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▣ ORIGINAL ARTICLE

Mitochondrial DNA Sequence Variability of *Spirometra* Species in Asian Countries

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Abstract: Mitochondrial DNA sequence variability of *Spirometra erinaceieuropaei* in GenBank was observed by reinvestigation of mitochondrial *cox1* and *cytb* sequences. The DNA sequences were analyzed in this study, comprising complete DNA sequences of *cox1* (n=239) and *cytb* (n=213) genes. The 10 complete mitochondrial DNA sequences of *Spirometra* species were compared with those of Korea, China and Japan. The sequences were analyzed for nucleotide composition, conserved sites, variable sites, singleton sites and parsimony-informative sites. Phylogenetic analyses was done using neighbor joining, maximum parsimony, Bayesian inference and maximum-likelihood on *cox1* and *cytb* sequences of *Spirometra* species. These polymorphic sites identified 148 (*cox1*) and 83 (*cytb*) haplotypes within 239 and 213 isolates from 3 Asian countries. Phylogenetic tree topologies were presented high-level confidence values for the 2 major branches of 2 *Spirometra* species containing *S. erinaceieuropaei* and *S. decipiens*, and *S. decipiens* sub-clades including all sequences registered as *S. erinaceieuropaei* in *cox1* and *cytb* genes. These results indicated that mitochondrial haplotypes of *S. erinaceieuropaei* and *S. decipiens* were found in the 3 Asian countries.

Key words: *Spirometra erinaceieuropaei*, *Spirometra decipiens*, mitochondria, DNA sequence variability

INTRODUCTION

The tapeworm *Spirometra erinaceieuropaei* is the most well-known species of the genus *Spirometra* tapeworms. Its plerocercoid larvae can infect the brain, eyes, breast, spinal cord, and subcutaneous tissue of humans [1,2]. Human sparganosis is a worldwide parasitic zoonosis, which causes serious clinical diseases. These diseases have been reported in more than 1,600 cases, mostly in east and Southeast Asia, and sporadic cases in South America, Europe and Africa [3]. Precise identification of these spirometrid tapeworms is therefore important in efforts to control the diseases caused by human sparganosis.

Species classification of the tapeworms in the genus *Spirometra* remains controversial. Identification of spirometrid species within the genus *Spirometra* species has been attempted by many researchers. Six *Spirometra* species were initially studied and morphologically identified [4]. The new species *S. mansonoides* derived from cat in the Syracuse region of the USA was then reported [5]. Fourteen *Spirometra* species were recognized by 2 separate groups as bresslauei and okumurai [6]. A genotype of all species in the genus *Spirometra* was the same as *S. erinaceieuropaei* [7]. The morphological difference in the uterine shape of the mature proglottids of *S. erinaceieuropaei* is due to differences in developmental larval stages [8]. *S. erinaceieuropaei*, *S. pretoriensis*, *S. theileri*, and *S. mansonoides* were the only valid species [9]. More recently, human sparganosis was identified by morphological and genetic analyses, and the complete mitochondrial genomes of *S. erinaceieuropaei* and *S. decipiens* have been recorded and compared in Korea [10,11].

Molecular identification has played an important role in studies on phylogenetics, parasite genetic variation and evolution over the decades. Mitochondrial DNA sequences have provided genetic markers for phylogenetic reconstruction, taxonomic identification, population genetics, and epidemiological investigations [12]. Investigations of phylogenetic relationships and genetic variation of the *Spirometra* tapeworms employed mitochondrial genomes or genes such as cytochrome c oxidase subunit 1 and 3 (*cox1* and *cox3*) and NADH dehydrogenase subunit 1, 3, and 4 (*nad1*, *nad3*, and *nad4*) [2,10,11,14-20].

The nucleotide sequence data employed by the aforementioned studies were obtained from public databases. The availability of nucleotide sequences of *Spirometra* in public databas-

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es has expanded since the 1990s and now includes the DNA DataBank of Japan (DDJB), European Molecular Biology Laboratory (EMBL) and GenBank (National Center for Biotechnology Information). These organizations provide a comprehensive public database of nucleotide sequences and supporting bibliographic and biological annotation of the submitted DNA sequence data from authors and from the bulk submission of expressed sequence tag (EST), genome survey sequence (GSS), whole-genome shotgun (WGS), and other high-throughput data from sequencing centers [21]. The sequence data in these public databases is made available by persons or groups that continue to make corrections to or update the data. Therefore, the speciation of sister or congeneric species may need to be reconsidered and revised based on any morphological analyses.

A total of 483,611 nucleotide sequences of *S. erinaceieuropaei* have been deposited in the GenBank database. Of those, 908 protein-coding DNA sequences of *S. erinaceieuropaei* are found in GenBank including nuclear and mitochondrial genomic DNA, as of November 2018. As indicated above, these DNA sequences may need to be revised following careful reconsideration based on morphological analyses of *Spirometra* tapeworms. The aim of this study was to observe mitochondrial DNA sequence variability of *S. erinaceieuropaei* in GenBank by reinvestigation of the sequence analysis of mitochondrial DNA based on species identified by morphological analyses of *S. erinaceieuropaei* and *S. decipiens*.

MATERIALS AND METHODS

DNA sequences of *Spirometra* spp. in GenBank

A total of 1,024 mitochondrial DNA sequences were extracted from GenBank in November 2018 for mitochondrial genes, *cox1* (n=514), *cox2* (n=10), *cox3* (n=73), *cytb* (n=213), *nad1* (n=60), *nad2* (n=10), *nad3* (n=10), *nad4* (n=74), *nad4L* (n=10), *nad5* (n=30), *nad6* (n=10), and *atp6* (n=10). Nineteen *cox1* sequences were obtained of human sparganosis from Australia, Japan, Korea and Thailand. The remaining *cox1*, *cox3*, *cytb*, *nad1*, and *nad4* sequences were obtained from various animals such as fox, dog, cat, snake, and frog. Four hundred and fifty-two DNA sequences were analyzed in this study, comprising DNA sequences of the *cox1* gene from Australia (n=12), China (n=360), India (n=1), Indonesia (n=3), Japan (n=30), Korea (n=3), Laos (n=16), New Zealand (n=1), Myanmar (n=2) and Thailand (n=14), and 213 DNA sequences of the *cytb* gene from China (n=209), Japan (n=2) and Korea (n=2). Among them, a total of 239 (*cox1*) and 213 (*cytb*) complete DNA sequences were inferred phylogenetic analysis (Table 1). The 10 complete mitochondrial DNA sequences of *Spirometra* species were compared with those of KJ599680 (*S. erinaceieuropaei*) and KJ599679 (*S. decipiens*), and comprised sequences from China (JQ267473, KY114886, KY114887, KY114888, KY114889 and KU852381) and Japan (AB374543 and AP017668), which were used as reference criteria for genetic observation of the GenBank database.

Table 1. Total number of mitochondrial DNA sequences of *Spirometra* species from GenBank as of November 2018

Genes		*cox1*	*cox2*	*cox3*	*cytb*	*nad1*	*nad2*	*nad3*	*nad4*	*nad4l*	*nad5*	*nad6*	*atp6*
Registered name of species	*S. erinaceieuropaei*	502	9	72	212	58	9	9	73	9	29	9	9
	S. decipiens	11	1	1	1	1	1	1	1	1	1	1	1
	S. ranarum	1				1							
Total (complete sequences)		514 (239)	10	73 (10)	213 (213)	60 (11)	10 (10)	10 (10)	74 (10)	10 (10)	30 (10)	10 (10)	10 (10)
Countries	Australia	12											
	China	360	6	69	209	55	6	6	70	6	26	6	6
	India	1											
	Indonesia	3											
	Japan	28	2	2	2	2	2	2	2	2	2	2	2
	Korea	3	2	2	2	3	2	2	2	2	2	2	2
	Lao PDR	16											
	New Zealand	1											
	Myanmar	2											
	Thailand	14											
	Others	74											
Total		514	10	73	213	60	10	10	74	10	30	10	10

MtDNA sequence analyses

DNA sequences were assembled using the Geneious 9.0 software program (Biomatter, Auckland, New Zealand) and than aligned using MAFFT methods in the Geneious 9.0 software program by comparison with DNA sequences of *S. erinaceieuropaei* and *S. decipiens* in the GenBank database. Molecular Evolution Genetics Analysis (MEGA) software version 7.0 was employed to analyze nucleotide composition, conserved sites, variable sites, singleton sites and parsimony-informative sites [22]. Evaluation of the number of haplotypes, nucleotide diversity and haplotype diversity were performed using DnaSP software version 6.12 [23].

Phylogenetic analyses

Phylogenetic analysis was evaluated using neighbor joining (NJ), maximum parsimony, Bayesian inference (BI) and maximum-likelihood (ML) using *cox1* (1,566 bp) and *cytb* (1,110 bp) sequences of *Spirometra* species. NL analysis was performed using MEGA version 7.0 [20]. MP analysis was performed in PAUP4b10 [24] using heuristic searches with tree bisection reconnection (TBR) branch swapping. ML analyses of *cox1* and *cytb* used RAxML version 7.3.1 [25] after the TRN+G substitution model was chosen with Modeltest using Partition Finder version 2.1.1 [26]. BI analyses were used in MrBayes 3.2 [27] after the HKY+G substitution model was chosen with Modeltest using Partition Finder version 2.1.1 and then running 2 independent MC^3 runs of 4 Markov chains each, for 10 million metropolis-coupled Markov chain Monte Carlo (MCMC) generations and discarding the first 25% generation as burn-in every 1,000 generations. Phylogenetic trees were constructed using the mitochondrial *cox1* and *cytb* DNA sequences of *Spirometra* species in GenBank and 3 taxa of Diphyllobothriidae represented by *Dibothriocephalus latum* (NC_008945) and *D. nihonkaiense* (NC_009463) as the outgroup to root the resulting trees.

RESULTS

Divergences of mt genomes among *Spirometra*

Mitochondrial genomes of *Spirometra* tapeworms have been reported for *S. erinaceieuropaei* 13,643 bp (AB374543; Japan), 13,603 bp (AP017668; Japan), 13,631 bp (JQ267473; China), 13,643 bp (KJ599680; Korea), 13,569 bp (KU852381; China), 13,609 bp (KY114887; China), 13,680 bp (KY114888; China), 13,643 bp (KY114886; China), 13,680 bp (KY113889; China), and for *S. decipiens* 13,641 bp (KJ599679, Korea). The mt genomes each contained 36 genes comprising 12 protein-coding genes, 22 tRNAs and 2 rRNAs.

A percentage pairwise comparison of sequence divergences of the 12 protein-coding genes between *S. erinaceieuropaei* (KJ 599680) and *S. decipiens* (the remaining mt genomes in this study) is shown in Table 2. The 12 protein-coding genes comprised 10,065 bp and 3,355 codons (*S. erinaceieuropaei*) and 10,067 bp and 3,355 codons (*S. decipiens*) of their respective mitochondrial genomes. The overall nucleotide sequence divergence of the 12 protein-coding genes between *S. erinaceieuropaei* and *S. decipiens* differed by 13.0% (Table 2). The divergence of amino acid sequences of the 12 protein-coding genes of *S. erinaceieuropaei* and *S. decipiens* ranged from as low as 2.3% (*nad4L*) to as high as 14.8% (*nad6*). The nucleotide sequence divergence of the most variable gene was the *nad6* and the most highly conserved gene was *cox1*, which was 18.8% and 9.4%, respectively, when comprising *S. erinaceieuropaei* and *S. decipiens* (Table 3). The nucleotide sequence difference of the 12 protein-coding genes within interspecies of *S. decipiens* between Sd and Sd* in Table 3 was

Table 2. Percentage whole mitochondrial DNA sequence homology of *Spirometra* species from GenBank as of November 2018

	KJ599680	AP017668	JQ267473	KJ599679	KY114888	KY114887	KY114889	KY114886	AB374543	KU852381
KJ599680		87.4	88.7	87.5	89	88.8	88.8	88.8	88.8	87.1
AP017668	87.4		98.7	98.2	98.3	98.1	98.1	98.1	98.1	97.6
JQ267473	88.7	98.7		98.7	98.8	98.8	98.8	99	99.1	99
KJ599679	87.5	98.2	98.7		99.1	99.3	99.4	99.4	99.4	98.7
KY114888	89	98.3	98.8	99.1		98.9	99.5	99.2	99.2	99.4
KY114887	88.8	98.1	98.8	99.3	98.9		99.2	99.5	99.6	99.8
KY114889	88.8	98.1	98.8	99.4	99.5	99.2		99.6	99.6	99.9
KY114886	88.8	98.1	99	99.4	99.2	99.5	99.6		99.9	99.9
AB374543	88.8	98.1	99.1	99.4	99.2	99.6	99.6	99.9		100
KU852381	87.1	97.6	99	98.7	99.4	99.8	99.9	99.9	100	

KJ599680, *S. erinaceieuropaei*; KJ599679, *S. decipiens*; Genbank No. AP017668, JQ267473, KY114886-114888, AB374543 and KU852381 are currently registered as *S. erinaceieuropaei* in GenBank.

Table 3. Nucleotides and amino acid divergence of 12 protein-coding genes in mt genomes

	Se	Sd	Sd*		Se	Sd	Sd*		Se	Sd	Sd*		Se	Sd	Sd*
cox1				*cox2*				*cox3*				*cytb*			
Se	-	9.4	8.8		-	10.4	10.4		-	12	10.4		-	10.9	11.2
Sd	2.9	-	2.2		3.2	-	0		5.6	-	1.4		4.1	-	2.4
Sd*	2.9	0.4	-		3.2	0	-		5.1	0.9	-		3.8	1.1	-
atp6				*nad1*				*nad2*				*nad3*			
Se	-	13.4	14.1		-	9.8	10		-	13.7	14		-	13	13.6
Sd	8.2	-	1.9		6.1	-	2.1		8.6	-	1.7		7.8	-	1.7
Sd*	8.2	1.2	-		5.4	0.7	-		7.6	1	-		7.8	0.9	-
nad4				*nad4L*				*nad5*				*nad6*			
Se	-	14	13.7		-	11.9	11.5		-	18.1	18.1		-	18.8	18.8
Sd	9.4	-	2.6		2.3	-	1.2		11.9	-	1.4		14.8	-	0
Sd*	9.1	1.4	-		2.3	0.2	-		12.1	0.8	-		14.8	0	-

Percentage pairwise divergences of nucleotides (above diagonal) and amino acids (below diagonal) of the 12 protein-coding genes of the *Spirometra* tapeworms; Se: *Spirometra erinaceieuropaei* (KJ599680), Sd: *S. decipiens* (KY114886-114889 are currently registered as *S. erinaceieuropaei* except KJ599679 in GenBank), Sd*: *S. decipiens* (AB374543, AP017668, JQ267473 and KU852381 are currently registered as *S. erinaceieuropaei* in GenBank).

Table 4. Genetic diversities of mitochondrial DNA sequences of *Spirometra* species in GenBank

	V-S	C-S	Sin-S	Parsi-S	Syn-S	NonSyn-S	Sq-D	Hd±S.D.	Pi±S.D.
cox1 (n=238)	198	1,364	32	166	363.02	1187.98	0-0.059	0.9936±0.0013	0.02484±0.00060
cytb (n=211)	93	1,017	24	69	270.64	836.36	0-0.036	0.931±0.013	0.01158±0.00065

V-S, variable site; C-S, conserved site; Sin-S, singleton site; Parsi-S, parsimony informative site; Syn-S, synonymous site; NonSyn-S, nonsynonymous site; Sq-D, sequence divergence; Hd, haplotype diversity; Pi, nucleotide diversity.

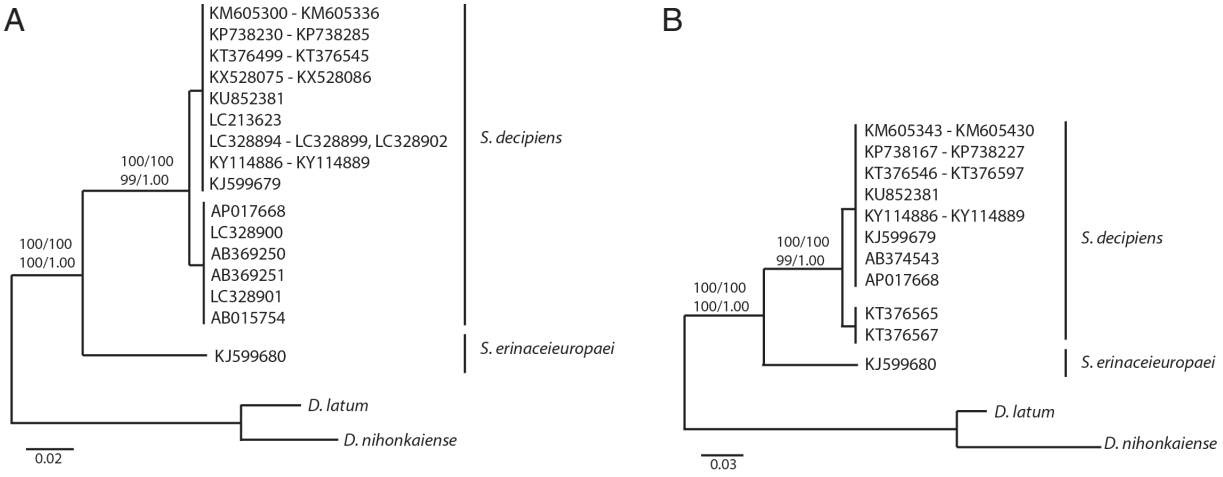

Fig. 1. Phylogenetic trees of *cox1* (A) and *cytb* (B) sequences of *Spirometra* species retrieved from GenBank. Trees were inferred by neighbor joining, maximum parsimony, maximum likelihood and Bayesian inference analyses. Numbers above nodes represent bootstrap values.

1.5% and ranged from as low as 0.0% (*cox2* and *nad6*) to as high as 2.6% (*nad4*) (Table 3).

Sequence analysis

The concatenated sequence analysis contained 239 (*cox1*) and 213 (*cytb*) sequences, of which 1,566 bp and 1,110 bp, respectively, comprised mitochondrial genes. Within the *cox1* and *cytb* genes was found 198 and 93 polymorphic sites, 166 and 69 parsimony informative sites, and 32 and 24 singleton variable sites, respectively. The polymorphic sites identified comprised 148 (*cox1*) and 83 (*cytb*) haplotypes within 239 and 213 isolates, respectively, from 3 Asian countries (China,

Japan and Korea). Non-synonymous substitution sites found were 1,187.98 (*cox1*) and 836.36 (*cytb*), while 363.02 and 270.64 represented synonymous substitution sites. The genetic divergence of *cox1* and *cytb* sequences of *Spirometra* species (*S. decipiens*) ranged from 0% to 5.9% and 0% to 3.6%, respectively. These 2 mitochondrial genes have high Hd (haplotype diversity) and Pi (nucleotide diversity) (Table 4).

Phylogenetic diversity

All haplotypes of *Spirometra* species were separated into 2 distinct clades in phylogenetic analyses based on neighbor joining, maximum parsimony, maximum likelihood and Bayesian inference. Clade I was *S. erinaceieuropaei* and clade II was *S. decipiens*. Phylogenetic tree topologies generated from the 4 analytic methods were identical and presented high-level confidence values for the 2 major branches of 2 *Spirometra* species for the *cox1* and *cytb* genes. The ML and BI analyses supported monophyly of *Spirometra* species and identified the species *S. erinaceieuropaei* and *S. decipiens* as 2 clades with bootstrap values of 100 and 1.00, respectively, based on *cox1* and *cytb* sequences (Fig. 1). The tree topologies of the NJ and MP analysis were congruent with those topologies in the ML and BI analyses. The bootstrap value of NJ and MP was 100 and 100, respectively. Both of tree in placing the 2 sub-clade were observed in *S. decipiens* clade by ranged 0-5.9% (*cox1*) and 0-3% (*cytb*) DNA sequences differences (Fig. 1).

DISCUSSION

Morphological and experimental studies for *Spirometra* species have been reported by Chandler [28] and Faust et al. (1929) [4]. Faust et al. (1929) reviewed *Spirometra* species (under the name *Diphyllobothrium*) with morphological and biological studies of *S. erinaceieuropaei* (Rudolphi, 1819), *S. decipiens* (Diesing, 1850), *S. ranarum* (Gastaldi, 1854), *S. mansoni* (Cobbold, 1882), *S. houghtoni* (=*S. mansoni*, Faust et al. [4], 1929) and *S. okumurai* (Faust et al. [4], 1929). *S. erinaceieuropaei* was first reported by Rudolphi (1819) and assigned the name *Dubium erinacei-europaei* from thoracic spargana of the hedgehog (*Erinaceus europaeus*). This larval tapeworm was recognized as a sparganum by Diesing (1854) and renamed to *Sparganum lanceolatum* by Moiln (1859). *S. decipiens* was described by Diesing (1854) and Lühe (1899) reexamined the original material with the recorded uterine character which facilitated its identification. Detailed morphological characteristics were then described by Chandler (1925) [4,28]. The only *Spirometra* species reported in North America was by Mueller (1935), who described the new species *S. mansonoides*, which was distinguished from *S. mansoni* distributed in the Asian region based on morphological characteristics [29].

The major difference in feature between *S. erinaceieuropaei* and *S. decipiens* is the spirally coiled uterus of *S. erinaceieuropaei*, which consists of 5-7 coils, while that of *S. decipiens* consist of 4-4.5 coils [4,10]. However, certain morphological characteristics are by *S. decipiens* and *S. mansoni*, such as their male and female reproductive organs. Morphological characteristics of *S. ranarum* include a uterine morphology that consists of 3-4.5 uterine coils, of which the posterior 2 are broader and more dilated than the terminal uterine ball [4,30]. One notable feature of *S. mansonoides* is the C-shaped outer loop of the uterus with its anterior limb constricted in the midline to form a lateral expulsion chamber [29].

Identification of *Spirometra* species has been reported from many endemic areas in Asian, South American and African countries as a result of molecular studies based on mitochondrial DNA sequences. Partial mitochondrial DNA sequence variations of *S. erinaceieuropaei* isolates from China, Japan and Indonesia ranged from 0.0-2.6% [31], 0.0-3.1% [14,15], 0.0-8.4% [16], and 0.0-7.4% [20] for *cox1*, 0.0-1.5% [15], 0.0-2.4% [16,20] for *cox3*, 0.0-2.8% [15] for *nad1*, and 0.0-2.7% [15], 0.0-1.4% [20] for *nad4*. Complete mitochondrial DNA sequence variations of *S. erinaceieuropaei* ranged from 0 to 4.9% for *cox1* and 0 to 3.6% for *cytb* [17,18]. African human sparganosis was found to differ from that of Asian and South American isolates by analysis of mitochondrial DNA sequence data [32]. Two *Spirometra* species, *S. theileri* (Baer, 1924) and *S. pretoriensis* (Baer, 1924) were reported in lions and hyenas in Africa [33]. South American isolates of *Spirometra* were neither *S. erinaceieuropaei*, *S. decipiens* nor *S. mansonoides* by *cox1* sequence analysis. Five *Spirometra* species comprising *S. decipiens*, *S. mansoni*, *S. gracile* (Baer, 1927), *S. longicollis* (Parodi and Widakowich, 1917) and *S. mansonoides* were reported from wild animals in South America [34]. These studies provoked epidemiological questions concerning the need to identify which *Spirometra* species are distributed in endemic areas and which *Spirometra* species induce human sparganosis. Many previous studies may need to be reexamined using a combination of molecular and morphological techniques to better understand the epidemiological status of *Spirometra* spp.

In this study, complete mitochondrial DNA sequence analy-

ses were performed using *cox1* and *cytb* genes and mitochondrial genomes derived from the GenBank database, thereby allowing for genetic variation and phylogenetic relationships to be determined. The nucleotide diversity (Pi) value is employed as a measure of genetic variation, and Pi > 0.01 is considered to indicate a comparatively large variation in animal phyla. The Pi values (genetic diversity) of the *cox1* and *cytb* genes were greater than 0.01 in this study, suggesting that nucleotide sequences of *Spirometra* species in the GenBank database have a large variation. The results of genetic divergence and polymorphic sites showed that there is moderate to high genetic differentiation. Sequence differences of *Spirometra* sp. haplotypes was shown to be greater than 5% for data in the GenBank database. These results indicated that mitochondrial haplotypes of *Spirometra* species were represented by at least 2 haplotypes within *S. erinaceieuropaei*. The genetic distance between *S. erinaceieuropaei* and *S. decipiens* differed by 12.4% in the 12 protein-coding genes, whereas the sequence difference for the complete mitochondrial sequences was 12.9% [35]. The phylogenetic analyses based on the *cox1* and *cytb* genes, and analyses of complete mt genome sequences based on ML, BI, NJ and MP methods, all suggested that mitochondrial DNA sequences of *Spirometra* species were separated into 2 distinct haplotypes isolated from China, Japan and Korea. Phylogenetic tree topologies showed 2 major branches of 2 *Spirometra* species containing *S. erinaceieuropaei* and *S. decipiens*, and *S. decipiens* sub-clades including all sequences registered as *S. erinaceieuropaei* in the *cox1* and *cytb* genes from GenBank.

This study has shown that the overall nucleotide sequence and amino acid divergence of 12 protein-coding genes between *S. erinaceieuropaei* and *S. decipiens* differed by 12.9% and 12.4%, respectively. Analyses of the *cox1* and *cytb* genes revealed 198 and 93 polymorphic sites, of which 166 and 69 were parsimony informative sites, and 32 and 24 were singleton variable sites, respectively. These polymorphic sites showed 148 (*cox1*) and 83 (*cytb*) haplotypes within 239 and 213 isolates from 3 Asian countries (China, Japan and Korea). Phylogenetic tree topologies generated from the 4 analytic methods were identical and presented high-level confidence values for the 2 major branches of 2 *Spirometra* species containing *S. erinaceieuropaei* and *S. decipiens*, and *S. decipiens* sub-clades including all sequences registered as *S. erinaceieuropaei* for the *cox1* and *cytb* genes from GenBank. These results indicated that mitochondria haplotypes of *S. erinaceieuropaei* and *S. decipiens* were found in Asian countries.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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