



Morphological and Molecular Identification of *Stellantchasmus dermogenysi* n. sp. (Digenea: Heterophyidae) in Thailand

Chalobol Wongsawad^{1,2,*}, Nattawadee Nantarat¹, Pheravut Wongsawad^{1,3}, Preeyaporn Butboonchoo^{1,2}, Jong-Yil Chai^{4,5}

¹Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50202, Thailand; ²Environmental Science Research Center (ESRC), Faculty of Science, Chiang Mai University, Chiang Mai 50202, Thailand; ³Economic Plant Genome Service Centre, Faculty of Science, Chiang Mai University, Chiang Mai 50202, Thailand; ⁴Institute of Parasitic Diseases, Korea Association of Health Promotion (KAHP), Seoul 07649, Korea; ⁵Department of Tropical Medicine and Parasitology, Seoul National University College of Medicine, Seoul 03080, Korea

Abstract: We tried a series of morphological and molecular approaches to identify a new species of *Stellantchasmus* (Digenea: Heterophyidae) originating from the wrestling half-beaked fish, *Dermogenys pusillus* of Thailand. Adult worm samples of the new species were recovered from hamsters experimentally infected with the metacercariae from *D. pusillus* in Thailand. Two isolates (Thai and Korean) of *Stellantchasmus falcatus* were used as comparative control groups. Worm samples of 3 *Stellantchasmus* groups were morphologically observed and molecularly analyzed with the mitochondrial cytochrome c oxidase 1 gene. The morphological characteristics of *S. dermogenysi* n. sp. are similar to *S. falcatus* originating from brackish water fish, but minor difference was noted including the absence of the prepharynx, position of the ovary near the ceca end, smaller body size, and shorter esophageal length. A phylogenetic tree derived from neighbor-joining and maximum-likelihood methods suggests that *S. dermogenysi* n. sp. is separated from *S. falcatus* supported by high bootstrap values. The relative divergences persist between these host-specific trematodes, which we suggest should be recognized as 2 distinct species. Comparisons of *S. dermogenysi* n. sp. with *S. falcatus* isolated from mullets in Thailand and Korea indicate a genetic divergence of mitochondrial DNA of 19.4% and 21.7%, respectively. By the present study, a new species, *Stellantchasmus dermogenysi* n. sp. (Digenea: Heterophyidae), is proposed in Thailand based on molecular evidences, in addition to minor morphological differences between *S. falcatus* and the new species.

Key words: *Stellantchasmus dermogenysi*, *Stellantchasmus falcatus*, heterophyid fluke, phylogeny, Thailand

INTRODUCTION

Stellantchasmus is a genus of minute intestinal flukes from the family Heterophyidae. This genus includes only 4 known species: *S. falcatus* Onji and Nishio, 1924, *S. aspinosus* Pearson, 1964, *S. gallinae* Oshmarin, 1970, and *S. batillans* Pearson, 1964, all of which have been clearly defined by their morphological characters [1]. The number and arrangement of testes and the presence of an armed or unarmed ventral sucker are used as the main characters for species identification [1]. *Stellantchasmus* has been found in both experimental and natural hosts: *S. falcatus* in natural and experimental cats; *S. aspinosus*

in the wild water rat and the experimental cat, rat, and chicken; *S. gallinae* in the domestic chicken; and *S. batillans* in the water rat and domestic duck [1,2].

S. falcatus was first reported in 1916 by Onji and Nishio [3]. Human infection by this parasite has been reported in Japan, the Philippines, the Republic of Korea (= Korea), Hawaii, and Thailand [4-7], and human infections also occur at a high rate in northern parts of Thailand [8]. So far only 1 species, *S. falcatus*, has been recorded in Thailand (and in some other countries) [4-8]. During infection, eggs can pass through the blood vessels to the brain or heart of the patient [9]. Pathology in humans includes mild to colicky pain, leading to death in severe cases (as seen in the Philippines) [7,9].

S. falcatus is widespread and can infect various types of hosts [3,10]. The first intermediate host of this parasite, reported in Thailand, is freshwater snails such as *Thiara scabra* and *Tarebia granifera* [11]. The metacercarial stage of *S. falcatus*, which infects humans, is found in several types of brackish water and

•Received 22 February 2019, revised 10 May 2019, accepted 10 May 2019.

*Corresponding author (wchalobol@gmail.com)

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freshwater fish, the second intermediate host [10,12]. There are only 2 species of fish, *Dermogenys pusillus* (half-beaked fish, freshwater species) and *Planiliza subviridis* (a species of mullet, brackish water species), that were reported to be second intermediate hosts of *S. falcatus* in Thailand [12]. Traditional identification of *Stellantchasmus* species has been based solely on morphological characteristics; however, numerous morphological similarities can result in misidentification.

Over the last decade, cryptic speciation has been discovered in other digenean parasitic species such as *Echinostoma*, *Phyllostomum*, and *Clinostomum* [13-17]. Mechanisms of speciation in parasites may be unswervingly connected to their hosts and their complex life cycles [13,18]. The presence of cryptic species arises through morphological similarities and appears to be widespread among parasites [18,19]. Speciation almost always occurs in parasite species that form a large diverse group of organisms, infecting various kinds of hosts [13,17,20]. There have been reports of morphological characteristics of *S. falcatus* from the fish *D. pusillus* that seems to be different from *S. falcatus* from the fish *P. subviridis* [7,9,10,12,21]. In this case, no exception exists in the ordinary cryptic speciation that may occur in *Stellantchasmus* species.

Molecular approaches are the most efficient tools currently used to understand phylogenetic relationships and to determine genetic variations in heterophyid flukes [22-25]. The conventional PCR and high annealing temperature-based random amplified polymorphic DNA (HAT-RAPD) methods have been used to discriminate *Stellantchasmus* species isolated from various hosts [22]. It has been suggested that different DNA fingerprints of *Stellantchasmus* worms acquired from different hosts may be due to distinct species [22]. To clarify this point, HAT-RAPD can be used to develop specific markers to detect *S. falcatus* infections [25]. Moreover, many genes, such as nuclear genes (including internal transcribed spacers 1 and 2; ITS1 and ITS2) and mitochondrial genes (including mitochondrial cytochrome *c* oxidase 1; CO1), have been used for identification and phylogenetic studies of various parasites [13,23,24]. ITS2 and CO1 regions have been useful for investigation and identification of *Stellantchasmus* sp. [23,24].

The aim of this study was to investigate and identify a new species of *Stellantchasmus* (Digenea: Heterophyidae) obtained from the wrestling half-beaked fish, *D. pusillus*, in Thailand based on both morphological and molecular approaches using CO1 gene.

MATERIALS AND METHODS

Ethical statements

All experimental hosts were managed according to the guidelines approved by the Animal Ethics Committee of the Faculty of Science, Chiang Mai University, Thailand, and the relevant document (no. RE 001/13) was approved by the committee. The guidelines for animal care were used according to the International Guiding Principles of Biomedical Research Involving Animals of Council for International Organizations of Medical Sciences (CIOMS).

Morphological investigation

The metacercariae of *Stellantchasmus* spp. were collected from different sites in Thailand. The hosts of this parasite were *P. subviridis* and *D. pusillus*, acquired from Chiang Mai and Chonburi Provinces in Thailand. Fish specimens were minced and digested using 1% pepsin solution for 2 hr at 37°C and rinsed with 0.85% NaCl. A hundred metacercariae of *Stellantchasmus* spp. were checked with light microscopy, and hamsters (*Phodopus campbelli*) were force-fed with the metacercariae samples. After 2 days, the adult-stage worms were collected from the hamsters. Total 57 specimens of *Stellantchasmus* were used for identification and morphological studies. The worms were processed for permanent slides. Briefly, they were flattened under a cover slip pressure and fixed in 4% formalin, stained with hematoxylin and/or Borax's carmine, dehydrated in alcohol series, and finally mounted in Permount. The specimens were checked with the descriptions of Onji and Nishio [3], Pearson [2], Kliks and Tantachamrun [5], Pearson and Ow-Yang [1], and Pubua and Wongsawad [12]. The body and organs of the worms were measured and analyzed. The holotype and paratypes of the new species are kept in the Natural History Museum, London (NHMUK). The worms were frozen at -20°C for DNA extraction.

DNA extraction

Genomic DNA from each *Stellantchasmus* species and related groups were collected from adult worms. *S. falcatus* from the laboratory of Prof. Jong-Yil Chai, Seoul National University College of Medicine and Korean Association of Health Promotion, Seoul, Korea, was used for molecular studies with the 2 Thai samples. Approximately, 2 mg of each trematode tissue sample was used for DNA extraction. The DNA extraction and purification steps were performed using 150 ml of 5% Chelex

(Fluka) solution containing 10 µl of 20 mg/ml proteinase K (Sigma-Aldrich, St. Louis, Missouri, USA): the mixture containing the trematode tissue was washed and incubated at 55°C for 1 hr, followed by heating at 95°C for 30 min, then gently mixed. The mixture was centrifuged for 10 sec at 13,000 rpm, after which the supernatant was removed and stored at -20°C until use.

Amplification and sequencing of the CO1 gene

The partial CO1 gene was amplified using the primers as follows: JB3 (5'-TTTTTGGGCATCCTGACGTTTAT-3') as a forward primer and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') as a reverse primer. The PCR amplifications were carried out as described above. The amplification procedure included a 3 min initial denaturation at 95°C, then 40 cycles of 1 min denaturation at 95°C, 1 min annealing at 50°C, 1 min extension at 72°C, and a 7 min final extension at 72°C. The PCR products were visualized on 1.0% agarose gels, purified using the Cleanup PCR Kit (Sigma-Aldrich), and directly sequenced in both directions by Ward Medic, Ltd. (Bangkok, Thailand).

Phylogenetic analyses

The molecular data in this study including the sequences from *Stellantchasmus* spp. and outgroup (*Metagonimus* spp.) were aligned using ClustalW, a subprogram of MEGA version 6.0 [26]. The sequences were checked for ambiguous nucleotide sites before being subjected to a phylogenetic analysis. The jModel test 2 [27] was used to find the most appropriate sub-

stitution model for the CO1 datasets. Phylogenetic trees of all taxa were constructed using neighbor-joining, maximum likelihood, and Bayesian inference. GTR+G model was applied for all analyses. The NJ analysis and the likelihood scores of dataset was carried out using PAUP* version 4.0b10 [28] with 1,000 bootstrap replicates. The ML analysis was undertaken using PHYML version 2.4.5 [29]. The tree searching system used for this method involved the heuristic procedure with tree-bisection-reconnection branch swapping. The bootstrap resampling [30,31] was done with 1,000 replicates that were assigned to support the particular branches. BI analysis was performed using MrBayes version 3.2.5 [32], where the Bayesian analysis was run for 5 million generations (heating parameter=0.15), sampling was done for every 100 generations, and the first 25% of trees were discarded with burn-in procedure. The final consensus tree was built using the last 15,002 trees. Support for nodes was defined as posterior probabilities (P).

RESULTS

Morphological data

The samples of *S. dermogenysi* were identified compared with the original description and holotype picture of *S. falcatu*s. In the morphology of *Stellantchasmus* spp. from different hosts [2-4], the differences in the presence or absence of the prepharynx, position of the ovary, body size, and esophagus length were distinguishing factors. The prepharynx was very short or absent in *S. dermogenysi* n. sp. but it was present in *S.*

Table 1. Helminth species used in this study and GenBank accession numbers for their corresponding CO1 sequence

Species	Host	Country	GenBank accession no.
<i>Stellantchasmus dermogenysi</i> n.sp. ^a	Experimental hamster	Chiang Mai, Thailand	-
<i>Stellantchasmus dermogenysi</i> n.sp.	Experimental hamster	Chiang Mai, Thailand	KU753571
<i>Stellantchasmus dermogenysi</i> n.sp.	Experimental hamster	Chiang Mai, Thailand	KU753572
<i>Stellantchasmus dermogenysi</i> n.sp.	Experimental hamster	Chiang Mai, Thailand	KU753573
<i>Stellantchasmus dermogenysi</i> n.sp.	Experimental hamster	Chiang Mai, Thailand	KU753574
<i>Stellantchasmus dermogenysi</i> n.sp.	Experimental hamster	Chiang Mai, Thailand	KU753575
<i>Stellantchasmus dermogenysi</i> n.sp.	Experimental hamster	Chiang Mai, Thailand	KU753576
<i>Stellantchasmus falcatu</i> s ^a	Experimental hamster	Chon Buri, Thailand	AY382320
<i>Stellantchasmus falcatu</i> s ^a	Korean human	Korea	-
<i>Stellantchasmus falcatu</i> s	Experimental hamster	Chon Buri, Thailand	KU753568
<i>Stellantchasmus falcatu</i> s	Experimental hamster	Chon Buri, Thailand	KU753569
<i>Stellantchasmus falcatu</i> s	Experimental hamster	Chon Buri, Thailand	KU753570
<i>Metagonimus yokogawi</i> ^b	Experimental hamster	Korea	AB470519
<i>Metagonimus takahashi</i> ^b	Experimental hamster	Korea	AF096231

^aQuery sequence generated.

^bUsed as the outgroup.

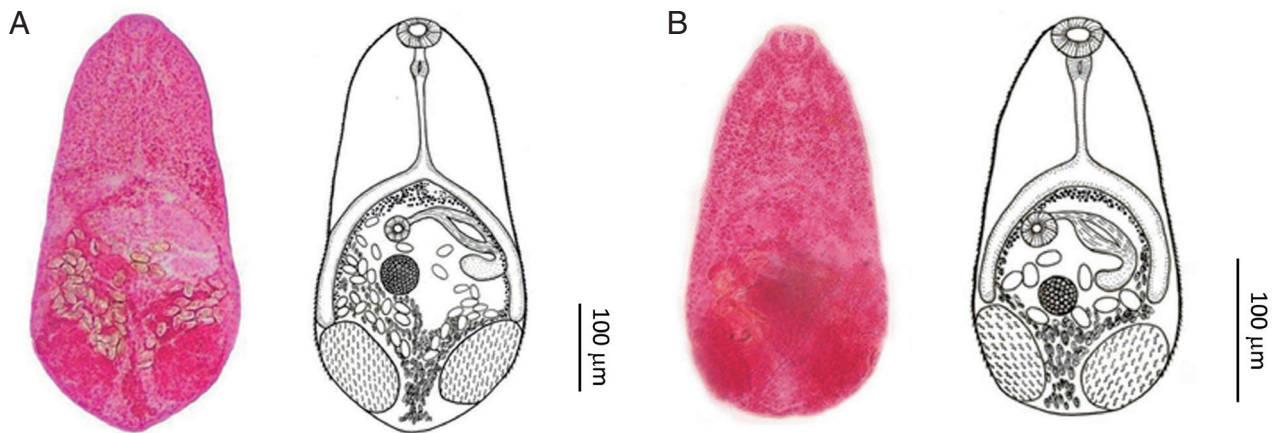


Fig. 1. (A) *Stellantchasmus falcatus* adult fluke originating from the brackish water fish *P. subviridis*. Ventral view. (B) *Stellantchasmus dermogenysi* n. sp. originating from the freshwater fish *D. pusillus*.

Table 2. Comparison of body and organ measurements between *Stellantchasmus falcatus* and *Stellantchasmus dermogenysi* n. sp.

Items	<i>Stellantchasmus falcatus</i>					<i>Stellantchasmus dermogenysi</i> n. sp. from <i>D. pusillus</i> (B)	Ratio A:B
	Onji & Nishio (1916)	Pearson (1964)	Seo et al. (1984)	From a Korean human ^a	From <i>P. subviridis</i> (A)		
No. worms measured	-	10	10	5	27	30	
Body length	440	480	700	526	520	374*	1.39
Body width	220	270	360	326	222	167*	1.33
Oral sucker length	55	40	62	52	42	36	1.17
Oral sucker width	55	46	61	51	48	42	1.14
Prepharynx length	-	Present	-	Present	Present	Almost absent	-
Pharynx length	35	24	44	36.2	30	27	1.11
Pharynx width	25	28	41	35.6	27	25	1.08
Esophagus length	70-150	94	108	87.6	117	56 ^b	2.09 ^c
Ventral sucker length	37	30	42	37	31	22 ^b	1.41
Ventral sucker width	37	29	42	37	31	24 ^b	1.29
Expulsor length	120	77	111	75	63	54	1.17
Expulsor width	30	26	60	33	32	26	1.23
Ovary length	50	46	71	65	54	53	1.02
Ovary width	50	42	70	58.3	42	49	0.86 ^c
Testis (R) length	96	70	100	88.8	104	90	1.16
Testis (R) width	78	57	71	85	56	58	0.97 ^c
Testis (left) length	88	70	96	102.5	105	91	1.15
Testis (left) width	75	57	66	96.5	58	57	1.02
Egg (uterine) length	27	23	27	27.1	21	21	1.00
Egg (uterine) width	14	13	12	13.8	12	11 ^b	1.09

^a*S. falcatus* from a Korean human used as a comparative species in both morphological and molecular analyses.

^bComparison of different characters between *S. dermogenysi* n. sp. and *S. falcatus*.

^cThe ratio of characteristic differences of *S. falcatus* from *P. subviridis* vs *S. dermogenysi* n. sp.

falcatus. The ovary of *S. dermogenysi* n. sp. is located near the ceca end, whereas in *S. falcatus* it is located in the middle of the ceca length. Of the new species, the body, esophagus, and ventral sucker were clearly smaller than those of *S. falcatus* (Table 2; Fig. 1). Similarity in the body shape, organ size, and their positions were observed, in addition to the ratios of the

body size (Table 2; Fig. 1). *S. falcatus* from *P. subviridis* revealed smaller values. Although the ratio of the body size of *S. falcatus* from *P. subviridis* to the new species was 1.33:1.39, ratios of the organs were found to be different, especially with regard to the esophagus length, ovary width, and the right testis width (Table 2).

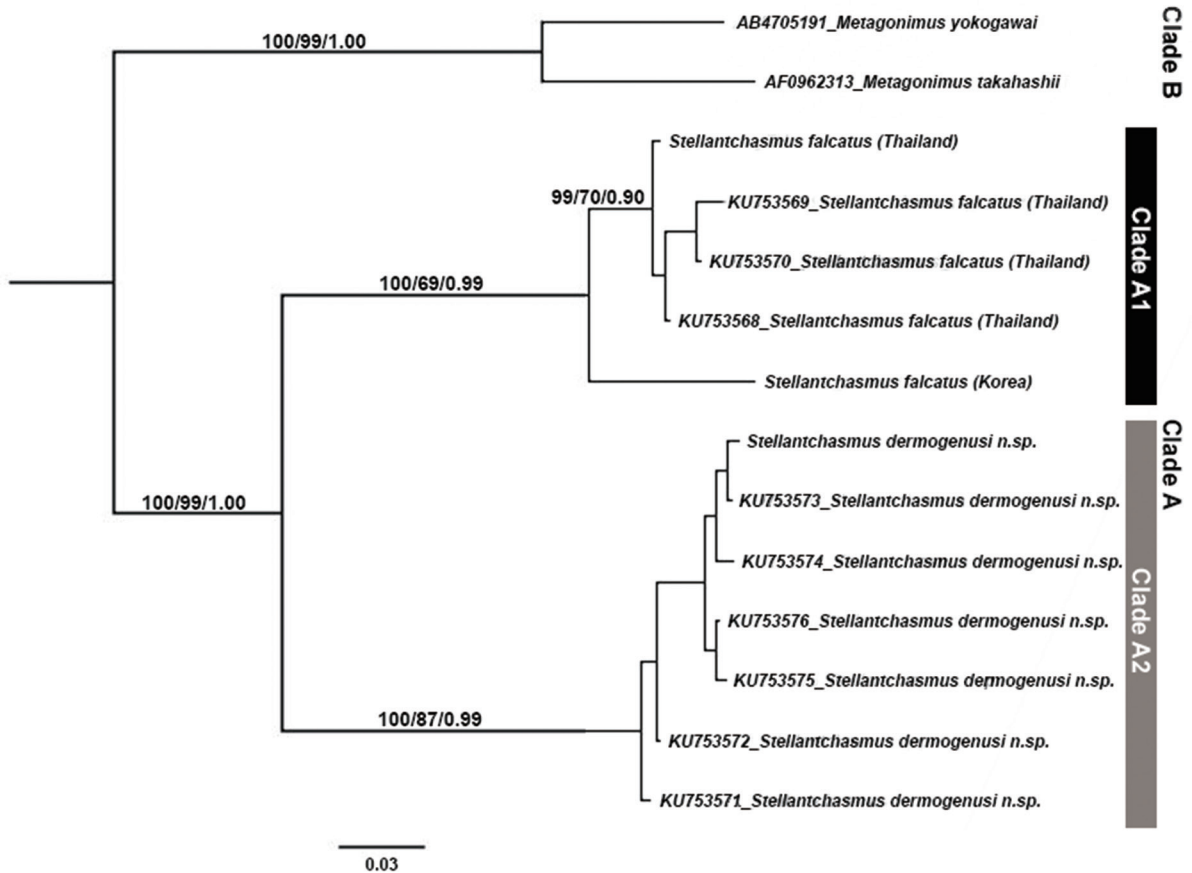


Fig. 2. A phylogenetic tree of *Stellantchasmus* spp. and related group based on CO1 gene. Statistics can be referred to in Materials and Methods.

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Descriptions: Adult specimens were obtained from experimentally infected hamsters (*P. campbelli*). All measurements below are given in micrometers (μm). Body pyriform, with a length of 374 (256-480), and forebody narrower than the hindbody, with a maximum width of 167 (136-220) across the testes. Almost the entire surface covered with scale-like spines extending to half of the posterior body. Oral sucker subterminal and 36 (26-46) long by 42 (32-54) wide; prepharynx 4 (2-10) long; pharynx 27 (22-40) long by 25 (20-34) wide; esophagus 56 (30-102) long, with bifurcation extending to the anterior border of testis. Ventral sucker 22 (20-30) long by 24 (20-30) wide. Testes paired, ovoid, opposite, and in the posterior part of the body, and measured 90 (68-116) long by 58 (40-80) wide (right). Left testis 90 (68-112) long by 57 (40-82) wide. Seminal vesicles thin-walled, located to the left of ovary, and connected to the expulsor lying dorsal and left of

cecum. Expulsor with muscle fibers 54 (26-80) long by 26 (20-36) wide, opened into the ventrogenital sac dorsally on the left side and anterior to ovary. Ovary slightly ovoid and positioned slightly right and anterior to testes, 53 (30-70) long by 49 (40-76). Uterus coiled in the posterior of the body and filled with operculate eggs. Vitelline follicles distributed at the inner intestine, with bifurcation extending to the posterior of the body. Eggs 21.6 (17.5-23.0) long by 11.5 (7.5-15.0) wide, thin-walled, possessing a smooth shell with a small operculum at anterior end.

Type materials: Type specimens have been deposited in the Natural History Museum, London, UK and the Applied Parasitology Research Laboratory, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. Holotype NHMUK 2015.1.7.1 (Fig. 1), paratypes NHMUK 2015.1.7.2 (1 individual) and CMUZ 01.1-01.28 (29 individuals).

Host: The metacercariae were collected from the freshwater fish *Dermogenys pusillus* and the adult stage in the experimental

Table 3. Genetic distance of *Stellantchasmus* spp. based on CO1 gene

Taxa	<i>S. falcatus</i>	<i>S. falcatus</i> ^a	<i>S. dermogenysi</i> n. sp.
<i>S. falcatus</i>	-	0.014	0.024
<i>S. falcatus</i> ^a	0.083	-	0.026
<i>S. dermogenysi</i> n. sp.	0.194	0.217	-

^aSpecimen from a Korean (human), distance value (below diagonal), and standard error (above diagonal).

hamster (*Phodopus campbelli*).

Type locality: Chiang Mai Province, Thailand

Etymology: The name is derived from their specific second intermediate host, the wrestling half-beaked fish (*Dermogenys pusillus*).

Phylogenetic analyses based on CO1 gene and genetic distance

The aligned 396 bp sequences of all the CO1 fragment codons had nucleotide frequencies of 0.173, 0.145, 0.290, and 0.392 for A, C, G, and T, respectively (41.1% GC content), with a 105 parsimony informative result and 126 variable sites. The K2P-distance between the taxa ranged from 0.005 to 0.217. Stop codons of all CO1 sequences were absent.

The phylogenetic trees were reconstructed using NJ, ML and BI methods (Fig. 2). All the trees showed similar tree topology with high statistical support. The tree could be divided into 2 main clades. The clade A was the *Stellantchasmus* group, and the clade B was the *Metagonimus* group (outgroup). The clade A was also subdivided into 2 subclades, including *S. falcatus* (clade A1) and *S. dermogenysi* n. sp. (clade A2). *S. dermogenysi* n. sp. (clade A2) was separated from both Thai and Korean *S. falcatus* isolates which had high bootstraps supported (100% and 99% for NJ and ML bootstrap replicates and 1.00 for BI posterior probability, respectively). The distance among the *Stellantchasmus* species complex is shown in Table 3. The distance between *S. dermogenysi* n. sp. and both Thai and Korean *S. falcatus* was 0.194 (19.4%) and 0.217 (21.7%), respectively, while that between Thai and Korean *S. falcatus* was lower (0.083 or 8.3%).

DISCUSSION

The occurrence of *S. dermogenysi* n. sp. has been reported previously in northern Thailand, under the name *S. falcatus* from the specific fish host *D. pusillus* [5,7,10,12,21]. Its morphology is clearly different from *S. batillans*, which has a single

testis. The arrangement of the testes is opposite in the new species, while in *S. gallinae* it is in tandem position. *S. aspinosus*, with an unarmed ventral sucker, is distinct from the new species, which has a ventral sucker with groups of tiny spines on the lip [1]. The morphological measurements are similar to those reported for *S. falcatus* from brackish water fish, but the difference in the length of the prepharynx, position of the ovary, body size, esophagus length, and different second intermediate host were distinguishing factors. The prepharynx is almost absent in *S. dermogenysi* n. sp., but present in *S. falcatus*, as described by Onji and Nishio in 1916 [3], Pearson in 1964 [2], and Chai et al. in 2016 [33] and 2017 [34], and in *S. falcatus* from humans in Korea (used as comparative specimens in this study). In *S. dermogenysi* n. sp., the ovary is located near the ceca end, whereas in *S. falcatus* it is located mid-way along the ceca. The body size, esophagus length, and ventral sucker size of the new species are clearly smaller than in *S. falcatus*. Most species of mullet fish serve as the second intermediate host for *S. falcatus*, including *Mugil* sp. in Korea [10], *Chelon macrolepis* in Cambodia [33] and Myanmar [34], and *P. subviridis* in Thailand (from this study). *Stellantchasmus* found in *D. pusillus*, from Thailand, was also reported under the name *S. falcatus*, but it was morphologically different from the *S. falcatus* originating from mullets [8,22]. Their prevalence (100%) and intensity (22-2,492 metacercaria/fish) were very high in *D. pusillus* [8].

We used molecular methods to confirm *S. dermogenysi* as a new species, and derived the name from their specific second intermediate host, the wrestling half-beaked fish (*D. pusillus*). Mitochondrial genes, especially the CO1 gene, have been widely used for identification of parasites and studies of phylogenetic relationships and genetic variations in the family Heterophyidae [23]. In our study, the CO1 gene revealed that the monophyletic tree of the *Stellantchasmus* group (clade A) was clearly distinct from the related outgroup (*Metagonimus* spp.), by NJ, ML, and BI methods. In addition, *S. dermogenysi* n. sp., originating from the freshwater fish *D. pusillus*, was also separated from both Thai and Korean *S. falcatus* isolates originating from brackish water mullets *P. subviridis* and humans, respectively. The intraspecific distance (K2P) based on the CO1 gene of the *Stellantchasmus* group was around 5%, while the distance (K2P) between *S. dermogenysi* n. sp. and 2 *S. falcatus* isolates averaged 19.9%. This distance was also high when compared with *Metagonimus* spp., at 7.5% [35]. This molecular evidence, along with different morphological characters,

clearly supports the distinction of the *Stellantchasmus* species. The present findings agree with Wongsawad and Wongsawad [22], who reported that differences in the morphology, habitat, DNA fingerprint, and phylogenetic relationships (based on HAT-RAPD) strongly suggested that *Stellantchasmus* from freshwater and brackish water habitats are different species.

The morphological and molecular characteristics obtained in this study allows us to confidently identify the parasite isolate originating from the freshwater fish *D. pusillus*, in Thailand, as a new species, *S. dermogenysi* n. sp. Although *S. dermogenysi* shares many morphological similarities with *S. falcatus*, the molecular evidence supports the distinction. The present findings will help a definitive diagnosis in humans, may affect treatment, prevention measures, and research into anti-parasitic agents.

ACKNOWLEDGMENTS

This research work was partially supported by Chiang Mai University, Chiang Mai, Thailand. We thank the Applied Technology for Biodiversity, Institute for Science and Technology; Applied Parasitology, Department of Biology, Faculty of Science; Economic Plant Genome Service Centre, Faculty of Science, Chiang Mai University, Thailand for the provision of instruments, facilities, and access to laboratories. This work was supported by National Research Council of Thailand to CW (grant no. 2559A10402051, 2016) and The Thailand Research Fund to NN (grant no. TRG5880053). Special thanks are extended to the Natural History Museum, London, and Senior Curator of Parasites and Vectors Division.

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