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Wildlife Science

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*

The family Brachycladiidae is composed of digenean parasites of cetaceans and pinnipedeans. 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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measurements and ratios of specimens from different hosts can be significantly variable [11]. To examine the variation between not clearly observed, the related measurements and ratios were treated as missing values. For species of brachycladiids, the calibrated digital image measurements software WraySpect (WRAIMER Inc., Osaka, Japan). If any organ or structure was (Mitsutoyo Corp., Kanagawa, Japan), and an additional 15 measurements of internal organs were taken under microscope using 172_5; 173_2, 3, 5, 6, 7, 8, 9, 10).

alum-carmine, dehydrated by ethanol series, cleared by xylene and creosote, and mounted with Canada balsam. Solution (ethanol/formalin/ACS/water=5/0.6/0.4/4). After fixation, worms were stained with Heidenhain’s iron hematoxylin or by flattening individuals between a slide glass and a cover slip, and then fixed with 70% ethanol or alcohol-formalin-acetic acid in freshwater and prepared for whole-mounted slides or preserved in 70% ethanol directly. Whole-mounted slides were made was opened and washed in tap water, and precipitation was inspected for parasites under a stereomicroscope. Worms were rinsed was immediately necropsied after being transported to the laboratory without freezing. During the necropsy, the whole intestine and biological characteristics of the hosts, as well as the number of worms used for morphology and molecular analysis from each already been dead at the time of found, and notified ministries and agencies according to Japanese regulation. The collection data drifted or by-caught in western Japan (Inland Sea, Ariake Sound, and Omura Bay) between 2011 and 2016. All of the animals had been collected from the intestines of Neophocaena asiaeorientalis stranded or by-caught in Japanese coast. Thus, in this study we first confirmed the identity of these specimens as Synthesium 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 Synthesium elongatum and S. tursionis.

MATERIALS AND METHODS

Specimens examined

Specimens of Synthesium sp. used in this study were collected from eight individuals of Neophocaena 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°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-carmine, dehydrated by ethanol series, cleared by xylene and creosote, and mounted with Canada balsam.

All specimens from Neophocaena 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 (−3 mm³) 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 µl 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'-GCTTAATT KKTAAAGC YTTGATCTC TCTACT-3') [13] and ND3 Primer 4 (5'-CTACTAG TCACA CACTCAAC (G/A) TAACC (T/C) T-3') [12]. The thermocycling profile for gene amplification was as follows: initial denaturation at 95°C for 5 min, 35 cycles
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. Testes in tandem, indistinguishable. Intestinal caeca H-shaped without lateral diverticula. Cirrus saccus 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 Mehlis’ 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, \( \alpha = 0.003 \)). 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. pontopor菅ae* 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

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**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).
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

### Table 3.
Measurements of Synthesium sp. obtained from specimens collected from 6 individuals of the narrow-ridged finless porpoise (Neophocaena asiaeorientalis) 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 | Synthesium sp. | S. elongatum | S. tursionis (redescription) |
|---------|----------------|--------------|-----------------------------|
| Reference | This study [27] | n=100 | n=15 |
| Body | | | |
| length [mm] | 31 | 22.7 | 24.1 | 14.6–28.4 | 17.5 |
| width [mm] | 31 | 1.4 | 1.4 | 0.73–1.9 | 20.1 |
| Oral sucker | | | |
| length [µm] | 31 | 532 | 544 | 390–676 | 15.1 |
| width [µm] | 31 | 471 | 476 | 324–650 | 16.8 |
| Ventral sucker | | | |
| length [µm] | 31 | 720 | 716 | 584–897 | 11.5 |
| width [µm] | 31 | 723 | 729 | 448–922 | 14.0 |
| Prepharynx [µm] | 31 | 989 | 956 | 357–1982 | 44.0 |
| Pharynx | | | |
| length [µm] | 31 | 542 | 554 | 403–678 | 14.6 |
| width [µm] | 31 | 324 | 320 | 222–410 | 15.3 |
| Pharynx shape [a) | | | |
| Pyriform | | | |
| Pear-shaped | | | |
| Intestinea) | | | |
| H-shaped | | | |
| H-shaped | | | |
| Cirrus [a) | | | |
| Armed with spines | | | |
| Ornamented with elaborate spines | | | |
| Arm ed | | | |
| With small, readily lost spines | | | |
| Anterior testis | | | |
| length [µm] | 31 | 1,081 | 1,086 | 659–1,555 | 21.6 |
| width [µm] | 31 | 845 | 836 | 506–1,276 | 22.4 |
| Posterior testis | | | |
| length [µm] | 31 | 1,205 | 1,164 | 701–1,838 | 23.7 |
| width [µm] | 31 | 824 | 859 | 543–1,183 | 20.5 |
| Testes shape [a) | | | |
| Lobed, indented, or ellipsoid | | | |
| Irregularly lobed, 4 to 7 | | | |
| Lobed | | | |
| Varying from oval to lobed | | | |
| Ovary | | | |
| length | 29 | 409 | 407 | 243–547 | 16.9 |
| width | 29 | 351 | 353 | 228–443 | 16.7 |
| Ovary shape [a) | | | |
| Globular | | | |
| Globular or slightly elongated | | | |
| Metraterma | | | |
| Unarmed | | | |
| Smooth | | | |
| Unarmed | | | |
| Anterior extent of vitellaria [a) | | | |
| Level of uterus or seminal vesicle | | | |
| A little behind the acetabulum | | | |
| Level of uterine field | | | |
| Posterior to cirrus sac | | | |
| Excretory vesicle [a) | | | |
| I-shaped | | | |
| Tubular | | | |
| Not reported | | | |
| Long, tubular | | | |
| Egg | | | |
| length [µm] | 31 | 52 | 52 | 48–60 | 5.1 |
| width [µm] | 31 | 32 | 33 | 27–37 | 8.7 |

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

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