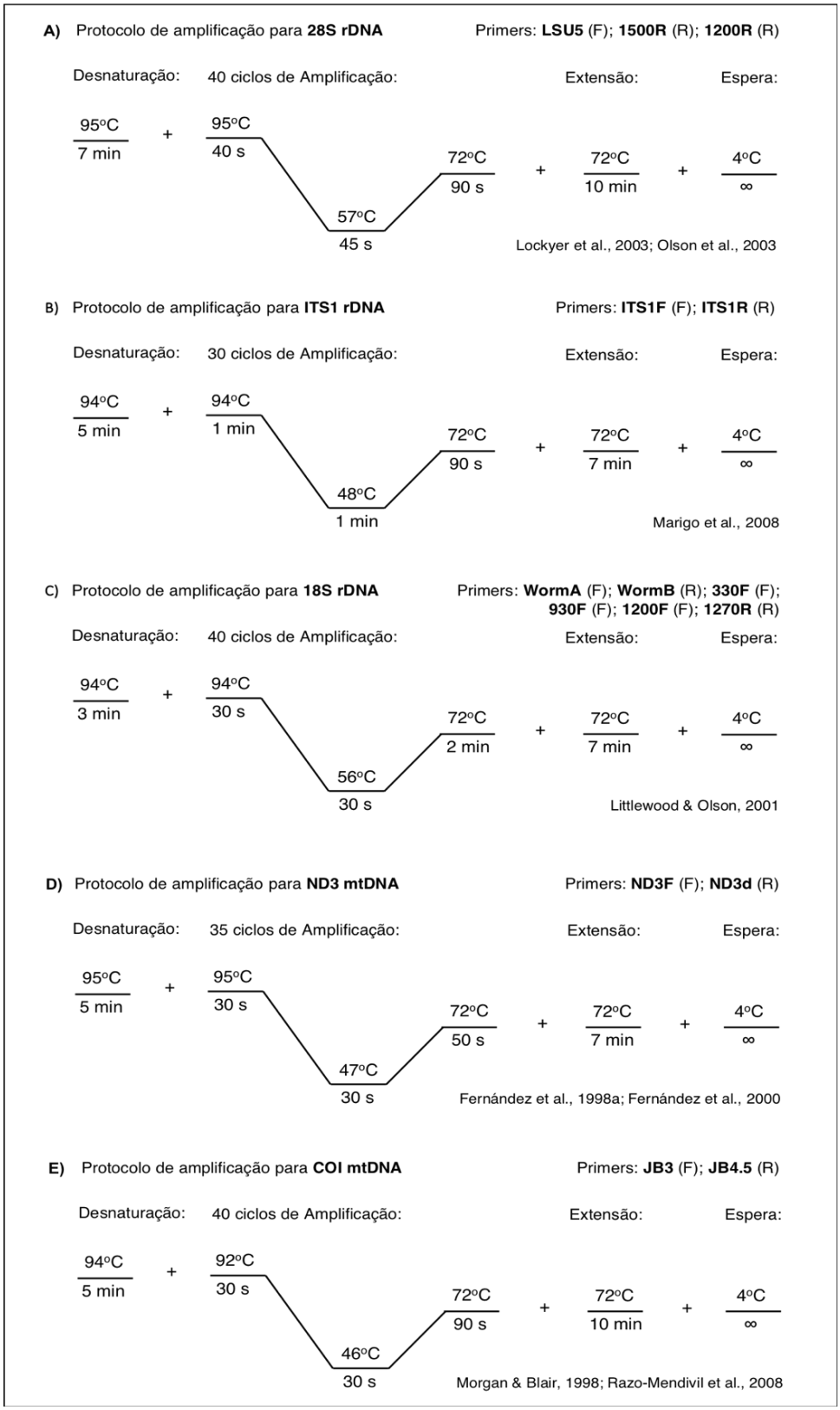


Figura 3. Perfis de termociclagem para a amplificação de; A) subunidade maior do rDNA (28S ou LSU rDNA); B) espaçador transcrito interno subunidade 1 (ITS1 rDNA); C) subunidade maior do rDNA (18S ou SSU rDNA); D) subunidade 1 do citocromo c oxidase do mtDNA (COI mtDNA) e; D) subunidade 3 da desidrogenase NADH do mtDNA (ND3 mtDNA).

A edição das sequências finais foi realizada utilizando o programa Sequencher v. 5.2.4 (Gene Codes, Ann Arbor, MI). Após edição, foram construídas matrizes de sequências para cada um dos marcadores. As matrizes foram então alinhadas no software MUSCLE contido no programa Geneious 7.1.3 (Kearse et al., 2012), utilizando configurações padrão.

O melhor modelo evolutivo para cada matriz alinhada foi calculado no programa jModelTest 2.1.1 (Posada 2008). Para as reconstruções filogenéticas foram utilizadas análises feitas sob modelos de Inferência Bayesiana (BI), usando o programa MrBayes v. 3.2.7 rodado na ferramenta XSEDE contido no portal CIPRES Science (www.phylo.org) (Miller et al., 2015), e Máxima verossimilhança (ML) usando o programa RAxML-HPC v.8 rodado na ferramenta XSEDE contido no portal CIPRES Science (www.phylo.org) (Miller et al., 2015). Os comandos e configurações específicas para cada tipo de análise estão descriminados nos capítulos correspondentes.

Para as análises de delimitação de espécies, foram aplicados os métodos de General Mixed Yule Coalescent (GMYC), realizado no programa BEAST v.1.6.2 (Drummond et al. 2012) e analisado online no servidor GMYC Species delimitation (http://species.h-its.org/gmyc/), e Distância Genética par a par (Pairwise Distance), calculadas utilizando o programa MEGA6 (Kimura, 1980; Tamura et al., 2013). Os comandos e configurações específicas para cada tipo de análise estão descriminadas nos capítulos correspondentes.

As árvores resultantes de cada análise foram visualizadas usando o programa FigTree v.1.3.1 (Rambaut, 2009) e editadas no programa CorelDraw X8.

Descritores ecológicos

Os descritores ecológicos foram calculados para cada espécie de hospedeiro de acordo com Bush et al. (1997), utilizando o programa BioEstat 5.0 (Ayres et al., 2007).

Foram calculados os seguintes índices:

- Abundância média (AM): número total de parasitas de uma determinada espécie, dividido pelo número total de hospedeiros de uma espécie examinados (infectados e não infectados);
- Intensidade média de infecção (IMI): número total de parasitas observados de

uma determinada espécie, dividido pelo número de hospedeiros infectados com esta mesma espécie de parasita.

Resultados e Discussão

RESULTADOS E DISCUSSÃO

Um total de 260 carcaças de cetáceos pertencentes à 11 espécies identificadas foi analisado quanto a presença de parasitos gastrointestinais. Destas, foram coletados 245 intestinos e 85 estômagos. Mais da metade das carcaças obtidas (57%) encontravam-se em grau de decomposição avançada (Código 4).

A espécie hospedeira mais abundante foi *S. guianensis* (43%), seguida por *P. blainvillei* (30%) e *T. truncatus* (13%). As demais, i.e., *Balaenoptera* sp., *B. acutorostrata*, *B. brydei, D. capensis*, *F. attenuata*, *P. crassidens*, *Stenella* sp., *S. frontalis*, *S. bredanensis* e *K. breviceps*, somaram 14% do total de espécies analisadas.

Foi coletado um total de 23.649 espécimes de parasitos. Os trematódeos foram o grupo mais abundante e especioso (n total = 23.210; 12 *taxa*), seguidos por acantocéfalos (n total = 290; 5 *taxa)*, nematoides (n total = 123; 2 taxa) e cestoides (n total = 26; 4 *taxa*).

Na Tabela 5, estão descriminadas as espécies de parasitos encontrados e seus respectivos hospedeiros, bem como seus índices parasitários e ecológicos.

Tabela 5. Espécies de parasitos encontrados em misticetos e odontocetos (Cetacea), coletados no litoral brasileiro. SI: Sítio de infestação (E = estômago; I = intestino); NTH: Numero total de parasitos; NHA: Número de hospedeiros analisados; NHP: Número de hospedeiros parasitados; A/AM: Abundância / Abundância média ± erro padrão; IMI: Intensidade média de infestação ± erro padrão; NRH: novo registro de hospedeiro; NRG: novo registro geográfico; NE: possível nova espécie.

Parasitos	Hospedeiros	SI	NTP	NHA	NHP	A/AM	IMI	NRH	NRG	NE
Trematoda										
Brachycladiidae Odhner, 1905										
Synthesium neotropicale Ebert et al. 2017	*Tursiops truncatus* (Montagu, 1821)	I	3.426	35	23	97,9 ± 34,9 (0-1.004)	148,9 ± 50,1 (2-1.004)			
Synthesium pontoporiae (Raga, 1984)	*Pontoporia blainvillei* (Gervais & d'Orbigny, 1844)	I	7.138	77	61	62,7 ± 19,9 (0-1.000)	117,0 ± 24,3 (1-1.000)			
Synthesium sp. 1	Delphinidae Gray, 1821	I	16	2	1	8 ± 8 (0-16)	16			X
	Sotalia guianensis (Van Beneden, 1864)	I	341	98	52	3,5 ± 0,6 (0-34)	6,6 ± 0,9 (1-34)			X
Synthesium sp. 2.	Delphinidae	I	2	2	1	1 ± 1 (0-2)	2			X
	S. guianensis	I	5	98	1	0,05 ± 0,05 (0-5)	5			X
	Stenella frontalis (Cuvier, 1829)	I	432	8	4	54,0 ± 37,2 (0-300)	108 ± 67,2 (2-300)			X
	Stenella sp.	I	647	8	5	80,9 ± 66,8 (0-546)	129,4 ± 104,5 (2-546)			X
Synthesium sp. *3*	*S. guianensis*	I	414	98	37	4,2 ± 1,3 (0-87)	11,2 ± 3,2 (1-87)			X
	S. frontalis	I	48	8	1	6,0 ± 6,0 (0-48)	48			X
	Steno bredanensis (Lesson, 1828)	I	154	3	2	51,3 ± 49,3 (0-150)	77,0 ± 73,0 (4-150)			X

Synthesium sp. 4	*S. bredanensis*	I	161	3	1	53,7 ± 53,7 (0-161)	161			X
	T. truncatus	I	2.315	35	6	66,1 ± 57,3 (0-2.000)	385,8 ± 324,8 (5-2.000)			X
Synthesium sp. 5	*Delphinus capensis* Cuvier, 1829	I	9	2	1	4,5 ± 4,5 (0-9)	9			X
Cyathocotylidae Mühling, 1898										
Braunina cordiformis Wolf, 1903	Delphinidae	E	16	2	2	8 ± 0 (0-8)	8 ± 0 (8-8)			
	Stenella sp.	E	4	3	2	1,3 ± 0,9 (0-3)	2,0 ± 1,0 (1-3)			
	S. frontalis	E	6	2	2	3,0 ± 1,0 (2,0-4,0)	3,0 ± 1,0 (2,0-4,0)			
	S. bredanensis	E	33	3	2	11,0 ± 9,5 (0-30)	16,5 ± 13,5 (3-30)			
	T. truncatus	E	10	6	4	1,7 ± 0,7 (0-4)	2,5 ± 0,6 (1-4)			
	S. guianensis	E	1.922	52	31	36,9 ± 13,5 (0-454)	62,0 ± 21,7 (1-454)			
Heminuridae Looss, 1899										
Hemiuridae Looss, 1899	*S. guianensis*	I	36	98	1	0,37 ± 0,37 (0-36)	36	X	X	
Heterophyidae Leiper, 1909										
Ascocotyle longa Ransom, 1920	*S. guianensis*	I	10	98	1	0,1 ± 0,1 (0-10)	10	X		
	S. bredanensis	I	2.500	3	2	833,3 ± 436,6 (0-1.476)	1.250,0 ± 226,0 (1.024-1.476)	X		
	T. truncatus	I	3.114	35	5	66,1 ± 57,3 (0-1.500)	622,8 ± 325,4 (2-1.500)	X		

Pholeter gastrophilus (Kossack, 1910)	*Feresa attenuata* Gray, 1874	E	6	1	1	6	1		X
	S. bredanensis	E	5	3	1	1,7 ± 1,7 (0-5)	5		X
	T. truncatus	E	2	6	1	0,33 ± 0,33 (0-2)	2		X
Notocotylidae Lühe, 1909									
Ogmogaster antarctica Johnston, 1931	Balaenopteridae Gray, 1864	I	33	1	1	33	33		
	Balaenoptera sp. Lacépède, 1804	I	303	2	2	151,5 ± 48,5 (103-200)	151,5 ± 48,5 (103-200)		
	Balaenoptera acutorostrata Lacépède, 1804	I	102	2	2	51,0 ± 38,0 (13-82)	51,0 ± 38,0 (13-82)	X	
Cestoda									
Diphyllobothriidae Lühe, 1910									
Diphyllobothrium latum (Linnaeus, 1758)	*Kogia breviceps* (de Blainville, 1838)	I	4	3	1	1,3 ± 0,9 (0-3)	2,0 ± 1,0 (1-3)	X	
Tetrabothriidae									
Trigonocotyle sexitesticulae Hoberg, 1990	*F. attenuata*	I	2	1	1	2	2		X
Tetrabothrius sp. Rudolphi, 1819	*S. frontalis*	I	14	8	3	1,8 ± 0,9 (0-6)	4,7 ± 0,9 (3-6)	X	
Tetrabothrius forsteri (Krefft, 1871)	*T. truncatus*	I	6	35	2	0,17 ± 0,15 (0-5)	3,0 ± 2,0 (1-5)	X	
Acantocefala									
Polymorphidae Meyer, 1931									
Bolbosoma sp. Porta, 1908	*S. frontalis*	I	3	8	1	0,37 ± 0,37 (0-3)	3		
	Stenella sp.	I	1	8	1	0,13 ± 0,13 (0-1)	1		
	T. truncatus	I	9	35	1	0,26 ± 0,26 (0-9)	9		
Bolbosoma capitatum (von Linstow, 1880) Porta, 1908	*Pseudorca crassidens* (Owen, 1846)	I	13	1	1	13	1		

Bolbosoma tuberculata Skrjabin, 1970	*Balaenoptera brydei* Anderson, 1878	I	250	1	1	250	250		X
Bolbosoma turbinella (Diesing, 1851) Porta, 1908	*K. breviceps*	I	6	3	1	2,0 ± 2,0 (0-6)	6	X	
Corynosoma cetaceum Johnston & Best, 1942	*T. truncatus*	I	7	35	2	0,17 ± 0,13 (0-4)	3,0 ± 1,0 (2-4)	X	
	P. blainvillei	I	1	14	1	0,07 ± 0,07 (0-1)	1		
Nematoda									
Anisakidae Railliet & Henry, 1912									
Anisakis paggiae. Mattiucci, Nascetti, Dailey, Webb, Barros, Cianchi & Bullini, 2005	*S. guianensis*	E	65	52	10	1,3 ± 1,1 (0-55)	8,2 ± 6,7 (1-55)	X	
	K. breviceps	I	4	3	1	1,3 ± 0,9 (0-3)	2,0 ± 1,0 (1-3)		
Pseudoterranova ceticola (Deardoff & Overstreet, 1981)	*K. breviceps*	I	54	3	2	18,0 ± 13,7 (0-45)	27,0 ± 18,0 (9-45)		X

Trematódeos

Os trematódeos relatados em cetáceos pertencem a quatro famílias: Brachycladiidae, Heterophyidae, Cyathocotylidae (=Brauninidae) e Notocotylidae, as quais foram representadas neste estudo por pelo menos uma espécie de parasito cada (Figura 4).

A família Brachycladiidae foi representada por apenas um gênero. *Synthesium* (Figura 4A) agrega parasitos intestinais de odontocetos, cosmopolitas e com ampla distribuição geográfica. O status taxonômico de *Synthesium* foi reavaliado diversas vezes (Gibson, 2005), incluindo realocações e sinonímias entre gêneros morfologicamente próximos, como *Hadwenius* Price, 1932 e *Leucasiella* Krotov & Delyamure 1952 (Gibson, 2005; WoRMS, 2019). A maioria dessas sinonímias foi feita baseada em caracteres morfológicos que muitas vezes são facilmente perdidos devido à decomposição ou difíceis de definir. Neste estudo, baseado nas análises morfológicas e moleculares, foram identificadas sete espécies de *Synthesium*, das quais duas já são conhecidas e registradas em águas brasileiras, *Synthesium neotropicale* e *S. pontoporiae* encontradas em *T. truncatus* e *P. blainvillei*, respectivamente, e cinco novas espécies do gênero (*Synthesium* sp. 1, 2, 3, 4 e 5), das quais uma, *Synthesium* sp. 1, é descrita neste trabalho (ver Capitulo 1)

Braunina cordiformis (Figura 4B), parasito de morfologia bastante peculiar, foi recentemente realocado na família Cyathocotylidae (Achatz et al., 2019). A espécie é registrada em diversos hospedeiros e regiões diferentes mundialmente. Neste estudo, *B. cordiformis* foi encontrado em pelo menos quatro espécies de odontocetos diferentes, i.e., *S. guianensis*, *S. bredanensis*, *T. truncatus* e *S. frontalis*, os quais já possuíam registros anteriores no Brasil (Luque et al., 2010).

Foram encontrados espécimes adultos pertencentes a família Hemiuridae (Figura 4C) no intestino de *S. guianensis*. A identificação especifica destes parasitos será concluída em estudos futuros. Parasitos da família Hemiuridae utilizam peixes como hospedeiros definitivos (Almeida et al., 2009) e não são considerados como parte da helmintofauna comum de cetáceos. No entanto, Hoss et al. (2017) relataram parasitos da família Hemiuridade em *P. blainvillei* no litoral Norte do Rio Grande do Sul, relacionando a presença do parasito à ingestão recente de peixes pelo odontoceto, uma vez que as cutículas do helminto se encontravam degradadas. Neste estudo, os espécimes adultos foram encontrados em *S. guianensis* e com morfologia integra (bom estado de conservação dos espécimes) (ver Tabela 5), o que pode

contribuir para novas investigações a respeito da relação destes parasitos com cetáceos como hospedeiros definitivos ou acidentais.

Pholeter gastrophilus (família Heterophyidae) (Figura 4D) é uma espécie comum em odontocetos. Distribuída mundialmente (Fraija-Fernández et al., 2017), essa espécie é encontrada em cistos na parede do estômago (principalmente pilórico, raramente no duodeno), às vezes gerando fibrose extensa. Apesar de amplamente distribuído, este é o primeiro registro da espécie no litoral brasileiro, o qual foi encontrado parasitando o estômago de três espécies de odontocetos diferentes, *T. truncatus*, *F. attenuata* e *S. bredanensis* (ver Anexo 3)

Outro heterofídeo, *Ascocotyle longa* (Figura 4E), foi identificado parasitando o intestino de três espécies de odontocetos diferentes, *T. truncatus*, *S. guianensis* e *S. bredanensis. Ascocotyle longa* é um pequeno parasito cosmopolita e potencialmente zoonótico, comumente encontrado no intestino de aves piscívoras e mamíferos (Pearson 2008). É considerado um dos agentes causadores da heterofíase humana (Simões et al. 2010), com casos já relatados no Brasil (Antunes e Almeida-Dias 1994). Apesar da grande variedade de hospedeiros definitivos de *A. longa*, este é o primeiro registro da espécie infectando cetáceos em geral (ver Anexo 2)

Heterophyidae era representada por somente uma espécie infectante de cetáceos, *P. gastrophilus*. Com a identificação de *A. longa* no intestino de odontocetos neste estudo, Heterophyidae passa a contar com duas espécies representantes que infectam cetáceos.

Ogmogaster antarctica (família Notocotylidae) (Figura 4F) é encontrado no intestino de baleias e pinípedes (Rausch e Fay, 1966). A espécie exibe uma distribuição mundial e foi relatada em diversas espécies de cetáceos de diferentes áreas geográficas. No entanto, no Atlântico Sul, poucos registros são conhecidos. O encontro de *O. antarctica* infectando um espécime de *B. acutorostrata* representa um novo registro de hospedeiro no Atlântico Sul (ver Anexo 1).

Cestoides

Três famílias de cestoides incluem espécies que infectam cetáceos: Tetrabothriidae, Diphyllobothriidae e Phyllobothriidae.

Duas espécies pertencentes aos gêneros *Trigonocotyle* e *Tetrabothrius* (família Tetrabothriidae) (Figura 5) foram identificadas neste estudo. Carvalho et al. (2010) registrou pela primeira vez o gênero *Trigonocotyle* em *S. longirostris* e *S. clymene* no

Brasil. Este estudo representa o primeiro registro de *Trigonocotyle sexitesticulae* (Figura 5 A-B) em cetáceos de águas brasileiras. A espécie *T. sexitesticulae* é um parasito descrito, até o momento, somente em *F. attenuata* (Hoberg, 1990). Já as espécies de *Tetrabothrius* são frequentemente registradas em cetáceos em nível global. No entanto, no Brasil, *Tetrabothrius* foi encontrado somente em *S. longirostris* e *S. clymene* (Carvalho et al., 2010). Neste estudo, *T. forsteri* (Figura 5 C, D) foi encontrado em *T. truncatus* e *Tetrabothrius* sp. em *S. frontalis*, sendo estes novos registros de hospedeiros para estas espécies parasitas no Brasil.

Diphyllobothrium latum (família Diphyllobothriidae) (Figura 5E-F) foi encontrado no intestino de *K. breviceps*. Este parasito é comumente encontrado infectando o homem, inclusive com relatos de caso no Brasil. No entanto, *D. latum* também foi identificado parasitando *P. phocaena* no Alaska, no Mar do Norte e no Mar Negro (Delyamure, 1955, 1957; Yablokov et al., 1976; Birkun, 2002). Esses registros, porém, foram considerados como “infecções acidentais” (Fraija-Fernández et al., 2016), nas quais estes golfinhos estariam servindo como hospedeiros paratênicos. Neste estudo, a presença de *D. latum* em um novo hospedeiro cetáceo, *K. breviceps*, traz novos questionamentos a respeito da relação desta espécie parasita com mamíferos marinhos.

Acantocéfalos

Dois gêneros de acantocéfalos, *Bolbosoma* e *Corynosoma* (família Polymorphidae) (Figura 6), ocorrem no intestino e ocasionalmente no estômago de baleias e golfinhos.

Neste estudo, três espécies do gênero *Bolbosoma* foram identificadas: *Bolbosoma capitatum* (Figura 6A-D), encontrada no intestino de *P. crassidens*, sendo este o segundo registro deste parasito neste hospedeiro no Atlântico; *Bolbosoma tuberculata* (Figura 6E-H), encontrada no intestino de *B. brydei*, sendo este um novo registro geográfico da espécie neste determinado hospedeiro; e *Bolbosoma turbinella* (Figura 6I-J) no intestino de *K. breviceps*, configurando um novo registro de hospedeiro para a espécie parasita. Espécimes de *Bolbosoma* também foram coletados do intestino de *S. frontalis*, *Stenella* sp. e *T. truncatus*, porém, não foi possível a identificação especifica.

Apenas uma espécie do gênero *Corynosoma*, *C. cetaceum* (Figura 6 K-L) foi encontrado nos intestinos de *T. truncatus* e *P. blainvillei*. Esta espécie é comumente

encontrada nestes hospedeiros (Yamaguti,1963; Andrade et al. 1996; Romero et al., 2014; Hoss et al., 2017), porém esta é a primeira vez que se registra o parasito em *T. truncatus* na costa brasileira.

Nematoides

A maioria dos nematoides infectantes do trato digestório de cetáceos pertencem a família Anisakidae e gêneros *Anisakis*, *Pseudoterranova* e *Contracaecum*. Anisaquídeos exibem ciclos de vida indiretos em ecossistemas aquáticos envolvendo vários hospedeiros em diferentes níveis da cadeia alimentar (Mattiucci e Nascetti, 2008). Cetáceos, pinípedes e aves piscívoras são os hospedeiros definitivos; peixes, lulas e outros invertebrados servem como hospedeiros intermediários ou paratênicos. As formas larvais, quando ingeridas através de peixes crus ou mal cozidos por humanos, causam a doença zoonótica conhecida como anisaquidose ou anisaquíase (Mattiucci e Nascetti, 2008). *Anisakis* é considerado o gênero de anisaquídeo mais importante no que diz respeito à potencial infecção humana (Audicana et al., 2002).

Os anisaquídeos são frequentemente encontrados livres ou aderidos à mucosa do estômago dos cetáceos, onde podem provocar úlceras e obstruções (Dhermain e Soulier, 2002). Esses parasitos provocam lesões que, apesar de raramente provocarem a morte, podem enfraquecer o animal e/ou deixa-lo susceptível a outras doenças (Dhermain e Soulier, 2002).

Neste estudo foram encontrados espécimes adultos de *Anisakis paggiae* (Figura 7A-B) infectando *K. breviceps* e *S. guianensis,* sendo este ultimo um novo registro de hospedeiro para esta espécie parasita. Já a espécie *Pseudoterranova ceticola* (Figura 7C-D) foi encontrada no intestino de *K. breviceps,* representando um novo registro geográfico de ocorrência deste parasito.

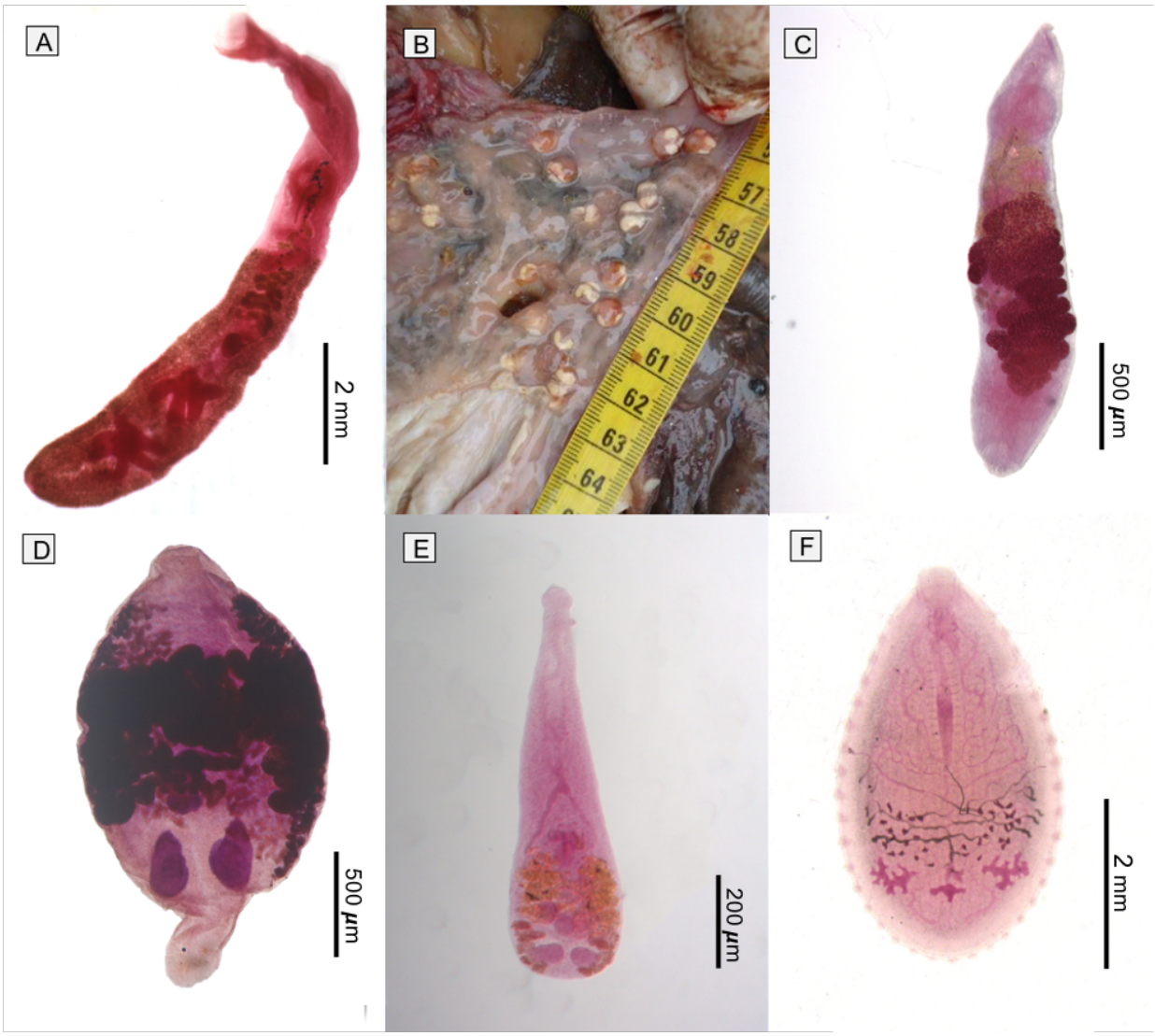


Figura 4. Trematódeos parasitos de cetáceos encontrados neste estudo. A) *Synthesium* sp. 1 coletado do intestino de *Sotalia guianensis;* B) *Braunina cordiformis* coletada da mucosa estomacal de *S. guianensis;* C) Hemiuridae coletado do intestino de *S. guianensis*; D) *Pholeter gastrophilus* coletado da mucosa estomacal de *Steno bredanensis*; E) *Ascocotyle longa* coletado no intestino de *Tursiops truncatus*; F) *Ogmogaster antarctica* coletado do intestino de *Balaenoptera acutorostrata*.

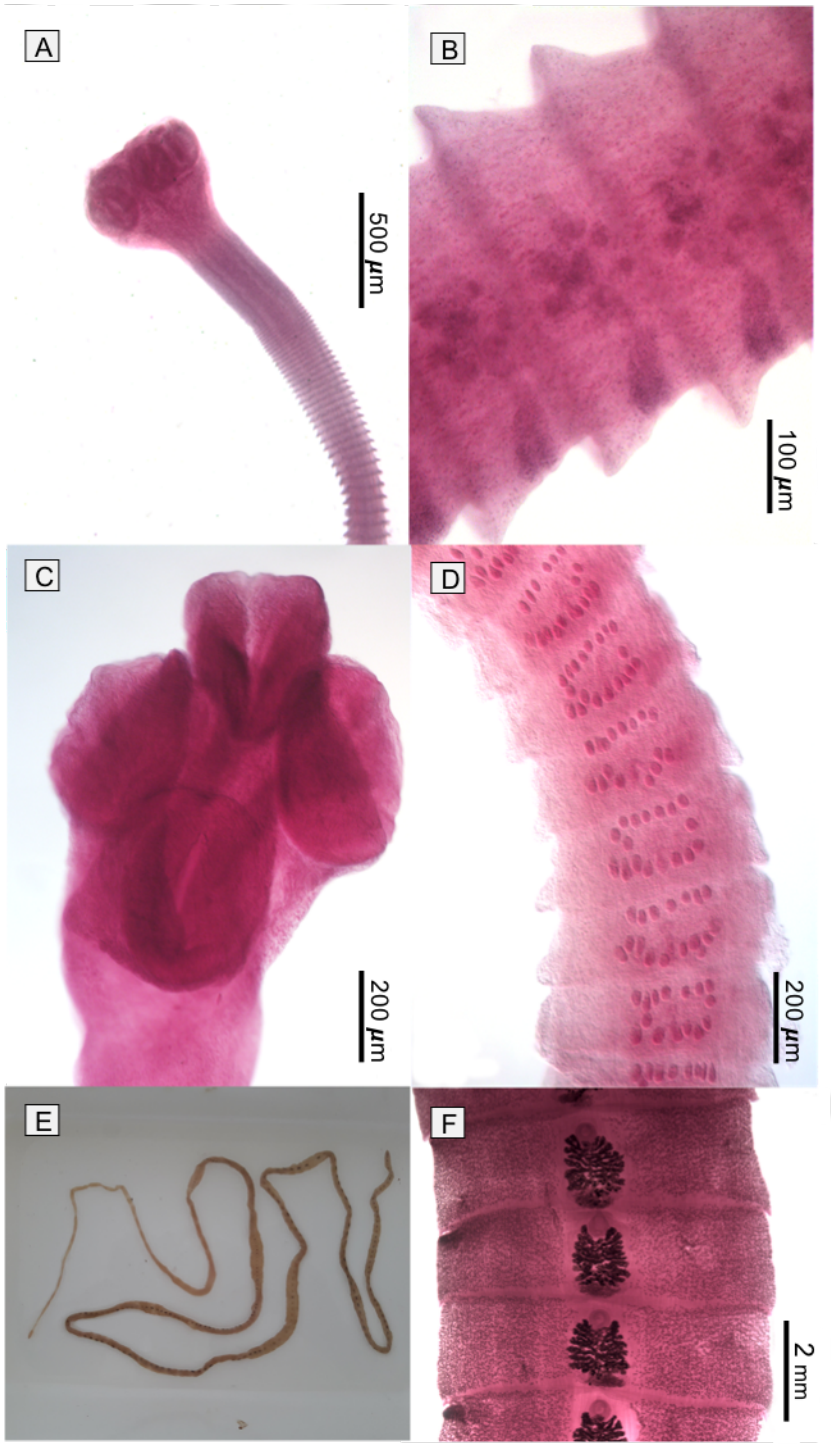


Figura 5. Cestoides parasitos de cetáceos encontrados neste estudo. A) Escólex de *Trigonocotyle sexitesticulae encontrado no intestino de Feresa attenuata;* B) Proglotes maduras de *T. sexitesticulae encontrado no intestino de F. attenuata;* C) Escólex de *Tetrabothrius forsteri* encontrado em *Tursiops. truncatus*; D) Proglotes de *T. forsteri* encontrado em *T. truncatus*; E) espécime adulto de *Diphyllobothrium latum* coletado no intestino de *Kogia breviceps*; F) Proglotes de *D. latum* coletado no intestino de *K. breviceps.*

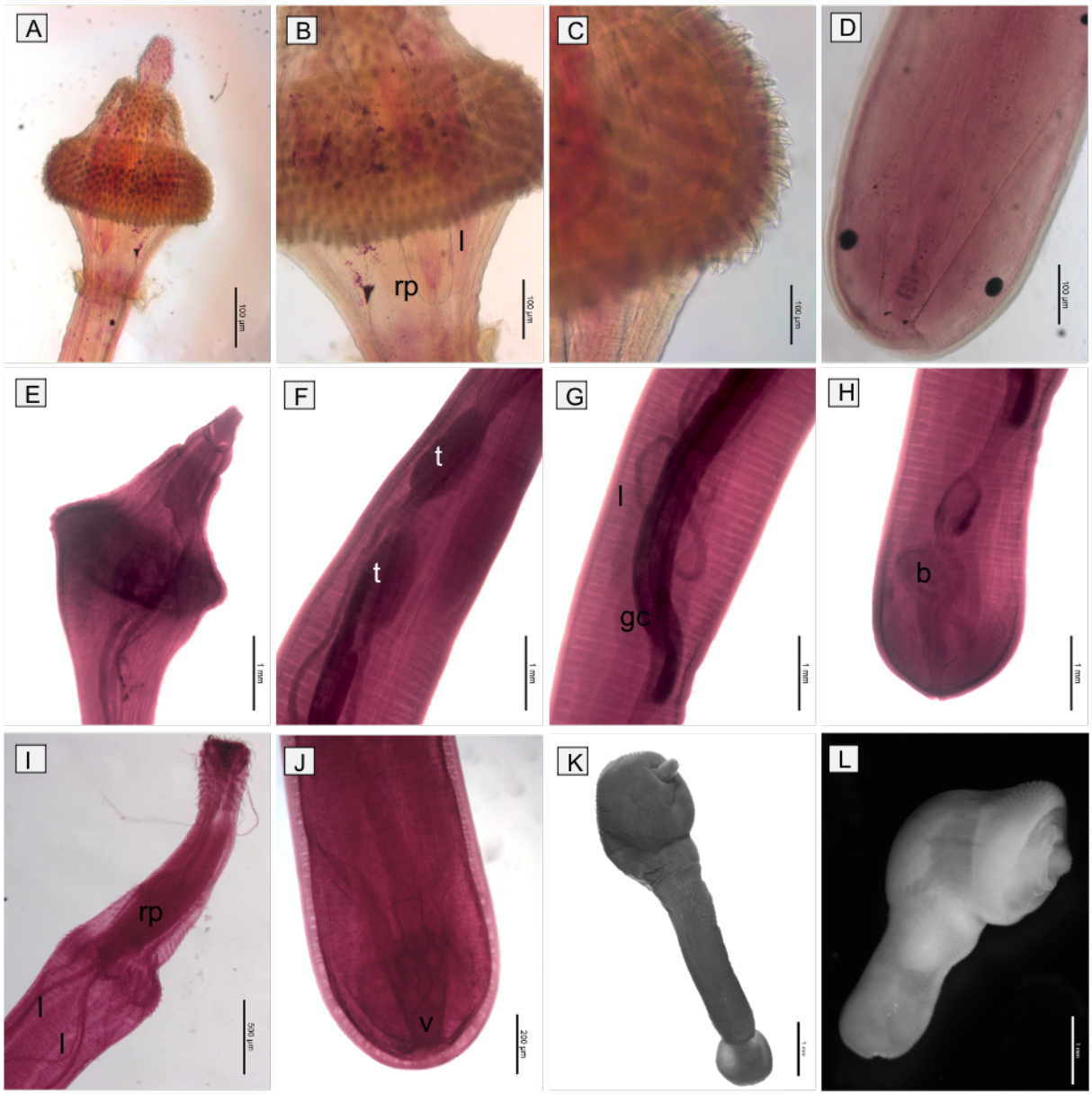


Figura 6. Acantocéfalos parasitos de cetáceos encontrados neste estudo. A) Região anterior de *Bolbosoma capitatum* encontrado no intestino de *Pseudorca crassidens*; B) Detalhe da região do bulbo cefálico evidenciando o receptáculo da probóscide (rp) e o lemnisco curto (l) de *B. capitatum;* C) Detalhe dos espinhos da região do bulbo cefálico de *B. capitatum;* D) Região posterior de um macho adulto de *B. capitatum;* E) Região anterior de *Bolbosoma tuberculata* encontrado no intestino *Balaenoptera brydei;* F) Região medial de um macho de *B. tuberculata* evidenciando os testículos (t); G) Região medial de um macho de *B. tuberculata* evidenciando o final do lemnisco (l) e as glândulas tubulares de cimento (gc). H) Região posterior de um macho de *B. tuberculata* com bursa (b)*;* I) Região anterior e probóscide de *Bolbosoma turbinella* encontrado no intestino *Kogia breviceps*, (rp) receptáculo da proboscide e (l) lemnisco; J) Região posterior de uma fêmea de *B. turbinella,* (v) vagina*;* K) Macho adulto com bursa evertida de *Corynosoma cetaceum* encontrado no intestino de *Tursiops truncatus*; L) Fêmea adulta de *C. cetaceum* encontrado no intestino de *T. truncatus*.

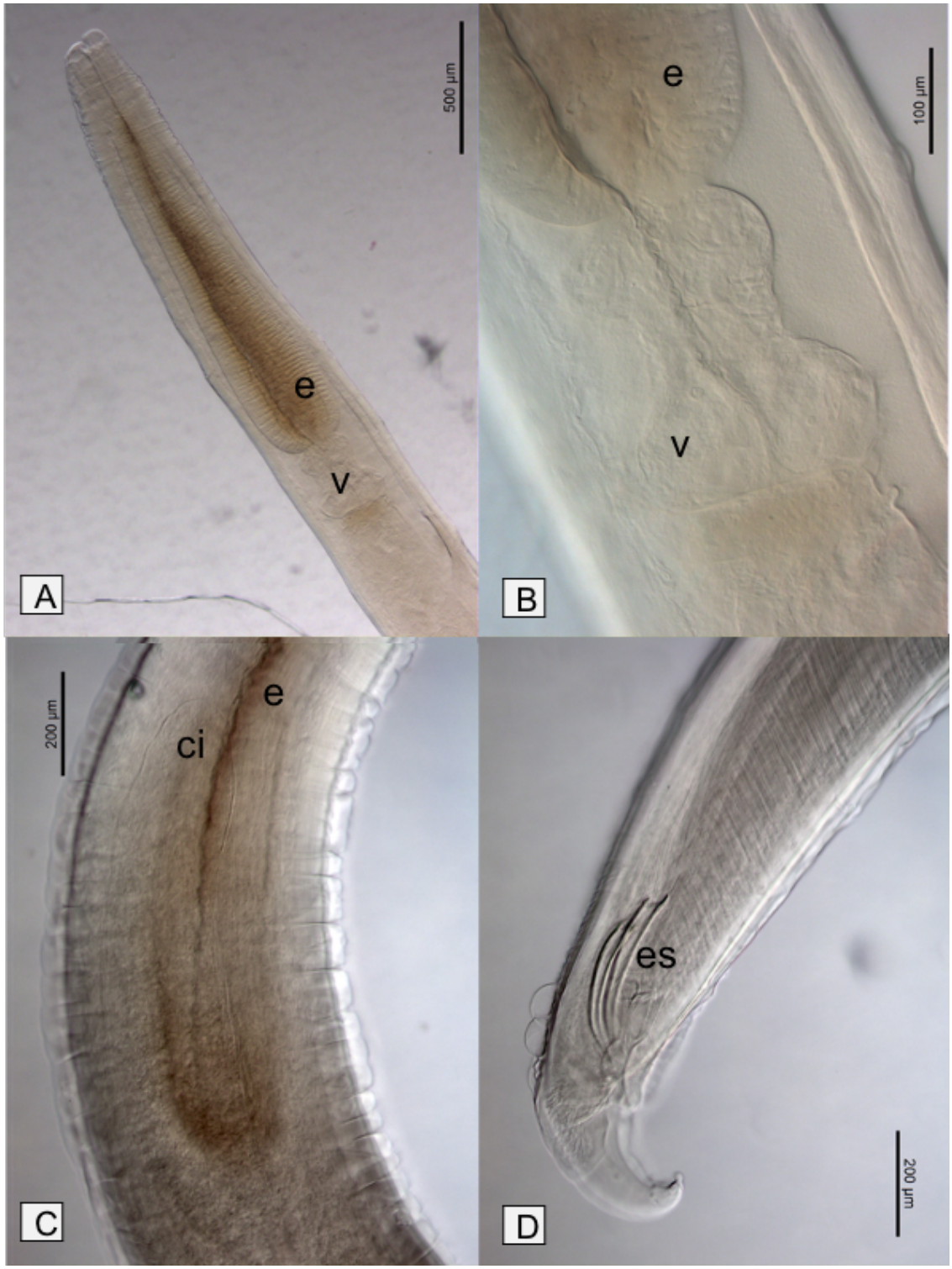


Figura 7. Nematoides parasitos de cetáceos encontrados neste estudo. A) Região anterior de *Anisakis paggiae* encontrado no intestino de *Kogia breviceps*, evidenciando o esôfago (e) e o ventrículo (v)*;* B) Detalhe da região anterior de *A. paggiae*. encontrado no intestino de *K. breviceps*, evidenciando o esôfago (e) e o ventrículo (v); C) Detalhe da região anterior de *Pseudoterranova ceticola* encontrado no intestino de *K. breviceps,* evidenciando o ceco intestinal (ci) e o esôfago; D) Detalhe da região posterior de *P. ceticola* encontrado no intestino de *K. breviceps,* evidenciando os espículos (es).

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Capítulo 1

Revision of *Synthesium tursionis* Marchi, 1873, the resurrection of *Synthesium longissimum* Poirier, 1887 and the description of a new species of *Synthesium* (Digenea, Brachycladiidae), parasites of cetaceans

***Artigo a ser submetido**

Revision of S*ynthesium tursionis* Marchi, 1873, the resurrection of *Synthesium longissimum* Poirier, 1887, and the description of a new species of *Synthesium* (Digenea, Brachycladiidae) parasites of cetaceans

Abstract

Synthesium tursionis is a cosmopolitan species frequently found in the intestines of dolphins worldwide. Its taxonomic status is still confusing and many authors have pointed out several morphological variations which were often attributed to intraspecific variability. To investigate the species boundaries, we analyzed, through morphological and molecular methods, specimens of *Synthesium* collected from 24 odontocete hosts found washed ashore off the Brazilian coast and the Mediterranean Coast of Spain, along with specimens deposited in museums. Sequences of the ribosomal (ITS1) and mitochondrial (ND3 and COI) DNA were obtained and analyzed under Bayesian and Maximum-likelihood methods for phylogenetic investigation and the General Mixed Yule Coalescent model (GMYC) for species delimitation. Our results showed morphological and molecular differences suggesting the existence of three separate species. Therefore, here we redescribe *S. tursionis* from *Tursiops truncatus* from the Mediterranean, resurrect *S. longissimum* from *T. truncatus* from the Mediterranean, and describe a new species, *Synthesium* sp. 1. n. sp. from *Sotalia guianensis* off the coast of Brazil.

Introduction

Digeneans of the family Brachycladiidae Odhner, 1905 are found in the hepatopancreatic ducts, air sinuses, lungs, and intestines of marine mammals, mainly cetaceans (Gibson, 2005). The family has a cosmopolitan distribution and is currently composed of more than 50 species distributed in nine genera (Fraija-Fernández et al., 2016a; WoRMS, 2019), i.e., *Brachycladium* Loss, 1899, *Campula* Cobbold, 1858, *Odhneriella* Skrjabin, 1915, *Orthosplanchnus* Odhner, 1905, *Oschmarinella* Skrjabin, 1947, *Synthesium* Stunkard & Alvey, 1930, *Zalophotrema* Stunkard & Alvey, 1929, *Cetitrema* Skrjabin, 1970, and *Nasitrema* Ozaki, 1935.

Among brachycladiids, the genus *Synthesium* is one of the most specious and widely distributed. Species of this genus are intestinal parasites of wide

ranging odontocete cetaceans from at least four families (Fernández et al.,1994, 1995, 1996), i.e., Pontoporiidae Gray, 1846, Delphinidae Gray, 1821, Monodontidae Gray, 1821, and Phocoenidae Gray, 1825. Currently, there are eight valid species of *Synthesium*: the type-species *S. tursionis* (Marchi, 1873), *S. pontoporiae* (Raga et al., 1994), *S. seymouri* (Price, 1932), *S. nipponicum* Yamaguti, 1951, *S. mironovi* Krotov & Delyamure, 1952, *S. subtile* Skrjabin, 1959, *S. delamurei* (Raga & Balbuena, 1988), and *S. neotropicale* Ebert, Mülller, Marigo, Valente, Cremer, & Silva, 2017 (WoRMS, 2019).

The taxonomic status of *Synthesium* has been revisited several times (Gibson, 2005), including synonymies between morphologically closely related genera such as *Hadwenius* Price, 1932 and *Leucasiella* Krotov & Delyamure 1952 (Gibson, 2005; WoRMS, 2019). Most of these synonyms were based on morphological characters that are often readily lost due to decomposition or difficult to define (e.g., presence or absence of spines in cirrus and shape and extension of vitellaria (Fernández et al.,1994, 1995). The species *S. nipponicum*, *S. mironovi*, *S. subtile,* and *S. delamurei* were originally described relying only on few specimens and/or lacking taxonomic revision since their descriptions. Other species such as *S. pontoporiae* and *S. seymouri* have been redescribed recently (Marigo et al., 2008). As for the type-species *S. tursionis*, since its first description made by Marchi (1873), many authors have pointed out several morphological variations, mainly regarding body size, shape and size of caeca, shape, and position of oral sucker, presence/absence of spines in cirrus, and shape of testes (Yamaguti, 1958; Skrjabin, 1976; Raga et al., 1985, Hafeezullah, 1986; Tantalean & Escalante, 1987; Lamothe-Argumedo, 1988; Fernández et al., 1994, 1995; Marigo et al., 2008). Such morphological variations have been often attributed to intraspecific variability or poor preservation of the specimens.

This study aimed to investigate the intraspecific boundaries of *S. tursionis* and provide a reliable morphological and molecular identification for the species. The specimens collected from stranded odontocetes found along the Brazilian coast and the Mediterranean coast of Spain were compared with other *S. tursionis* deposited in parasite collections and museums. Based on the morphological and molecular variation found between the analyzed specimens, here we present a taxonomic revision of *S. tursionis*, the revalidation of the species *S. longissimum,* and the description of a new *Synthesium* species.

Material and methods

Parasite collection

Samples of *Synthesium* were obtained from 18 *Sotalia guianensis* Van Bénéden, 1864 and one unidentified Delphinidae Gray, 1821 from the southeastern and southern marine coastline of Brazil, between the states of Rio de Janeiro (22°58'00.1"S; 42°50'50.4"W) and Santa Catarina (28°33'30.0"S; 48°46'39.6"W). We have also obtained samples of *Synthesium* from five *T. truncatus* off the coast of Spain in the Mediterranean Sea (38°02'05.2"N; 0°20'15.5"W). The dolphins were found already deceived and washed ashore along the coastline. During necropsy, the gastrointestinal tract of the dolphins was removed and immediately analyzed or stored at -20 °C until further examination. The intestines were cut open, washed in tap water over a 150 µm sieve, and their content examined under a stereomicroscope. All trematodes were cleaned in saline 10% or tap water, fixed, and preserved directly in 70% and 96% ethanol for morphological and molecular analyses, respectively.

To morphologically compare our specimens, we obtained: high-quality photographs of *S. tursionis* collected from *Phocoena sinus* McFarland e Norris, 1958 from the Gulf of California, Mexico, which were analyzed by Lamothe-Argumedo (1988), and deposited at the Helminth National Collection of Mexico (CNHE) under number 240-10; and high-quality photographs of *S. tursionis* collected from *T. truncatus* from the Mediterranean coast of Spain (catalog number 2745503) deposited at the Natural History Museum, London.

Morphological examination and morphometrical analyses

Specimens of *Synthesium* were identified following specific literature (Marchi, 1873; Poirier, 1886; Parona, 1896; Price, 1932; Raga & Balbuena, 1988; Raga et al., 1994; Fernández et al., 1994; Gibson, 2005; Marigo et al., 2008; Ebert et al., 2017; Shiozaki et al., 2019).

We examined 49 specimens for morphological and morphometrical analyses using light microscopy. The specimens were stained with chloridric carmine, dehydrated through a graded ethanol series, cleared with eugenol, and mounted as permanent preparations. A total of 27 diagnostic morphometric

measurements was taken based on previous studies (Fernández et al., 1995), in a computerized system for image analysis (Qwin Lite 3.1, Leica Microsystems, Wetzlar, Germany).

Molecular analyses

A total of 23 specimens of *Synthesium* was selected for molecular analyses, i.e., three from *T. truncatus* from the Mediterranean coast of Spain, 19 from *S. guianensis,* and one from an unidentified Delphinidae, collected from the Brazilian coast (Table 1).

To provide context for molecular analyses, we have also selected other brachycladiids, which were acquired during ongoing surveys carried out by our team to obtain sequences. These include *Brachycladium atlanticum* (Abril, Balbuena & Raga, 1991) Gibson, 2005 from *Stenella coeruleoalba* (Meyen, 1833) from Mediterranean sea, Spanish coast; *Campula oblonga* Cobbold, 1858 from *Phocoena phocoena* (Linnaeus, 1758) from the North sea; *Nasitrema delphini* Neiland, Rice & Holden, 1970 from *T. truncatus* collected off the Canary Island; *Nasitrema attenuatum* Neiland, Rice & Holden, 1970 from *Sotalia guianensis* collected off southern São Paulo coast, Brazil; *S. neotropicale* from *T. truncatus* collected off Santa Catarina coast, Brazil; *Nasitrema* sp. Ozaki, 1935 from *Delphinus delphis* Linnaeus, 1758 collected off the Canary Island; *S. delamurei* from *Globicephala melas* Traill, 1809 from the North Sea; and *Oschmarinella rochebruni* (Poirier, 1886) Gibson & Bray, 1997) from *S. coeruleoalba* from the Mediterranean sea, Spanish coast (Table 1).

Table 1. Parasite species, cetacean hosts, locality of collection, and their GenBank accession numbers for the ITS1 rDNA, ND3 mtDNA, and COI mtDNA sequences used in this study. New sequences are highlighted in bold.

*Locality: US California, USA; WM Western Mediterranean, Spain; NS North Sea, United Kingdom; UK United Kingdom; BS Bohai Sea, China; CI Off Canary Islands; CC Off Chilean Coast; GR Qaanaaq, Greenland; FR Scandola, Corsica, France; CRJ Central Rio de Janeiro state coast, Brazil; SRJ Southern Rio de Janeiro state coast, Brazil; NSP Northern São Paulo state coast, Brazil; SSP Southern São Paulo state coast, Brazil; NPR Northern Paraná state coast, Brazil; NSC Northern Santa Catarina state coast, Brazil; SSC Southern Santa Catarina state coast, Brazil; SBR Southern/South Brazilian Coast; SLE St. Lawrence estuary, Canada; ISJ Inland Sea of Japan; OB Omura Bay, Japan; HOK Samani coast, Hokkaido, Japan; ARG Argentinean coast.

**Sequences attributed as *Synthesium tursionis* by Ebert et al. (2017)

*** Sequence attributed as *Synthesium tursionis* by Fernández et al. (1998)

Parasite Species	**Delphinid Host**	**Locality***	**GenBank Accession numbers**			**Reference**
			ITS1 rDNA	**ND3 mtDNA**	**COI mtDNA**	
Brachycladium atlanticum	*Stenella coeruleoalba*	WM	**MN245241**			**This study**
	S. coeruleoalba	WM		AF034551		Fernández et al., 1998
	S. coeruleoalba	WM		KT180217		Fraija-Fernández et al., 2015b
Brachycladium goliath	*Balaenoptera acutorostrata*	NS	KR703279	KR703278	KR703278	Briscoe et al., 2016
Brachycladium sp.	*Stenella coeruleoalba*	WM		AF123439		Fernández et al., 2000
Campula oblonga	*Phocoena phocoena*	UK				Fraija-Fernández et al., 2015b
	Phocoena phocoena	NS	**MN245242**			**This study**
	P. phocoena	NS		AF034554		Fernández et al., 1998
	P. phocoena	NS		KT180214		Fraija-Fernández et al., 2015b
Nasitrema attenuatum	*Sotalia guianensis*	SSP	**MN245243**	**MN264280**	**MN273251**	**This study**
Nasitrema delphini	*Tursiops truncatus*	CI	**MN245244**			**This study**
	Delphinus delphis	CI		KT180216		Fraija-Fernández et al., 2015b
Nasitrema globicephalae	*Globicephala melas*	CC		AF034557		Fernández et al., 1998

Nasitrema sp.	*Delphinus delphis*	CI	**MN245245**			**This study**
Orthosplanchnus arcticus	*Pusa hispida*	GR		MG763083-5		Andersen-Ranberg et al., 2018
Orthosplanchnus fraterculus	*Enhydra lutris*	WM		AF034555		Fernández et al., 1998
Oschmarinella macrorchis	*Mesoplodon carlhubbsi*	HOK		LC326064		Nakagun et al., 2018
Oschmarinella rochebruni	*Stenella coeruleoalba*	WM	**MN245246**			**This study**
	S. coeruleoalba	WM		AF034556		Fernández et al., 1998
	S. coeruleoalba	WM		KT180215		Fraija-Fernández et al., 2015b
Synthesium sp. *1* n. sp.	*Sotalia guianensis*	SSP	**MN245251**	**MN264295-303**	**MN273268-74**	**This study**
	S. guianensis	NPR		**MN264304-5**		**This study**
	S. guianensis	CRJ		**MN264306**	**MN273275**	**This study**
	S. guianensis	NSC		**MN264307**	**MN273276-7**	**This study**
	S. guianensis	NSP		**MN264308**		**This study**
	S. guianensis	SRJ			**MN273278-9**	**This study**
	Delphinidae	NSC		**MN264309**	**MN273280**	**This study**
	Sotalia guianensis	SSP	KY595990**		KY612258**	Ebert et al., 2017
Synthesium delamurei	*Globicephala melas*	WM	**MN245247**	**MN264281**	**MN273252-3**	**This study**
	G. melas	WM		KY612255		Ebert et al., 2017
Synthesium longissimum	*Tursiops truncatus*	WM	**MN245252**	**MN264310-1**	**MN273281-2**	**This study**
	T. truncatus	WM		AF034552***		Fernández et al., 1998
Synthesium neotropicale	*Tursiops truncatus*	NSC	**MN245248**	**MN264288-90**	**MN273254-9**	**This study**
	T. truncatus	SSC		**MN264284-7**	**MN273260-3**	**This study**
	T. truncatus	CRJ		**MN264282**		**This study**
	T. truncatus	SSP		**MN264283**	**MN273264**	**This study**
	T. truncatus	NSC	KY595988	KY612256	KY612257	Ebert et al., 2017

Synthesium pontoporiae	*P. blainvillei*	SSP	JX644084			Marigo et al., 2011
	P. blainvillei	SBR		JX644086, 89, 90-1, 98, 100-1, 6-7, 10, 13, 16; FJ829472	JX644123-8; JX644130-51	Marigo et al., 2015
	P. blainvillei	ARG		JX644118, 21-1	JX644152-6	Marigo et al., 2015
Synthesium tursionis	*Neophocaena asiaeorientalis*	ISJ		MH634347-9		Shiozaki et al., 2019
	Tursiops truncatus	WM		KT180218		Fraija-Fernández et al., 2015b
	T. truncatus	WM	**MN245253**		**OK181933**	**This study**
Tormopsolus orientalis	*Seriola dumerli*	FR				Bray et al., 2005
	S. dumerli	WM	**MN273310**			**This study**
	S. dumerili	WM		KT180219		Fraija-Fernández et al., 2015b

Genomic DNA from the worms was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's recommended protocol and adjusted to a final volume of 30 µl. DNA fragments of the ITS1 rDNA, ND3 mtDNA, and COI mtDNA genes were amplified with the primers and cycling conditions presented in Table 2. PCR amplifications were performed on a final volume of 25 µl using Ready-to-Go PCR beads (GE Healthcare), with 3.0 to 5.0 µl of extracted DNA and 1.0 µl of each PCR primer. 2.0 µl of PCR product were run on an agarose gel (1%) using GelRed™ fluorescent nucleic acid dye and loading buffer to confirm amplicon size and yield. PCR amplicons were purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. Automated sequencing was performed directly on purified PCR products using BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction kit onto an ABI 3500 DNA genetic sequencer (Applied Biosystems). Sequences were assembled and edited with Sequencher v.5.2.4 (Gene Codes, Ann Arbor, MI, USA), subjected to BLAST analysis to confirm identity, and submitted to GenBank.

Table 2. Primers, cycling conditions used during PCR, and respective references.

Gene/Region	Primer Sequences 5'-3'	Cycling conditions	References
ITS1 rDNA	**ITS1F** GACGACCAAACTTGATCATT **ITS1R** TGCGCTCTTCATCGACACACGA	Initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min and 30 s; Final extension at 72°C for 7 min	Marigo et al., 2015
ND3 mtDNA	**ND3F** GCTTAATTKKTAAAGCYTTGRATTCTTACT **ND3D** CTACTAGTCCCACTCAACRTAACCYT	Initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30s, 47°C for 30s and 72°C for 50s; Final extension at 72°C for 7 min	Fernández et al., 1998a; Fernández et al., 2000
COI mtDNA	**COIPRA** TGGTTTTTTGTGCATCCTGAGGTTTA **COIPRB** AGAAGAACGTAATGAAAATGAGCAAC	Initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 47°C for 30 s and 72°C for 1 min; Final extension at 72°C for 7 min	Bessho et al., 1992

All newly generated sequences of the ITS1 rDNA, ND3 mtDNA, and COI mtDNA genes were aligned separately with other published sequences of the Brachycladiidae downloaded from GenBank (Table 1). The alignments were performed using the MUSCLE algorithm implemented on Geneious 7.1.3 (Kearse et al., 2012), with default settings. *Tormopsolus orientalis* Yamaguti, 1934 (Acanthocolpidae Lühe, 1906) from *Seriola dumerlli* (Risso, 1810), collected at the Mediterranean coast of Spain, was included as outgroup for the ITS1 rDNA and ND3 mtDNA alignments. The presence of

stop codons and indels for the ND3 mtDNA and COI mtDNA alignments were verified with the amino acid translation using the trematode mitochondrial code table (Frame 1 for both datasets) on Geneious 7.1.3 (Kearse et al., 2012).

GTR+I+G was selected as the best-fitting model for nucleotide substitution for the ITS1 rDNA and COI mtDNA datasets and HKY+I+G for the ND3 mtDNA dataset, as suggested by the Akaike information criterion in the jModelTest software (Posada, 2008). Phylogenies were reconstructed for each alignment under Bayesian Inference (BI) on MrBayes v. 3.2 and Maximum-likelihood (ML) on RAxML v. 8 (Stamatakis, 2014), both implemented on the CIPRES web portal (Miller et al., 2015). The BI was run using four Markov chain Monte Carlo (MCMC) searches with 10,000,000 generations and sampling tree topologies every 100 generations. The first 25% of generations were discarded as burn-in and the consensus trees were estimated using the remaining topologies; only nodes with posterior probabilities (pp) greater than 90% were considered well supported. The ML analyses were estimated using random starting trees with the best-fitting model selected for each dataset and 1,000 bootstrap replicates to investigate the support of each node in the most likely topologies; only nodes with bootstrap values (bv) greater than 70% were considered well supported. Resultant trees were visualized and edited using FigTree v.1.3.1 (Rambaut, 2009) and CorelDraw X6.

For species delimitation, we applied the method of General Mixed Yule Coalescent (GMYC). The GMYC method delimits independently evolving species using single-locus data (Pons et al., 2006), which considers that the mutations arising in one species cannot readily spread into another (De Queiroz, 2007). The method relies on the prediction that independent evolution leads to the appearance of distinct genetic clusters, separated by longer internal branches (Fujisawa & Barraclough, 2013). For conducting the analyses, a Bayesian ultrametric tree for the ND3 mtDNA and COI mtDNA genes was estimated separately employing an uncorrelated lognormal relaxed clock and birth-death speciation process on an arbitrary timescale implemented on BEAST v.1.6.2 (Drummond et al., 2012). The Bayesian topologies reconstructions started with UPGMA trees and the MCMC were run for 10^7 generations, sampling one tree every 100 generations. The first 1,000,000 trees were discarded as burn-in. The generated ultrametric trees were used as an input for the GMYC analysis with a single threshold method implemented online at http://species.h-its.org/gmyc/.

Results

Synthesium tursionis comb. n. (Marchi, 1873) Ebert, Fernández & Silva (Figs.1-2)

Taxonomic summary:
Definitive host: *Tursiops truncatus* Montagu, 1821, Bottlenose dolphin.
Site: Small intestine.
Type locality: Guardamar del Segura, Alicante, Spain, Mediterranean Sea.
Type specimens: Coleção Helmintológica do Instituto Oswaldo Cruz - Fundação Oswaldo Cruz (CHIOC-Fiocruz) Rio de Janeiro, RJ, Brazil under number XXXX (numbers will be available after acceptance of the manuscript).
Other representative specimens: Colección Nacional de Helmintos (CNHE), Mexico, under number 240-10 (Lamothe-Argumedo, 1988); Coleção Helmintológica do Instituto de Biociências de Botucatu - UNESP (CHIB-UNESP), Brazil, under number XXXX (numbers will be available after acceptance of the manuscript).
Representative DNA sequences: ITS1 rDNA - MN245253 (This study); ND3 mtDNA gene - KT180218 (Fraija-Fernández et al., 2015b), MH634347-8 (Shiozaki et al., 2019); COI mtDNA gene - OK181933 (this study).
Representative descriptions in literature: Lamothe-Argumedo, 1988
Synonyms: *Distoma tursionis* Marchi, 1873; *Hadwenius tursionis* (Marchi, 1873).

Observations and measurements based on two whole-mounted adult specimens collected from a *T. truncatus* from the Mediterranean, and unstained one photographed specimen collected from a *T. truncatus* from the Mediterranean, which originated the DNA sequences MN245253 (ITS1 rDNA) and OK181933 (COI mtDNA); and two photographed whole-mounted adult specimens collected from *P. sinus* from the Gulf of California, Mexico, which were analyzed and deposited in the CNHE under number 240-10 by Lamothe-Argumedo (1988). Measurements are shown as the range followed by the mean in parentheses and are expressed in millimeters unless otherwise stated.

Body elongated, slender, dorsoventrally flattened, 7.26 - 18.65 (11.96) long 0.92 - 1.04 (0.98) wide, covered with small spines at body surface, at least at anterior portion of

body. Oral sucker muscular, round, subterminal, with a longitudinal aperture in middle section, 0.33 - 0.59 (0.43) long 0.31 - 0.49 (0.38) wide. Prepharynx 0.20 - 0.85 (0.48) long 0.08 - 0.16 (0.11) wide. Pharynx pyriform, strongly muscular, 0.21 - 0.52 (0.35) long 0.17 - 0.26 (0.20) wide. Oesophagus short or almost indistinguishable. Intestinal caeca H-shaped, with two anterior branches beginning almost immediately after posterior margin of pharynx, reaching medial level of oral sucker; posterior branches ending blindly close to posterior extremity of body. Ventral sucker muscular, round, larger than oral sucker, 0.57 - 0.64 (0.59) long 0.55 - 0.60 (0.58) wide. Distance between oral and ventral suckers 1.07 - 4.03 (2.34). Cirrus pouch extends beyond ventral sucker posterior margin, 0.35 - 0.45 (0.39) long, containing a seminal vesicle at its extremity, 0.12 - 0.16 (0.13) long 0.04 - 0.07 (0.06) wide, pars prostatica with numerous glands, cirrus without spines. Cirrus pouch opens into a genital pore, immediately anterior to the ventral sucker. Ovary round to oval, 0.26 - 0.34 (0.29) long 0.18 - 0.25 (0.21) wide, postacetabular, pretesticular. Mehlis' gland large, preovarian. Vitelline reservoir ovoid, in front or close to the anterior margin of ovary. Laurer´s canal opening dorsally at ovary's anterior region. Seminal receptacle absent. Distance between ovary and anterior testis 0.08 - 0.89 (0.43). Testes lobed, presenting five lobes in anterior testis and six lobes in posterior testis, in mature specimens. Anterior testis 0.42 - 0.87 (0.58) long and 0.52 - 0.74 (0.59) wide, slightly smaller than posterior testis 0.54 - 0.99 (0.73) long x 0.43 - 0.61 (0.55) wide. Distance between testes 0.24 - 0.68 (0.45). Distance from posterior testis to posterior extremity of body 2.33 - 6.04 (3.82). Vitellaria arranged in acinous bunches, with anterior margin beginning close to posterior extremity of seminal vesicle, extending to posterior extremity of body. Distance between anterior margin of vitellaria and ventral sucker 0.72 - 1.07 (0.88). Uterine coils intercaecal, widening into an unarmed and muscular metraterm before opening into genital pore. Eggs oval, slightly flattened at opercular pole, triangular in cross-section, 50-57 (55) x 30-36 (33) µm.

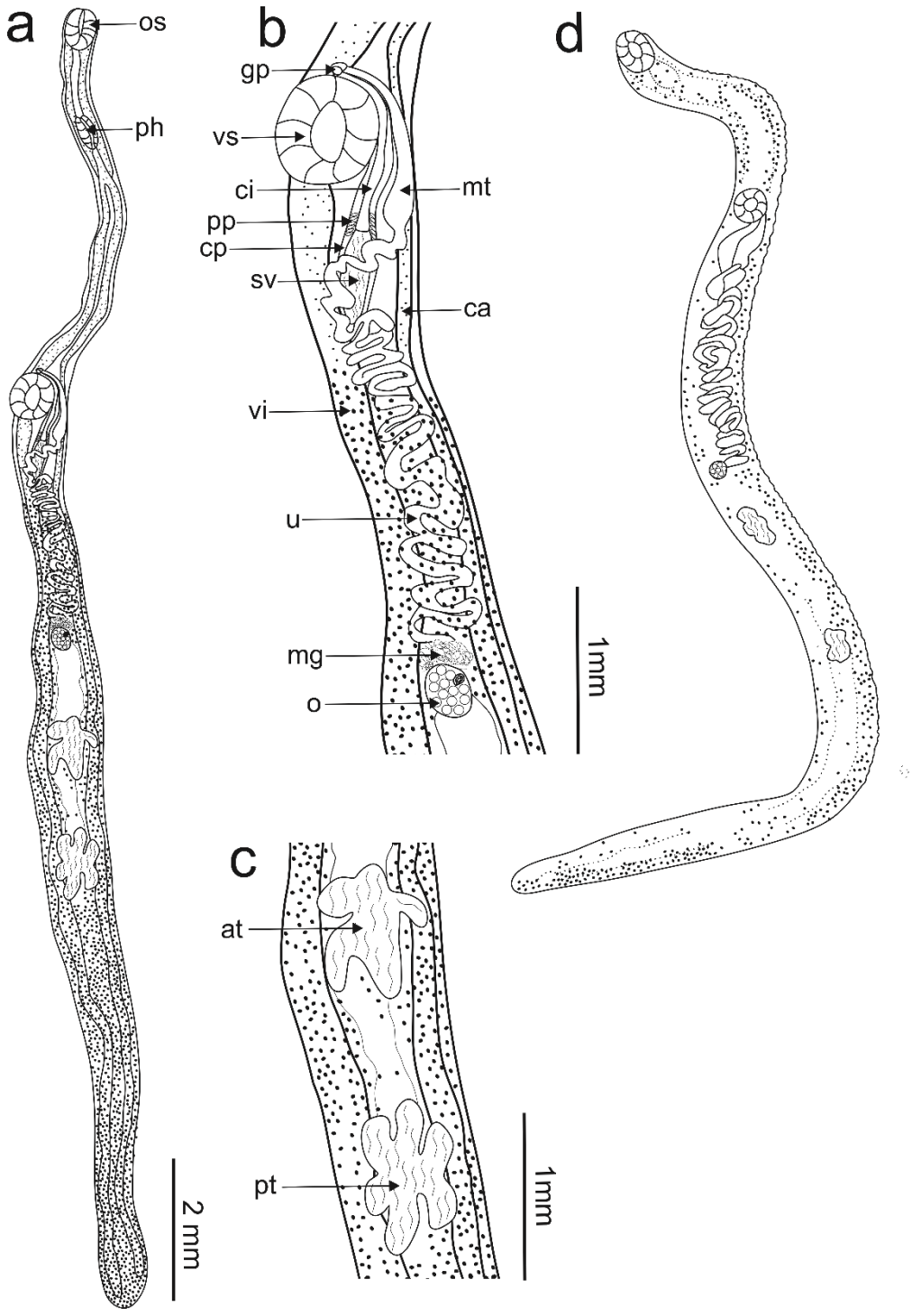


Fig 1. *Synthesium tursionis* comb. n. (Marchi, 1873) Ebert, Fernández & Silva. a) Whole worm, collected from *Tursiops truncatus* from Spain, Mediterranean Sea; b) Detail of medial region of the worm, showing ventral sucker, cirrus pouch without spiny cirrus, seminal vesicle, uterus, and ovary; c) Posterior part of worm showing anterior and posterior lobed testes; d) Whole worm, redrawn and adapted after the original provided by Marchi (1873), without a scale (not provided by the original author). at = anterior testis; ca = caeca; ci = cirrus; cp = cirrus pouch; gp = genital pore; mg = Mehlis' gland; mt = metraterm; o = ovary; os = oral sucker; ph = pharynx; pt = posterior testis; sv = seminal vesicle; u = uterus; vi = vitellaria; vs = ventral sucker.

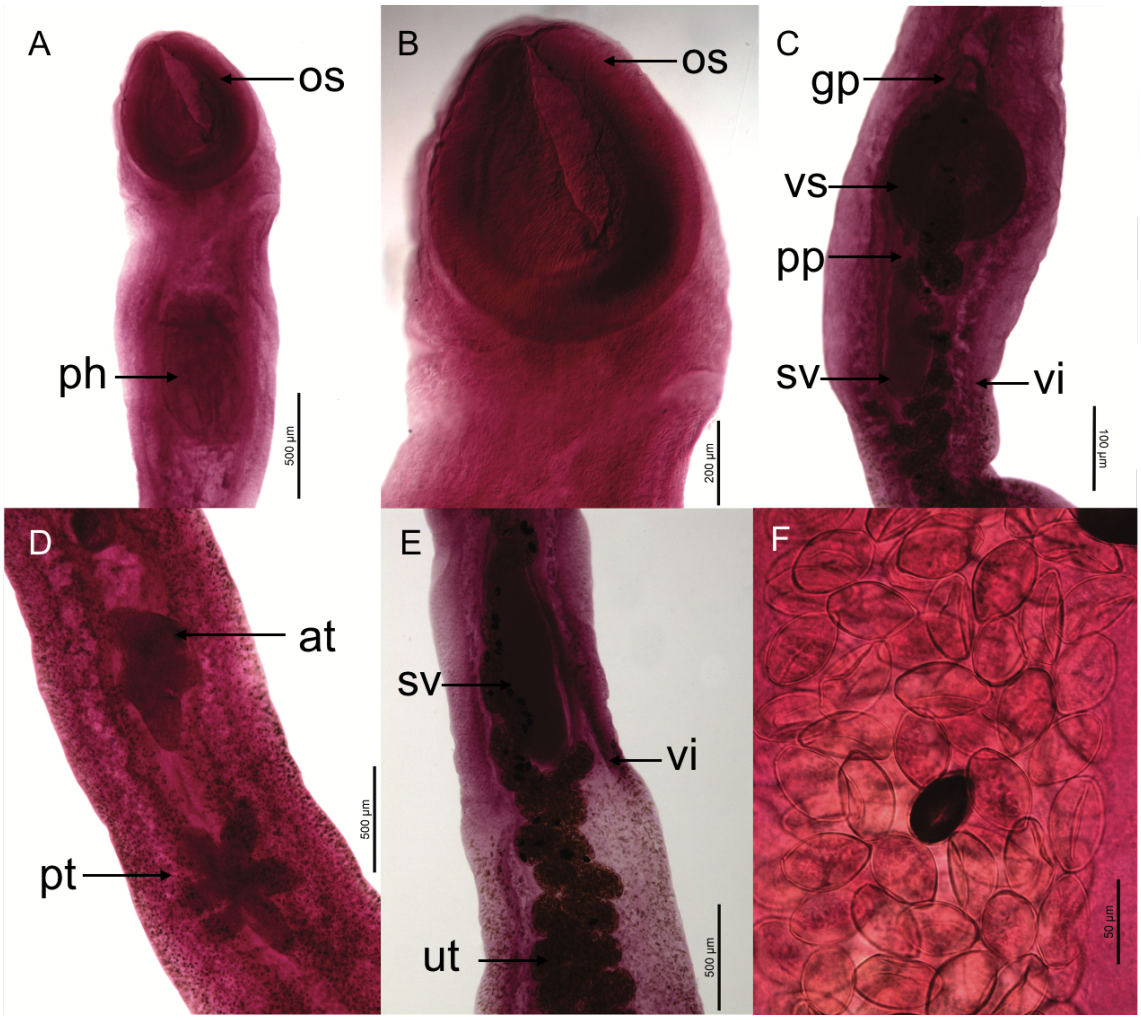


Fig 2. *Synthesium tursionis* comb. n. (Marchi, 1873) Ebert, Fernández & Silva, collected from *Tursiops truncatus* from Spain, Mediterranean Sea; A) Anterior portion of the worm, showing the round-shaped oral sucker and pharynx. B) Detail of the anterior portion of worm showing muscular round-shaped oral sucker with an aperture in the middle section. C) Medial portion of the worm, showing the genital pore located immediately anterior to the ventral sucker, cirrus pouch, and anterior margin of vitellaria at the region of the posterior extremity of the seminal vesicle. D) Detail of anterior and posterior testes. E) Detail of seminal vesicle and anterior margin of vitellaria at the region of the posterior extremity of the seminal vesicle. F) Eggs in metraterm. at = anterior testis; gp = genital pore; os = oral sucker; ph = pharynx; pp = pars prostatica; pt = posterior testis; sv = seminal vesicle; ut = uterus; vi = vitellaria; vs = ventral sucker.

Remarks

Synthesium tursionis was first described by Marchi (1873) as *Distomum tursionis* from the intestine of a *Delphinus tursio* (probably *T. truncatus*). In the original description, which was rather incomplete and accompanied by a very sketchy illustration (Fig. 1d), the author described his specimens as “body with 20.00 mm long

and 1.00 mm wide, covered with small spines; oral and ventral sucker “globuliform”, being the oral sucker slightly smaller than the ventral sucker (0.75 mm x 0.80 mm in diameter, respectively), separated one from the other by 4 mm”. By observing Marchi’s illustration combined with the description of the species, we assume that the oral sucker of his specimens was probably round-shaped with the mouth opening subterminally, as he meant by “globuliform”; the shape of the testes was most round with irregular margin, but not nearly completely lobed; and the posterior testis was positioned at a relatively long distance from the posterior extremity of the body.

Some years later, Poirier (1886) described *Distomum longissimum* also from the intestine of a *Delphinus tursio* (probably *T. truncatus*). Poirier (1886) affirmed that *D. longissimum* was distinguished from *D. tursionis* described by Marchi, by “its body surface lacking spines, by the enlarged shape of its oral sucker, by its lobed testicles and by the shape of its eggs clearly acuminated at the posterior extremity”. A while later, Parona (1896) after analyzing three of the *D. tursionis* specimens from Marchi’s original material and comparing it with the description and illustration of *D. longissimum* provided by Poirier (1886), decided that “the two species described by Marchi (1873) and Poirier (1886) should be considered as the same, which the name, considering the priority rule of the INZC, should be *D. tursionis*”. Parona (1896) stated that; the enlarged shape of the oral sucker in *D. longissimum* - described by Poirier (1886) - was a consequence of “slight compression of the coverslip, which influenced on the shape of the oral sucker of *D. longissimum* shown in the figure drawn by Poirier (1886), which was enlarged at its edges, appearing to be larger than the indicated by Marchi (1873)”; the spines in the body surface of Marchi’s specimens were “rare, very small, and limited to the anterior half of the body”, meaning that the lack of spines in Poirier’s specimens should be certainly missed by that author; and the eggs of *D. tursionis* were “identical to the ones described by Poirier (e.g. operculated, acuminated at the posterior pole and presenting the same dimensions). Parona (1896) also pointed out that the testes of the specimens he examined were “evidently lobed, and quite similar to the ones of *D. longissimum*, and that they even possessed the same number of lobes (e.g., anterior testis with five lobes and posterior testis with six lobes), precisely as affirmed by Poirier”. However, Parona (1896) claimed that the smallest specimen analyzed by him, which he considered to be an immature specimen, had “the anterior testis with only three lobes and the posterior one with four lobes”; therefore the species could present from mostly round testes in immature specimens to lobed testes in

mature specimens. Parona (1896) also mentioned that *D. longissimum* from Poirier (1886) had the "body wider than *S. tursionis* from Marchi (1873)" (1.5 mm x 1.0 mm, respectively), but considered this difference as insignificant. After the synonymization of *D. tursionis* and *D. longissimum* by Parona (1896), the species went through several taxonomic rearrangements.

Most redescriptions of the species were made largely after Poirier's description and illustration, which was reproduced by many authors (Price, 1932; Delyamure, 1955; Delamure and Serdyukov, 1966; Dawes, 1968; Skrjabin, 1976). As the species was progressively being reported worldwide, many authors presented new morphological observations for it. Price (1932) gave his contribution by describing the species with a spiny cirrus ("cirrus as in *Orthosplanchnus*, according to Odhner"). Delamure and Serdyukov (1966) reported *S. tursionis* with H-shaped caeca. After that, Raga et al. (1985) and Haffezulah (1986) recorded *S. tursionis* in *T. truncatus* from the Mediterranean Sea and in *Neophocaena phocaenoides* from the Arabian sea, respectively, reporting the species, between other observations, as presenting: oral sucker funnel-shaped, terminal, well developed and equal in size as the ventral sucker; and both testes deeply lobed with five lobes on the anterior testis and six lobes on the posterior testis, even in immature specimens. Raga et al. (1985) and Haffezulah (1986) also presented new illustrations that were similar to the one provided by Poirier (1886) from his *D. longissimum*. Posteriorly, Tantalean and Escalante (1987) and Lamothe-Argumedo (1988) described *S. tursionis* from a *Delphinus delphis* off the Peruvian Coast and from a *Phocoena sinus* off the Gulf of California, in Mexico, respectively. Those authors indicated that their specimens presented: oral sucker round, subterminal, with the aperture of the mouth at the middle of the sucker, which was smaller than the ventral sucker; testicles deeply lobed, possessing five lobes on the anterior testis and six lobes on the posterior testes, however, besides being lobed, those were smaller than the ones described by Raga et al. (1985) and Haffezulah (1986), if comparing the authors provided measurements and illustrations. The descriptions given by Tantalean and Escalante (1987) and Lamothe-Argumedo (1988) of *S. tursionis* were likely to be referring to specimens morphologically most similar to the ones described by Marchi (1873) of his *S. tursionis* although, in Tantalean and Escalante (1987) specimens, the authors identified the cirrus covered with spines. Fernández et al. (1994) also described *S. tursionis* collected from *T. truncatus* off the Mediterranean coast of Spain as possessing a terminal oral sucker and lobed testes

and noticed a large variability in the numbers of lobes of the testes, which was later attributed to intraspecific variability (Fernández et al., 1995). For last, Marigo et al. (2008) intended to redescribe *S. tursionis* from a *T. truncatus* off the Brazilian coast. However, when closely comparing her description and illustration, it is most likely that the authors mistakenly described specimens of *S. neotropicale* (which was erected years later by Ebert et al., 2017) than a specimen of *S. tursionis*.

Therefore, based on the miscellaneous descriptions of *S. tursionis* found in literature until now and our morphological observations, it seems evident that Parona (1896) must erroneously synonymize two *Synthesium* species that are clearly different, *S. tursionis* from Marchi (1873) and *S. longissimum* from Poirier (1886). As a consequence, the following authors dealing with *S. tursionis* tended to mislead their morphological interpretations of the species, delivering combined descriptions of different species that were considered the same until now.

We tried to locate the original *S. tursionis* holotype deposited by Marchi (1873), as well as the *S. longissimum* holotype deposited by Poirier (1886), in several European and American institutions; however, we could not obtain any answer or the material could not be located. Therefore, sadly, we consider that these exemplars are lost to science.

Marchi (1873) did not mention the type-locality of his specimens, but considering his original manuscript and affiliation (Museum of Physics and Natural History in Florence, Italy), we assume that the Mediterranean Sea is the type locality of *S. tursionis* and *T. truncatus* is the type-host. Thereby, here we present the redescription of *S. tursionis,* amending Marchi's original description, of specimens collected from *T. truncatus* off the Mediterranean coast of Spain and specimens collected from *P. sinus* from the Gulf of California, Mexico, provided by Lamothe-Argumedo (1988).

Synthesium longissimum **comb. n. (Poirier, 1886) Ebert, Fernández & Silva (Figs. 3-4)**

Taxonomic summary:

Definitive host: *Tursiops truncatus* Montagu, 1821, Bottlenose dolphin.

Site: Small intestine.

Type locality: Guardamar del Segura, Alicante, Spain, Mediterranean Sea.

Type specimens: Coleção Helmintológica do Instituto Oswaldo Cruz - Fundação Oswaldo Cruz (CHIOC-Fiocruz) Rio de Janeiro, RJ, Brazil under number XXXX (numbers will be available after acceptance of the manuscript).
Other representative specimens: Natural History Museum, London (catalog number 2745503); Coleção Helmintológica do Instituto de Biociências de Botucatu - UNESP (CHIB-UNESP), Botucatu, SP, Brazil under number XXXX (numbers will be available after acceptance of the manuscript).
Representative DNA sequences: ITS1 rDNA - KY595990 (Ebert et al., 2017), MN245252 (this study); ND3 mtDNA gene - AF034552 (Fernández et al., 1998), MN264310-1 (this study); COI mtDNA gene - KY612258 (Ebert et al., 2017), MN273281-2 (this study).
Representative descriptions in literature: Raga et al. (1985); Haffezulah (1986); Fernández et al. (1995).
Synonims: *Distomum tursionis* Marchi, 1873; *Distomum longissimum* Poirier (1886) nec *D. longissimum* von Linstow, 1896; *Distomum* (*Dicrocoelium*) *tursionis* (Marchi, 1873) Parona, 1896.

Observations and measurements based on 15 whole-mounted adult specimens collected from 4 different *T. truncatus* hosts from the Mediterranean coast of Spain. Measurements are shown as the range followed by the mean in parentheses and are expressed in millimeters, unless otherwise stated.

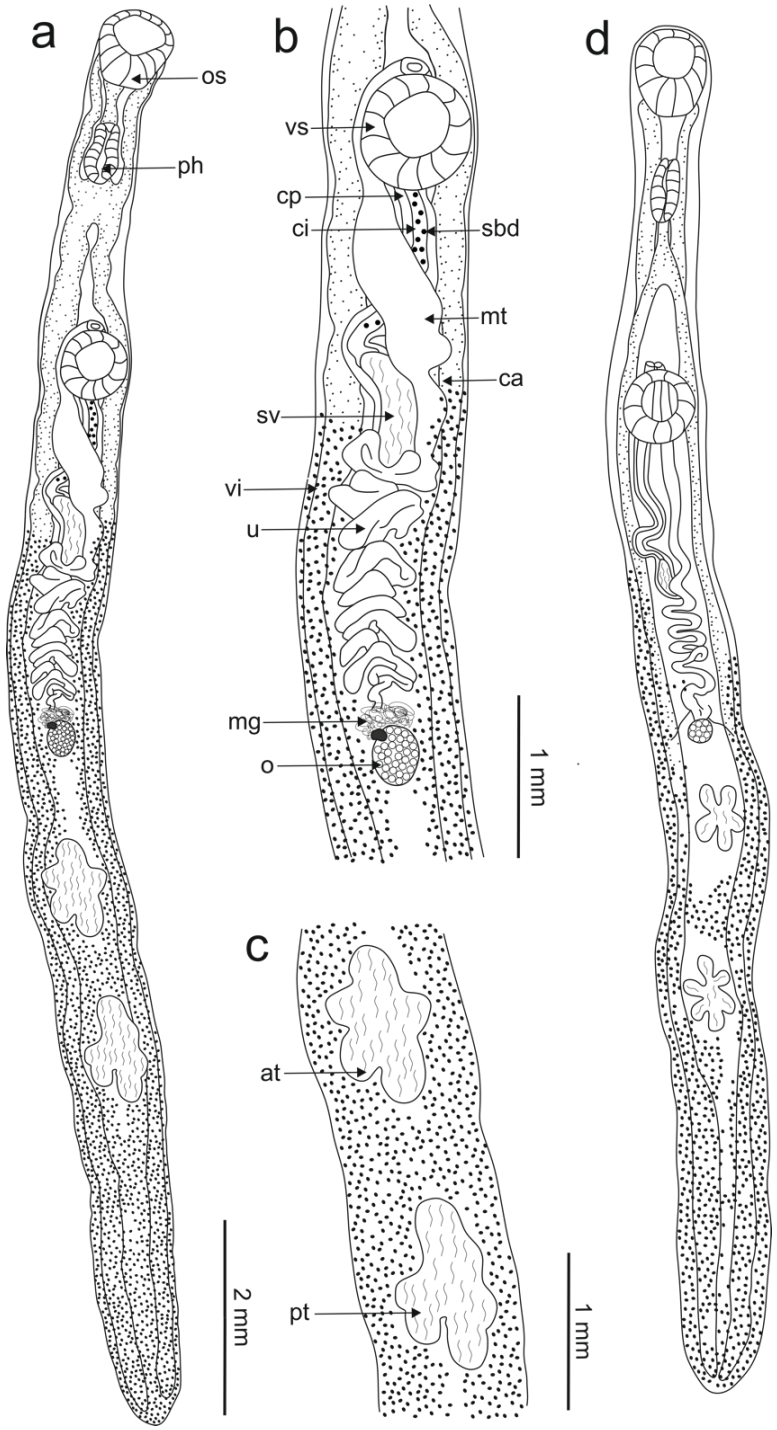


Fig 3. *Synthesium longissimum* comb. n. (Poirier, 1886) Ebert, Fernández & Silva. a) Adult entire worm collected from *Tursiops truncatus* from Mediterranean; b) Detail of medial region of the worm, showing ventral sucker, cirrus pouch containing spiny cirrus and seminal vesicle, uterus and ovary; c) Posterior part of worm showing anterior and posterior lobed testes; d) Whole worm, redrawn and adapted after the original provided by Poirier (1886), without a scale (not provided by the original author). at = anterior testis; ca = caeca; ci = cirrus; cp = cirrus pouch; gp = genital pore; mg = Mehlis' gland; mt = metraterm; o = ovary; os = oral sucker; ph = pharynx; pt = posterior testis; sbd = spine with basal disc; sv = seminal vesicle; u = uterus; vi = vitellaria; vs = ventral sucker.

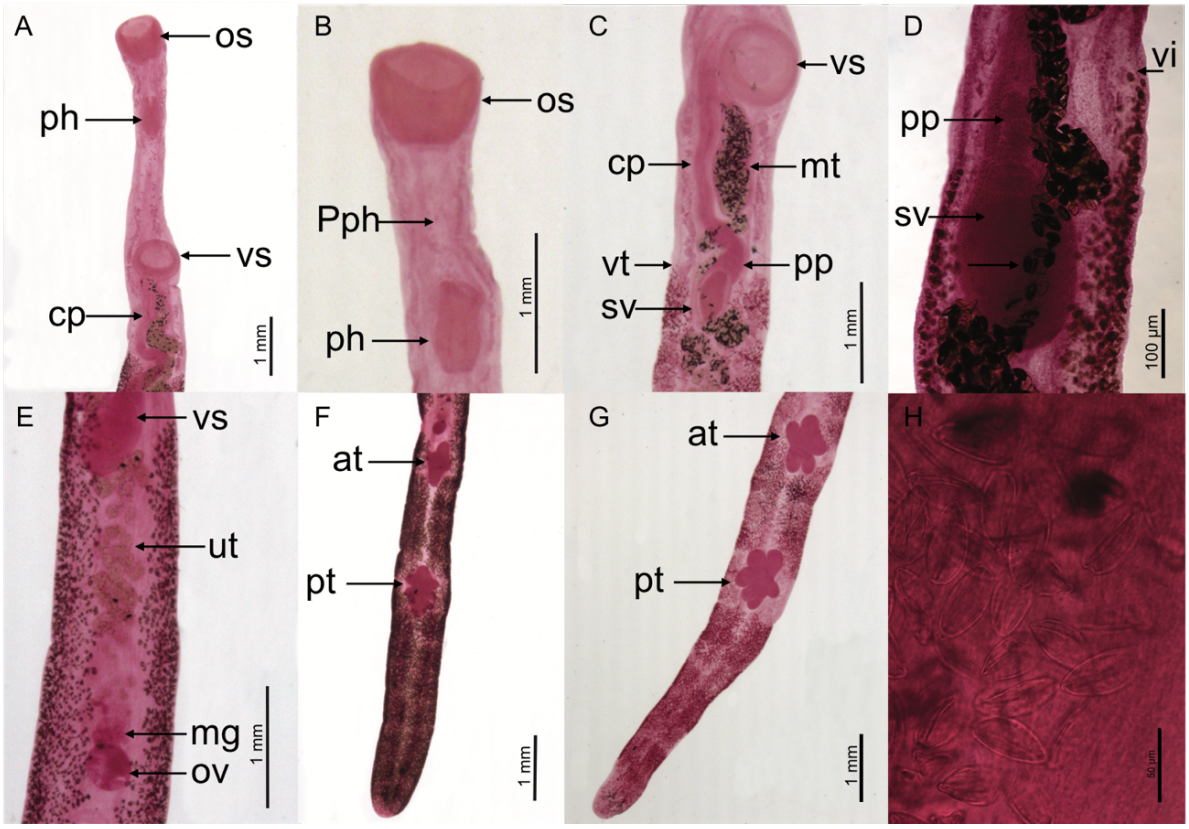


Fig 4. *Synthesium longissimum* comb. n. (Poirier, 1886) Ebert, Fernández & Silva collected from *Tursiops truncatus* from Mediterranean. A) Anterior extremity of the body showing terminal oral sucker, pharynx, ventral sucker, and cirrus pouch. B) Detail of terminal cup-shaped oral sucker, prepharynx, and pharynx. C) Detail of the medial portion of the worm, showing ventral sucker, cirrus pouch, metraterm, pars prostatica, anterior limit of vitellaria, and seminal vesicle. D) Detail of the medial portion of the worm, showing the region of pars prostatica, seminal vesicle, and the anterior limit of vitellaria. E) Detail of ovary and the preovarian Mehli's gland, uterus, and seminal vesicle. F and G) Posterior extremity of worm showing the anterior testis and posterior testis and the distance between posterior testis and posterior extremity of the worm. H) Eggs in metraterm. at: anterior testis; cp = cirrus pouch; mg = Mehlis' gland; mt = metraterm; ov = ovary; os = oral sucker; Pph = prepharynx; ph = pharynx; pt = posterior testis; ut = uterus; vt = vitellaria; vs = ventral sucker.

Body elongated, slender, dorsoventrally flattened, 11.70 - 22.02 (15.61) long, with maximum width at level of posterior testis, 0.73 - 1.32 (1.01) wide. Spines at body surface not observed. Oral sucker strongly muscular, cup-shaped, with large terminal aperture, 0.61 - 1.04 (0.74) long 0.57 - 0.99 (0.73) wide. Prepharynx variable in length, but mostly long, 0.21 - 0.96 (0.55) long 0.14 - 0.22 (0.16) wide. Pharynx pyriform, strongly muscular, 0.37 - 0.77 (0.58) long 0.19 - 0.41 (0.30) wide. Oesophagus very short or almost indistinguishable. Intestinal caeca H-shaped, with two anterior branches beginning almost immediately after posterior margin of pharynx and reaching

medial level of oral sucker; posterior branches ending blindly close to posterior extremity of body. Ventral sucker muscular, round, equal in size to oral sucker, 0.48 - 0.99 (0.73) long 0.49 - 0.94 (0.71) wide. Distance between oral and ventral suckers 1.58 - 4.89 (2.88). Cirrus pouch 2.15 - 4.89 (2.90) long, extending well beyond ventral sucker posterior margin, containing a large seminal vesicle located at its extremity, 0.60 - 0.79 (0.69) long and 0.26 - 0.44 (0.34), pars prostatica with numerous glands, cirrus covered with small spines attached to it by basal discs that can be easily seen. Cirrus pouch opens into a genital pore located immediately anterior to ventral sucker. Ovary round to slightly oval, 0.20 - 0.40 (0.31) long 0.15 - 0.39 (0.27) wide, postacetabular, pretesticular. Mehlis' gland large, preovarian. Vitelline reservoir ovoid, located in front or close to anterior margin of ovary. Laurer´s canal not observed. Seminal receptacle absent. Distance between ovary and anterior testis 0.45 - 1.25 (0.72). Testes lobed, with short and wide lobes in both testes. Number of lobes in each testis variable; 3-5 in anterior testis and 5-6 in posterior testis. Anterior testis 0.63 - 1.22 (0.95) long 0.43 - 0.97 (0.68) wide, slightly smaller than posterior testis 0.74 - 1.43 (1.00) x 0.48 - 0.90 (0.68). Distance between testes 0.50 - 1.75 (0.99). Distance from posterior testis to posterior extremity of body 2.77 - 5.11 (3.61). Vitellaria arranged in acinous bunches, with anterior margin beginning close to anterior extremity of seminal vesicle, extending until posterior extremity of body. Distance from anterior margin of vitellaria to ventral sucker 0.75 - 2.00 (1.27). Distance from anterior margin of vitellaria to posterior extremity of body 7.82 - 13.13 (9.78). Uterine coils intercaecal, widening into a large, unarmed and very muscular metraterm, 1.26 - 1.40 (1.33) long 0.35 - 0.48 (0.42) wide, before opening into genital pore. Eggs oval, slightly flattened at opercular pole, triangular in cross-section, 47 - 66 (55) x 28 - 41 (34) µm.

Remarks

Synthesium longissimum differs from *S. tursionis* by possessing a robust body, terminal cup-shaped oral sucker equal in size to the ventral sucker, longer cirrus pouch, larger testes with shorter and wider lobes which are variable (3-5 in the anterior testis and 5-6 in the posterior testis, following Fernández et al. (1995) and our observations), vitellaria initiating at the level of the glandular area of the pars prostatica or anterior extremity of the seminal vesicle, and a spiny cirrus

Synthesium sp. 1 n. sp. Ebert, Marigo & Silva
(Figs. 5-6)

Taxonomic summary:
Definitive host: *Sotalia guianensis* Van Benédén 1864, Guiana dolphin.
Site: Small intestine.
Type locality: Trapandé Bay, Cananéia district, southeastern São Paulo state, Brazil, South Atlantic Ocean (25°02'52.6"S, 47°55'28.2"W).
Additional localities: Babitonga Bay, São Francisco do Sul district, northeastern Santa Catarina state, Brazil, South Atlantic Ocean (26°11'37.3"S, 48°36'20.4"W); Paranaguá Bay, Guaraqueçaba district, northeastern Paraná state, Brazil, South Atlantic Ocean (25°27'10.0"S, 48°23'33.8"W); Praia Grande beach, Praia Grande district, northeastern São Paulo state, Brazil, South Atlantic Ocean (24°04'20.2"S, 46°32'01.4"W); Maranduba beach, Ubatuba district, northeastern São Paulo state, Brazil, South Atlantic Ocean (23°33'01.9"S, 45°11'47.7"W); Sepetiba bay, Sepetiba district, southeastern Rio de Janeiro state, Brazil, South Atlantic Ocean (23°00'10.2"S, 43°46'52.5"W).
Type specimens: Coleção Helmintológica do Instituto Oswaldo Cruz - Fundação Oswaldo Cruz (CHIOC-Fiocruz) Rio de Janeiro, RJ, Brazil under number XXXX (numbers will be available after acceptance of the manuscript).
Other representative specimens: Coleção Helmintológica do Instituto de Biociências de Botucatu - UNESP (CHIB-UNESP), Botucatu, SP, Brazil under number XXXX (numbers will be available after acceptance of the manuscript).
Representative DNA sequences: ITS1 rDNA - MN245251 (this study); ND3 mtDNA gene - MN264295-309 (this study); COI mtDNA gene - MN273268-80 (this study).

Observations and measurements based on 33 whole-mounted specimens collected from 15 different *S. guianensis* hosts from Brazil. Measurements are shown as the range followed by the mean in parentheses expressed in millimeters, unless otherwise stated.

Body elongated, slender, dorsoventrally flattened, 12.53 - 23.06 (17.02) long, with the maximum width at posterior testis level, 0.77 - 1.51 (1.22) wide. Body surface covered

with small spines, at least at the anterior portion of body. Oral sucker very muscular, cup-shaped, with terminal large aperture, 0.78 - 1.31 (1.04) long 0.57 - 1.04 (0.80) wide. Prepharynx variable in length, but mostly long, 0.60 - 2.20 (1.16) long 0.15 - 0.42 (0.25) wide. Pharynx pyriform, strongly muscular, 0.52 - 0.87 (0.70) long 0.27 - 0.46 (0.34) wide. Oesophagus very short or almost indistinguishable. Intestinal caeca H-shaped, with two anterior branches reaching medial level of oral sucker and two posterior branches ending blindly close to posterior extremity of body. Ventral sucker muscular, round, smaller than oral sucker, 0.71 - 1.00 (0.83) long 0.52 - 0.99 (0.78) wide, at posterior region of first third of body. Distance between oral and ventral sucker 1.20 - 6.20 (3.87). Cirrus pouch long 2.32 - 5.67 (3.76), extending well beyond ventral sucker posterior margin. Large seminal vesicle 0.43 - 1.20 (0.80) long 0.23 - 0.62 (0.38). Glandular pars prostatica imediatelly anterior from seminal vesicle. Cirrus covered with spines, 66 µm - 88 µm (75 µm) long. Spines in cirrus attached by small basal discs, 16 µm - 25 µm (19 µm) in diameter. Cirrus pouch opening into the genital pore, immediately anterior to ventral sucker. Ovary round to oval, 0.11 - 0.50 (0.37) long 0.10 - 0.46 (0.32) wide, postacetabular, pretesticular. Mehlis' gland preovarian. Vitelline reservoir ovoid, above or laterally close to ovary. Laurer´s canal not observed. Seminal receptacle absent. Distance between ovary and anterior testIs 0.74 - 2.80 (1.38). Testes deeply lobed, in tandem, close to posterior extremity of body. Anterior testis presenting five well defined, slender, elongated lobes, 0.78 - 1.56 (1.14) long 0.56 - 1.19 (0.84) wide. Posterior testis with six well defined, slender, elongated lobes, 0.93 - 1.76 (1.24) long 0.60 - 1.22 (0.90) wide. Anterior testis slightly smaller than posterior testis Distance between the testes 0.58 - 2.08 (1.23). Distance from posterior testis to posterior extremity of body 1.20 - 5.39 (2.37). Vitellaria arranged in acinous bunches, profuse, commencing at level of glandular area of pars prostatica or anterior extremity of seminal vesicle, extending until posterior extremity of body. Distance between anterior margin of vitellaria and ventral sucker 0.88 - 3.50 (1.34). Distance between anterior margin of vitellaria and posterior extremity of body 7.00 - 12.77 (9.42). Uterine coils intercecal, widening into an enlarged, muscular and unarmed metraterm, before opening into genital pore. Eggs oval, slightly flattened at opercular pole, triangular in cross-section, 34 - 60 (50) µm long and 21 - 33 (28) µm wide.

Remarks

The new species clearly differs from all of its congeners, except *S. longissimum,* mainly by the robust body, the cup-shaped terminal oral sucker, and the shape and position of the markedly lobed testes, always presenting five well defined, slender, and elongated lobes on the anterior testis and six well defined, slender and elongated lobes on the posterior testis, which are positioned close to the posterior extremity of the body, at a larger distance from the ovary (see measurements above). *Synthesium* sp. 1 n. sp. closely resemble *S. longissimum,* however, the two species differ in the size of the terminal cup-shaped oral sucker, which is more robust and longer in the new species. *Synthesium* sp. 1 n. sp. also presents the oral sucker bigger than the ventral sucker, while in *S. longissimum,* both suckers are equal in size. The testes lobes in the new species are more elongated, furrowed, and narrower than that of *S. longissimum*, and are located closer to the posterior extremity of the body.

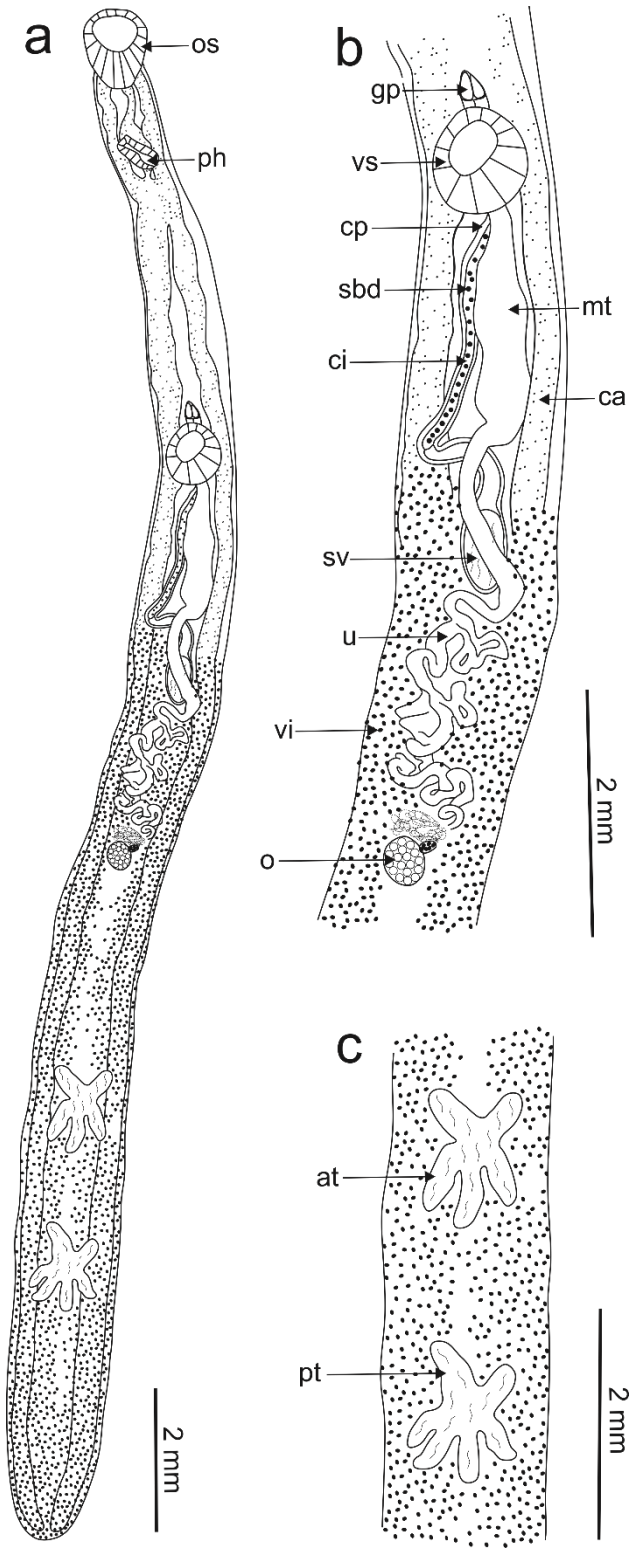


Fig 5. *Synthesium* sp.1 n. sp. Ebert, Marigo & Silva from *Sotalia guianensis* from Brazil; b) Detail of medial region of the worm, showing ventral sucker, cirrus pouch containing spiny cirrus and seminal vesicle, uterus and ovary; c) Posterior part of worm showing anterior and posterior lobed testes. at: anterior testis; ca = caeca; ci = cirrus; cp = cirrus pouch; gp = genital pore; mg = Mehlis' gland; mt = metraterm; o = ovary; os = oral sucker; ph = pharynx; pt = posterior testis; sbd = spine with basal disc; sv = seminal vesicle; u = uterus; vi = vitellaria; vs = ventral sucker.

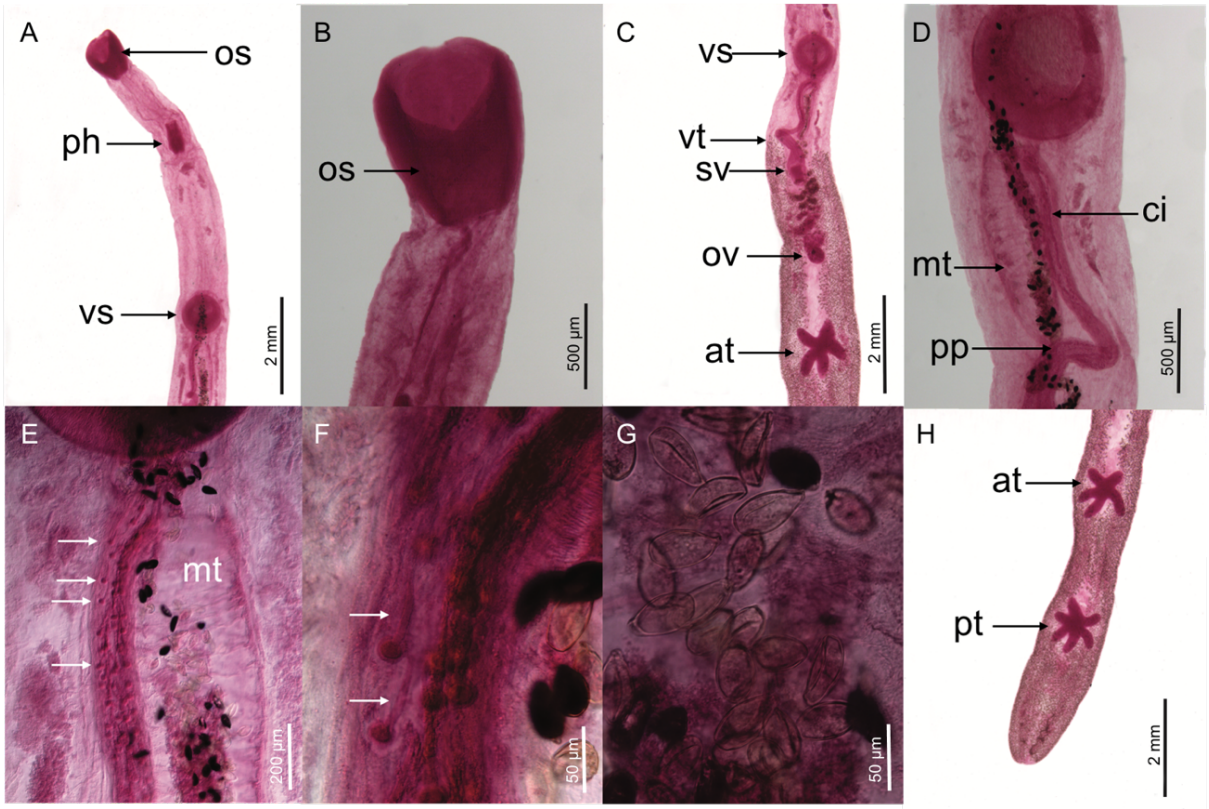


Fig 6. *Synthesium* sp.1 n. sp. Ebert, Marigo & Silva collected from *Sotalia guianensis* from Brazil; A) Anterior extremity of the body showing terminal oral sucker, pharynx, and ventral sucker; B) Detail of terminal cup-shaped oral sucker; C) Detail of the medial portion of the worm, showing ventral sucker, anterior limit of vitellaria, seminal vesicle, uterus, ovary, and the anterior testis; D) Detail of the medial portion of the worm, showing cirrus, region of pars prostatica glands and metraterm; E) Detail of cirrus pouch armed with spines (white arrows) and muscular metraterm; F) Detail of spines attached in cirrus by basal discs (white arrows); G) Eggs in metraterm; H) Posterior part of the worm showing anterior and posterior lobed testes, close to the extremity of the body. at: anterior testis; ci = cirrus; mt = metraterm; ov = ovary; os = oral sucker; ph = pharynx; pp = pars prostatica; pt = posterior testis; sv = seminal vesicle; vt = vitellaria; vs = ventral sucker.

Phylogenetic analyses

We generated a total of 69 new sequences of species of the Brachycladiidae (GenBank accession numbers in Table 1).

The ITS1 rDNA final alignment included 16 sequences and 547 positions. In the ITS rDNA BI and ML phylogenetic reconstruction (Fig. 7), all *Synthesium* spp. sequences clustered in a highly supported monophyletic clade (posterior probability (pp) = 1; bootstrap value (bv) = 100), except for *S. delamurei*, which was placed as sister to all the other Brachycladiidae sequences (pp = 1). The *Synthesium* clade was divided into two subclades, one composed by all sequences of *S. pontoporiae* with *S.*

neotropicale as its sister taxon (pp = 0.77; bv = 87), and another composed by *S. tursionis* as a sister taxon of *S. longissimum* and *Synthesium* sp. 1 n. sp. (pp = 0.68; bv = 93).

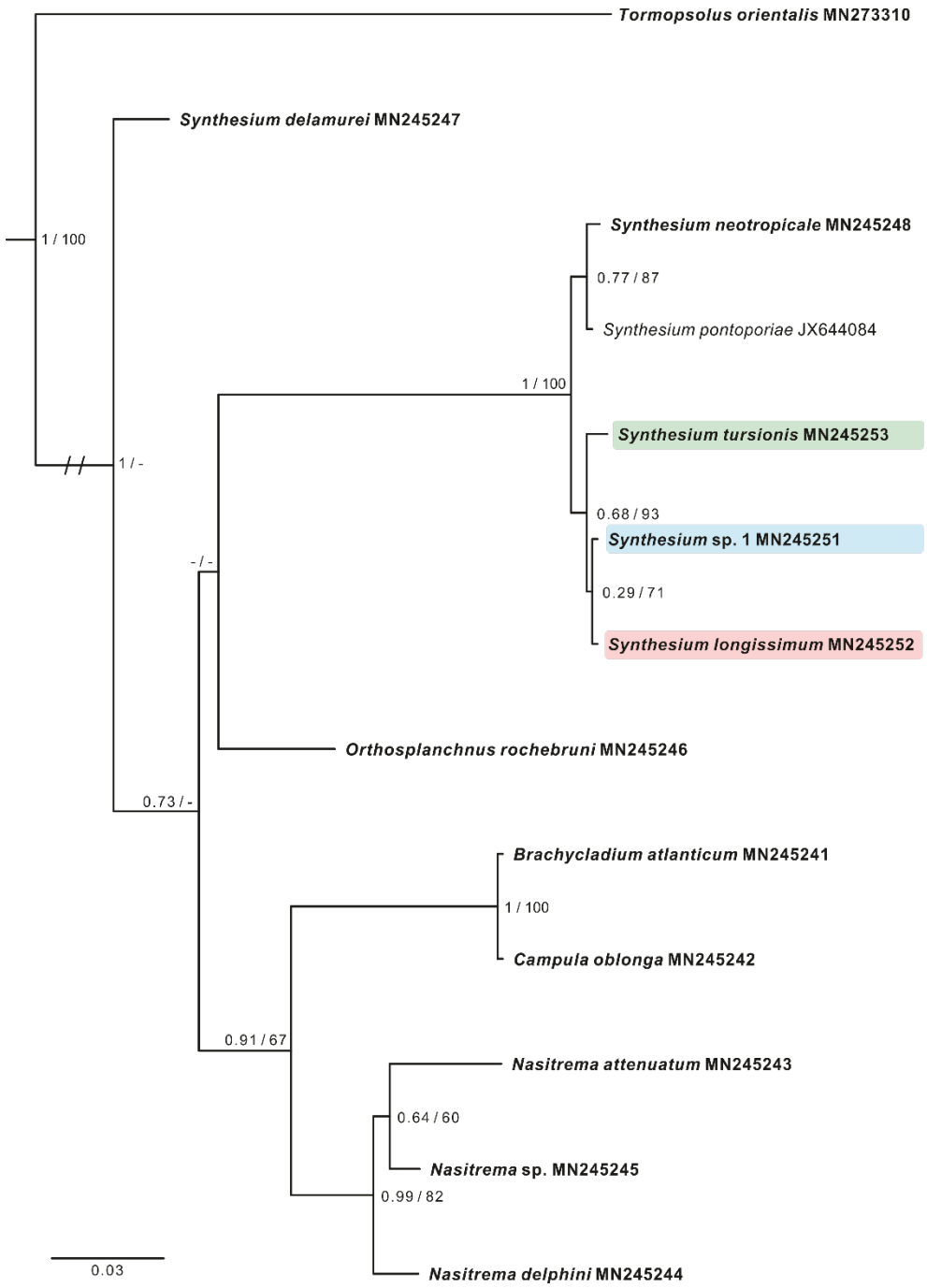


Fig 7. Bayesian topology of the Brachycladiidae species obtained with ITS1 rDNA data. The support values at the branching points are shown as the Bayesian posterior probabilities (pp) followed by Maximum-likelihood bootstrap values (bv). The branch length scale bar indicates the mean number of substitutions per site. New sequences are in bold.

The ND3 mtDNA final alignment included 67 sequences and 334 positions. Both the BI and ML analyses (topologies not shown; pp and bv values shown in Fig. 8) resulted in consensus trees with similar results, recovering all sequences of *Synthesium* spp. in a well-supported monophyletic clade (pp = 0.99, bv = 95), except for *S. delamurei*, which was placed as sister to *Orthosplanchnus* spp. + *Oschmarinella* spp. + *Brachycladium* spp. + *Campula oblonga* (pp = 0.74, bv = 28). The *Synthesium* clade was subdivided into two clades, as shown by the ITS1 rDNA analyses: one clade grouped together the sequences of *S. pontoporiae* and *S. neotropicale* (pp = 0.95, bv = 84), and the other the sequences of *S. longissimum* + *S. tursionis* + *Synthesium* sp. 1 n. sp.. Here, as a difference, the BI analysis recovered *S. longissimum* as a sister taxon of *S. tursionis* + *Synthesium* sp. 1 n. sp. (pp = 0.43), while the ML analysis recovered *S. tursionis* as a sister taxon of *Synthesium sp. 1 n. sp.* + *S. longissimum* (bv = 68). However, none of them was highly supported.

The COI mtDNA final alignment included 66 sequences and 416 positions. The BI and ML analyses (topologies not shown; pp and bv values shown in Figure 9) also resulted in consensus trees with similar results, with all sequences of *Synthesium* spp. grouped together in a well-supported monophyletic clade (pp = 1, bv = 100), except for *S. delamurei*, as shown in the ITS1 rDNA and ND3 mtDNA. Once again, the *Synthesium* clade subdivided into two well supported clades; *S. pontoporiae* + *S. neotropicale* (pp = 1, bv = 93) and *S. tursionis* + *S. longissimum* + *Synthesium* sp. 1 n. sp. (pp = 1, bv = 98). As in the ND3 mtDNA, the BI and ML analyses exhibited different topologies regarding this last clade; the BI analysis recovered *S. tursionis* (pp = 1) as sister to *Synthesium* sp. 1 n. sp. and *S. longissimum* (pp = 0.73), while in the ML analysis, *S. tursionis* clustered together with *Synthesium* sp. 1 n. sp. (bv = 29), being *S. longissimum* their sister taxon (bv = 98).

The ND3 mtDNA GMYC analysis (Fig. 8) suggested a total of 17 species, five of which were composed of only one sequence. The analyses recognized six distinct species of *Synthesium*; *S. pontoporiae*, *S. neotropicale, S. tursionis* (MH634349), *S. tursionis* (MH634347-8 and KT180218), *S. longissimum,* and *Synthesium* sp. 1 n. sp.

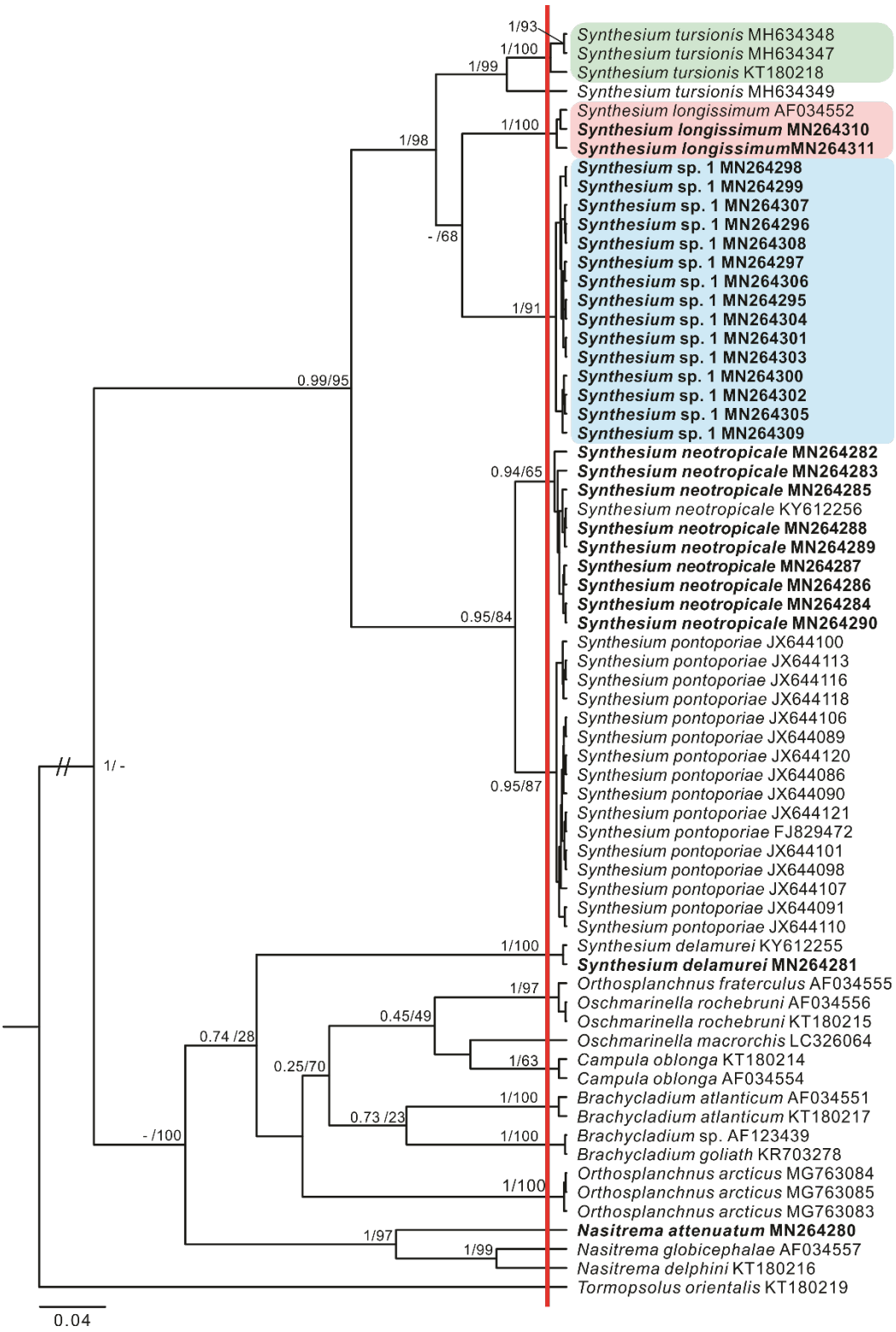


Fig 8. Bayesian General Mixed Yule Coalescent (GMYC) tree of the Brachycladiidae species obtained with ND3 mtDNA data. The support values at the branching points are shown as the Bayesian posterior probabilities (pp) followed by Maximum-likelihood bootstrap values (bv). The clades to the right of the red line (threshold) represent independent species as estimated by the single-threshold model in the GMYC test. The branch length scale bar indicates the mean number of substitutions per site.

The COI mtDNA GMYC analysis (Fig. 9) suggested a similar tree topology to the one obtained for the ND3 mtDNA dataset, recognizing a total of eight species. The analysis delimited five genetically distinct species within *Synthesium*, i.e., *S. pontoporiae*, *S. neotropicale*, *S. tursionis*, *S. longissimum,* and *Synthesium* sp. 1 n. sp.

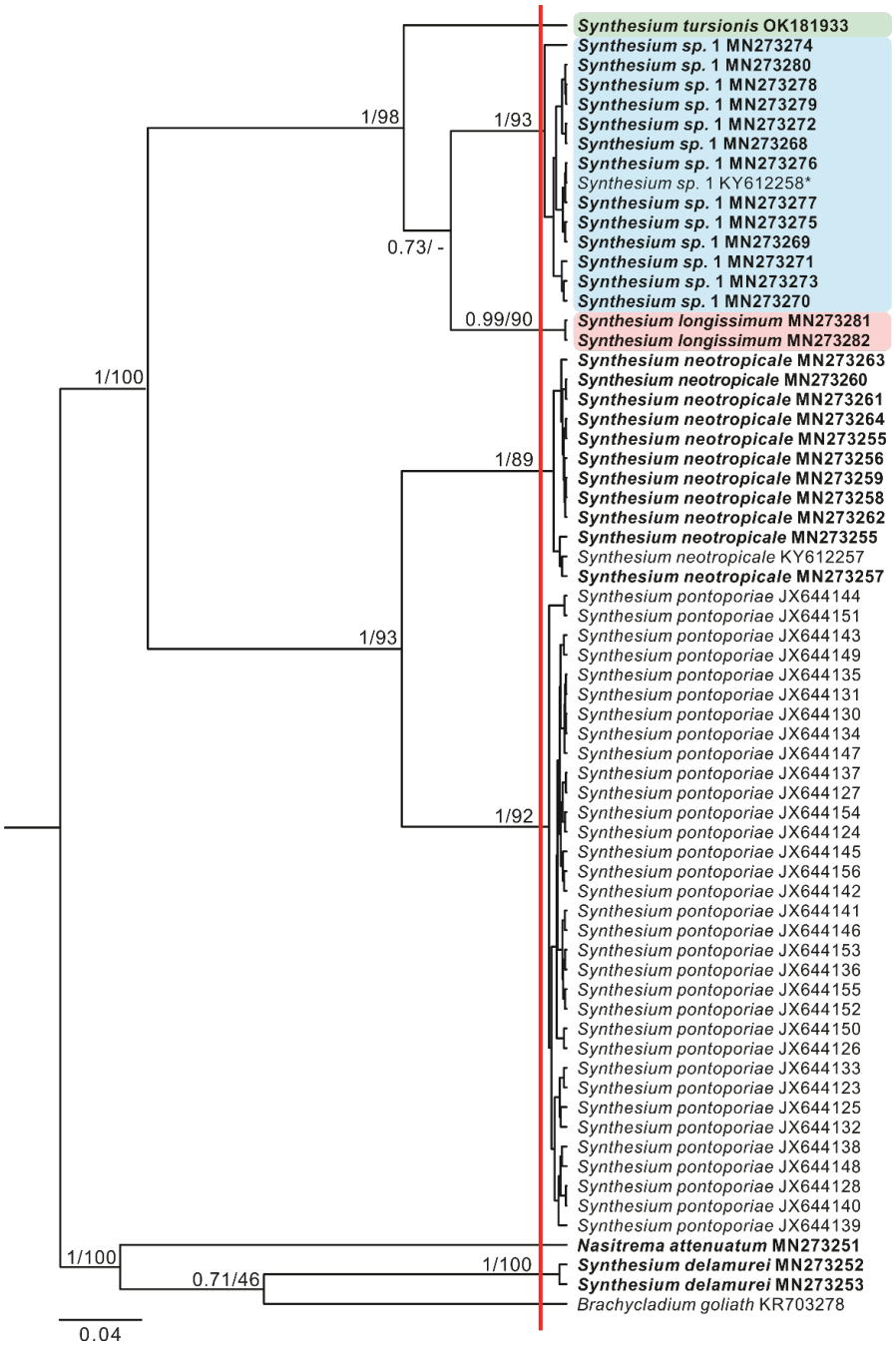


Fig 9. Bayesian General Mixed Yule Coalescent (GMYC) tree of the Brachycladiidae species obtained with COI mtDNA data. The support values at the branching points are shown as the Bayesian posterior probabilities (pp) followed by Maximum-likelihood bootstrap values (bv). The clades to the right of the red line (threshold) represent independent species as estimated by the single-threshold model in the GMYC test. The branch length scale bar indicates the mean number of substitutions per site.

Discussion

Our study, which integrates morphological survey, phylogenetic analyses of ribosomal and mitochondrial DNA, and species delimitation method (GMYC), shed light on the confusing taxonomy of *S. tursionis*, separating the species into two different species which were interpreted as a single one until now; *S. tursionis* and *S. longissimum.* Based on our results, we were also able to describe a new species of *Synthesium*.

Our phylogenetic reconstructions were congruent with previous studies for the family Brachycladiidae (Fernández et al., 1998a, 1998b, 2000; Marigo et al., 2011, 2015; Fraija-Fernández et al., 2016b; Ebert et al., 2017; Shiozaki et al., 2019), corroborating *Synthesium* as monophyletic, except for *S. delamurei*, which might not represent a *Synthesium* species, as previously proposed by Ebert et al. (2017) and Colombo (2018) and, therefore, we recommend an extensive morphological and molecular revision of this species in future studies to properly assign it to its correct genera.

The ND3 and COI mtDNA GMYC analyses recognized the same distinct species of *Synthesium*, i.e., *S. pontoporiae*, *S. neotropicale*, *S. longissimum*, *Synthesium* sp. 1 n. sp., and *S. tursionis*, as well as corroborated by our morphological analyses. However, in the ND3 mtDNA GMYC analysis, the sequence of *S. tursionis* MH634349 from Japan was recognized as a different species from S. *tursionis* MH634347-8 from Japan and KT180218 from the Mediterranean. Recently, Shiozaki et al. (2019) synonymized *S. elongatum* with *S. tursionis* due to morphological similarities presented by these two species. Nevertheless, as showed by this study, *S. tursionis* presented highly problematic morphological identification until now. The sequences from *S. tursionis* MH634347-9 were used by Shiozaki et al. (2019) in their proposed synonymisation. As shown by our molecular analyses, there is a possibility that the sequence MH634349 considered as *S. tursionis* by Shiozaki et al. (2019) represent a different species from the sequences of S. *tursionis* MH634347-8 from Japan and S. *tursionis* KT180218 from the Mediterranean. Therefore, we advise careful morphological and molecular analyses using more specimens and different markers to corroborate the synonymy of *S. elongatum* with *S. tursionis* proposed by Shiozaki et al. (2019).

Synthesium tursionis is known as a cosmopolitan species, exhibiting a worldwide distribution throughout the Atlantic and Pacific Oceans and occurring in, at

least, 10 species of odontocete hosts (Fernández, 1996). However, the recognition of the mistake committed by Parona (1896) opens a new window to the actual ecological aspects of the species. We suggest an extensive revision of the literature involving records of this species, as well as a detailed analysis of hosts and geographic distribution, to correctly identify host-parasite relationships. Likewise, ecological and cophylogenetic studies may help to understand the associations between the *Synthesium* species and their hosts from different geographical localities.

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Anexo 1

Ogmogaster antarctica (Digenea: Notocotylidae) infecting a dwarf minke whale *Balaenoptera acutorostrata* (Cetartiodactyla: Balaenopteridae) from the Southwestern Atlantic Ocean

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Ogmogaster antarctica (Digenea: Notocotylidae) infecting a dwarf minke whale *Balaenoptera acutorostrata* (Cetartiodactyla: Balaenopteridae) from the Southwestern Atlantic Ocean

Abstract

Digeneans of the genus *Ogmogaster* Jägerskiöld, 1891 are intestinal parasites of whales and pinnipeds. Due to the difficulty in recovering these parasites from opportunistic stranding events of their hosts, very little morphological and molecular data are available on the species of this genus. During a beach monitoring survey on the Southern Brazilian coast, a dwarf minke whale *Balaenoptera acutorostrata* (Lacépède, 1804) was necropsied and some digeneans were found in its intestine. Morphological and molecular analyses based on the ribosomal DNA SSU and the mitochondrial DNA COI genes were conducted. The morphological data along with the phylogenetic reconstructions allowed the identification of *Ogmogaster antarctica* Johnston, 1931. This is the first report of *O. antarctica* infecting a *B. acutorostrata* in the South Atlantic Ocean. The morphological data, the molecular characterization and the phylogenetic positioning of *O. antarctica* presented in this study contribute to the knowledge of the helminth diversity of large whales.

Introduction

To better understand host-parasite relationships, detailed reports on helminth diversity are broadly needed. Currently, the availability of parasites from large whales relies almost exclusively on their strandings and subsequent necropsy to collect helminths (Briscoe et al. 2016). Recovering these organisms from such opportunistic stranding events of their large hosts is a challenging task and, as a result, they lack on detailed morphological descriptions along with molecular information.

Minke whales *Balaenoptera acutorostrata* Lacépède, 1804 are cosmopolitan worldwide distributed species (Perrin and Brownell 2002). Their taxonomic status is yet to be resolved, but three different lineages have already been suggested based on morphological and genetic differences (Wada et al. 1991; Rice 1998; Pastene 1994, 2010). The species lineage occurring in the Southern hemisphere and in Antarctic waters is commonly referred to as dwarf minke whale (Best 1985; Kasamatsu et al. 1993; Rice 1998).

The digenean *Ogmogaster antarctica* Johnston, 1931 is found in the intestine of baleen whales and lobodont pinnipeds (Rausch and Fay 1966). The species exhibits a worldwide distribution and has been reported in several host species from different geographical areas (for a detailed review see Leiper and Atkinson 1914; Johnston 1931; Yamaguti 1942; Delyamure 1955; Rausch and Fay 1966; Skrjabin 1970; Yamaguti 1971; Dailey and Brownell 1972; Margolis and Dailey 1972; Shults 1979; Raga and Balbuena 1986; Malatesta et al. 1998; Muniz-Pereira et al. 1999; Dailey et al. 2000; Munoz and Olmos 2008; Fraija-Fernández et al. 2016; Rolbiecki et al. 2019; Marcer et al. 2019). In South Atlantic waters, few records on *O. antarctica* are known. The digenean was first reported from carcasses of a fin whale *Balaenoptera physalus* (Linnaeus, 1758) and a sei whale *Balaenoptera borealis* Lesson, 1828 found washed ashore on the Brazilian coast in the 1960s (Muniz-Pereira et al. 1999). A while later, this trematode was once again found infecting a *B. borealis* off the Argentinian coast (Leonardi et al. 2011). The helminth fauna of *B. acutorostrata* from the South Atlantic Ocean still remains poorly documented (Zerbini et al. 1996; Perrin and Brownell 2002) and parasitological records are restricted to nematodes of the genera *Anisakis* Dujardin, 1845 and *Pseudoterranova* Mozgovoi, 1951 (Dawson and Slooten 1990; Secchi et al. 2003), and cestodes of the genus *Pyllobothrium* Van Beneden, 1850 (Dawson and Slooten 1990).

In this study, we provide the first reference of *O. antarctica* infecting a specimen of *B. acutorostrata* from South Atlantic waters. The new record extends the knowledge on the helminth fauna of this unexplored whale. Additionally, we contribute with information on the morphology of the digenean species with a new descriptive drawing, and provide the molecular characterization and phylogenetic positioning of the species based on the ribosomal DNA SSU and mitochondrial DNA COI genes.

Material and methods

Collection of samples

In October 2016 a 4.5 m long adult male of *B. acutorostrata* was found washed ashore at the São Francisco do Sul district, Santa Catarina State, South Brazil, Southwestern Atlantic (26°09'43.0" S, 48°34'01.6" W). During necropsy, the whale's small intestine was examined for helminths. The digeneans were collected, cleaned in tap water to remove debris and then transferred directly to 70% ethanol.

Morphological analyses

Unflattened specimens were stained with chloride carmine, dehydrated in a graded ethanol series, cleared with creosote, and mounted as temporary preparations. The parasites were identified based on specific references (Johnston 1931; Rausch and Fay 1966; Malatesta et al. 1998; Barton and Blair 2005; Rolbiecki et al. 2019). Morphometric analyses were made using the Qwin Lite 3.1 (Leica) computerized system for image analysis. Drawing was made with a drawing tube attached to a microscope and edited on CorelDRAW v. 18. Voucher specimens were deposited at the Helminthological Collection of the Institute of Biosciences (CHIBB), UNESP, Botucatu, São Paulo state, Brazil, under numbers 8839 and 8840.

Molecular analyses

The genomic DNA of three adult worms was extracted using the DNeasy® Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol, in a final volume of 30 µl. Partial fragments of the ribosomal DNA SSU and the mitochondrial DNA COI genes were amplified. PCR amplifications were performed using 3 µl of genomic DNA, 1.0 µl of each set of primers and Ready-to-Go PCR beads (Pure Taq™Ready-to-Go™PCR beads, GE Healthcare) in a final volume of 25 µl. The primers used to obtain partial fragments of nuclear and mitochondrial markers were: rDNA SSU, WormA (5'-GCGAATGGCTCATTAAATCAG-3') and WormB (5'-CTTGTTACGACTTTTACTTCC-3') (Littlewood and Olson 2001) and sequencing primers 300F (5'-AGGGTTCGATTCCGGGAG-3'), 930F (5'-GCATGGAATAATGGAATAGG-3'), 1200F (5'-CAGGTCTGTGATGCCC-3') and 1270R (5'-CCGTCAATTCCTTTAAGTTT-3') (Littlewood and Olson 2001); mtDNA COI, JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT- 3') (Morgan and Blair 1998) and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') (Razo-Mendivil et al. 2008). The thermocycling profile for the rDNA SSU amplification included initial denaturation at 94 °C for 3 min, followed by 40 cycles of amplification at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min, and a final extension step at 72 °C for 7 min (Littlewood and Olson 2001). For the mtDNA COI amplification, the initial denaturation was at 94 °C for 5 min, followed by 40 cycles of amplification at 92 °C for 30 s, 46 °C for 45 s and 72 °C for 90 s, and a final extension step at 72 °C for 10 min (Morgan and Blair 1998). PCR amplicons (3 µl) were separated electrophoretically in 1% agarose gel with gel red (1 µl) (Biotium Inc) and DNA bands were visualized under UV light. PCR products were

purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol and sequenced on an Applied Biosystems ABI 3500 automated DNA sequencer using Big Dye v.3.1 Terminator (Applied Biosystems, Foster City, CA, USA).

Forward and reverse sequences were assembled and edited using Sequencher v. 5.2.4. The new rDNA SSU and mtDNA COI consensus sequences were aligned separately with selected sequences belonging to members of close related families recovered from GenBank database, using MUSCLE algorithm implemented on Geneious v. 7.1 program, with default settings (rDNA SSU: *Catatropis indicus* Srivastava, 1935 (AY222114), Pronocephaloidea sp. Looss, 1899 (EU371601), *Notocotylus pacifera* (Noble, 1933) Harwood, 1939 (AY245765), *Notocotylus* sp. Diesing, 1839 (AJ287547), *Ogmogaster antarctica* (KM258669; KY945915) [Notocotylidae], *Rhabdiopoeus taylori* Johnston, 1913 (AY222113), *Taprobanella bicaudata* Crusz & Fernand, 1954 (AY222112) [Rabdiopoeidae], *Labicola* cf. *elongata* Blair, 1979 (AY222115) [Labicolidae], *Lankatrema mannarense* Crusz & Fernand, 1954 (AY222116), *Opisthotrema dujonis* Fischer, 1883 (AY222117) [Ophistotrematidae], *Macrovestibulum obtusicaudum* Mackin, 1930 (AY222111) [Pronocephalidae], *Chiorchis fabaceus* (Diesing, 1838) Fischoeder, 1901 (MF370224) [Cladorchiidae], *Diplodiscus japonicus* Yamaguti, 1936 (KX506855), *Diplodiscus mehrai* Pande, 1937 (KX506857), *Diplodiscus subclavatus* (Pallas, 1760) (AJ287502) [Diplodiscidae], *Heronimus mollis* (Leidy, 1856) (AY222118) [Heronimidae] (Cribb et al. 2001; Olson et al. 2003; Adema et al. 2009; Fraija-Fernández et al. 2015); mtDNA COI: *Ogmocotyle sikae* (Yamaguti, 1933) (KR006934; NC027112), *Ogmocotyle* sp. Skrjabin & Shulz, 1933 (KR006935), *Tristriata anatis* Belopolskaia, 1953 (KX833003; KX833006), *Hippocrepis hippocrepis* (Diesing, 1850) (MN268535; MN268536), *O. antarctica* (KY945916) [Notocotylidae], *Paramphistomum cervi* Schrank, 1790 (KF475773) [Paramphistomidae] (Yan et al. 2013; Ma et al. 2015; Gonchar and Galaktionov 2017; Assis et al. 2019).

Phylogenetic relationships were inferred by Maximum Likelihood (ML) and Bayesian Inference (BI), applying the model GTR + I + G selected as the best fitting model of nucleotide evolution by jModelTest v.2.1 for both datasets. ML analyses were carried out using RAxML v.8 software on CIPRES web portal, with bootstrap support values calculated using 1000 replications. BI were made using MrBayes v.3.2 software implemented on CIPRES web portal, with the analyses performed by running two

independent MCMC runs of four chains for 10^7 generations and sampling tree topologies every 10^2 generations, the first 25% of trees discarded as burn-in and the remaining trees used for calculating the Bayesian posterior probabilities. The obtained ML and BI trees were visualized using FigTree v.1.3.1 and edited using CorelDRAW v.18. Genetic distances were calculated with MEGA v.7 using Kimura-2-parameter model considering the number of nucleotide differences between sequences.

Results

Eighty-nine adult digeneans were found in the lumen of the whale's intestine. Based on morphological and molecular features described below, we have assigned the specimens as *Ogmogaster antarctica*.

Morphological description

The following observations and measurements were made based on 19 whole-mounted specimens. Measurements are shown as the range followed by the mean in parentheses and are expressed in millimeters unless otherwise stated. The main morphological features observed to morphologically identify our specimens were the body size, the presence/absence of spines, the number of ventral-longitudinal ridges, the extension of the cirrus pouch, the size and shape of the gonads and the location and number of the vitelline glands.

Phylum Platyhelminthes Gegenbaur, 1859
Class Trematoda Rudolphi, 1808
Subclass Digenea Carus, 1863
Order Plagiorchiida La Rue, 1957.
Family Notocotylidae Lühe, 1909
Genus *Ogmogaster* Jägerskiöld, 1891

***Ogmogaster antarctica* Johnston, 1931 (Fig. 1)**

Body strongly muscular, oval, flattened, with the margins turned ventrally, slightly narrowed at the anterior extremity. Lateral margin of the body with well-defined crenulations (number from 38 to 40). Spiny tegument, with parallel longitudinal ridges

on the ventral surface (number from 13 to 15). Body length 3.91-6.30 (5.33) and width 1.90-3.40 (2.73). Oral sucker terminal, muscular, 0.40-0.61 (0.51) long and 0.40-0.63 (0.54) wide. Esophagus indistinguishable. Intestinal caeca extending posteriorly to the level of the ovary, curved along its entire length, with small lateral branches. Testes symmetrically positioned, deeply lobed to dendritic, extracaecal, situated in the posterior half of the body. Right testis 0.35 -0.75 (0.59) long and 0.39-0.89 (0.69) wide. Left testis 0.35-0.77 (0.59) long and 0.44-0.93 (0.70) wide. Vas efferent joining anteriorly to Mehlis' gland and forming a single medial vas deferens dorsal to the uterus, which forms an external seminal vesicle before reaching the level of the posterior end of the cirrus sac. Cirrus sac wide, in the midline of the body, 1.41-2.29 (1.85) long and 0.11-0.41 (0.21) wide, containing internal seminal vesicle, prostate glands, and cirrus covered with small spines. Genital pore opening immediately posterior to oral sucker. Ovary lobed, medially located between testes, 0.32-0.71 (0.56) long and 0.17-0.42 (0.32) wide. Mehlis' gland preovarian, almost lobed. Vitellaria in groups of 15 to 18 follicles limited to each side of the body, located between testes and the posterior end of the cirrus sac. Uterine field extending between anterior margins of testes and region posterior to the genital pore, with numerous coils positioned extracaecally. Metraterm short, wide and muscular, with small spines. Eggs 15.20-22.70 µm (19.24) long, 8.30-13.20 µm (10.84) wide, with 2 polar filaments 62.10-100.20 µm (73.48) long.

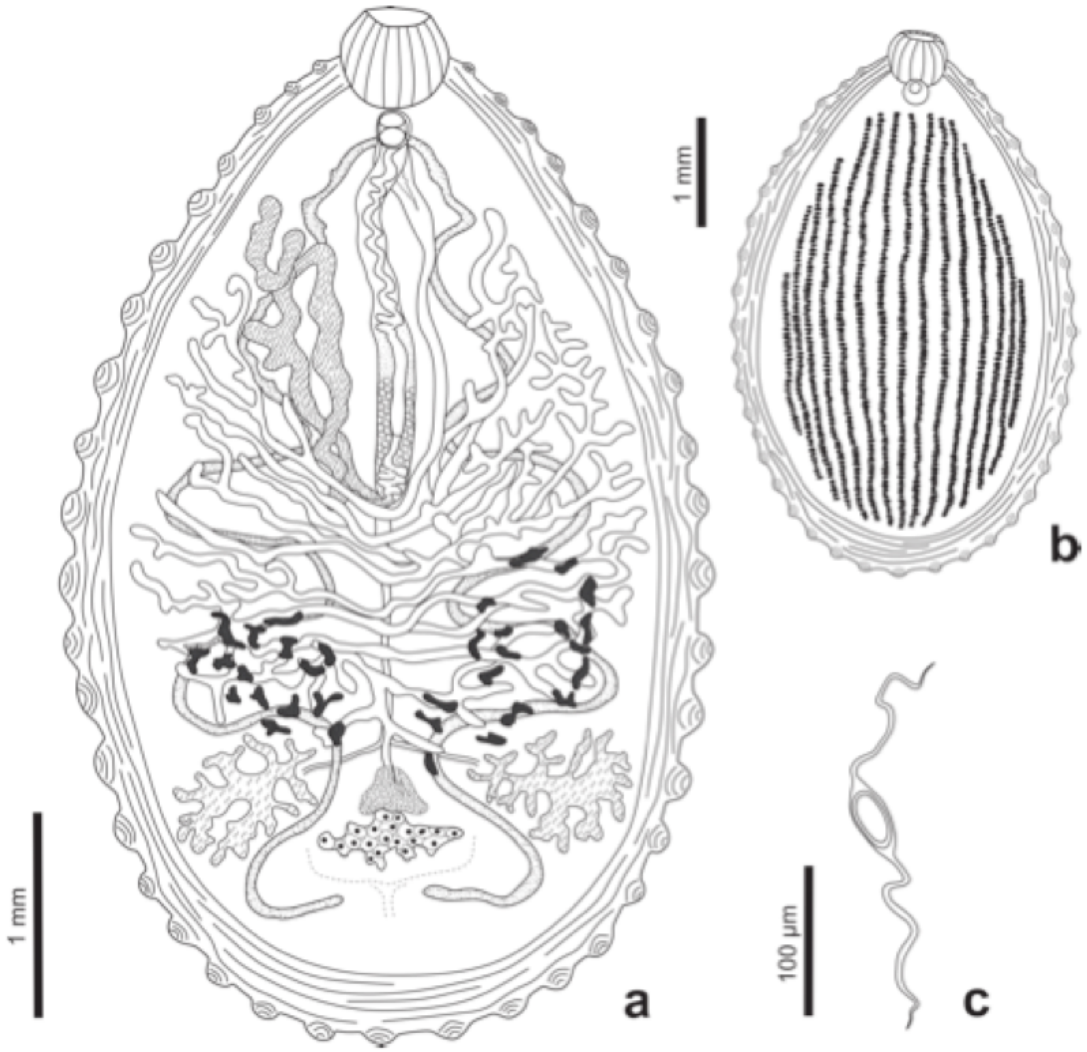


Fig. 1 *Ogmogaster antarctica* from the intestine of a dwarf minke whale *B. acutorostrata* from Southwestern Atlantic Ocean. **a** Whole worm, ventral view; **b** whole worm, disposition of ventral ridges; **c** egg, with polar filaments

Phylogenetic reconstructions

One partial rDNA SSU sequences (1488 bp) and 3 partial mtDNA COI sequences (447 bp; 445 bp; 371bp) of *O. antarctica* were obtained. The aligned datasets of the newly generated sequences with those previous selected on Genbank included 18 sequences comprising 1279 nucleotides positions for rDNA SSU and 11 sequences comprising 352 nucleotides positions for mtDNA COI.

For the rDNA SSU dataset, both BI and ML analyses resulted in consensus trees with identical topologies (Fig. 2). Despite poorly supported in both analyses, the newly generated sequence formed a monophyletic clade with the other sequences of *O. antarctica* (KM258669 and KY945915). The intraspecific genetic divergence in this

clade was 0.1%. The *O. antarctica* clade formed by the three sequences clustered as sister to the group formed by all other members of the family Notocotylidae used in the analyses.

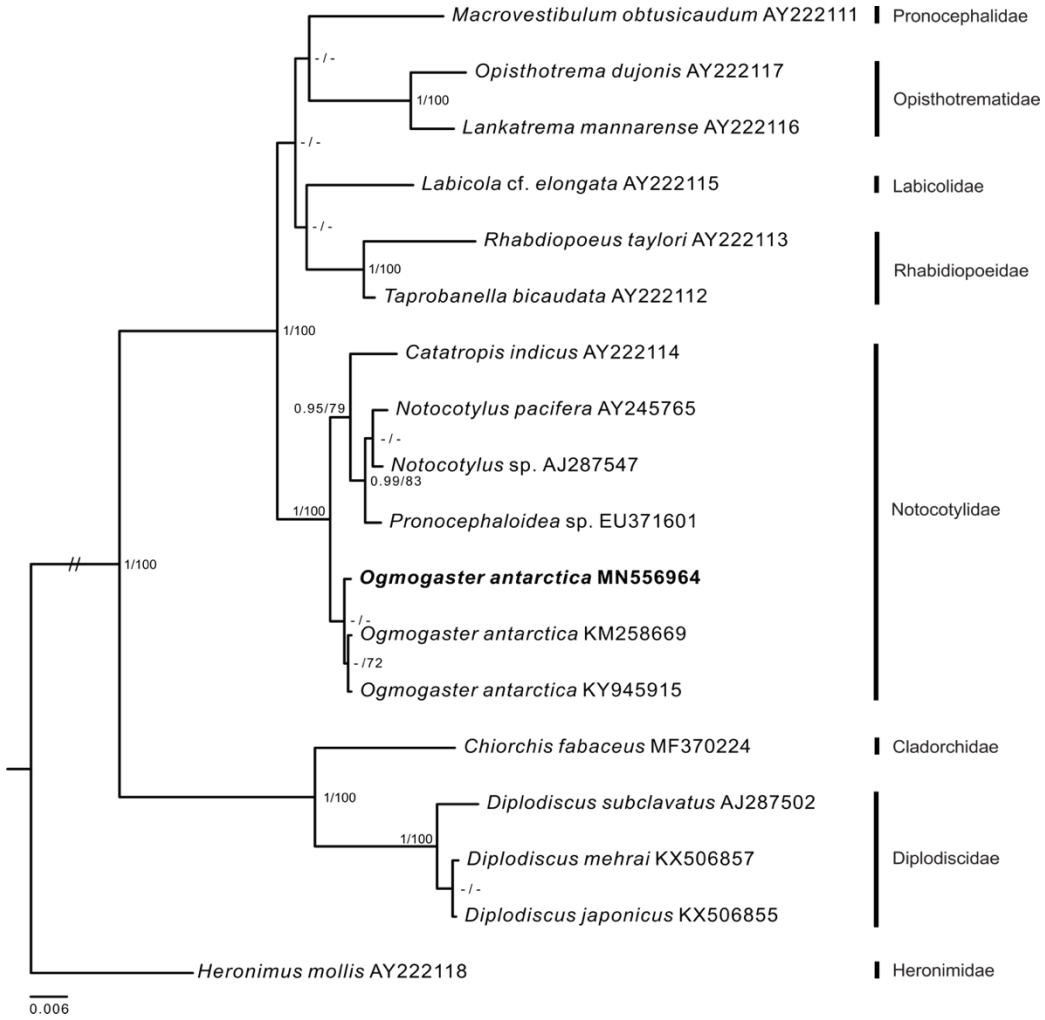


Fig. 2 Bayesian phylogenetic tree based on partial rDNA SSU sequences of Notocotylidae and close related families. The support values at the branching points are shown as the Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses (pp < 0. 95; bootstrap < 70%). New sequence obtained by this study is highlighted in bold

The topologies resulting from ML and BI analyses of the mtDNA COI dataset were also identical (Fig. 3). The three new generated sequences clustered together and along with another *O. antarctica* sequence available in GenBank (KY945916) in a well-supported monophyletic clade (Bayesian inference PP = 0.98, Maximum likelihood bootstrap = 97%), which was also placed within the Notocotylidae. The genetic divergence between *O. antarctica* sequences ranged from 0.0% (*O. antarctica*

MN562653 x *O. antarctica* MN562654; both collected from *B. acutorostrata* from Southwestern Atlantic) to 4.6% (*O. antarctica* KY945916 from *B. borealis* from Chilean Patagonia x *O. antarctica* MN562654 from *B. acutorostrata* from Southwestern Atlantic).

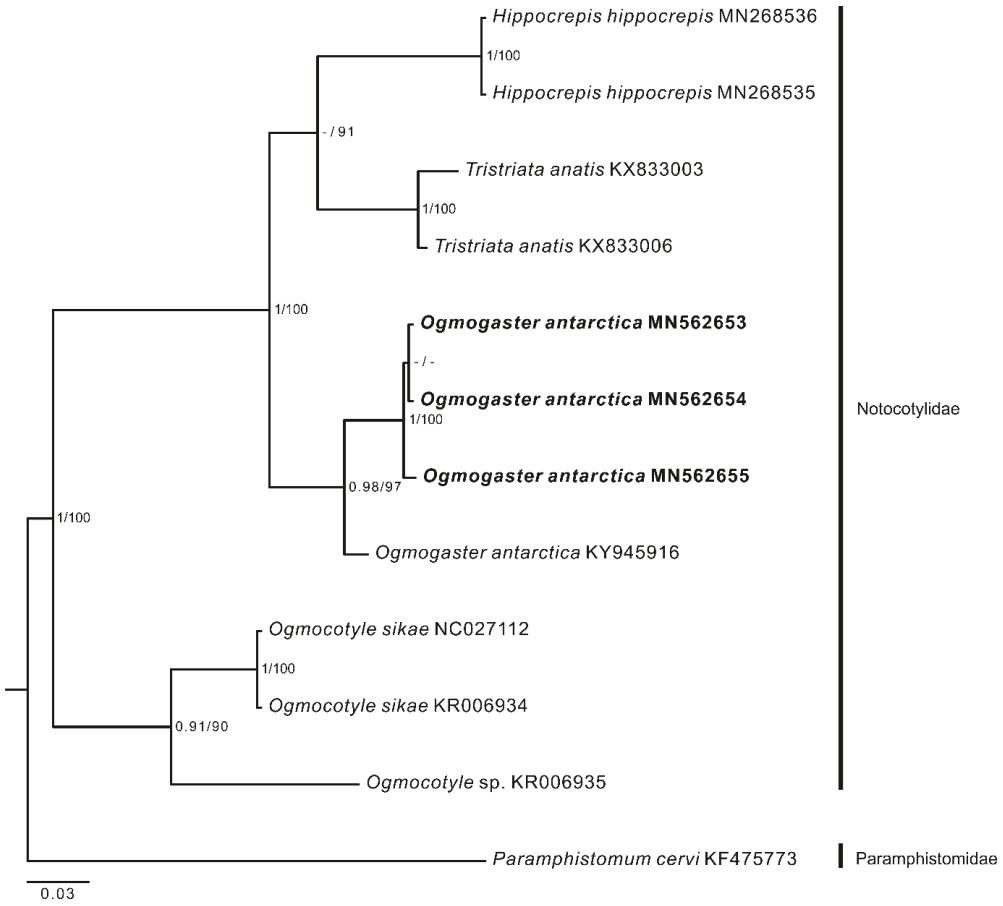


Fig. 3 Bayesian phylogenetic tree based on partial mtDNA COI sequences of Notocotylidae. The support values at the branching points are shown as the Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses (Bayesian posterior probability values < 0. 95; 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

Discussion

The present study is the first report on *O. antarctica* infecting the dwarf minke whale *B. acutorostrata* in the South Atlantic Ocean. Whilst there is a bulk of scientific literature regarding the helminth fauna of large whales, only a few records are related to *B. acutorostrata*, especially in South Atlantic waters. To date, only one punctual

parasitological record is known at this locality for this cetacean species (Secchi et al. 2003). Therefore, the new report on the occurrence of this digenean species extends our knowledge on the distribution and biodiversity of helminths infecting whales.

The genus *Ogmogaster* presently includes six species: *O. antarctica*, *Ogmogaster grandis* Skrjabin, 1969, *Ogmogaster heptalineata* Carvajal, Duran & George-Nascimento, 1983, *Ogmogaster pentalineata* Rausch & Fay, 1966, *Ogmogaster plicata* (Creplin, 1829) Jägerskiöld, 1891, and *Ogmogaster trilineata* Rausch & Rice, 1970 (Worms 2020), which are mainly distinguished one from the other based on the number of ventral-longitudinal ridges, the presence or absence of spines or scales and the size of the body (Rausch and Fay 1966; Raga et al. 1986). We were able to differentiate our specimens of *O. antarctica* from its congeners mainly by their body size, the number of ventral ridges (13 to 15), the extension of the cirrus pouch which is restricted to the anterior portion of the body, and the location of the vitelline glands between the base of the cirrus pouch and the anterior margin of the deeply lobed testes.

Most references on *O. antarctica* are aged records and/or from specimens recovered in the Northern hemisphere. The species have been revised (Skrjabin 1953; Rausch and Fay 1966) but quality drawings and detailed diagnostic descriptions were still lacking. Reliable helminth identifications depend on high quality and detailed descriptions, thus the morphological data presented here contributes greatly to the diagnosis of the species.

The phylogenetic reconstructions based on the rDNA SSU and mtDNA COI datasets consistently showed that the new sequences studied herein are certainly ascribed to its correct assigned family (Notocotylidae) and genus (*Ogmogaster*), thus corroborating with previous phylogenetic studies on trematodes (Fraija-Fernández et al. 2015). The low rDNA SSU genetic divergence (0.1%) found among the three sequences of *O. antarctica* recovered from different hosts from contrasting geographical locations (e.g., *B. borealis* from Mediterranean x *B. borealis* from Chilean Patagonia x *B. acutorostrata* from South Atlantic) was expected since this gene is presumed to be very conserved. However, although the phylogenetic positioning and distance values recovered using the rDNA SSU could indicate the correct assignation of the specimens taxonomic genus and family, this gene by itself could not support the species identification. The mtDNA COI values of distances between *O. antarctica* collected from *B. borealis* from Chilean Patagonia and those collected from *B.*

acutorostrata from the South Atlantic (4.6%) were higher than the expected values for mtDNA COI intraspecific divergence rates in trematodes. According to Vilas et al. (2005), the maximum intraspecific variation in platyhelminths ranges between 0.3% and 2.2% and, individuals considered conspecific which diverge by more than 5% should be investigated as congeneric species. This suggests that the *O. antarctica* sequences used in this study might belong to diverging *Ogmogaster* species. Despite the sequence of the mtDNA COI gene of *O. antarctica* collected from a *B. borealis* in Chilean Patagonia (KY945916) is properly submitted to Genbank, it remains unpublished; thus, no phylogenetic analysis nor any morphological description regarding the specimen used to generate that sequence is available. Based on our results of morphological analyses, we assume that our rDNA SSU and mtDNA COI sequences are representing data from *O. antarctica*; however, it is difficult to affirm if we are actually comparing our sequences with ones which represent the same morphological species as ours, since they are lacking information. On the other hand, *O. antarctica* is a cosmopolitan species worldwide distributed that infects a variety of hosts and consequently is involved in many ecological and evolutionary interactions, which could influence the species gene flow and drift processes of speciation. Therefore, we suggest further taxonomic and phylogenetic investigations using additional *Ogmogaster* spp. sequences to unravel the species boundaries.

Here we provide new rDNA SSU and mtDNA COI sequences of morphologically identified *O. antarctica* along with phylogenetic analyses. Our new molecular data and the analyses of genetic distances provide extra comparative data, which can be applied in further investigations on the taxonomic status and the diversity among species of *Ogmogaster* and, consequently, parasite-host relationships.

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licensing division of the Brazilian environmental agency (IBAMA), for the exploration of oil and gas by Petrobras at the Santos Basin pre-salt province.

Ethical standards: Procedures performed in this study were in accordance with the ethical standards of the institutions in which they were conducted. Permission to collect and transport deceived stranded dolphins was given by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA/SISBIO) under registration 11980-1.

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Anexo 2

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

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

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 southeastern 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

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Availability of data and material Permission to collect and transport deceived stranded dolphins was given by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) under registration 11980-1 (SISBIO) and 5820094 (ABIO).

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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, 1999, 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 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 Bénedén, 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 trichome 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 & 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'-TAGGTCGACCCGCTGAAYTTAAGCA-3') and 1500R (5'-GCTATCCTGAGGGAAACTTCG-3') (Olson et al. 2003); COI, JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') (Morgan and Blair 1998) and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-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 10^6 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 1,000 bootstrap replicates. Nodes with bootstrap values greater than 70% were considered well supported.

Results and discussion

We found a total of 5,563 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 1,498 and 1,005 specimens, respectively; and three out of 22 *T. t. gephyreus* were infected with 1,323, 1,482, and 230 specimens, respectively. A total of 93 digeneans (29 from *S. bredanensis* identification number 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.

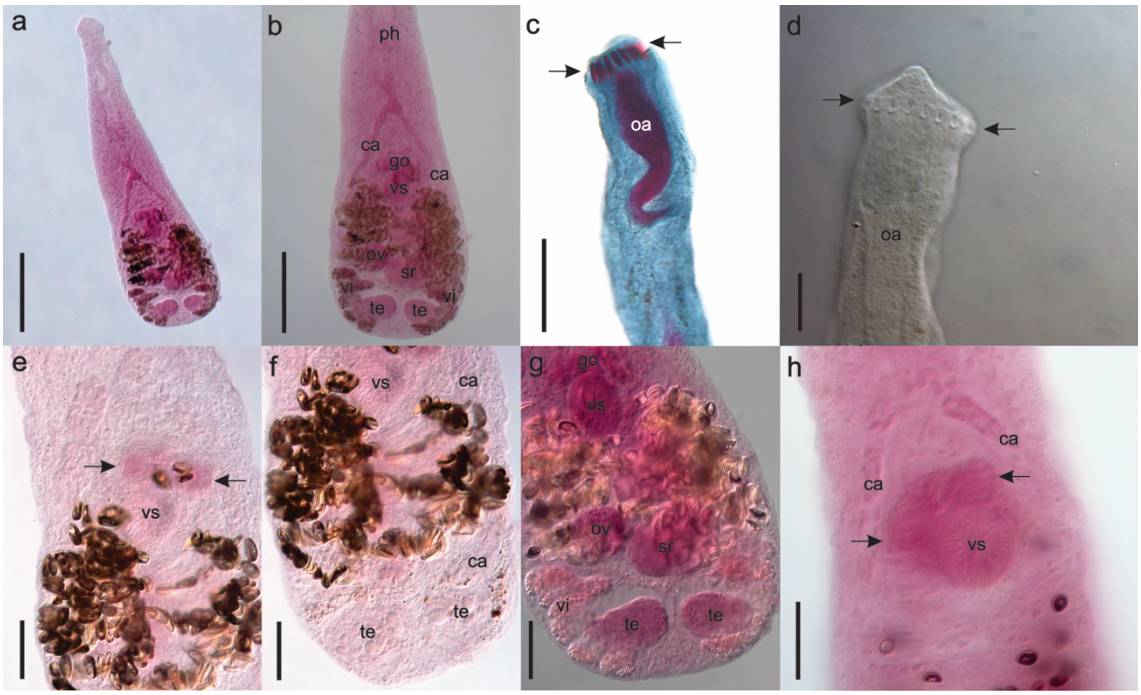


Fig. 1 Adult *Ascocotyle longa* collected from *Otaria flavescens* (a, b, d, e, f, g, and h) and *Steno bredanensis* (c) from the southern Brazilian coast. a) Whole worm, scale bar = 200 µm. b) Posterior extremity of the worm, scale bar = 100 µm. c) Anterior extremity of the worm; arrow indicates the circumoral spines stained with Gomori's trichome, scale bar = 50 µm. d) Anterior extremity of the worm; arrow indicates the circumoral spines; Hoyer medium, scale bar = 50 µm. e) Detail of the bipartite gonotyl; arrows indicate pad-like lobes, scale bar = 50 µm. f) Posterior extremity of the worm showing the end of caeca reaching up the pretesticular area, scale bar = 50 µm. g) Posterior extremity of the worm, scale bar = 50 µm. h) Detail of the bipartite gonotyl; arrows indicate pad-like lobes, scale bar = 200 µm. ca = caeca; go = gonotyl; ov = ovary; oa = oral appendage; ph = pharynx; sr = seminal receptacle; te = teste; vi = vitellarium; vs = ventral sucker.

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 1,111 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 & 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 grey 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*.

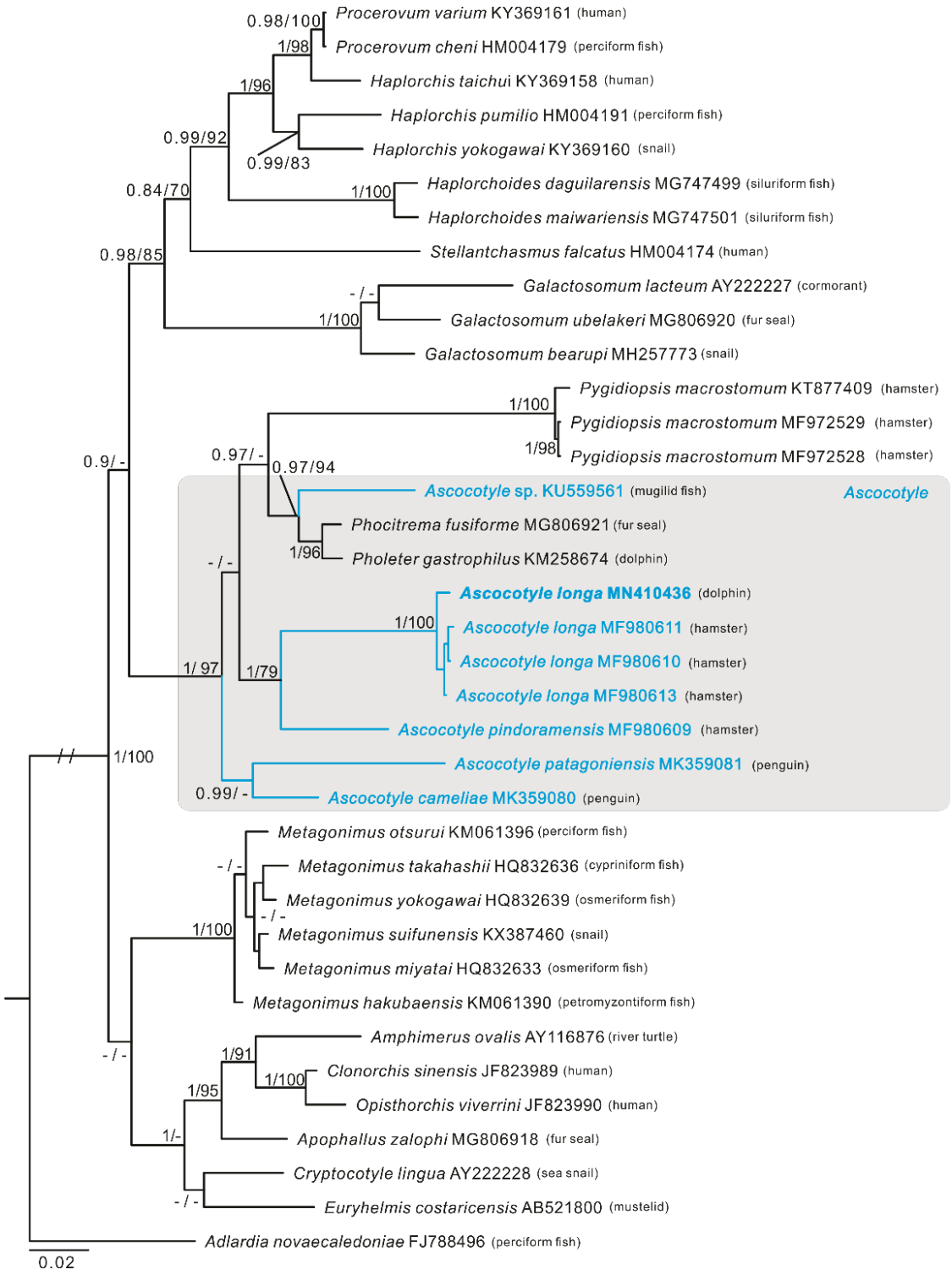


Fig. 2 Bayesian phylogenetic topology of Heterophyidae and closely related families constructed with the 28S rDNA gene dataset. Support values at branching points are shown as Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the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

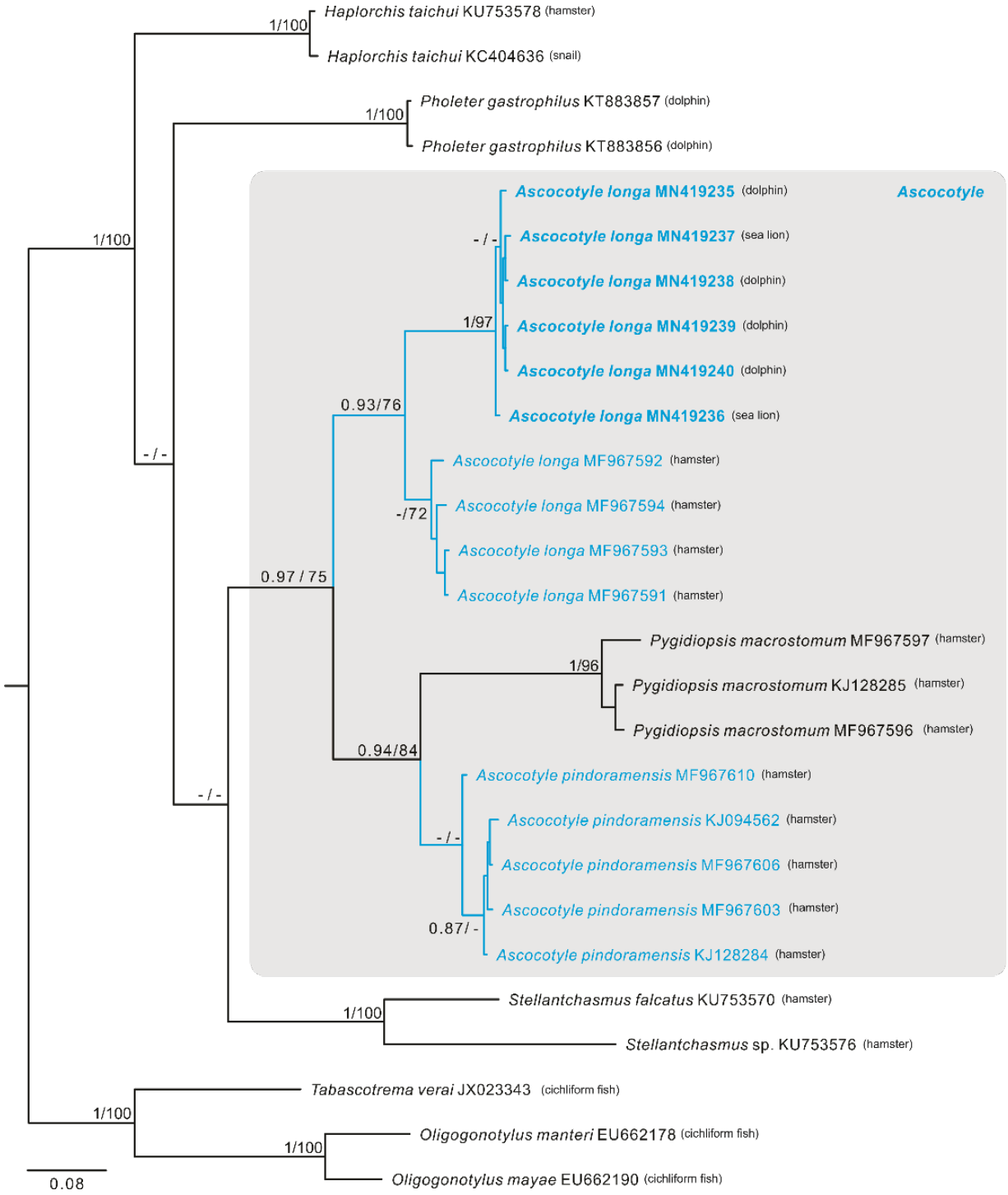


Fig. 3 Bayesian phylogenetic topology based on partial COI sequences of Heterophyidae. Support values at branching points are shown as Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses. Branch length scale bar indicates mean number of substitutions per site. New sequences are highlighted in bold

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 intraspecific 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 harbour 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 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.

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Supplementary table 1 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 and the GenBank accession numbers (28S and COI). (EI) = recovered from an experimentally infected host

Digenean species	Host species	Locality	Accession numbers		References
			28S rDNA	COI mtDNA	
Heterophyidae Leiper, 1909					
Amphimerus ovalis Barker, 1911	*Trionyx muticus (river turtle)*	Mississippi, USA	AY116876		Olson et al. 2003
Apophallus zalophi Price, 1932	*Callorhinus ursinus* (northern fur seal)	St. Paul Island, Alaska, USA	MG806918		Kuzmina et al. 2018
Ascocotyle cameliae Hernández-Orts et al. 2019	*Spheniscus magellanicus* (magellanic penguin)	San Matias Gulf, Argentina	MK359080		Hernández-Orts et al. 2019
Ascocotyle longa Ransom, 1920	***Steno bredanensis* (rought-theeth dolphin)**	**Santa Catarina, Brazil**	**MN410436**	**MN419235; MN419239**	**This study**
	***Tursiops truncatus* (bottlenose dolphin)**	**Santa Catarina, Brazil**		**MN419238; MN419240**	**This study**
	***Otaria flavescens* (Patagonian sea lion)**	**Rio Grande do Sul, Brazil**		**MN419236-7**	**This study**
	Mesocricetus auratus (golden hamster) (EI)	Brazil	MF980610-1-3	MF967591-4	Santos and Borges 2020
Ascocotyle patagoniensis Hernández-Orts et al. (2012)	Spheniscus magellanicus (magellanic penguin)	San Matias Gulf, Argentina	MK359081		Hernández-Orts et al. 2019
Ascocotyle pindoramensis (Travassos, 1928)	Mesocricetus auratus (golden hamster) (EI)	Brazil	MF980609; MF980223	MF967603-6-10	Santos and Borges 2020
	Mesocricetus auratus (golden hamster) (EI)	Brazil		KJ128284; KJ094562	Borges et al. 2017
Ascocotyle sp.	*Chelon labrosus (grey mullet)*	Sardinia, Italy	KU559561		Masala et al. 2016
Cryptocotyle lingua (Creplin, 1825)	Littorina littorea (sea snail)	Germany	AY222228		Olson et al. 2003
Euryhelmis costaricensis Brenes, Arroyo & Jiménez-Quirós, 1960	Martes melampus (Japanese marten)	Japan	AB521800		Sato et al. 2010

Galactosomum bearupi Pearson, 1973	Clypeomorus batillariaeformis (necklace cerith)	Heron Island, Australia	MH257773		Huston et al. 2018
Galactosomum ubelakeri Dailey, 1969	Callorhinus ursinus (northern fur seal)	St. Paul Island, Alaska, USA	MG806920		Kuzmina et al., 2018
Galactosomum lacteum (Jägerskiöld, 1896)	Phalacrocorax carbo (great cormorant)	Ukraine	AY222227		Olson et al. 2003
Haplorchis pumilio Looss, 1896	*Trichogaster trichopterus (three-spotted gourami)*	Thailand	HM004191		Thaenkham et al. 2010
Haplorchis taichui (Nishigori, 1924)	Homo sapiens (Human)	Vietnam	KY369158		Le et al. 2017
	hamster (EI)	Thailand		KU753578	Wongsawad et al. 2017
	Snail	Thailand		KC404636	Chontananarth et al. (Unpublished)
Haplorchis yokogawai (Katsuta, 1932)	Snail	Vietnam	KY369160		Le et al. 2017
Haplorchoides daguilarensis Hostettler, Cutmore & Cribb, 2018	Neoarius graeffei (australian salmon-tailed catfish)	Queensland, Australia	MG747499		Hostettler et al. 2018
Haplorchoides maiwariensis Hostettler, Cutmore & Cribb, 2018	Neoarius graeffei (australian salmon-tailed catfish)	Queensland, Australia	MG747501		Hostettler et al. 2018
Metagonimus hakubaensis Shimazu, 1999	Lethenteron reissneri (eastern lamprey)	Japan	KM061390		Pornruseetairatn et al. 2015
Metagonimus miyatai Saito, Chai, Kim, Lee & Rim, 1997	Plecoglossus altivelis (ayu fish)	Miyakoda River, Japan	HQ832633		Pornruseetairatn et al. 2015
Metagonimus otsurui Saito & Hori, 1962	Rhinogobius flumineus (japanese goby)	Japan	KM061396		Pornruseetairatn et al. 2015
Metagonimus suifunensis Shumenko,Tatonova & Besprozvannykh, 2017	*Juga tegulata (snail)*	Komarovka, Russia	KX387460		Shumenko et al. 2017
Metagonimus takahashii Takahashi, 1929	Carassius auratus langsdorfii (ginbuna)	Japan	HQ832636		Pornruseetairatn et al. 2015

Metagonimus yokogawai (Katsurada, 1912)	Plecoglossus altivelis (ayu fish)	Tenryu River, Japan	HQ832639		Pornruseetairatn et al. 2015
Phocitrema fusiforme Goto & Ozaki, 1930	Callorhinus ursinus (northern fur seal)	St. Paul Island, Alaska, USA	MG806921		Kuzmina et al. 2018
Pholeter gastrophilus (Kossack, 1910)	Tursiops truncatus (bottlenose dolphin)	Spain	KM258674	KT883856-7	Fraija-Fernández et al. 2015
Procerovum cheni Hsu, 1950	Anabas testudineus (climbing perch)	Thailand	HM004179		Thaenkham et al. 2010
Procerovum varium Onji & Nishio, 1916	Homo sapiens (Human)	Vietnam	KY369161		Le et al. 2017
Pygidiopsis macrostomum Travassos, 1928	Mesocricetus auratus (golden hamster) (EI)	Brazil	KT877409	KJ128285	Borges et al. 2017
	Mesocricetus auratus (golden hamster) (EI)	Brazil	MF972528-9	MF967596-7	Santos and Borges 2020
Stellantchasmus falcatus Onji & Nishio, 1916	Homo sapiens (Human)	Vietnam	HM004174		Thaenkham et al. 2010
	hamster (EI)	Thailand		KU753570	Wongsawad et al. 2017
Stellantchasmus sp.	hamster (EI)	Thailand		KU753576	Wongsawad et al. 2017
Opisthorchiidae Looss, 1899					
Clonorchis sinensis Looss, 1907	Homo sapiens (Human)	Vietnam	JF823989		Thaenkham et al. (Unpublished)
Opisthorchis viverrini Poirier, 1886	Homo sapiens (Human)	Thailand	JF823990		Thaenkham et al. (Unpublished)
Cryptogonimidae Ward, 1917					
Adlardia novaecaledoniae Miller, Bray, Goiran, Justine & Cribb 2009	Nemipterus furcosus (rosy threadfin bream)	New Caledonia	FJ788496		Bray et al. 2009
Oligogonotylus manteri Watson, 1976	Cichlasoma urophthalmus (Mayan cichlid)	Yucatan Peninsula, Mexico		EU662178	Razo-Mendivil et al. 2008

Oligogonotylus mayae Razo-Mendivil, Rosas-Vaidez & Perez-Ponce de Leon, 2008	Cichlasoma urophthalmus (Mayan cichlid)	Yucatan Peninsula, Mexico	EU662190	Razo-Mendivil et al. 2008
Tabascotrema verai Lamothe-Argumedo & Pineda-López, 1990	Petenia splendida (bay snook)	Belize, Mexico	JX023343	Razo-Mendivil et al. 2015

Anexo 3

Pholeter gastrophilus (Trematoda: Heterophyidae), a parasite of dolphins from Brazilian waters

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Pholeter gastrophilus (Trematoda: Heterophyidae), a parasite of dolphins from Brazilian waters

Abstract

This is the first confirmed report of *Pholeter gastrophilus* infecting three delphinid species from Brazilian waters, *Steno bredanensis*, *Tursiops truncatus*, and *Feresa attenuata*. The worms were identified based on morphological and molecular data using the 28S rDNA and COI mtDNA genes. The present study increases the distribution area of this trematode species and provides new molecular data to be used in future phylogenetic studies of the group, thus contributing to scientific knowledge on marine parasites.

Keywords: Digenea, gastric parasite, *Feresa attenuata*, *Steno bredanensis*, *Tursiops truncatus*, 28S rDNA gene, COI mtDNA gene, southwestern Atlantic Ocean

Ethical standards Procedures performed in this study were in accordance with the ethical standards of the institutions in which they were conducted. Permission to collect and transport deceived stranded dolphins was given by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) under registration 11980-1 (SISBIO) and 5820094 (ABIO).

Introduction

Pholeter gastrophilus (Kossack, 1910) Odhner, 1914 (Heterophyidae Leiper, 1909) is a generalist and geographically widespread gastric digenean exclusive to cetaceans (Pearson and Courtney 1977; Pearson 2008). The adult worms are found encysted within submucosal fibrotic nodules in the wall of glandular chambers of the stomach of cetaceans (Woodard et al. 1969; Migaki et al. 1971), which can contain a variable number of individuals, but most commonly two or three (Pons-Bordas et al. 2021).

The species has been reported in at least 23 species of oceanic and coastal dolphins and porpoises (Conti and Frolic 1894; Aznar et al. 1992; Fraija-Fernández et al. 2016; Pons-Bordas et al. 2021) from the Mediterranean, Black, North, and Red seas, as well as from the Pacific (Peruvian coast and South Australia) and Atlantic oceans (west coast of the USA, Canada, and Argentina) (Fraija-Fernández et al.

2016, 2017 and references therein; Conti and Frolic 1894). Despite its wide geographic distribution, Fraija-Fernández et al. (2017), after an extensive survey to gather all registers of *P. gastrophilus*, considered the species virtually absent in Brazilian waters, due to lack of information from this particular region.

Here we provide the first confirmed case of *P. gastrophilus* infecting delphinids from the Brazilian coast. The new records extend the knowledge on the distribution patterns of this digenean. Additionally, we provide new molecular data (28S rDNA gene and COI mtDNA gene) from *P. gastrophilus* that should be used as a complement in future phylogenetic studies for the species and the Heterophyidae in general, thus contributing to helminth scientific knowledge.

Material and methods

We analyzed the stomachs of 37 dolphins (14 franciscana dolphins *Pontoporia blainvillei* (Gervais & D' Orbigny, 1844), 11 Guiana dolphins *Sotalia guianensis* (Van Bénedén, 1864), five bottlenose dolphins *Tursiops truncatus* (Montagu, 1821), two rough-toothed dolphins *Steno bredanensis* Lesson, 1828, one pygmy killer whale *Feresa attenuata* (Gray, 1875), one Atlantic spotted dolphin *Stenella frontalis* (Cuvier, 1829), and three *Stenella* sp. Gray, 1866), which were found dead and washed ashore at the southeastern and southern Brazilian coast between July 2016 and October 2018. The carcasses were in moderate (code 3) to advanced decomposition (code 4), according to Geraci and Lounsburry (2005) decomposition codes for cetaceans. During necropsy, the gastrointestinal tract of the dolphins was removed and stored at -20 °C until further examination. The worms were collected from the stomach of only three of the analyzed dolphins: one *S. bredanensis* (2.60 m long, adult, male) from the municipality of São Francisco do Sul, Santa Catarina State (26°09'43.0" S, 48°34'01.6" W), one *T. truncatus* (adult, 1.95 m long, female) and one *F. attenuata* (2.35 m long, adult, male), both from the municipality of Cananéia, São Paulo State (25°00'57.2" S, 47°55'31.0" W). During necropsy, the stomachs of all dolphins were removed and washed. The mucosa of all three chambers of the stomach of the cetaceans was examined macroscopically to search for fibrotic nodules which contained the worms. Each nodule was gently opened with scissors to remove the parasites. The collected worms were cleaned in tap water to remove debris and then transferred directly to 70% ethanol until further morphological and molecular analyses.

The worms were stained with chloride carmine, dehydrated in a graded ethanol series, 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 parasites were identified based on specific references (Dawes 1968; Pearson and Courtney 1977; Pearson 2008). Voucher specimens were deposited at the Helminthological Collection of the Institute of Biosciences (CHIBB), UNESP, Botucatu, São Paulo state, Brazil, under numbers 635L – 637L and 9199. The access to the genetic data was authorized by the Brazilian Ministry of Environment (Sisgen A69E199).

The genomic DNA of the worms was extracted using the DNeasy® Blood and Tissue Kit (Qiagen). Fragments of the 28S rDNA and the COI mtDNA genes were amplified using Pure TaqTM Ready-to-GoTM PCR beads (GE Healthcare). The primers used for amplification were: 28S rDNA, LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3') and 1500R (5'-GCTATCCTGAGGGAAACTTCG-3') (Olson et al. 2003); COI mtDNA, JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') (Morgan and Blair 1998) and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-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 mtDNA 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 products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced on the BigDye v.3.1 Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were read with an ABI 3730xl Genetic Analyzer (Applied Biosystems). Forward and reverse sequences were assembled and edited using Sequencher v. 5.2.4.

The new 28S rDNA and the COI mtDNA consensus sequences were aligned separately with sequences belonging to members of closely related families recovered from the GenBank database (Table 1), using the MUSCLE algorithm implemented on Geneious v. 7.1.3 program, with default settings. The presence of stop codons and indels for the COI mtDNA alignment was also verified on Geneious 7.1.3.

Phylogenetic relationships were inferred under Bayesian Inference (BI) and Maximum-likelihood (ML), applying the model GTR + I + G, which was selected as the

best-fitting model of nucleotide evolution by jModelTest v.2.1 for both datasets. The BIs were run on the Cipres Science Gateway using MrBayes v.3.2.7a program on the XSEDE tool. The Markov chain Monte Carlo (MCMC) chains were run with 106 generations and sampling tree topologies every 100th generations. The first 25% of trees were discarded as burn-in and the remaining trees were used for calculating the Bayesian posterior probabilities. The ML analyses were also estimated on the Cipres Science Gateway using RAxML-HPC v.8 on the XSEDE tool, with 1,000 bootstrap replications.

Results

All worms were collected from inside fibrotic nodules in the wall of the glandular chamber of the stomachs of the cetaceans (Fig. 1). Each nodule contained at most two worms. A total of 27 adult digeneans were collected; *T. truncatus* was infected with two worms, *S. bredanensis* was infected with 19 worms, and *F. attenuata* with six worms.

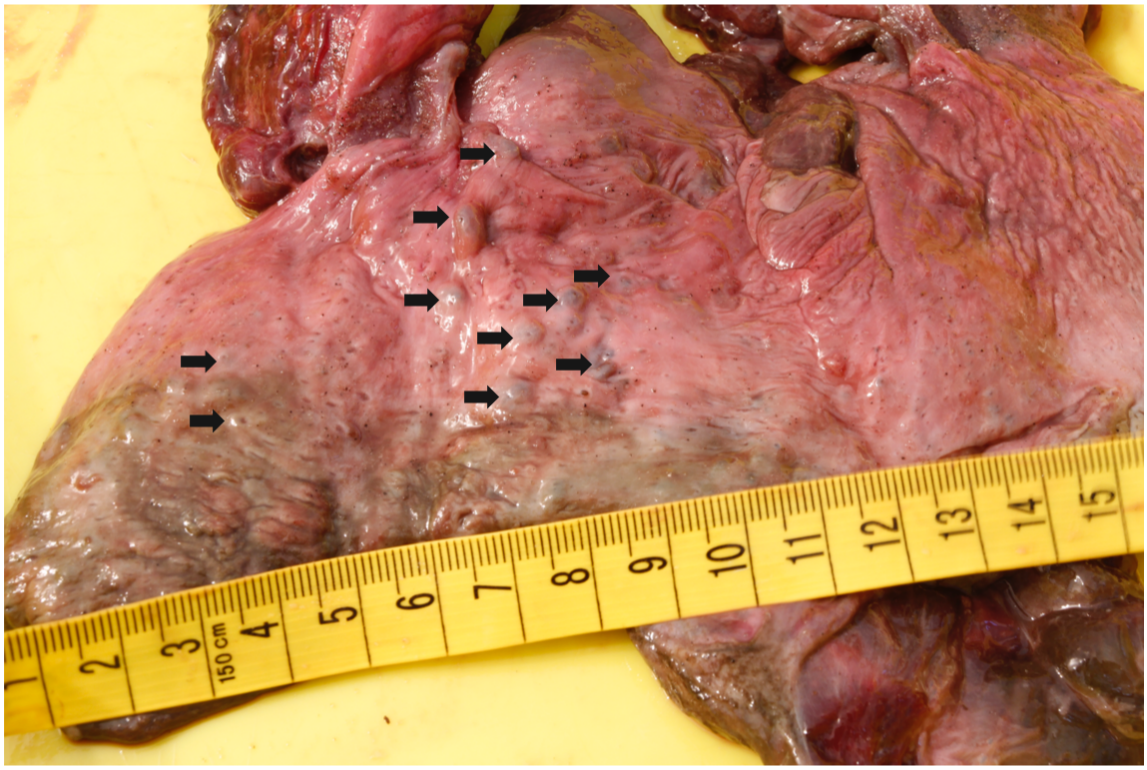


Fig. 1 Fibrotic nodules (arrows) containing adult specimens of *Pholeter gastrophilus* in the wall of the glandular chamber of the stomachs of *Steno bredanensis* from Brazil. Scale presented in centimeters

Based on the observed morphological features and morphometric analyses, we have confirmed our worms as *P. gastrophilus* (Fig. 2). The main morphological characteristics considered to identify the species were: caeca extending to the level of the middle of testes, laterodorsal vitelline follicles arranged in rosettes extending from the middle of testes forwarding to a point half-way between the bifurcation of caeca and the ventral sucker, entire elliptical testes in opposite, deeply lobed ovary, and coils of uterus positioned ventrally to the caeca extending from the level of ovary, or of testes laterally, extending beyond ventral sucker. The tegumental and circumoral spines were not seen, which were probably detached during the process of collection of the worms.



Fig. 2 Adult specimen of *Pholeter gastrophilus* collected from *Steno bredanensis* from southern Brazilian coast

We successfully extracted genomic DNA from three adult worms; two specimens from *S. bredanensis* and one specimen from *F. attenuata*. We failed to obtain good quality DNA from worms collected from *T. truncatus*, probably because of the poor preservation of the specimens. A total of four new sequences were obtained; two of the 28S rDNA gene from *P. gastrophilus* from *S. bredanensis* (MZ165007; MZ169920); and two of the COI mtDNA from *P. gastrophilus* from *S. bredanensis* (MZ165033) and *F. attenuata* (MZ165034). The 28S rDNA and COI mtDNA final alignments included 39 sequences and 1,111 positions and 45 sequences and 323 positions, respectively.

The BI and the ML analyses of the 28S rDNA alignment yielded identical topologies with most clades well supported. In the 28S rDNA phylogenetic reconstruction (Fig. 3) our newly generated sequences clustered in a highly supported monophyletic clade with the other *P. gastrophilus* sequence recovered from *T. truncatus* from the western Mediterranean Sea (KM258674), thus confirming its specific identity. The sequence of *Phocitrema fusiforme* Goto & Ozaki, 1930 (MG806921) collected from *Callorhinus ursinus* (Linnaeus, 1758) from Alaska was recovered as their sister taxa.

For the COI alignment, the BI and the ML phylogenetic reconstructions (Fig. 4) recovered slightly different topologies, mostly related to the positioning of the sequences of genera *Stellantchasmus* Onji & Nishio, 1916 and *Haplorchis* Looss, 1899. The new sequences of *P. gastrophilus* clustered together with the other available sequences from the species which were included in the study in a highly supported clade, also confirming its specific identity; however, its position inside Heterophyidae differed in each phylogenetic analyses; in the ML analyses *P. gastrophilus* was recovered as sister taxa to *Ascocotyle* Looss, 1899 + *Pygidiopsis* Looss, 1907 clade, corroborating with the 28S rDNA topology; as for the BI, the analyses recovered *P. gastrophilus* as sister taxa to *Ascocotyle* + *Pygidiopsis* + *Stellantchasmus* clade. Nevertheless, both hypotheses presented unsupported values of bootstrap and posterior probability.

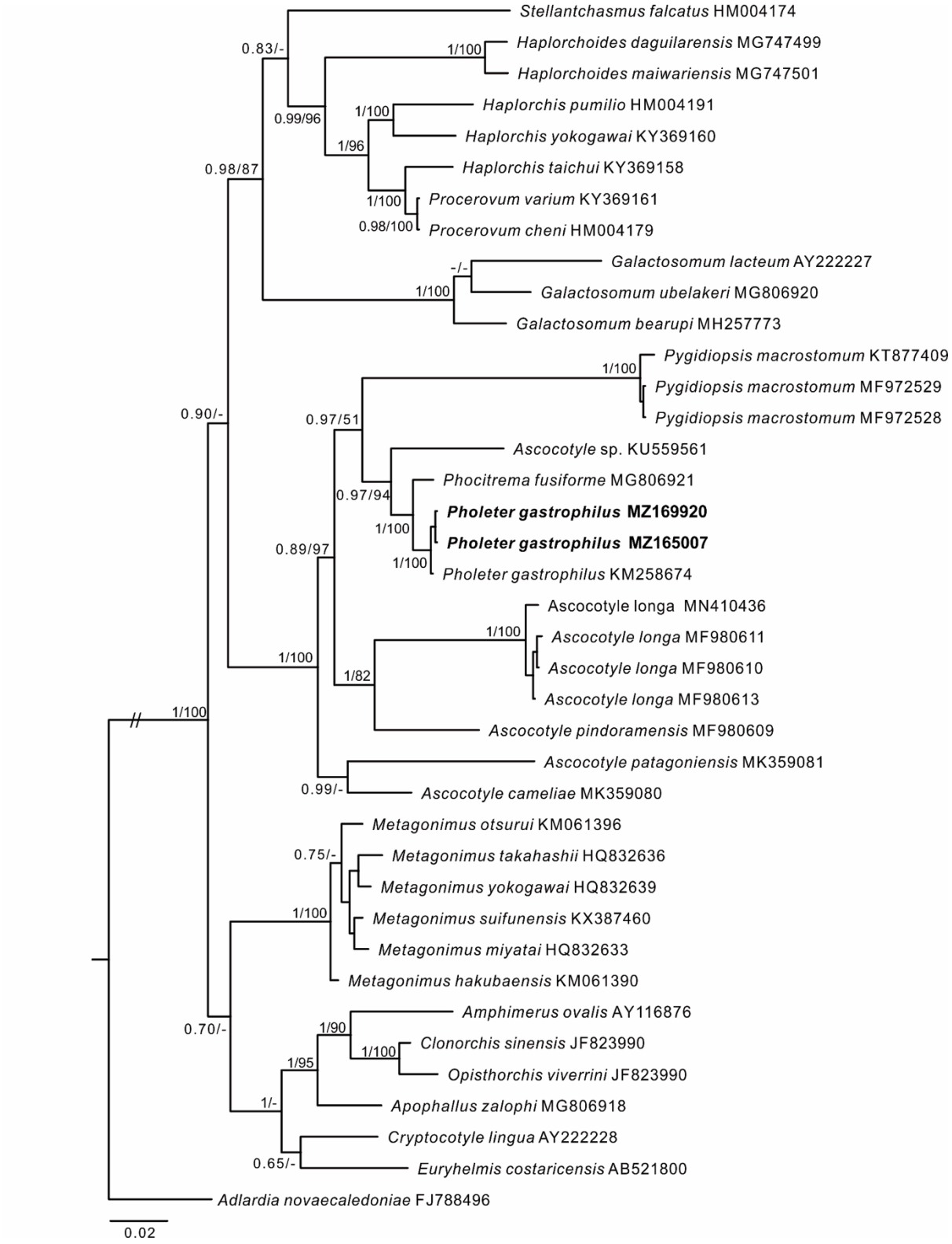


Fig. 3 Bayesian phylogenetic topology of Heterophyidae and closely related families constructed with the 28S rDNA gene dataset. Support values at branching points are shown as Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses. Branch length scale bar indicates mean number of substitutions per site. New sequences are highlighted in bold

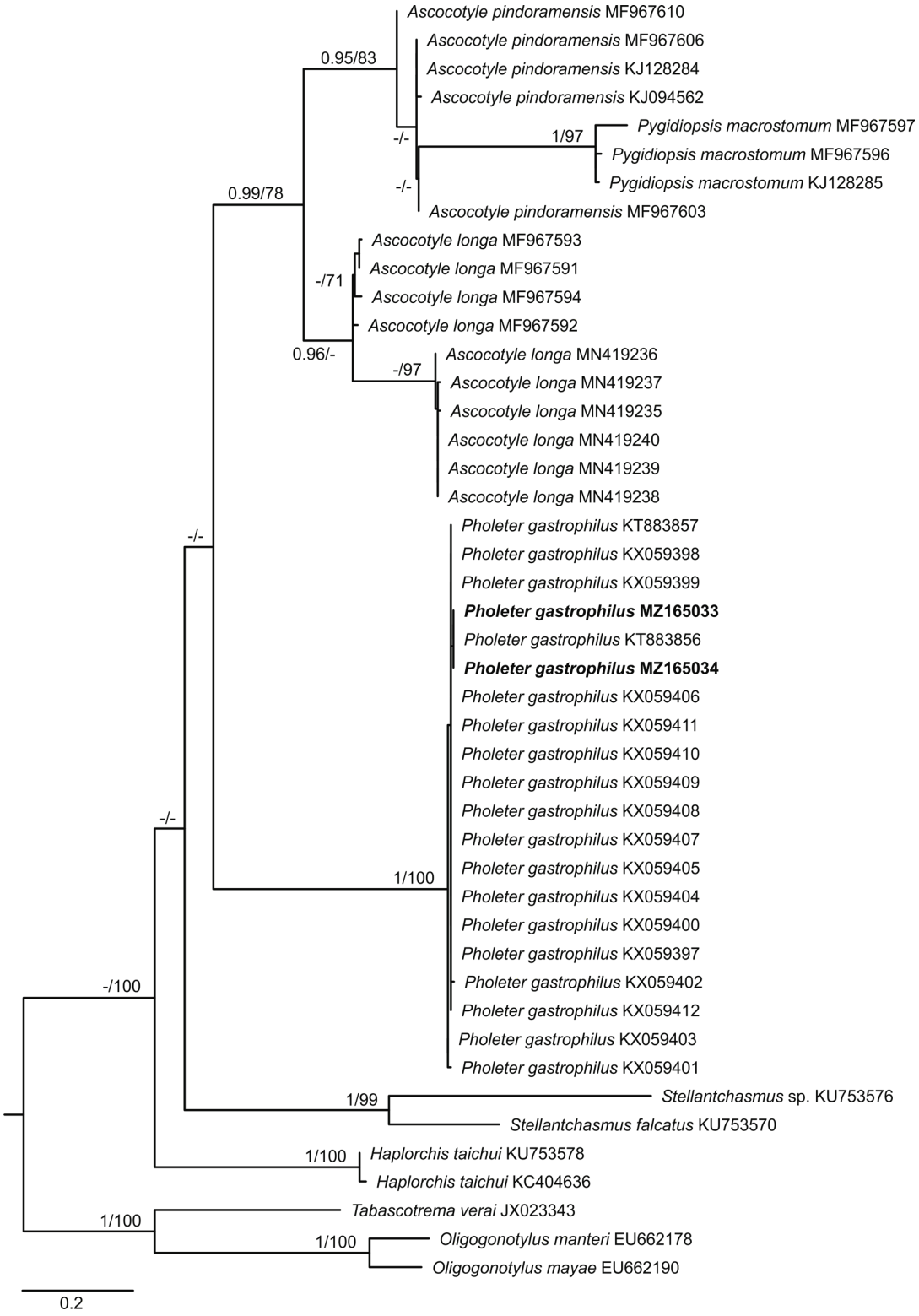


Fig. 4 Maximum-Likelihood phylogenetic topology based on partial COI mtDNA sequences of Heterophyidae. Support values at branching points are shown as Bayesian posterior probabilities followed by Maximum-likelihood bootstraps. Dashes are shown for branches not supported by the analyses. Branch length scale bar indicates mean number of substitutions per site. New sequences are highlighted in bold

Discussion

As a generalist and cosmopolitan species, *P. gastrophilus* have been recorded in at least 23 cetacean species inhabiting coastal, oceanic, and even freshwater habitats from the Mediterranean, Black, North and Red seas, the Pacific (Peruvian coast and South Australia), as well as the North (USA, Canada, Canary Islands, and Britain), and South (Argentina) Atlantic oceans (Conti and Frolic 1894; Fraija-Fernández et al. 2016, 2017 and references therein; Pons-Bordas et al. 2021). Recently, Groch et al. (2018) found numerous trematode eggs which they attributed as belonging to *P. gastrophilus*, however without any other information on parasitological analyses, in the pyloric stomach of *S. guianensis* from southeastern Brazil. Nevertheless, Valente and Ebert (2014) recorded the species in *S. bredanensis* also from southeastern Brazil, however no morphological or molecular data were presented. Therefore, this is the first confirmed report of molecular and morphologically identified adult *P. gastrophilus* worms infecting three delphinid species from Brazilian waters, *S. bredanensis*, *T. truncatus*, and *F. attenuata*.

The genus *Pholeter* contains only two valid species: *P. gastrophilus*, which is exclusive to cetaceans, and *Pholeter anterouterus* (Fischthal & Nasir, 1974), which is found encysted in the intestinal wall of fish-eating birds such as pelicans (Pearson and Courtney 1977; Pearson 2008). Among other differences between the two species, those considered taxonomically significant are: the caeca reaching up to the anterior border of the testes in *P. gastrophilus* versus the caeca not reaching the area of the testes in *P. anterouterus*; the uterus positioned ventrally to the caeca in *P. gastrophilus* versus the uterus positioned dorsally to the caeca and exceeding the bifurcation of the caeca anteriorly in *P. anterouterus*; testes subterminal in *P. gastrophilus* versus testes terminal in *P. anterouterus*; and a single row of 28 circumoral spines in *P. gastrophilus* versus two rows of circumoral spines, with about 23 spines in each row in *P. anterouterus* (Pearson and Courtney 1977; Pearson 2008). The combinations of these morphological characteristics enabled us to assign the specimens found in this study as *P. gastrophilus*, despite we could not visualize the circumoral spines in our specimens, which were probably detached.

In our study, we found a low prevalence of infected hosts and intensity of infection. This pattern seems to be common, at least, in cetaceans from South Atlantic. In Argentinian Patagonia, *P. gastrophilus* was also found in low prevalence infecting *P. blainvillei*, *Delphinus delphis* Linnaeus, 1758, *Lagenorhynchus obscurus* (Gray,

1828), and *T. truncatus* (Aznar et al. 1994; Dans et al. 1999; Berón-Vera et al. 2007; Romero et al. 2014). By contrast, in the Mediterranean and the North seas, it seems to be more frequently registered in higher numbers (Raga and Carbonell 1985; Rogan et al. 1997; Connaglia et al. 2000; Aznar et al. 2006). Further studies should be made to investigate rather this parasite is a common species in the South Atlantic.

Our phylogenetic analyses using 28S rDNA and COI mtDNA genes recovered *P. gastrophilus* as a member of Heterophyidae and clustered all sequences of the species in monophyletic clades, thus corroborating its current taxonomic assignation. However, we recovered divergent topologies when comparing our results using the 28SrDNA and COI mtDNA (see Figs. 2 and 3). Nevertheless, we believe that this inconsistency may be due to the lack of available COI mtDNA sequences for the Heterophyidae, which may cause disrupting results regarding the phylogenetic position of genera. This problem may be overcome with the inclusion of new sequences from more genera in future analyses. Therefore, our new molecular data serve as preliminary information that should be accessed in future studies to help unravel phylogenetic relationships in Heterophyidae and in the genus *Pholeter*, thus contributing to the scientific knowledge of digeneans as a whole.

Besides several studies regarding gastric parasites of cetaceans have already been made in Brazil, none of them have ever mentioned *P. gastrophilus*. The fibrotic nodules in which *P. gastrophilus* are found can be detected through a careful visual and tactile screening of the cetacean's stomach wall. Most nodules are prominent and better identifiable; however, some of them, especially the small ones, can be confounded and easily overlooked during parasitological examinations, unless done by careful specialists. We draw attention here to the importance of systematized routine parasitological surveys in marine mammals to overcome this situation.

Here we extend the knowledge on the distribution patterns of *P. gastrophilus* by adding new information which will contribute to further analyses regarding host-parasite relationships, especially in the marine environment.

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Table 1. 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 and the GenBank accession numbers (28S rDNA and COI mtDNA)

Digenean species	Host species	Locality	Accession numbers		References
			28S rDNA	COI mtDNA	
Heterophyidae Leiper, 1909					
Amphimerus ovalis Barker, 1911	*Trionyx muticus Lesueur 1827* (river turtle)	Mississippi, USA	AY116876		Olson et al. 2003
Apophallus zalophi Price, 1932	*Callorhinus ursinus* (Linnaeus, 1758) (northern fur seal)	St. Paul Island, Alaska, USA	MG806918		Kuzmina et al. 2018
Ascocotyle cameliae Hernández-Orts et al. 2019	*Spheniscus magellanicus* (Forster, 1781) (magellanic penguin)	San Matias Gulf, Argentina	MK359080		Hernández-Orts et al. 2019
Ascocotyle longa Ransom, 1920	*Steno bredanensis* Lesson, 1828 (rought-theeth dolphin)	Santa Catarina, Brazil	MN410436	MN419235; MN419239	Ebert et al. 2020
	Tursiops truncatus (Montagu, 1821) (bottlenose dolphin)	Santa Catarina, Brazil		MN419238; MN419240	Ebert et al. 2020
	Otaria byronia (de Blainville, 1820) (Patagonian sea lion)	Rio Grande do Sul, Brazil		MN419236-7	Ebert et al. 2020
	Mesocricetus auratus Waterhouse, 1839 (golden hamster)	Brazil	MF980610-1-3	MF967591-4	Santos and Borges 2020
Ascocotyle patagoniensis Hernández-Orts et al. (2012)	*S. magellanicus* (magellanic penguin)	San Matias Gulf, Argentina	MK359081		Hernández-Orts et al. 2019
Ascocotyle pindoramensis (Travassos, 1928)	*M. auratus* (golden hamster)	Brazil	MF980609; MF980223	MF967603-6-10	Santos and Borges 2020
	M. auratus (golden hamster)	Brazil		KJ128284; KJ094562	Borges et al. 2017
Ascocotyle sp.	*Chelon labrosus* (Risso, 1827) (grey mullet)	Sardinia, Italy	KU559561		Masala et al. 2016
Cryptocotyle lingua (Creplin, 1825)	*Littorina littorea* (Linnaeus, 1758) (sea snail)	Germany	AY222228		Olson et al. 2003
Euryhelmis costaricensis Brenes, Arroyo & Jiménez-Quirós, 1960	*Martes melampus* (Wagner, 1841) (Japanese marten)	Japan	AB521800		Sato et al. 2010

Galactosomum bearupi Pearson, 1973	*Clypeomorus batillariaeformis* Habe & Kosuge, 1966 (necklace cerith)	Heron Island, Australia	MH257773		Huston et al. 2018
Galactosomum ubelakeri Dailey, 1969	*C. ursinus* (northern fur seal)	St. Paul Island, Alaska, USA	MG806920		Kuzmina et al., 2018
Galactosomum lacteum (Jägerskiöld, 1896)	*Phalacrocorax carbo* (Linnaeus, 1758) (great cormorant)	Ukraine	AY222227		Olson et al. 2003
Haplorchis pumilio Looss, 1896	*Trichogaster trichopterus* Pallas, 1770 (three-spotted gourami)	Thailand	HM004191		Thaenkham et al. 2010
Haplorchis taichui (Nishigori, 1924)	*Homo sapiens* Linnaeus, 1758 (Human)	Vietnam	KY369158		Le et al. 2017
	hamster	Thailand		KU753578	Wongsawad et al. 2017
	Snail	Thailand		KC404636	Chontananarth et al. (Unpublished)
Haplorchis yokogawai (Katsuta, 1932)	Snail	Vietnam	KY369160		Le et al. 2017
Haplorchoides daguilarensis Hostettler et al. 2018	*Neoarius graeffei* (Kner & Steindachner, 1867) (australian salmon-tailed catfish)	Queensland, Australia	MG747499		Hostettler et al. 2018
Haplorchoides maiwariensis Hostettler et al. 2018	*N. graeffei* (australian salmon-tailed catfish)	Queensland, Australia	MG747501		Hostettler et al. 2018
Metagonimus hakubaensis Shimazu, 1999	*Lethenteron reissneri* (Dybowski, 1869) (eastern lamprey)	Japan	KM061390		Pornruseetairatn et al. 2015
*Metagonimus miyatai S*aito et al. 1997	*Plecoglossus altivelis* (Temminck & Schlegel, 1846) (ayu fish)	Miyakoda River, Japan	HQ832633		Pornruseetairatn et al. 2015
Metagonimus otsurui Saito & Hori, 1962	*Rhinogobius flumineus* (Mizuno, 1960) (japanese goby)	Japan	KM061396		Pornruseetairatn et al. 2015
Metagonimus suifunensis Shumenko et al. 2017	*Koreoleptoxis tegulata* (Martens, 1894) (snail)	Komarovka, Russia	KX387460		Shumenko et al. 2017
Metagonimus takahashii Takahashi, 1929	*Carassius auratus langsdorfii* (ginbuna)	Japan	HQ832636		Pornruseetairatn et al. 2015

Metagonimus yokogawai (Katsurada, 1912)	*P. altivelis* (ayu fish)	Tenryu River, Japan	HQ832639		Pornruseetairatn et al. 2015
Phocitrema fusiforme Goto & Ozaki, 1930	*C. ursinus* (northern fur seal)	St. Paul Island, Alaska, USA	MG806921		Kuzmina et al. 2018
***Pholeter gastrophilus* (Kossack, 1910)**	***Feresa attenuata* Gray, 1874 (pigmy killer whale)**	**São Paulo, Brazil**		**MZ165034**	**This study**
	***Steno bredanensis* Lesson, 1828 (rought-theeth dolphin)**	**Santa Catarina, Brazil**	**MZ165007; MZ169920**	**MZ165033**	**This study**
	T. truncatus (bottlenose dolphin)	Spain	KM258674	KT883856-7, KX059397-8, KX059400-12	Fraija-Fernández et al. 2015
Procerovum cheni Hsu, 1950	*Anabas* testudineus (Bloch, 1792) (climbing perch)	Thailand	HM004179		Thaenkham et al. 2010
Procerovum varium Onji & Nishio, 1916	*H. sapiens* (Human)	Vietnam	KY369161		Le et al. 2017
Pygidiopsis macrostomum Travassos, 1928	*M. auratus* (golden hamster)	Brazil	KT877409	KJ128285	Borges et al. 2017
	M. auratus (golden hamster)	Brazil	MF972528-9	MF967596-7	Santos and Borges 2020
Stellantchasmus falcatus Onji & Nishio, 1916	*H. sapiens* (Human)	Vietnam	HM004174		Thaenkham et al. 2010
	hamster	Thailand		KU753570	Wongsawad et al. 2017
Stellantchasmus sp.	hamster	Thailand		KU753576	Wongsawad et al. 2017
Opisthorchiidae Looss, 1899					
Clonorchis sinensis Looss, 1907	*H. sapiens* (Human)	Vietnam	JF823989		Thaenkham et al. (Unpublished)
Opisthorchis viverrini Poirier, 1886	*H. sapiens* (Human)	Thailand	JF823990		Thaenkham et al. (Unpublished)
Cryptogonimidae Ward, 1917					
Adlardia novaecaledoniae Miller et al. 2009	*Nemipterus furcosus* (Valenciennes, 1830) (rosy threadfin bream)	New Caledonia	FJ788496		Bray et al. 2009

Oligogonotylus manteri Watson, 1976	*Cichlasoma urophthalmus* (Günther, 1862) (Mayan cichlid)	Yucatan Peninsula, Mexico	EU662178	Razo-Mendivil et al. 2008
Oligogonotylus mayae Razo-Mendivil et al. 2008	*C. urophthalmus* (Mayan cichlid)	Yucatan Peninsula, Mexico	EU662190	Razo-Mendivil et al. 2008
Tabascotrema verai Lamothe-Argumedo & Pineda-López, 1990	*Petenia splendida* Günther, 1862 (bay snook)	Belize, Mexico	JX023343	Razo-Mendivil et al. 2015

Considerações finais

CONSIDERAÇÕES FINAIS

A partir dos resultados obtidos neste estudo nota-se que a helmintofauna de odontocetos no Brasil é ainda subestimada. Estudos parasitológicos em cetáceos ainda são escassos, principalmente na costa brasileira, devido a falta de incentivo e conhecimento dos pesquisadores a respeito destes organismos.

Neste trabalho, as informações e análises morfológicas e moleculares dos espécimes encontrados permitiram discussões importantes a respeito da filogenia, evolução e distribuição de parasitos de cetáceos. A identificação correta bem como estudos agregando dados ecológicos, biológicos e moleculares destes parasitos e seus hospedeiros contribuem para o conhecimento não somente da mortalidade destes animais, mas também de aspectos evolutivos e demográficos de ambos.

De maneira geral, este trabalho aumentou o conhecimento acerca das relações dos parasitos com seus hospedeiros através do levantamento da biodiversidade e da identificação das espécies de parasitos encontrados em cetáceos de diferentes espécies do litoral brasileiro. Neste estudo foram identificadas cinco novas espécies de *Synthesium*. Além disso, o encontro de algumas espécies de parasitos representam novos registros para o país e ampliam a ocorrência de espécies de parasitos para novos hospedeiros. Os dados aqui agrupados irão constituir uma linha de base para futuras pesquisas sobre diversidade de parasitos em cetáceos, além de incentivar pesquisadores a uma maior conscientização sobre a importância biológica da helmintofauna de cetáceos.