



# Revision of the taxonomic status of *Synthesium elongatum* (Ozaki, 1935) (Brachycladiidae), an intestinal digenean of narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*)

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**ABSTRACT.** *Synthesium elongatum* (Brachycladiidae) is an intestinal digenean described from the finless porpoise (*Neophocaena asiaeorientalis*) in Japan. Few records of this species exist and there is a remarkable morphological similarity between *S. elongatum* and *S. tursionis*, such that a synonymy between the species has been suggested previously. However, no morphological and/or molecular analysis has been carried out to clarify the taxonomic status of *S. elongatum*. In this study, we collected specimens of *Synthesium* sp. from *N. asiaeorientalis* in western Japan. The specimens possess lobed testes within the third quarter of the body, a round ovary, and vitellaria extending to level of uterine field, which are diagnostic characters for both *S. elongatum* and *S. tursionis*. They were morphologically identified to *S. elongatum* or *S. tursionis* due to the fact that the available morphometric data for both species overlap remarkably. A molecular analysis of the mitochondrial ND3 gene showed that the pairwise nucleotide distances between these specimens and *S. tursionis* were small, and phylogenetic analysis showed that these specimens and *S. tursionis* are in the same clade. Therefore, it was indicated that *S. elongatum* and *S. tursionis* are the same species and, consequently, *S. elongatum* is a synonym of *S. tursionis*.

**KEY WORDS:** Brachycladiidae, *Synthesium elongatum*, *Synthesium tursionis*

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The family Brachycladiidae is composed of digenean parasites of cetaceans and pinnipeds. The collection of worms of this family is usually limited to stranded or by-caught animals, and the helminths from long-dead hosts are often in poor condition. Thus, the taxonomy of the family and the taxonomic assignment of its species have traditionally been controversial [16]. One of its members, *Synthesium elongatum* (Ozaki, 1935) is an intestinal digenean first found in a finless porpoise in Japan [27]. Until now, this species has been taxonomically rearranged several times, being originally described as *Orthosplanchnus elongatus* [27], and later transferred to the genus *Odhneriella* [32] and then to the genus *Hadwenius* [2]. After the most recent taxonomic revision of the family, the genus *Hadwenius* was considered a synonym of *Synthesium* [16], leading to the species now accepted as *S. elongatum*. Also, the taxonomic status of the host species, the finless porpoise, has been revised, and two species are distinctively recognized. Indo-Pacific finless porpoise (*Neophocaena phocaenoides*) is distributed through the Indian Ocean up to South-China Sea, and, narrow-ridged finless porpoise (*N. asiaeorientalis*) in eastern Asia [19]. Thus the specimens used for the original description of *S. elongatum* by Ozaki [27] were collected from what it is now recognized as the narrow-ridged finless porpoise (*N. asiaeorientalis*).

There is a remarkable morphological resemblance between *S. elongatum* and *S. tursionis* (Marchi, 1873). The reported morphological characteristics of both species in previous studies well overlap with each other. In fact, Hafeezullah [18] reported *S. tursionis* from an Indo-Pacific finless porpoise (*N. phocaenoides*) in the Arabian Sea, and pointed out the probable synonymy with *S. elongatum*. So far, *S. elongatum* has been treated as a distinct species from *S. tursionis*, without any comparative study of both species [9, 10].

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**Table 1.** Host found date, location, type of event, length, weight and sex of *Neophocaena asiaeorientalis* from western Japanese waters. The number of specimens of *Synthesium* sp. used from each host and the type of analysis carried out are also shown

Host No.	Date	Location	Type of event	Length (cm)	Weight (kg)	Sex	Number of specimens in the analysis	
							morphology	molecular
1	2011/11/3	Inland Sea, Kanda, Fukuoka	By-caught	114.6	22.9	Female	-	1
2	2014/2/20	Inland Sea, Usa, Oita	Stranded	134.4	26.6	Female	8	-
3	2015/1/8	Inland Sea, Saijo, Ehime	Stranded	130.2	35.0	Male	3	2
4	2015/7/7	Inland Sea, Kiduki, Oita	Stranded	168.5	58.2	Female	8	-
5	2015/12/4	Ariake Sound, Kamiamakusa, Kumamoto	Stranded	145.2	39.2	Female	3	-
6	2016/1/22	Omura Bay, Togitsu, Nagasaki	Drifted	142.2	37.1	Female	8	-
7	2016/4/1	Omura Bay, Togitsu, Nagasaki	Stranded	139.8	36.6	Male	-	2
8	2016/5/17	Omura Bay, Higashisonogi, Nagasaki	By-caught	127.9	34.7	Female	1	-
Total							31	4

In this study, we aimed to verify the taxonomic identity of *S. elongatum* by using morphological and molecular data. The reported morphological information of *S. elongatum* is only that in Ozaki's description and no molecular analysis have been conducted. The type specimen of *S. elongatum* was lost during or after World War II from the Hiroshima University (Dr. Norio Shimizu, associate professor at the Hiroshima University Museum personal communication). However, we had access to several specimens of *Synthesium* sp., which were recently collected from the intestines of *N. asiaeorientalis* stranded or by-caught in Japanese coast. Thus, in this study we first confirmed the identity of these specimens as *S. elongatum* or *S. tursionis* by using whole-mounted specimens and determined the morphological variation within the species. Second, we compared these specimens with *S. tursionis* by molecular analysis using ethanol-preserved specimens. As a result of these comparisons, we suggest the synonymy of *S. elongatum* and *S. tursionis*.

## MATERIALS AND METHODS

### Specimens examined

Specimens of *Synthesium* sp. used in this study were collected from eight individuals of *N. asiaeorientalis* that were stranded, drifted or by-caught in western Japan (Inland Sea, Ariake Sound, and Omura Bay) between 2011 and 2016. All of the animals had already been dead at the time of found, and notified ministries and agencies according to Japanese regulation. The collection data and biological characteristics of the hosts, as well as the number of worms used for morphology and molecular analysis from each host are shown in Table 1. Porpoise carcasses were stored at  $-25$  to  $-18^{\circ}\text{C}$  until necropsy, except for one animal (No. 8), which was immediately necropsied after being transported to the laboratory without freezing. During the necropsy, the whole intestine was opened and washed in tap water, and precipitation was inspected for parasites under a stereomicroscope. Worms were rinsed in freshwater and prepared for whole-mounted slides or preserved in 70% ethanol directly. Whole-mounted slides were made by flattening individuals between a slide glass and a cover slip, and then fixed with 70% ethanol or alcohol-formalin-acetic acid solution (ethanol/formalin/ACS/water=5/0.6/0.4/4). After fixation, worms were stained with Heidenhain's iron hematoxylin or alum-carmin, dehydrated by ethanol series, cleared by xylene and creosote, and mounted with Canada balsam.

All specimens from *N. asiaeorientalis* are deposited at the Marine Mammal Research Laboratory, Nagasaki University (NU\_MMRL\_Parasite\_Coll. No. Dig. 93\_3, 9, 10, 14, 15, 16; 94\_13, 15; 142\_7, 9, 10; 156\_2, 3, 4, 6, 7, 8, 9, 10; 164\_1; 171\_1; 172\_5; 173\_2, 3, 5, 6, 7, 8, 9, 10).

### Morphological observation and molecular analysis

Body length and maximum width were measured from whole-mounted specimens by calibrated digimatic caliper CD-15PSX (Mitsutoyo Corp., Kanagawa, Japan), and an additional 15 measurements of internal organs were taken under microscope using calibrated digital image measurements software WraySpect (WRAIMER Inc., Osaka, Japan). If any organ or structure was not clearly observed, the related measurements and ratios were treated as missing values. For species of brachycladiids, the measurements and ratios of specimens from different hosts can be significantly variable [11]. To examine the variation between infrapopulations, the morphometric data of specimens from different hosts harboring 8 worms (i.e., host no. 2, 4, 6 in Table 1) were compared using a Kruskal-Wallis test for each variable using the statistical package R (3.4.0) [29]. Significance level was set at 0.003 based on Bonferroni adjustment.

Genomic DNA was extracted from a small piece of tissue ( $\sim 3\text{ mm}^2$ ) from each of the ethanol-preserved specimens using the Isolate II Genomic DNA Kit (Bioline, London, U.K.), following the manufacturer's recommendations. Before DNA extraction, ethanol from each sample was replaced with 500  $\mu\text{l}$  of TE buffer (0.001 M TrisHCl, pH 7.5, 0.001 M EDTA, pH 8). Partial mitochondrial NDH dehydrogenase subunit 3 (ND3) was amplified with primers ND3F (5'-GCTTAATTKKTAAGCYTTGRATTCTTACT-3') [13] and ND3 Primer 4 (5'-CTACTAGTCCCACTCAAC (G/A) TAACC (T/C) T-3') [12]. The thermocycling profile for gene amplification was as follows: initial denaturation at  $95^{\circ}\text{C}$  for 5 min, 35 cycles

**Table 2.** List of species, hosts, collection sites and GenBank accession numbers of the mitochondrial ND3 sequences used in this study. New sequences generated in this study are emphasized in bold

Species	Host and locality	Accession no.	Reference
<i>Tormopsolus orientalis</i> (Outgroup)	<i>Seriola dumerili</i> (Mediterranean Sea)	KT180219	[14]
<i>Brachycladium</i> sp.	<i>Balaenoptera acutorostrata</i> (North Sea)	AF123439	[13]
<i>Brachycladium atlanticum</i>	<i>Stenella coeruleoalba</i> (Mediterranean Sea)	KT180217	[14]
<i>Brachycladium atlanticum</i>	<i>Stenella coeruleoalba</i> (Mediterranean Sea)	AF034551	[12]
<i>Brachycladium goliath</i>	<i>Balaenoptera acutorostrata</i> (North Sea)	KR703278	[4]
<i>Campula oblonga</i>	<i>Phocoena phocoena</i> (Baltic Sea)	AF034554	[12]
<i>Campula oblonga</i>	<i>Phocoena phocoena</i> (North Sea)	KT180214	[14]
<i>Nasitrema delphini</i>	<i>Delphinus delphis</i> (Off Canary Islands)	KT180216	[14]
<i>Nasitrema globicephalae</i>	<i>Globicephala melas</i> (Southern Pacific Ocean)	AF034557	[12]
<i>Synthesium delamurei</i>	<i>Globicephala melas</i> (Mediterranean Sea)	KY612255	[9]
<b><i>Synthesium</i> sp. in the present study</b>	<b><i>Neophocaena asiaorientalis</i> (Inland Sea of Japan)</b>	<b>MH634348</b>	<b>Present study</b>
<b><i>Synthesium</i> sp. in the present study</b>	<b><i>Neophocaena asiaorientalis</i> (Inland Sea of Japan)</b>	<b>MH634349</b>	<b>Present study</b>
<b><i>Synthesium</i> sp. in the present study</b>	<b><i>Neophocaena asiaorientalis</i> (Inland Sea of Japan)</b>	<b>MH634350</b>	<b>Present study</b>
<b><i>Synthesium</i> sp. in the present study</b>	<b><i>Neophocaena asiaorientalis</i> (Omura Bay)</b>	<b>MH634347</b>	<b>Present study</b>
<i>Synthesium neotropicalis</i>	<i>Tursiops truncatus</i> (South Atlantic Ocean)	KY612256	[9]
<i>Synthesium pontoporiae</i>	<i>Pontoporia blainvillei</i> (Off Brazilian coast)	FJ829472	[23]
<i>Synthesium tursionis</i>	<i>Tursiops truncatus</i> (Mediterranean Sea)	AF034552	[12]
<i>Synthesium tursionis</i>	<i>Tursiops truncatus</i> (Mediterranean Sea)	KT180218	[14]
<i>Orthosoplanchmus fraterculus</i>	<i>Enhydra lutris</i> (North Pacific Ocean)	AF034555	[12]
<i>Oschmarinella macrorchis</i>	<i>Mesoplodon carlhubbsi</i> (North Pacific Ocean)	LC326064	[26]
<i>Oschmarinella rochebruni</i>	<i>Stenella coeruleoalba</i> (Mediterranean Sea)	AF034556	[12]

of 95°C for 30sec, 50°C for 30sec and 72°C for 50sec, and a final extension at 72°C for 7 min [12]. Amplicons were purified with a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and sequenced in both directions with an Applied Biosystems ABI 3730 XL automated sequencer by Macrogen Inc. Europe (Amsterdam, The Netherlands). Contigs were edited and assembled with BioEdit 7.0.5.3 [17] and Sequencher™ 5.1 (Gene Codes Corp., Ann Arbor, MI, U.S.A.) and submitted to GenBank (see Table 2 for accession numbers).

New sequences from *Synthesium* sp. in the present study were aligned with other 16 sequences of the Brachycladiidae available from GenBank using the online version of Mafft (<https://mafft.cbrc.jp/alignment/software/>). *Tormopsolus orientalis* (Acanthocolpidae) was also included in the alignment and used as an outgroup according to previous phylogenetic hypotheses of the family [14]. The Hasegawa, Kishino and Yano model with gamma distribution and invariant sites (HKY+G+I) was selected as the best model that fit the nucleotide alignment according to the Akaike Information Criteria (AIC) applied in JModelTest 2.1.4 [7]. A phylogenetic tree was constructed through Maximum Likelihood (ML) using a successive approximation approach starting on a tree estimated by Neighbour-Joining. A heuristic search strategy was performed using model parameters from the previous analysis based on nearest-neighbor-interchange (NNI) first, subtree-pruning-regrafting (SPR) second, and tree-bisection-reconnection (TBR) last, until the topology remained stable. ML bootstrap values for 100 replicates were estimated using Genetic Algorithm for Rapid Likelihood Inference (GARLI 0.942) [33] using default settings. A Bayesian inference analysis was performed on the protein-translated dataset using the JTT+G+F model as suggested by ProtTest 2.4 [1]. Posterior probabilities (PP) were calculated after 1,000,000 generations and a “burnin” of 4,600. Clades were considered to have high nodal support when ML bootstrap values were >80% and PP >90%. Pairwise genetic distances as the number of base differences per site between sequences were obtained with MEGA 6 [31].

## RESULTS

General morphological characteristics based on 31 gravid specimens of *Synthesium* sp. are as follows (Fig. 1, Table 3).

Body slender. Tegument unarmed. Oral and ventral sucker circular, located subterminally and around one fifth of the body, respectively. Oral/ventral sucker width ratio 0.5 to 0.8. Prepharynx length variable. Pharynx pyriform. Esophagus very short or undistinguishable. Intestinal caeca H-shaped without lateral diverticula. Anterior caeca reaching posterior margin of oral sucker and posterior caeca reaching posterior extremity. Testes tandem, situated 3/4 of body. Anterior testis 4–5 lobed in 77% of specimens, indented in 10%, and ellipsoid in 13%. Posterior testis 5–7 lobed in 71%, indented in 16%, and ellipsoid in 13%. Testicular type (lobed, indented, ellipsoid) usually consistent in both testes. Vas deferens extending anteriorly from each testis, connecting seminal vesicle together. Cirrus sack long and passing dextral or dorsal to ventral sucker, including seminal vesicle, pars prostatica and cirrus. Cirrus armed with spines (45 µm long on average). Ovary globular. Uterus coiled between Mehli's gland and seminal vesicle. Metraterm unarmed. Genital pore located just anterior to the ventral sucker. Vitellaria commencing at level of uterus (42%) or seminal vesicle (58%), distributing lateral field of body, confluent in post testicular region. Eggs ovoid, triangular in transverse section. Excretory vesicle I-shaped and not forming uroproct.

In regards to the similarities between *S. elongatum* and *S. tursionis*, there are many common characteristics and overlapping between ranges of measurements (Table 3). Specifically, lobed testes are situated in the third quarter of body, both species have a round ovary and vitellaria is limited anteriorly to level of uterine field. These characteristics were observed in our specimens. There was no significant infrapopulation variation in our specimens, except for a variation observed in the pharynx length (Kruskal-Wallis test,  $P < 0.003$ ). Most of the measurements from our specimens widely overlapped with those previously available for *S. elongatum* and *S. tursionis* (Table 3). Therefore, the specimens could be assigned either to *S. elongatum* or *S. tursionis*, as these species closely resembled morphologically.

Regarding the molecular analysis, length of the ND3 sequences ranged from 234 to 324 bp, yielding 78 to 108 amino acids, respectively. The shortest sequence (GenBank Accession No. MH634350) was excluded from subsequent analyses. The working nucleotide alignment was 297 bp long and had 127 parsimony-informative characters, whereas the protein-translated alignment yielded 99 characters, which were used for the Bayesian inference. Pairwise nucleotide distances between the three *Synthesium* sp. sequences analyzed ranged between 0.3 and 6.5%, whereas genetic distances with the rest of *Synthesium* species ranged between 1.7 and 20.5% (Table 4). The smallest genetic distance between *Synthesium* sp. and any other *Synthesium* species occurred with *S. tursionis* (1.7–10.6%), and the largest genetic distance with *S. delamurei* (18.4–20.5%). Nucleotide divergence between *Synthesium* sp. and *S. neotropicalis* and *S. pontoporiae* ranged between 14.3 and 16.7% (Table 4). When compared to the rest of the species of the Brachycladiidae, the genetic divergence with *Synthesium* sp. ranged between 18.4% (with *Brachycladium atlanticum*) and 25.3% (with *Nasitrema globicephalae*) (data not shown).

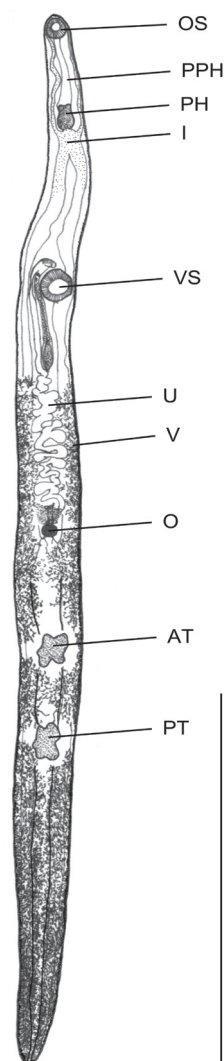
Similar tree topologies were obtained for the ML and Bayesian inference hypotheses for the Brachycladiidae (Fig. 2). All *Synthesium* species were clustered together in a single and highly supported clade (PP=100%; ML bootstrap=95%), except for *S. delamurei*. The three sequences of *Synthesium* sp. and the two of *S. tursionis* grouped in a single clade (PP=100%; ML bootstrap=81%). However, the two sequences of *S. tursionis* did not cluster together.

## DISCUSSION

The morphological characteristics and measurements of the specimens used in this study were comparable to those of the description of *S. elongatum* by Ozaki [27] and the redescription of *S. tursionis* [10, 21]. The ranges of body length, width, and every internal organ dimensions shown in this study overlapped with those of the two species (Table 3). However, body length and testes shape of our specimens were more variable than *S. elongatum*. Ozaki omitted the number of specimens observed, and the individual variation of the morphology of *S. elongatum* is unclear. In other species of *Synthesium*, a large individual variation of testes shape from lobed to ellipsoid or oval has been reported [10, 21]. In addition, the worms that Ozaki [27] observed were alive at the time of collection; however, our specimens came from frozen hosts. Therefore, the larger body could be accounted for by individual variation and/or the postmortem changes of the worm, and varied testes shape might be individual variation. Considering that the morphological characters are the same, and furthermore that the host species and locality are the same as for the original, our specimens could be identified as the same species as that of Ozaki [27].

Regarding morphological characteristics, there seemed to be no significant differences between *S. elongatum* and *S. tursionis* on considering the morphological data obtained from our specimens (Table 3). When comparing species of trematodes, we are aware that differences in fixation and preparation methods of worms may greatly influence the morphology and morphometrics of whole-mounted specimens, making the comparison difficult [6, 15]. In addition, sampling of digeneans of cetaceans is usually limited to accidentally stranded or by-caught animals, and their freshness varies according to host condition [16]. Therefore, this study made much account of molecular comparison between closely resembled species of *Synthesium*.

In this study, the species boundaries between our specimens and *S. tursionis* were not clearly delimited from the molecular



**Fig. 1.** Whole-mounted specimen of *Synthesium* sp. collected from a narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*), scale bar=10 mm; Abbreviations: AT (anterior testis); I (intestinal caeca); O (ovary); OS (oral sucker); PH (pharynx); PT (posterior testis); PPH (prepharynx); U (uterus); VS (ventral sucker); V (vitellaria).



**Table 3.** Measurements of *Synthesium* sp. obtained from specimens collected from 6 individuals of the narrow-ridged finless porpoise (*Neophocaena asiaorientalis*) in Japan. CV (%) means coefficient of variation. The reported morphology of *S. elongatum* and *S. tursionis* in the original description and re-descriptions of the species, respectively, are also listed

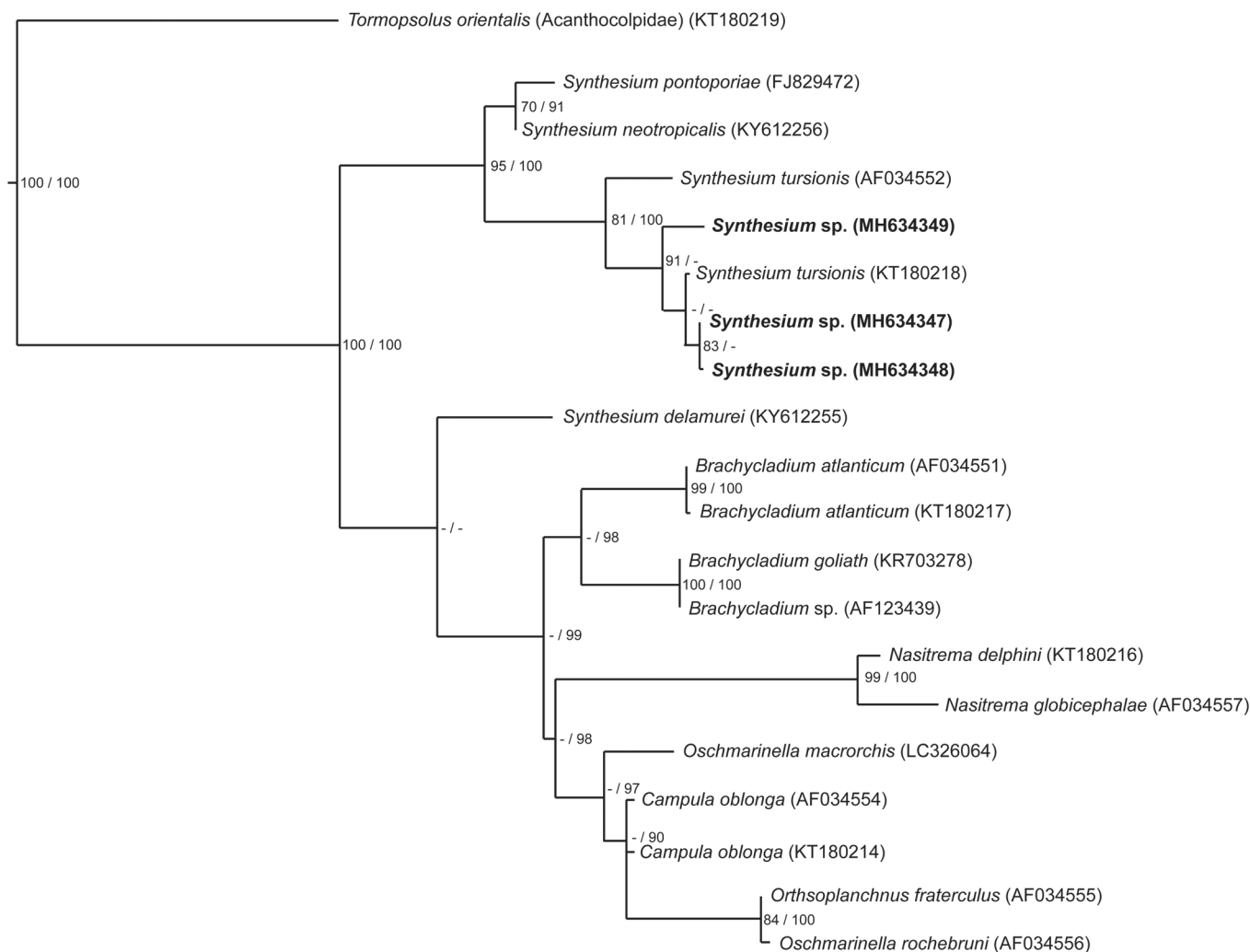
Species		<i>Synthesium</i> sp.					<i>S. elongatum</i>	<i>S. tursionis</i> (redescription)	
Reference		This study					[27]	[10]	[21]
		n	Average	Median	Range	CV (%)	Specimen number not reported	n=100	n=15
Body	length [mm]	31	22.7	24.1	14.6–28.4	17.5	13–18	6.2–30.8	8.9–21.3
	width [mm]	31	1.4	1.4	0.73–1.9	20.1	1.0–2.1	0.39–1.5	0.48–0.85
Oral sucker	length [ $\mu$ m]	31	532	544	390–676	15.1	400–550	309–814	431–722
	width [ $\mu$ m]	31	471	476	324–650	16.8	-	230–728	462–675
Ventral sucker	length [ $\mu$ m]	31	720	716	584–897	11.5	550–700	257–824	400–760
	width [ $\mu$ m]	31	723	729	448–922	14.0	-	233–754	428–684
Prepharynx [ $\mu$ m]		31	989	956	357–1,982	44.0	500–520	0–2,191	200–789
Pharynx	length [ $\mu$ m]	31	542	554	403–678	14.6	450–600	210–615	409–678
	width [ $\mu$ m]	31	324	320	222–410	15.3	310–350	91–330	185–333
Pharynx shape <sup>a)</sup>		Pyriform					Pear-shaped	Pyriform	Pyriform
Intestine <sup>a)</sup>		H-shaped					H-shaped	H-shaped	H-shaped
Cirrus <sup>a)</sup>		Armed with spines					Ornamented with elaborate spines	Armed	With small, readily lost spines
Anterior testis	length [ $\mu$ m]	31	1,081	1,086	659–1,555	21.6	Undescribed	306–1,074	409–1,093
	width [ $\mu$ m]	31	845	836	506–1,276	22.4	-	174–681	276–618
Posterior testis	length [ $\mu$ m]	31	1,205	1,164	701–1,838	23.7	Undescribed	341–1,100	475–1,112
	width [ $\mu$ m]	31	824	859	543–1,183	20.5	-	219–715	266–589
Testes shape <sup>a)</sup>		Lobed, indented, or ellipsoid					Irregularly lobed, 4 to 7	Lobed	Varying from oval to lobed
Ovary	length	29	409	407	243–547	16.9	200–330	93–361	143–339
	width	29	351	353	228–443	16.7	-	74–287	124–247
Ovary shape <sup>a)</sup>		Globular					Globular or slightly elongated	Oval	Round to oval
Metratem <sup>a)</sup>		Unarmed					Smooth	Unarmed	Unarmed
Anterior extent of vitellaria <sup>a)</sup>		Level of uterus or seminal vesicle					A little behind the acetabulum	Level of uterine field	Posterior to cirrus sac
Excretory vesicle <sup>a)</sup>		I-shaped					Tubular	Not reported	Long, tubular
Egg	length [ $\mu$ m]	31	52	52	48–60	5.1	47–55	35–47	51–55
	width [ $\mu$ m]	31	32	33	27–37	8.7	25–31	19–27	28–32

a) Morphological characteristics of two species were quoted from each reference.

**Table 4.** Pairwise genetic distances between each pair of *Synthesium* species. The three sequences obtained in this study are emphasized in bold. Lower half shows the percentage of base differences per site between sequences and upper half shows their standard error estimates

		1	2	3	4	5	6	7	8	
1	<i>S. pontoporiae</i>	FJ829472		0.02	0.02	0.01	0.02	<b>0.02</b>	<b>0.02</b>	<b>0.02</b>
2	<i>S. tursionis</i>	AF034552	14.7		0.02	0.02	<b>0.02</b>	<b>0.02</b>	<b>0.02</b>	
3	<i>S. tursionis</i>	KT180218	14.3	10.9		0.02	<b>0.01</b>	<b>0.01</b>	<b>0.01</b>	
4	<i>S. neotropicalis</i>	KY612256	4.4	11.9	14.7		<b>0.02</b>	<b>0.02</b>	<b>0.02</b>	
5	<i>S. delamurei</i>	KY612255	17.4	19.1	18.1	16.4		<b>0.02</b>	<b>0.02</b>	
6	<i>Synthesium</i> sp.	<b>MH634347</b>	<b>14.3</b>	<b>10.6</b>	<b>1.7</b>	<b>14.7</b>	<b>18.4</b>	<b>0</b>	<b>0.01</b>	
7	<i>Synthesium</i> sp.	<b>MH634348</b>	<b>14.7</b>	<b>10.6</b>	<b>2</b>	<b>14.7</b>	<b>18.4</b>	<b>0.3</b>	<b>0.01</b>	
8	<i>Synthesium</i> sp.	<b>MH634349</b>	<b>16.7</b>	<b>10.6</b>	<b>6.1</b>	<b>14.7</b>	<b>20.5</b>	<b>6.5</b>	<b>6.5</b>	

analysis. Two main arguments support this hypothesis: first, the nucleotide genetic distance ranged from 1.7 to 10.6% between *Synthesium* sp. and *S. tursionis* for the mitochondrial ND3 (mtND3) gene (Table 4). Species boundaries based on genetic yardsticks have been criticized based on empirical and theoretical grounds [5, 25]. However, closely related species of the family Brachycladiidae, and specifically of the genus *Synthesium*, have been found to have higher genetic distances in the mtND3 gene than the ones found in this study (e.g., 17.8% between *S. tursionis* and *S. pontoporiae*, and 14.0% between *S. tursionis* and *S. neotropicalis*) [9]. Second, neither the ML hypothesis nor the Bayesian inference conformed to reciprocal monophyletic clades for *Synthesium* sp. and *S. tursionis* (Fig. 2), an important criterion for delimiting species boundaries [8, 20]. Therefore, *S. elongatum*



**Fig. 2.** Maximum likelihood hypothesis inferred from 19 mitochondrial ND3 sequences from species of the Brachycladiidae and *Tormopsolus orientalis* (Acanthocolpidae). New sequences of *Synthesium* sp. in the present study are highlighted in bold. Support values for each node are expressed as ML bootstraps after 100 replicates and PP obtained after 1,000,000 generations on the protein-translated dataset; scores below 80% for ML bootstraps and 90% for PP are not shown. Branch length scale bar indicates the number of substitutions per site.

and *S. tursionis* should be considered as the same species, with *S. elongatum* becoming a synonym of *S. tursionis*.

*Synthesium tursionis* is a cosmopolitan species frequently found in bottlenose dolphin (*Tursiops truncatus*) and other odontocetes species, and has been reported in the Mediterranean and Black Seas, the Atlantic, Pacific, and Indian Oceans [3, 10, 18, 21, 22, 24, 28, 30]. The Japanese waters and narrow-ridged finless porpoise are now added to the distribution and host range of the species.

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## REFERENCES

1. Abascal, F., Zardoya, R. and Posada, D. 2005. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21**: 2104–2105. [Medline] [CrossRef]
2. Adams, A. M. and Rausch, R. L. 1989. A revision of the genus *Orthosplanchnus* Odhner, 1905 with consideration of the genera *Odhneriella*

- Skriabin, 1915 and *Hadwenius* Price, 1932 (Digenea: Campulidae). *Can. J. Zool.* **67**: 1268–1278. [CrossRef]
3. Aguilar-Aguilar, R., Moreno-Navarrete, R. G., Salgado-Maldonado, G. and Villa-Ramírez, B. 2001. Gastrointestinal helminths of spinner dolphins *Stenella longirostris* (Gray, 1828) (Cetacea: Delphinidae) stranded in La Paz Bay, Baja California Sur, Mexico. *Comp. Parasitol.* **68**: 272–274.
  4. Briscoe, A. G., Bray, R. A., Brabec, J. and Littlewood, D. T. J. 2016. The mitochondrial genome and ribosomal operon of *Brachycladium goliath* (Digenea: Brachycladiidae) recovered from a stranded minke whale. *Parasitol. Int.* **65**: 271–275. [Medline] [CrossRef]
  5. Cognato, A. I. 2006. Standard percent DNA sequence difference for insects does not predict species boundaries. *J. Econ. Entomol.* **99**: 1037–1045. [Medline] [CrossRef]
  6. Cribb, T. H. and Bray, R. A. 2010. Gut wash, body soak, blender and heat-fixation: approaches to the effective collection, fixation and preservation of trematodes of fishes. *Syst. Parasitol.* **76**: 1–7. [Medline] [CrossRef]
  7. Darriba, D., Taboada, G. L., Doallo, R. and Posada, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* **9**: 772. [Medline] [CrossRef]
  8. De Queiroz, K. 2007. Species concepts and species delimitation. *Syst. Biol.* **56**: 879–886. [Medline] [CrossRef]
  9. Ebert, M. B., Müller, M. I., Marigo, J., Valente, A. L. S., Cremer, M. J. and da Silva, R. J. 2017. A new *Synthesium* species (Digenea: Brachycladiidae) from the bottlenose dolphin *Tursiops truncatus* (Cetacea: Delphinidae) in Southwestern Atlantic waters. *Parasitol. Res.* **116**: 1443–1452. [Medline] [CrossRef]
  10. Fernández, M., Balbuena, J. A. and Raga, J. A. 1994. *Hadwenius tursionis* (Marchi, 1873) n. comb. (Digenea, Campulidae) from the bottlenose dolphin *Tursiops truncatus* (Montagu, 1821) in the western Mediterranean. *Syst. Parasitol.* **28**: 223–228. [CrossRef]
  11. Fernández, M., Balbuena, J. A., Pertusa, J. F. and Raga, J. A. 1995. Biometric variability of *Hadwenius tursionis* (Marchi, 1873) (Digenea, Campulidae) from the intestine of the bottlenose dolphin *Tursiops truncatus* (Montagu, 1821). *Syst. Parasitol.* **30**: 67–76. [CrossRef]
  12. Fernández, M., Aznar, F. J., Latorre, A. and Raga, J. A. 1998. Molecular phylogeny of the families Campulidae and Nasitremitidae (Trematoda) based on mtDNA sequence comparison. *Int. J. Parasitol.* **28**: 767–775. [Medline]
  13. Fernández, M., Aznar, F. J., Raga, J. A. and Latorre, A. 2000. The origin of *Lecithodesmus* (Digenea: Campulidae) based on ND3 gene comparison. *J. Parasitol.* **86**: 850–852. [Medline] [CrossRef]
  14. Fraija-Fernández, N., Aznar, F. J., Fernández, A., Raga, J. A. and Fernández, M. 2016. Evolutionary relationships between digeneans of the family Brachycladiidae Odhner, 1905 and their marine mammal hosts: A cophylogenetic study. *Parasitol. Int.* **65**: 209–217. [Medline] [CrossRef]
  15. Gibson, D. I. 1984. Technology as applied to museum collections: the collection, fixation and conservation of helminths. *Syst. Parasitol.* **6**: 241–241. [CrossRef]
  16. Gibson, D. I. 2005. Family brachycladiidae odhner, 1905. pp. 641–652. In: Keys to the Trematoda, Vol. 2 (Jones, A., Bray, R. A. and Gibson, D. I. eds.), CABI and the Natural History Museum, London.
  17. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**: 95–98.
  18. Hafeezullah, M. 1986. On a trematode (Digenea: Campulidae) of a marine mammal from Arabian Sea. *Rec. Zool. Surv. India* **83**: 41–48.
  19. Jefferson, T. A. and Wang, J. Y. 2011. Revision of the taxonomy of finless porpoises (genus *Neophocaena*): the existence of two species. *J. Mar. Anim. Ecol.* **4**: 3–16.
  20. Kizirian, D. and Donnelly, M. A. 2004. The criterion of reciprocal monophyly and classification of nested diversity at the species level. *Mol. Phylogenet. Evol.* **32**: 1072–1076. [Medline] [CrossRef]
  21. Marigo, J., Vicente, A. C., Valente, A. L., Measures, L. and Santos, C. P. 2008. Redescription of *Synthesium pontoporae* n. comb. with notes on *S. tursionis* and *S. seymouri* n. comb. (Digenea: Brachycladiidae Odhner, 1905). *J. Parasitol.* **94**: 505–514. [Medline] [CrossRef]
  22. Marigo, J., Ruoppolo, V., Rosas, F. C. W., Valente, A. L. S., Oliveira, M. R., Dias, R. A. and Catão-Dias, J. L. 2010. Helminths of *Sotalia guianensis* (Cetacea: Delphinidae) from the south and southeastern coasts of Brazil. *J. Wildl. Dis.* **46**: 599–602. [Medline] [CrossRef]
  23. Marigo, J., Thompson, C. C., Santos, C. P. and Iñiguez, A. M. 2011. The *Synthesium* Brachycladiidae Odhner, 1905 (Digenea) association with hosts based on nuclear and mitochondrial genes. *Parasitol. Int.* **60**: 530–533. [Medline] [CrossRef]
  24. Mignucci-Giannoni, A. A., Hoberg, E. P., Siegel-Causey, D. and Williams, E. H. Jr. 1998. Metazoan parasites and other symbionts of cetaceans in the Caribbean. *J. Parasitol.* **84**: 939–946. [Medline] [CrossRef]
  25. Nadler, S. A. 2002. Species delimitation and nematode biodiversity: phylogenies rule. *Nematology* **4**: 615–625. [CrossRef]
  26. Nakagun, S., Shiozaki, A., Ochiai, M., Matsuda, A., Tajima, Y., Matsuishi, T., Watanabe, K., Horiuchi, N. and Kobayashi, Y. 2018. Prominent hepatic ductular reaction induced by *Oschmarinella macrorchis* in a Hubbs' beaked whale *Mesoplodon carlhubbsi*, with biological notes. *Dis. Aquat. Organ.* **127**: 177–192. [Medline] [CrossRef]
  27. Ozaki, Y. 1935. Trematode parasites of Indian porpoise *Neophocaena phocaenoides* Gray. *J. Sci. Hiroshima Univ.* **3**: 115–138.
  28. Quiñones, R., Giovannini, A., Raga, J. A. and Fernández, M. 2013. Intestinal helminth fauna of bottlenose dolphin *Tursiops truncatus* and common dolphin *Delphinus delphis* from the western Mediterranean. *J. Parasitol.* **99**: 576–579. [Medline] [CrossRef]
  29. R Core Team 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/> [accessed on October 27, 2018]
  30. Romero, M. A., Fernández, M., Dans, S. L., García, N. A., González, R. and Crespo, E. A. 2014. Gastrointestinal parasites of bottlenose dolphins *Tursiops truncatus* from the extreme Southwestern Atlantic, with notes on diet composition. *Dis. Aquat. Organ.* **108**: 61–70. [Medline] [CrossRef]
  31. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729. [Medline] [CrossRef]
  32. Yamaguti, S. 1958. CAMPULIDAE odhner, 1926. pp. 845–853. In: Systema Helminthum, Vol. 1. The digenetic trematodes of vertebrates (Yamaguchi, S. ed.), Interscience Publishers, New York.
  33. Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas at Austin.