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A new *Synthesium* species (Digenea: Brachycladiidae) from the bottlenose dolphin *Tursiops truncatus* (Cetacea: Delphinidae) in Southwestern Atlantic waters

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Abstract A new species of *Synthesium* from the bottlenose dolphin *Tursiops truncatus* in South Brazilian waters is described. Morphological and molecular identification was performed, and phylogenetic analyses were carried out using the ribosomal small subunit and internal transcribed spacer 1 and the mitochondrial NDH dehydrogenase subunit 3 and cytochrome c oxidase subunit 1 genes. The main characteristics of the new species are the subterminal round-shaped oral sucker, the anterior distribution of vitellaria reaching the level of the ovary and the oval-shaped testes. The results obtained with the molecular markers supported the inclusion of the specimens into the genus *Synthesium*. The nucleotide divergence detected for the mitochondrial genes among the new species and others of the same genus supported the erection of a new species. This is the ninth species assigned to the genus and the third *Synthesium* species recorded in the South Atlantic Ocean.

Keywords *Tursiops truncatus* · Brachycladiidae ·
Synthesium · Morphology · Molecular identification · South
Atlantic Ocean

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Introduction

The bottlenose dolphin *Tursiops truncatus* Montagu, 1821, is a cosmopolitan species found primarily in coastal and inshore regions in tropical and temperate oceans worldwide (Jefferson et al. 1993). This species is distributed along the Brazilian coast, in the Southwestern Atlantic Ocean (SWA), inhabiting a variety of natural environments (Pinedo et al. 1992). In southern Brazil, bottlenose dolphins are observed in coastal waters, bays, and offshore of the Santa Catarina and Rio Grande do Sul states (Cremer et al. 2009; Fruet et al. 2014).

The helminth fauna associated with *T. truncatus* has been surveyed in several geographical regions, mostly in the Northern Hemisphere (Dailey 1976; Raga et al. 1985; Fernández et al. 1994; Aznar et al. 2006, 2007; Kuwamura et al. 2007; Fauquier et al. 2009; Quiñones et al. 2013), in the Caribbean Sea (Mignucci-Giannoni et al. 1998; Colón-Llavina et al. 2009; Oliveira et al. 2011), and scarcely in the South Atlantic (Tomo et al. 2010; Romero et al. 2014). Some studies regarding the diversity and composition of the helminth communities of different odontocete species, including bottlenose dolphins, are also recorded in Brazilian waters (Santos et al. 1996; Marigo et al. 2008, 2011; Carvalho et al. 2010).

In this study, we describe a new species of the genus *Synthesium* Stunkard & Alvey, 1930, family Brachycladiidae Odhner, 1905, collected from bottlenose dolphins *T. truncatus* off the Brazilian coast, SWA. The diagnosis is based on morphological and molecular analyses.

Material and methods

Parasite sampling

The small intestines of three adult *T. truncatus*, two males and one female, stranded at the São Francisco do Sul district, Santa Catarina state, southern Brazil (26° 14′ 36″ S, 48° 38′ 17″ W), between 2012 and 2014, were analyzed. Permission to collect and transport dead stranded dolphins was given by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA/SISBIO) under registration 11980-1. During the necropsy, the gastrointestinal tracts were removed and stored at −20 °C for later examination. After thawing, the intestines were cut open and washed in tap water over a 150-µm sieve and the contents examined under a stereo microscope. A total of 1174 trematodes were recovered, cleaned in tap water, and then fixed and preserved in 70% ethanol for both morphological and molecular analyses.

Morphological examination and morphometric analyses

Thirty-five trematode specimens were studied by light microscopy. The worms were stained with chloridric carmine, dehydrated in a graded ethanol series, cleared with eugenol, and mounted as temporary preparations. Morphometric analyses were done according to Fernández et al. (1995) in a computerized system for image analysis (Qwin Lite 3.1, Leica Microsystems, Wetzlar, Germany). Drawings were made with the aid of a camera lucida. Three additional specimens were also histologically analyzed. The helminthes were placed in 2-hydroxyethyl-methacrylate (7022 18500 Leica historesin embedding kit). Transverse and longitudinal serial sections with a 4 µm thickness were made (Microtome Leica, model RM2165). These sections were stained with hematoxylin-eosin (HE).

Molecular characterization

Molecular analyses were conducted on five specimens collected from three stranded *T. truncatus* in Southern Brazil. Additionally, we obtained DNA from three specimens identified as *Synthesium tursionis* (Marchi, 1873) Stunkard & Alvey, 1930, collected from the small intestines of an adult Guiana dolphin *Sotalia guianensis* (Van Bénéden, 1864) stranded on the São Paulo coast of Brazil in 2012 and two specimens identified as *Synthesium delamurei* (Raga & Balbuena, 1988) collected from a long-finned pilot whale *Globicephala melas* (Traill, 1809) stranded on the Mediterranean coast of Spain in 2007.

Genomic DNA was extracted from each worm using the DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol in a final volume of 30 µl. DNA fragments were amplified using primers for the ribosomal small subunit (SSU), internal transcribed spacer 1 (ITS1), NDH dehydrogenase subunit 3 (mtND3), and cytochrome c oxidase subunit 1 (mtCOI). The primers and the cycling conditions are shown in Table 1.

PCR amplifications were carried out using 3 to 5 µl of genomic DNA and 1.0 µl (for SSU, mtND3, and mtCOI genes) or 1.25 µl (for ITS1) of each set of primers and Ready-to-Go PCR Beads (PuReTaq™ Ready-to-Go™ PCR Beads, GE Healthcare). The solution consisted of stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of PuReTaq DNA polymerase, and a reaction buffer. With the reconstituted bead to a final volume of 25 µl, the concentration of each dNTP was 200 µM in 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl, and 1.5 mM MgCl₂. Aliquots (3 µl) of individual PCR products were separated by electrophoresis using agarose gels (1%), stained with gel red (1 µl) (Biotium Inc.), and visualized using ultraviolet transillumination. Gel images were captured electronically and analyzed using the program Multi-Analyst (v.1.1, Bio-Rad).

PCR amplicons were purified using a QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. Automated sequencing was directly performed on the purified PCR products using a BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) for cycle sequencing. The sequences were run on an Applied Biosystems ABI 3500 DNA genetic sequencer.

Contiguous sequences from each molecular marker were assembled and edited in Sequencher v. 5.2.4 (Gene Codes, Ann Arbor, MI). All sequences were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov) to confirm their identity. All alignments were made using the MUSCLE software implemented in Geneious version 7.1.3 (Kearse et al. 2012).

Phylogenetic analyses

Genetic divergence between sequences of the SSU, ITS1, mtND3, and mtCOI of species of Brachycladiidae was calculated within the aligned portion of each gene using the Kimura two-parameter distance model (Kimura 1980) in MEGA6 (Tamura et al. 2013). Only sequences of the mtND3 were used for phylogenetic inferences, due to the considerable number of other Brachycladiidae sequences available in GenBank.

Phylogeny was inferred by neighbor-joining (NJ) analyses using MEGA6, Bayesian inference (BI) carried out in BEAST (Drumond et al. 2012), and maximum likelihood (ML) performed with PhyML v3.0 (Guindon et al. 2010). The NJ analyses were performed using the Kimura two-parameter model and 2000 bootstrap replicates. Prior to the ML analyses, the best fitting model of nucleotide substitution was determined based on the Akaike information criteria (AIC) using jModelTest 2.1.1 (Posada 2008) as TPM3uf+I+G. Supports

Table 1 Primers, cycling conditions for PCR, and bibliographic sources

Primer	Sequences 5′–3′	Cycling conditions	Source
SSU	18SF CGTATCTTTCAAATGTCTGCCC 18SR CCGATGACCTTGCTAAACC	Initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 2 min; final extension at 72 °C for 10 min	This study (adapt. from Fernández et al. 1998b)
ITS1	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
COI	COIPRA TGGTTTTTTGTGCATCCTGAGGTTTA COIPRB AGAAGAACGTAATGAAAATG AGCAAC	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
ND3	ND3F GCTT AATTKKTAAAGCYTTGRATTCTT- ACT ND3D CTACTAGTCCCACT CAACRTAACCYT	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

for ML were determined by performing 100 bootstrap replicates. The BI analyses were run with a GTR nucleotide substitution model available in BEAST. BI Markov chain Monte Carlo (MCMC) chains were run for 300 million generations, the log-likelihood scores were plotted, and only the final 70% of the trees were used to produce the consensus tree by setting the "burn-in" parameter at 3×10^7 generations. The phylogenetic trees were generated and edited in FigTree v1.3.1 (Rambaut 2009). The species, hosts, and accession numbers used in this study are summarized in Table 2.

Results

Description of *Synthesium neotropicalis* n. sp.

The observations and measurements were based on 35 whole-mounted and three serially sectioned specimens (see Fig. 1). Measurements (length × width) are shown as the range, with the mean in parentheses followed by the standard deviation, and are expressed in millimeters, unless otherwise stated.

Body elongated, slender, dorsoventrally flattened 11.43–29.61 (22.90 ± 5.42), maximum width behind posterior testes 0.36–0.73 (0.49 ± 0.08). Body spines not observed. Oral sucker subterminal, muscular, round-shaped, slightly oval 0.33–0.74 (0.56 ± 0.09) × 0.25–0.62 (0.44 ± 0.09). Ventral sucker muscular located in posterior region of the first third of body 0.31–0.59 (0.49 ± 0.07) × 0.33–0.61 (0.45 ± 0.09). Oral sucker/ventral sucker length ratio 1:1.14. Distance between suckers 2.32–7.83 (5.12 ± 1.60). Prepharynx variable in

length, mostly long 0.38–2.18 (1.40 ± 0.53). Pharynx pyriform, strongly muscular 0.37–0.81 (0.50 ± 0.09) × 0.13–0.34 (0.20 ± 0.05). Esophagus very short or almost indistinguishable. Intestine H-shaped with anterior caeca reaching medial level of oral sucker and posterior caeca ending blindly close to posterior extremity of body. Uroproct absent. Genital pore preacetabular. Cirrus pouch long 1.84–3.31 (2.65 ± 0.42), extending well beyond ventral sucker, containing large seminal vesicle located at its extremity 0.33–1.07 (0.61 ± 0.20) × 0.10–0.30 (0.21 ± 0.04), pars prostatica and unarmed cirrus. Cirrus pouch opening into genital pore. Ovary round to oval 0.14–0.45 (0.30 ± 0.08) × 0.09–0.30 (0.17 ± 0.05), postacetabular, pretesticular. Mehlis' gland preovarian. Vitelline reservoir ovoid, close to ovary. Laurer's canal not observed. Seminal receptacle absent. Distance between ovary and anterior testes 0.07–1.82 (0.66 ± 0.37). Testes oval-shaped, tandem, with anterior testes situated in posterior region of middle third of body and posterior testes situated in anterior region of posterior third of body. Anterior testes 0.35–0.80 (0.60 ± 0.11) × 0.13–0.37 (0.30 ± 0.05) slightly smaller than posterior testes 0.37–1.06 (0.71 ± 0.15) × 0.14–0.45 (0.30 ± 0.07). Intertesticular distance 0.63–3.19 (1.74 ± 0.61). Distance between posterior testes and extremity of body 2.77–9.12 (6.00 ± 1.67). Vitellaria arranged in acinous bunches, profuse, commencing anteriorly to ovary and extending to posterior extremity of body. Distance between anterior margin of vitellaria and ovary 0.25–2.48 (1.13 ± 3.54). Distance between anterior margin of vitellaria and ventral sucker 2.00–11.80 (4.21 ± 2.42). Distance between anterior margin of vitellaria and extremity of body 5.64–16.10

Table 2 Parasites and host species, GenBank accession numbers, and bibliographic sources of sequences used for phylogenetic analyses

Parasite species (family)	Host species (common name)	GenBank accession no.	Source
SSU rDNA			
Synthesium pontoporiae (Brachycladiidae)	*Pontoporia blainvillei* (Franciscana)	FJ357162	Marigo et al. unpub. data
Synthesium neotropicalis n. sp. (Brachycladiidae)	*Tursiops truncatus* (Bottlenose dolphin)	KY595987	This study
Synthesium tursionis (Brachycladiidae)	*Tursiops truncatus* (Bottlenose dolphin)	FJ357163	Marigo et al. unpub. data
ITS1 rDNA			
Synthesium neotropicalis n. sp. (Brachycladiidae)	*Tursiops truncatus* (Bottlenose dolphin)	KY595988	This study
Synthesium pontoporiae (Brachycladiidae)	*Pontoporia blainvillei* (Franciscana)	JX644084	Marigo et al. 2015
Synthesium tursionis (Brachycladiidae)	*Sotalia guianensis* (Guiana dolphin)	KY595990	This study
ND3 mtDNA			
Brachycladium atlanticum (Brachycladiidae)	*Stenella coeruleoalba* (Striped dolphin)	AF034551	Fernández et al. 1998a
Brachycladium atlanticum (Brachycladiidae)	*Stenella coeruleoalba* (Striped dolphin)	KT180217	Fraija-Fernández et al. 2016
Brachycladium goliath (Brachycladiidae)	*Balaenoptera acutorostrata* (Minke whale)	KR703278	Briscoe et al. 2016
Brachycladium sp. (Brachycladiidae)	*Balaenoptera acutorostrata* (Minke whale)	AF123439	Fernández et al. 2000
Campula oblonga (Brachycladiidae)	*Phocoena phocoena* (Harbor porpoise)	AF34554	Fernández et al. 1998a
Campula oblonga (Brachycladiidae)	*Phocoena phocoena* (Harbor porpoise)	KT180214	Fraija-Fernández et al. 2016
Nasitrema globicephalae (Brachycladiidae)	*Globicephala melas* (Long-finned pilot whale)	AF034557	Fernández et al. 1998a
Nasitrema delphini (Brachycladiidae)	*Delphinus delphis* (Common dolphin)	KT180216	Fraija-Fernández et al. 2016
Oschmarinella rochebruni (Brachycladiidae)	*Stenella coeruleoalba* (Striped dolphin)	KT180215	Fraija-Fernández et al. 2016
Oschmarinella rochebruni (Brachycladiidae)	*Stenella coeruleoalba* (Striped dolphin)	AF034556	Fernández et al. 1998a
Orthosplanchnus fraterculus (Brachycladiidae)	*Enhydra lutris* (Sea Otter)	AF034555	Fernández et al. 1998a
Synthesium pontoporiae (Brachycladiidae)	*Pontoporia blainvillei* (Franciscana)	FJ829472	Marigo et al. 2011
Synthesium neotropicalis n. sp. (Brachycladiidae)	*Tursiops truncatus* (Bottlenose dolphin)	KY612256	This study
Synthesium tursionis (Brachycladiidae)	*Tursiops truncatus* (Bottlenose dolphin)	AF034552	Fernández et al. 1998a
Synthesium delamurei (Brachycladiidae)	*Globicephala melas* (Long-finned pilot whale)	KY612255	This study
Tormopsolus orientalis (Acanthocolpidae)	*Seriola dumerili* (Greater amberjack)	KT180219	Fraija-Fernández et al. 2016
COI mtDNA			
Synthesium neotropicalis n. sp. (Brachycladiidae)	*Tursiops truncatus* (Bottlenose dolphin)	KY612257	This study
Synthesium pontoporiae (Brachycladiidae)	*Pontoporia blainvillei* (Franciscana)	JX644156	Marigo et al. 2015
Synthesium tursionis (Brachycladiidae)	*Sotalia guianensis* (Guiana dolphin)	KY612258	This study

(11.29 ± 2.93). Uterus coils intercecally, widening into un-armed metraterm before opening into genital pore. Eggs oval slightly flattened at opercular pole, triangular in cross section, 47–66 (55) × 28–41 (34) μm.

Taxonomic summary:

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

Site: Small intestine.

Type locality: Babitonga Bay, São Francisco do Sul district, Santa Catarina state, Brazil, South Atlantic Ocean.

Specimens deposited: Holotype and paratypes were deposited at Coleção Helmintológica do Instituto Oswaldo Cruz-Fundação Oswaldo Cruz (CHIOC-Fiocruz) Rio de Janeiro, RJ, Brazil, under numbers 38383a and 38383b, respectively. Paratypes were also deposited at Coleção Helmintológica do Instituto de Biociências de Botucatu-UNESP (CHIB-UNESP), Botucatu, SP, Brazil, under number 7880.

Etymology: The specific epithet "*neotropicalis*" refers to the Neotropical region, location where the new species was first collected.

Remarks

The general morphology of the specimens analyzed allowed its inclusion in the family Brachycladiidae and the subfamily Brachycladiinae Odhner, 1905, according to Gibson (2005). The following features placed the specimens into the genus *Synthesium*: body very elongated, well-developed pyriform pharynx, intestines with anterior and posterior caeca without diverticula, with posterior caeca ending blindly close to
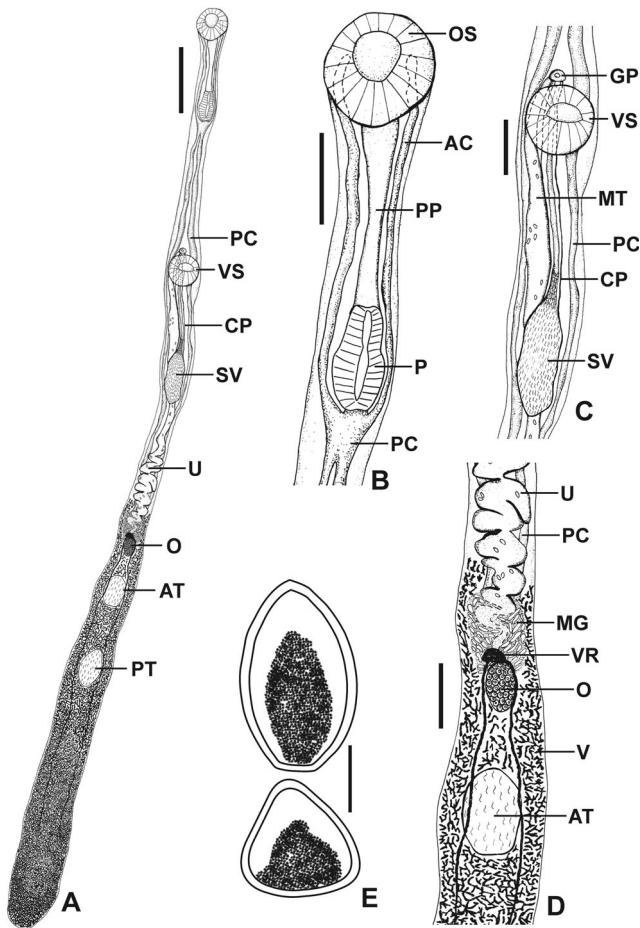
Fig. 1 *Synthesium neotropicalis* n. sp. from the small intestine of *Tursiops truncatus* from Brazil. Ventral view. *A* Whole individual—scale bar = 1 mm. *B* Detail of anterior extremity of body with anterior caeca reaching lateral of oral sucker—scale bar = 200 μm. *C* Detail of cirrus pouch, seminal vesicle, and metraterm—scale bar = 200 μm. *D* Detail of anterior extent of vitellaria, ovary, and anterior testes—scale bar = 200 μm. *E* Detail of eggs—scale bar = 20 μm. Abbreviations: *AC* anterior caeca, *AT* anterior testis, *CP* cirrus pouch, *GP* genital pore, *M* metraterm, *MG* Mehli's gland, *O* ovary, *OS* oral sucker, *P* pharynx, *PC* posterior caeca, *PP* prepharynx, *PT* posterior testes, *SV* seminal vesicle, *U* uterus, *V* vitellaria, *VR* vitelline reservoir, *VS* ventral sucker

posterior extremity of body, ventral sucker in anterior third of the body, presence of cirrus sac, unarmed metraterm, testes in middle third or third quarter of body, and vitellaria entirely in hindbody.

Currently, the genus *Synthesium* includes eight species, the type-species *S. tursionis*, *S. pontoporiae* (Raga, Aznar, Balbuena & Dailey, 1994) Marigo, Vicente, Valente, Measures & Santos, 2008, *S. seymouri* (Price, 1932) Marigo, Vicente, Valente, Measures & Santos, 2008, *S. elongatum* (Osaki, 1935), *S. nipponicum* Yamaguti, 1951, *S. mironovi* (Krotov & Delyamure, 1952), *S. subtile* (Skrjabin, 1959), and *S. delamurei* (see Gibson 2014). The principal morphological features that differentiate *S. neotropicalis* n. sp. from the other *Synthesium* species are a combination of characteristics such as the subterminal round-shaped oral sucker, the

anterior distribution of vitellaria reaching the level of ovary, and the oval-shaped testes.

Synthesium neotropicalis n. sp. differs from *S. seymouri*, *S. nipponicum*, *S. mironovi*, and *S. subtile* in the shape of oral sucker (round versus cup-shaped) and its position (subterminal versus terminal), the morphology of the pharynx (pyriform versus oval), the anterior distribution of vitellaria (at the level of the ovary in *S. neotropicalis* n. sp.), and the egg size (smaller than in the other species). *Synthesium neotropicalis* n. sp. differs from *S. tursionis* because the latter has a terminal cup-shaped oral sucker, lobed testes with shorter distance from the end of the body, and anterior distribution of vitellaria, reaching the seminal vesicle level. *Synthesium neotropicalis* n. sp. can also be distinguished from *Synthesium elongatum* because the latter presents lobe-shaped testes and vitellaria commencing at the seminal vesicle level. The new species resembles *S. pontoporiae* and *S. delamurei* in the shape and position of the oral sucker (round subterminal) and the shape of the testes (oval). However, *S. pontoporiae* shows a wider body size, vitellaria commencing at the seminal vesicle level, and smaller oral and ventral suckers. Finally, *S. delamurei* presents gonads positioned close to the extremity of the body, anterior extent of vitellaria at the seminal vesicle level, and longer and wider testes and eggs compared to the new species. The morphological features differentiating all of the *Synthesium* species are summarized in Table 3.

Phylogenetic analyses

Partial SSU alignment (816 bp) comprised a newly generated sequence from *S. neotropicalis* n. sp. (KY595987) and the sequences from *S. pontoporiae* (FJ357162) and *S. tursionis* (FJ357163) retrieved from GenBank. The partial SSU pairwise distance analysis revealed that the *S. neotropicalis* n. sp., *S. tursionis*, and *S. pontoporiae* sequences included in the alignment are identical.

The ITS1 alignment (534 bp) included the newly generated sequences of *S. neotropicalis* n. sp. (KY595988) and *S. tursionis* (KY595990), and *S. pontoporiae* (JX644084) retrieved from GenBank. The ITS1 pairwise distance analysis showed only one nucleotide (0.2%) of difference between *S. neotropicalis* n. sp. and *S. pontoporiae*. The nucleotide divergence between *S. neotropicalis* n. sp. and *S. tursionis* was also small (four nucleotides, 0.9% of divergence). Additionally, *Synthesium pontoporiae* and *S. tursionis* diverged from each other by three nucleotides (0.7% of divergence).

The mtCOI alignment (406 bp) consisted of newly generated sequences of *S. neotropicalis* n. sp. (KY612257) and *S. tursionis* (KY612258) and a sequence of *S. pontoporiae* (JX644156) retrieved from GenBank. The mtCOI pairwise distance analysis showed a nucleotide divergence between *S. neotropicalis* n. sp. and *S. pontoporiae* of 9.1% (35

Table 3 Mean (range) of morphological measurements of *Synthesium neotropicalis* n. sp. compared to other species of the genus

	S. neotropicalis n. sp.	*S. delamurei*	*S. pontoporiae*	*S. elongatum*	*S. tursionis*	*S. seymouri*	*S. nipponicum*	*S. mironovi*	*S. subtile*
No. of specimens	(n = 35)	(n = 4)	(n = 20)	(n = 2)	(n = 15)	(n = 10)	(n = 10)	(n = 2)	(n = 20)
Host	*T. truncatus*	*G. melas*	*P. blainvillei*	*N. phocoenoides*	*T. truncatus*	*D. leucas*	*P. dalli; P. phocoena*	*D. leucas*	*O. orca; D. leucas; G. melas*
Geographical distribution	Brazil	Mediterranean Sea	Argentina	Japan	Mediterranean Sea	Alaska	Japan, USA (Pacific)	North Pacific	North Atlantic
References	This study	Raga and Balbuena 1988	Raga et al. 1994	Ozaki 1935	Price 1932; Fernández et al. 1994	Price 1932	Yamaguti 1951; Ching and Robinson 1959	Delyamure 1955	Balbuena et al. 1989
Body length	22.9 (11.4–29.6)	12.1 (9.7–16.8)	5.0 (3.6–7.1)	13.0–18.0	14.3 (8.85–21.31)	34.1 (27.2–38.1)	14.0 (13–15.9)	8.9–12.9	33.3 (14.0–38.3)
Max. width	0.5 (0.4–0.7)	0.7 (0.6–0.8)	0.5 (0.3–0.7)	1–2.1	0.6 (0.5–0.8)	0.8 (0.6–1.0)	0.9 (0.8–1.0)	0.7–1.2	1.5 (1.2–2.0)
Oral sucker position	Subterminal	Subterminal	Subterminal	Subterminal	Terminal	Terminal	Terminal	Terminal	Terminal
Oral sucker	0.6 × 0.4 (0.3–0.7 × 0.3–0.6)	0.5 × 0.4 (0.4–0.5 × 0.3–0.5)	0.2 × 0.1 (0.1–0.2 × 0.1–0.2)	0.4 × 0.5	0.6 × 0.5 (0.4–0.7 × 0.4–0.7)	2.0 × 1.6 (1.8–2.6 × 1.3–2.1)	0.8 × 0.6 (0.8–0.9 × 0.6–0.7)	0.3–1.0 × 0.5–1.1	1.5 × 1.8 (1.4–2.2 × 1.2–1.8)
Prepharynx length	1.40 (0.4–2.2)	0.5 (0.3–0.7)	0.2 (0.7–0.3)	0.5–0.5	0.5 (0.2–0.8)	0.8 (0.4–1.3)	0.4 (0.3–0.6)	0.3	0.5 (0.05–1.8)
Pharynx shape	Pyriform	Pyriform	Pyriform	Pyriform	Pyriform	Oval	Oval	–	Oval
Pharynx	0.5 × 0.2 (0.4–0.8 × 0.1–0.3)	0.2 (0.1–0.2)	0.6 × 0.1 (0.1–0.3 × 0.03–0.2)	0.4–0.6 × 0.3–0.3	0.5 × 0.2 (0.5–0.7 × 0.2–0.3)	1.0 × 0.8 (0.9–1.2 × 0.7–1.3)	0.3 × 0.3 (0.3–0.4 × 0.3–0.3)	0.3–0.4 × 0.3–0.4	0.7 × 0.7 (0.5–0.8 × 0.5–0.9)
Ventral sucker position	Anterior 1/3	Anterior 1/3	Anterior 1/3	Anterior 1/3	Anterior 1/3	Anterior 1/3	Anterior 1/3	Anterior 1/3	Anterior 1/3
Ventral sucker	0.5 × 0.4 (0.3–0.6 × 0.3–0.6)	0.6 × 0.5 (0.5–0.8 × 0.4–0.6)	0.3 × 0.3 (0.2–0.4 × 0.2–0.4)	0.7 × 0.5	0.6 × 0.5 (0.4–0.8 × 0.4–0.7)	1.3 × 1.00 (1.1–1.5 × 0.8–1.4)	0.7 × 0.7	0.6–1.0 × 0.6–0.8	0.8 × 0.9 (0.7–1.3 × 0.7–1.1)
Cirrus pouch length	2.6 (1.8 × 3.3)	2.6 (2.0–3.4)	0.9 (0.7–1.1)	0.9–1.5	2.2 (1.4–2.9)	3.2 (1.3–4.1)	1.2 (1.0–1.4)	–	4.4 (2.4–6.1)
Testes shape	Oval	Oval	Oval	Lobed	Lobed	Oval	Oval	Oval	Oval
Ovary	0.3 × 0.2 (0.1–0.4 × 0.1–0.3)	0.3 × 0.2 (0.3–0.4 × 0.1–0.2)	0.2 × 0.1 (0.1–0.3 × 0.06–0.2)	0.3–0.4 × 0.2–0.3	0.2 × 0.2 (0.1–0.3 × 0.1–0.2)	0.3 × 0.2 (0.2–0.4 × 0.1–0.3)	0.22 × 0.22 (0.2–0.3 × 0.2–0.3)	0.1–0.3	0.4 × 0.3 (0.2–0.5 × 0.2–0.4)
Gonads position	Medial 1/3	Posterior 1/3	Medial 1/3	Medial 1/3	Medial 1/3	Medial 1/3	Medial 1/3	Anterior 1/3	Medial 1/3
Anterior testes	0.6 × 0.3 (0.3–0.8 × 0.1–0.4)	1.0 × 0.3 (0.8–1.2 × 0.2–0.4)	0.3 × 0.2 (0.2–0.5 × 0.1–0.2)	–	0.7 × 0.3 (0.5–1.1 × 0.3–0.6)	1.5 × 0.7 (1.3–1.8 × 0.5–1.0)	0.7 × 0.3 (0.6–0.8 × 0.2–0.3)	0.6–0.9 × 0.2–0.3	1.2 × 0.6 (0.7–1.8 × 0.4–0.8)
Posterior testes	0.7 × 0.3 (0.4–1.0 × 0.1–0.4)	1.0 × 0.3 (0.9–1.1 × 0.3–0.4)	0.3 × 0.2 (0.2–0.5 × 0.1–0.3)	–	0.7 × 0.4 (0.5–1.1 × 0.3–0.6)	1.6 × 0.7 (1.3–1.9 × 0.5–0.8)	0.7 × 0.3 (0.6–0.8 × 0.3–0.4)	0.5–1.0 × 0.2–0.5	1.3 × 0.6 (0.8–1.9 × 0.4–0.9)

Table 3 (continued)

	S. neotropicalis n. sp.	S. delamurei	S. pontoporiae	S. elongatum	S. tursionis	S. seymouri	S. nipponicum	S. mironovi	S. subtile
		Seminal vesicle	Seminal vesicle	Uterine field	Seminal vesicle	Anterior testes	Anterior testes	Anterior testes	Seminal vesicle
Anterior extent of vitellaria	Ovary								
Egg size (μm)	55 × 34 (47–68 × 28–41)	67 × 40 (57–72 × 32–50)	53 × 26 (46–60 × 23–28)	47–55 × 25–31	53 × 29 (51–55 × 28–32)	91 × 55 (83–97 × 51–55)	70–82 × 35–45	72–90 × 33–37	89 × 49 (75–98 × 44–55)

Values are given as length × width in millimeters unless otherwise stated

nucleotides) and between *S. neotropicalis* n. sp. and *S. tursionis* of 14.6% (55 nucleotides). The divergence between *S. pontoporiae* and *S. tursionis* was also of 14.6% (55 nucleotides).

The mtND3 alignment (276 bp) included 13 Brachycladiidae sequences retrieved from GenBank, newly generated sequences from *S. neotropicalis* n. sp. (KY612256) and *S. delamurei* (KY612255), and *Tormopsolus orientalis* (KT180219) as an outgroup. The mtND3 pairwise distance analysis revealed that the sequences of *S. neotropicalis* n. sp. differed from *S. pontoporiae* in 14 nucleotides (5.3% of divergence), from *S. tursionis* in 35 nucleotides (14% of divergence), and from *S. delamurei* in 49 nucleotides (20.8% of divergence). The difference between *S. tursionis* and *S. delamurei* was 53 nucleotides (22.4%) and that between *S. pontoporiae* and *S. delamurei* was 51 nucleotides (21.8% of divergence). Additionally, the sequences of *S. pontoporiae* and *S. tursionis* are different in 43 nucleotides (17.8% of divergence). The genetic divergence estimated among Brachycladiidae genera had a mean of 19.6%, ranging from 11.9% (*Brachycladium atlanticum* (Abril, Balbuena & Raga, 1991) Gibson, 2005 versus *Campula oblonga* Cobbold, 1858) to 27.4% (*S. pontoporiae* versus *Nasitrema globicephalae* Neiland, Rice & Holden, 1970).

The mtND3 phylogenetic analyses (Fig. 2) based on the BI, NJ, and ML trees showed identical topologies with most of the nodes well-supported by posterior probability and bootstrap values (≥0.7/70 respectively). In all topologies, *S. neotropicalis* n. sp. and *S. pontoporiae* are sister taxa and cluster with *S. tursionis* in a monophyletic group. However, *S. delamurei* is placed in a different clade apart from all other *Synthesium* species.

Discussion

In the past, the genus *Synthesium* has undergone various taxonomic rearrangements with several synonymies among morphologically closely related genera such as *Leucasiella* Krotov & Delyamure, 1952, *Hadwenius* Price, 1932, and *Odhneriella* Skrjabin, 1915 (see Gibson 2005, 2014). Yamaguti (1958) recognized *Leucasiella* as a synonym of *Hadwenius*. Later, Gibson (2005) considered *Hadwenius* as a synonym of *Synthesium*, based on the priority criteria. The genus *Odhneriella* remains accepted with some species transferred to *Synthesium* (Adams and Rausch 1989; Gibson 2005). *Odhneriella* can be easily distinguished from *Synthesium* due to the presence of a uroproct, large and elongate testes, and a metraterm armed with spines (Gibson 2005).

The presence of spines in the cirrus is a diagnostic characteristic of the genus *Synthesium* (Gibson, 2005). *Synthesium neotropicalis* n. sp. presents all morphological features of the
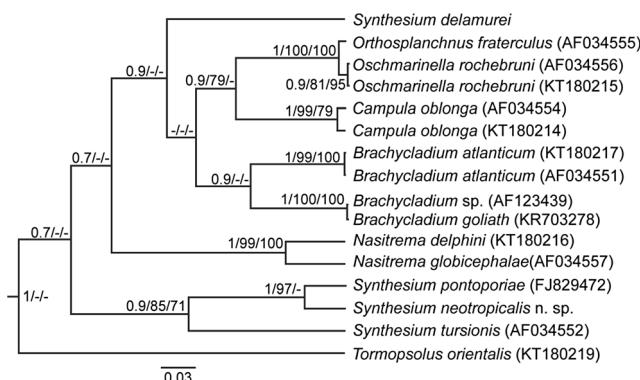
Fig. 2 Bayesian tree based on the mtND3 sequences. Species names are followed by the GenBank accession numbers in parentheses. The support values at the branching points are shown as follows: Bayesian posterior probability/neighbor-joining bootstraps/maximum likelihood bootstraps. *Dashes* are shown for branches not supported by the analyses (scored <70%). The branch length *scale bars* indicate mean number of substitutions per site

genus *Synthesium*, except for an armed cirrus. It is noteworthy that spines can be lost due to poor preservation, but it is unlikely that we have missed the spines or, at least, their basal disks in the cirrus, given the large number of specimens examined herein. Nevertheless, two other *Synthesium* species, namely, *S. delamurei* and *S. mironovi*, are described as possessing an unarmed cirrus. Originally, these species were described as belonging to the genus *Leucasiella* due to the structure of the vitellaria and the lack of spines in the cirrus. Until now, no redescriptions of *S. delamurei* and *S. mironovi* have been done and a morphological reassessment could reveal new observations that are different from their original descriptions. Because the genus *Synthesium* currently aggregates species with both armed and unarmed cirrus, we think that this diagnostic characteristic could not be relevant and should be revised.

In the South Atlantic Ocean, only two *Synthesium* species have been reported parasitizing odontocetes until now: *S. pontoporiae*, hosted by the endemic and endangered dolphin Franciscana *Pontoporia blainvillei* (Gervais & d'Orbigny, 1844) from Argentinian and Brazilian waters (Raga et al. 1994; Silva and Cousin 2004; Marigo et al. 2002, 2008), and *S. tursionis*, found in *S. guianensis* and *T. truncatus* from Brazil (Marigo et al. 2008, 2010) and Argentina (Romero et al. 2014). *Synthesium* spp. have also been reported in the intestine of *Cephalorhynchus commersonii* (Lacépède, 1804), *Lagenorhynchus obscurus* (Gray, 1828), and *Lagenorhynchus cruciger* (Quoy & Gaimad, 1824) off Argentina (Dans et al. 1999; Berón-Vera et al. 2001; Fernández et al. 2003). *Synthesium neotropicalis* n. sp. is the third *Synthesium* species recorded in the South Atlantic Ocean.

Regarding the molecular analyses, the sequences of the partial SSU did not exhibit any genetic variation between *S. neotropicalis* n. sp., *S. pontoporiae*, and *S. tursionis*.

Thus, interspecific differences could not be inferred from this marker. The nucleotide variations in the ITS1 within our alignment were less than 1%. For the ITS region, previous studies have shown that the intraspecific divergence for species of digeneans is below 1% (Vilas et al. 2005; Nolan and Cribb 2005), with some exceptions (Galazzo et al. 2002; Jousson and Bartoli 2002). Nevertheless, the rate of change in a region of DNA may vary from one group to another. Marigo et al. (2015) found identical ITS1 sequences among *S. pontoporiae* specimens, suggesting a single lineage of this species along the SWA coast. The nucleotide divergence observed in ITS1 for the species analyzed here was quite low and should be interpreted with caution, once this marker should not be useful for species delimitation in the genus *Synthesium*. Although the ribosomal markers used in this study did not completely diagnose the *Synthesium* species employed here, the data generated will contribute to future phylogenetic analyses of the group.

The mitochondrial genetic divergence found among the species analyzed in this study (i.e., *S. neotropicalis* n. sp., *S. pontoporiae*, *S. tursionis*, and *S. delamurei*) ranged from 9.1 to 14.6% for mtCOI and from 5.3 to 22.4% for mtND3. Previous studies have suggested that trematodes show maximum intraspecific divergence of up to 2% for mtDNA to be considered a single species (Vilas et al. 2005). This is below the observed values for the *Synthesium* species analyzed here. Moreover, the two recognized species coexisting along the Brazilian coast, namely, *S. pontoporiae* and *S. tursionis*, presented interspecific genetic differences of 14.6% in mtCOI and 17.8% in mtND3. These results might be interpreted as a threshold to narrow the divergence between *Synthesium* species. Therefore, comparing the genetic divergence found between both species and *S. neotropicalis* n. sp., we recognize the latter as a different taxon.

The phylogenetic position of *S. delamurei* caught our attention because we expected it to be placed within the *Synthesium* clade. The mtND3 genetic divergence between *S. delamurei* and its congeners (22.4%) was also higher than among the other *Synthesium* species. From our results, the mean mtND3 genetic divergence between the genera of the Brachycladiidae family is 19.6%. Fernandéz et al. (2000) also found genetic divergence in mtND3 between brachycladiids from separate genera such as *N. globicephalae* and *S. tursionis* to be 26.5%. The specimens used here were morphologically identified as *S. delamurei* according to Raga and Balbuena (1988). However, given the phylogenetic inference and genetic distance results, a detailed study should be conducted to elucidate whether *S. delamurei* is correctly assigned to the genus *Synthesium*.

A combination of morphological traits and molecular tools using different genes is highly recommended for describing new species. The morphostructural and morphometrical features combined with the phylogenetic reconstruction and

genetic distances presented here support the erection of a new parasite species infecting *T. truncatus*. The mtND3 phylogenetic inferences indicate the correct assignment of the new species into the genus *Synthesium*. We also added new SSU, ITS1, mtND3, and mtCOI gene sequences to the limited available Brachycladiidae sequence collection.

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