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Phylogenetic analysis reveals cryptic species diversity within minute intestinal fluke, *Stellantchasmus falcatus* Onji and Nishio, 1916 (Trematoda, Heterophyidae)

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ABSTRACT

Objective: To examine the taxonomic boundaries in the *Stellantchasmus* populations in different hosts and reveal the cryptic speciation of the *Stellantchasmus* in Thailand based on both morphological and molecular approaches.

Methods: *Stellantchasmus falcatus* (*S. falcatus*) s.l. was collected from different hosts throughout Thailand. The worms were examined and measured. The nuclear *ITS2* gene and the mitochondrial *COI* gene were used to investigate the *S. falcatus* s.l. from *Dermogenus pusillus* and *Liza subviridis*.

Results: *Stellantchasmus* was one of food-borne trematode that widely distribute throughout Thailand. The comparison of *S. falcatus* s.l. from *Dermogenus pusillus* and *Liza subviridis* indicated a genetic divergence of nuDNA with 3.6% and mtDNA with 19.3%, respectively. Morphological characteristics were fairly different in the ratio of body size, length of prepharynx, and ratio of organ size.

Conclusions: Our results provide initial evidence that *S. falcatus* s.l. from different hosts tend to be a different species based on both molecular and morphological characters. Cryptic species complexes are generally found among parasites that tend to have large populations and/or rapid evolution. The degree of genetic diversity existing would suggest the practice of targeted regimes to design and minimize these lections of anthelmintic resistance in the future.

1. Introduction

Stellantchasmus is a genus of the minute intestinal fluke in family Heterophyidae. The first report on *Stellantchasmus falcatus* (*S. falcatus*) was uncovered in 1916 by Onji and Nishio [1]. Human infection of this parasite has been reported in Japan, the Philippines, Korea, Hawaii and Thailand [2–5]. Thus far, there is only one species, '*S. falcatus*' that has been recorded in Thailand. The pathogenicity revealed mild to colicky pain until death, at which time eggs pass through blood vessels to the brain or heart [6]. The first intermediate hosts of this parasite that had been reported in Thailand are *Thiara granifera* and *Tarebia granifera*. The metacercarial stage of *Stellantchasmus* sp. in the second intermediate hosts are present in the northern part of Thailand at a high rate of infection [7]. There are only two species of fish, *Dermogenus pusillus* (*D. pusillus*) and *Liza subviridis* (*L. subviridis*) were reported as the second intermediate host of *S. falcatus* in Thailand [5,8]. The traditional identification of the *Stellantchasmus* species has been based on only morphological characteristics. However, a large number of morphological similarities can result in misidentification.

In the present day, molecular approaches are the most efficient tools that are used to understand the relationships and determine the genetic variations of many parasites [9–13]. Various genes have been approved as effective identification tools such as *ITS2* and *COI* gene [14,15]. The identified molecular tools have been determined to be valuable for the purposes of more accurate estimate and to discriminate for *Stellantchasmus* diversity [7,16].

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The taxonomic study of *Stellantchasmus* in Thailand has still not been fully developed. Consequently, this study aims to investigate the taxonomic boundaries in *Stellantchasmus* populations, revealing cryptic speciation of the *Stellantchasmus* in Thailand based on both morphological and molecular approaches using *ITS2* and *COI* genes.

2. Materials and methods

2.1. 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, 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).

2.2. Morphological investigation

The metacercariae of the *Stellantchasmus* species complex were collected from different sites in Thailand. The hosts of this parasite were *L. subviridis* and *D. pusillus* acquired. Fish specimens were minced and digested using 1% pepsin solution over 2 h at 37 °C and then were rinsed with 0.85% NaCl. The adult stage of the worm was collected from hamsters, 2 d after being force-fed with separate metacercaria samples. Fifty-seven worms of the *Stellantchasmus* species complex were used for identification and description according to order. The worms were processed for making permanent slides, fixed in 4% formalin, stained with Haematoxylin and/or Borax's carmine, dehydrated in alcohol series and finally mounted with Permount. The specimens were checked with the descriptions was based on Onji and Nishio [1], Pearson [17], Kliks and Tantachamrun [3], Pearson and Ow-Yang [18], Pubua and Wongsawad [8]. The body and organs of the worm were measured and analyzed. For molecular analysis, the worms were frozen at −20 °C for DNA extraction.

2.3. DNA extraction, amplification and sequencing

Genomic DNA of each *Stellantchasmus* species complex and the related groups were collected from adult worms. Approximately, 20 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 that contained 10 mL of 20 mg/mL proteinase K (Sigma). The mixture containing the trematode tissue was washed and treated at 55 °C for 1 h, followed by being heating at 95 °C for 30 min and then gently mixed. The mixture was centrifuged for 10 s at 13 000 rpm. After that, the supernatant was removed and stored at −20 °C until being used.

The internal transcribed spacer 2 (*ITS2*) region was amplified by using the primers as follows: *BD1* (5′-GCT GTA ACAAGG TTT CCG TA-3′) as a forward primer and *BD2* (5′-TAT GCT TAAATT CAG CGG GT-3′) as a reverse primer. The PCR amplifications were implemented at a final volume of 50 μL, including 50 ng of DNA template, 1.5 mM of $MgCl_2$, 200 μM of each dNTPs, 50 pM of each primer and 0.5 unit of Taq DNA polymerase. The amplification procedure included 2 min for an initial denaturation step at 94 °C, followed by 40 cycles of 1 min for a denaturation step at 94 °C, 1 min for an annealing step at 57 °C, and 1 min for the extension step at 72 °C and finally 10 min for a final extension step at 72 °C.

The partial of cytochrome c oxidase sub-unit I mitochondrial (*COI*) region was amplified by using the primers as follows: *JB3* (5′- TTTTTTGGGCATCCTGACGTTTAT-3′) as a forward primer and *JB4.5* (5′ –TAAAGAAAGAACATAATGAAAA TG-3′) as a reverse primer. The PCR amplifications were carried out as described above. The amplification procedure included 3 min for an initial denaturation step at 95 °C, then 40 cycles of 1 min for a denaturation step at 95 °C, 1 min for an annealing step at 50 °C, 1 min for an extension step at 72 °C, and then 7 min for a final extension step at 72 °C. The PCR products were visualized on 1.0% agarose gel, purified using the Cleanup PCR Kit (Sigma) and were directly sequenced in both directions by Ward Medic, Ltd.

2.4. Phylogenetic analyses

Sequences were edited with reference to the electropherograms and initially aligned using MUSCLE version 3.6 [19,20], a sub program of MEGA 6.0.6 [21]. The alignments were improved manually where necessary. All base frequencies and molecular character statistics were calculated using MEGA 6.0.6 [21,22].

The sequences were checked for ambiguous nucleotide sites, saturation, and phylogenetic signals before being subjected to phylogenetic analysis. For saturation tests, the 1st, 2nd, 3rd and all *COI* positions were performed using DAMBE v. 4.5.33 [23]. The analyses were run for each gene fragment separately and the concatenated dataset of the *ITS2* and *COI* gene. The jModel test 2 [24] was used to find the most appropriate substitution model for all datasets.

Phylogenetic trees of all taxa were constructed using neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI). GTR + G model was applied for all analyses. The NJ analysis and the likelihood scores of different data partitions were carried out using PAUP* version 4.0b10 [25]. The NJ tree was carried out to assign support to the actual branches by bootstrap re-sampling [26,27] with 1 000 replicates.

The ML analysis was undertaken using PHYML version 2.4.5 [28]. The tree searching system used for this method involved the heuristic procedure with tree-bisention-reconnection branch swapping. The bootstrap resampling [26,27] was done with 1 000 replicates that were assigned to support the particular branches.

BI analysis was performed using MrBayes version 3.2.5 [29], where the tree space was explored using four chains of a Markov chain Monte Carlo algorithm (MCMC). The Bayesian analysis was run for 5 million generations (heating parameter = 0.15), sampling was done for every 100 generations and then the first 25% 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).

2.5. Nucleotide sequence accession numbers

Nucleotide sequences of this study have been deposited in GenBank under accession number KU753568–KU753597.

3. Results

3.1. Morphological data

The samples of the *Stellantchasmus* species complex were identified based on the original description and compared to the picture of the holotype to confirm their identification. In terms of comparing the morphology of *Stellantchasmus* species complex [1,17,30] with the others/from different hosts, a similarity of almost all body shapes, organs and positions was shown except with regard to the ratio of body size, distance of prepharynx, and ratio of organ size. *Stellantchasmus* species complex from *L. subviridis* revealed smaller values. Although the body-size ratio of *Stellantchasmus* species complex from *L. subviridis*: *Stellantchasmus* species complex from *D. pusillus* was 1.33–1.39, the ratios of the organs were found to be different especially with regard to the prepharynx, esophagus length, ovary width and testis right width. The morphological data of *Stellantchasmus* species complex from *D. pusillus* was shown to be different from the previous reports on the *Stellantchasmus* species complex from *L. subviridis* [5,7,8,17,30,31].

Table 1

Ranges of genetic divergence (K2P-distance) of two *S. falcatus* clades and related species based on *ITS2* gene (above diagonal) and *COI* gene (below diagonal).

Taxa	Clade A: *S. falcatus*	Clade B: *S. falcatus*	*Haplorchis taichui* (*H. taichui*)	*Haplorchoides* sp.	*Fasciola hepatica* (*F. hepatica*)
Clade A: *S. falcatus*	–	0.036	0.299	0.209	0.407
Clade B: *S. falcatus*	0.193	–	0.291	0.223	0.397
H. taichui	0.244	0.222	–	0.259	0.410
Haplorchoides sp.	0.281	0.256	0.221	–	0.380
F. hepatica	0.323	0.295	0.290	0.271	–

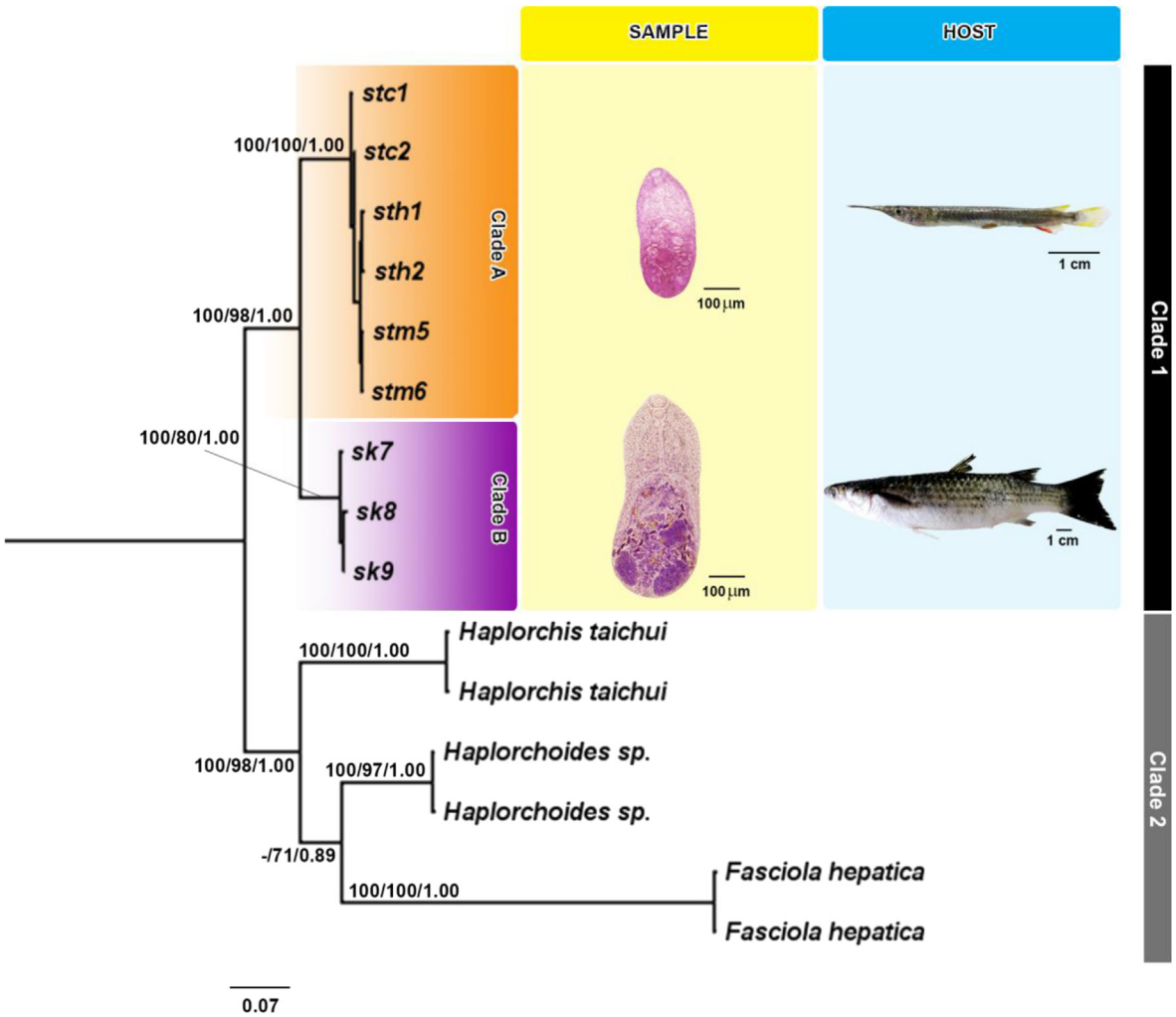

Figure 1. Phylogenetic tree of the *Stellantchasmus* species complex.

The phylogenetic tree of the *Stellantchasmus* species complex and related groups reconstructed using maximum-likelihood analysis of 800 nucleotide sites of the concatenate genes of *ITS2* and all *COI* using the GTR + G model. Statistic support values for individual nodes are shown on the tree (based on NJ/ML/BI method).

3.2. DNA sequence variation and distance

The *ITS2* gene aligned fragment (404 bp), had nucleotide frequencies of 0.200, 0.245, 0.274 and 0.281 for A, C, G and T, respectively (51.47% GC content), with 174 (43.1%) parsimony informative results. The K2P-distance between the taxa ranged from 0.000 to 0.410.

The aligned 396 bp sequence of all the *COI* fragment codons had nucleotide frequencies of 0.176, 0.130, 0.282, 0.412 for A, C, G and T, respectively (40.8% GC content), with a149 (37.6%) parsimony informative result and 153 variable sites (38.6%). The K2P-distance between the taxa ranged from 0.003 to 0.329. Stop codons of all *COI* sequences were absent. The results of the saturation test using DAMBE v. 4.5.33 showed ISS values included the third codon position, which were in all cases significantly lower than the ISS.c. Consequently, we decided not to exclude the third codon position, with the third codon position revealing 149 parsimony informative characters against 47 for the first and second positions. The K2P-distances derived from the *COI* fragment were lower than those from the *ITS2*, which suggested that the *COI* fragment was more conservative than the *ITS2*.

The concatenated dataset of ITS2 combined with all codon positions of COI (800 bp) revealed nucleotide frequencies of 0.188, 0.188, 0.278 and 0.345 for A, C, G and T, respectively (38.8% GC content), with 323 (40.38%) parsimony informative results and 327 (40.88%) variable sites. The uncorrected p-distance between the taxa ranged from 0.001 to 0.367. The discrete Gamma Distributions [32] for the *COI* fragment (all codons), *ITS2*, and the concatenated data set of *ITS2* with all *COI* were recorded as 0.616 0, 0.630 3 and 0.378 1 respectively. The K2P-distance between the taxa ranges of *ITS2* and *COI* are shown in Table 1.

3.3. mtDNA and ncDNA phylogenetic analyses

The model for the three datasets was the GTR + G model (*ITS2*, All codon of *COI* and concatenate of *ITS2* and all codon of *COI*). The phylogenetic trees reconstructed by the NJ, ML and BI analyses were greatly consistently producing almost identical topologies and the same supported nodes for all major clades and all datasets.

The tree (Figure 1) was separated into two main principal clades as clade 1 and clade 2. Clade 1 included the *Stellantchasmus* species complex (100% and 98% for NJ and ML bootstrap replicates, and a posterior probability of 1 in BI). Clade 2 included *H. taichui*, *Haplorchoides* sp. and *F. hepatica*, all of which are members of Heterophyidae (100% and 98% for NJ and ML bootstrap replicates and a posterior probability of 1 in BI). Clade 1 was the *Stellantchasmus* species complex acquired from Chiang Mai and Chonburi Provinces in Thailand. However, it was also separated into two sub-clades (clades A and B) by their hosts with strong support (100% and 98% for NJ and ML bootstrap replicates and a posterior probability of 1 in BI). Clade A was made up of the *Stellantchasmus* species complex from *D. pusillus* (100% and 100% for NJ and ML bootstrap replicates and a posterior probability of 1 in BI) and clade B was made up of the *Stellantchasmus* species complex from *L. Subviridis* (100% and 80% for NJ and ML bootstrap replicates, and a posterior probability of 1 in BI).

4. Discussion

The two DNA regions (*ITS2* and *COI*) have been determined to be useful for the species identification of Heterophyidae. A phylogenetic tree showed a monophyletic tree of trematodes in the Family Heterophyidae. The result is congruent with previous reports on the PCR-RFLP and RAPD studies and the phylogenetic relationship of heterophyid trematodes [33–35]. In this study, we were able to reveal the phylogenetic results based on multiple loci of *S. falcatus* species complex. The two lineages (host 1; *D. pusillus*; Clade A and host 2; *L. subviridis*; Clade B) may actually represent two different species. Moreover, the average K2P-distance among 2 clades (Clade A, B) of *ITS2* (nuDNA) was 3.6% and *COI* (mtDNA) was 19.3%. The similar genetic distances have been inferred to be indicative of distinct species in studies of other heterophyidae. As in the genus *Apohallus*, the genetic distances appear to reveal certain differences that exist between species based on the *COI* gene >12% [36]. These results are also supported by the morphological characteristics that can also be used to distinguish them from each other as a ratio of body size, distance of prepharynx, as well as a ratio of organ size.

Populations of trematodes that are widely distributed may lead to a reduced gene flow or a complete lack of genetic exchanges between the trematode populations [13,37–39]. In this case, the host of the *S. falcatus* s.l. in this study is distributed in different types of geographic areas (freshwater fish, *D. pusillus* and coastal marine waters fish *L. subviridis*) and due to the high levels of host-specificity reported for the worm, it is likely to show a lower level/lack of gene flow between the trematode population [13,37,38]. These spatial isolations will probably lead to being a new species over time. The scenarios frequently observed are referred to as unexpected cryptic divergence within a nominal species that is caused by allopatric speciation [15,38,40,41]. Cryptic species complexes are common in parasites, especially; a large population of parasitic worms and through rapid evolution can live in different hosts. There have been many reports that have described the co-evolution and co-speciation that exist between parasites and their hosts. However, it seems that the main factors are multi-host systems, host specific details, extinction and host-switching [9,13,38,39]. Our results seem to confirm these hypotheses and provide the first evidence of cryptic speciation, which might have occurred within the *S. falcatus* s.l.

In conclusion, our recent study has been based on both molecular and morphological approaches and has suggested that these difference host populations of *Stellantchasmus falcatus* s.l. trend to be a cryptic species. Despite numerous studies having earlier investigated the genetic variability and structure of *S. falcatus* s.l., the present information is the most comprehensive evidence of *S. falcatus* s.l. to be produced in Thailand to date. The current findings will have important implications for future research using this model to test the impacts of parasites on host evolution. Moreover, the findings of the study would be useful for food-borne trematode disease management and medical treatments administered in Thailand as well as in other countries. Further genetic markers are needed to confirm the presence of these cryptic *Stellantchasmus* species complexes, potentially leading to more in-depth genetic, ecological and evolutionary analyses on this multi-host-parasite system.

Conflict of interest statement

We declare that we have no conflict of interest.

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