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▣ ORIGINAL ARTICLE

Mitochondrial Genome Sequences of *Spirometra erinaceieuropaei* and *S. decipiens* (Cestoidea: Diphyllobothriidae)

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Abstract: The present study was performed to compare the mitochondrial genomes between 2 *Spirometra* tapeworms, *Spirometra erinaceieuropaei* and *Spirometra decipiens* (Cestoidea: Diphyllobothriidae), which larval stages are important etiological agents of sparganosis in humans. For each species, the full mitochondrial genome was amplified in 8 overlapping fragments using total genomic DNA purified from a single worm as the template. The mitochondrial genomes were 13,643 bp (*S. erinaceieuropaei*) and 13,641 bp (*S. decipiens*) in length and contained 36 genes; 12 protein-coding genes, 2 ribosomal RNA (rRNA, small and large subunits), and 22 transfer RNAs (tRNAs). The 12 protein-coding genes constituted 10,083 bp (*S. erinaceieuropaei*) and 10,086 bp (*S. decipiens*) of their respective mitochondrial genomes. The tRNA genes, ranging in length from 56 to 70 bp, were identified based on putative secondary structures such as the typical cloverleaf shape. A total of 23 intergenic sequences, varying from 1 to 204 bp in size, were interspersed in *S. erinaceieuropaei* (total, 504 bp) and *S. decipiens* (total, 496 bp) mtDNA. The 12 protein-coding genes of *S. erinaceieuropaei* and *S. decipiens* differed by 12.4%, whereas the overall difference in mtDNA sequence between *S. erinaceieuropaei* and *S. decipiens* was 12.9%. Thus, from the standpoint of the mitochondrial genome, *S. decipiens* represents a valid species that can be distinguished from *S. erinaceieuropaei*.

Key words: *Spirometra erinaceieuropaei*, *Spirometra decipiens*, mitochondrial genome

INTRODUCTION

Spirometra erinaceieuropaei and *S. decipiens* are pseudophyllidean cestodes. In humans, these worms can induce sparganosis, and their medical importance is well- established. Humans can be infected by procercoid larvae in cyclops (the first intermediate host) and plerocercoid larvae in reptiles or amphibians (paratenic hosts). The genus *Spirometra* was established by Mueller [1], but this name had been used earlier by Faust et al. [2], who proposed dividing the genus into 2 subgenera, *Diphyllobothrium* and *Spirometra*.

S. erinaceieuropaei was first reported by Rudolphi as *Dubium erinaceieuropaei* from hedgehogs (*Erinaceus europaeus*) in 1819. Diesing in 1853 recognized this larva as a sparganum and named it *Sparganum erinaceieuropaei*. Molin in 1895 renamed it as *Sparganum lanceolatum*, which is regarded as a synonym. These names refer to the larval stage of spirometrid species. Based on morphological characteristics described by Diesing [2], *S. decipiens* is synonymous with 4 species; *Bothriocephalus felis* Creplin, 1825, *Bothriocephalus maculatus* Leukart, 1848, *Dibothrium decipiens*, and *Bothriocephalus decipiens* Diesing, 1850. In 1929, Faust, Campbell and Kellogg reviewed the morphological characteristics of *S. erinaceieuropaei* and *S. decipiens* by monitoring the experimental development of adults from spargana in humans and other vertebrates.

Currently, the species classification in the genus *Spirometra* is controversial. Despite the availability of reliable diagnostic morphological characters for species identification, the species-level taxonomy of *Spirometra* spp. remains obscure. Therefore, it is necessary to devise a reliable taxonomic criterion based on morphological characteristics in combination with molecular analysis of mitochondrial DNA sequences. Mitochondrial DNA sequencing is now considered a useful molecular tool for inferences of evolutionary analysis, phylogenetic reconstruction, taxonomic identification, biogeography, population genetics, and epidemiological investigation [3,4]. The com-

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plete mitochondrial genomes of order Pseudophyllidea have been recently published for *Diphyllobothrium latum* [5], *Diphyllobothrium nihonkaiense* [6], and *S. erinaceieuropaei* [7].

Within the genus *Spirometra*, mitochondrial DNA sequences have also been used as genetic makers for identification and characterization of genetic variation of species [8,9]. The mtDNA *cox1* gene sequences revealed that *S. erinaceieuropaei* and *S. proliferum* are distinct species [10]. More recently, the complete mitochondrial genome of *S. erinaceieuropaei* isolated from China was reported and compared to the mitochondrial genome of a Japanese isolate [7]. However, the speciation of the parasite may need to revise careful reconsideration based on morphological analyses of *Spirometra* tapeworms. The aim of this study was to provide information useful for classification of *Spirometra* spp., by the mitochondrial genome of *S. erinaceieuropaei* and *S. decipiens* and the mitochondrial genomes with those of other cestodes.

MATERIALS AND METHODS

Worm samples

Plerocercoid larvae of *S. decipiens* were collected from *Rhabdophis tigrinus tigrinus*, which were donated by the Association of Wildlife Protection, and then used to infect a cat, in April 2008. Plerocercoid larvae of *S. erinaceieuropaei* were collected from a 58-year-old woman, and then used to infect a dog in May 2009. Eight weeks after the infection, the cat and the dog were sacrificed, and adult worms were recovered from their intestines. Experimental animals were used following the ethical guidelines in commission of laboratory animals in Chungbuk National University (2008). The tapeworms were pressed and fixed in alcohol-formalin-acetic acid (AFA) for carmine staining. The following anatomical features of mature and gravid proglottids were observed, based on the morphological data of Faust et al. [2]; vaginal opening, uterus, uterine pore, cirrus, genital pore, testes, and vitellaria. Gravid proglottids were preserved at -70˚C in 70% ethanol until use. A single proglottid was chopped into small pieces, and total genomic DNA was extracted using the DNeasy tissue Kit (Qiagen, Valencia, California, USA).

PCR and DNA sequencing

For each species, the full mitochondrial genome was amplified in 8 overlapping fragments using total genomic DNA purified from a single worm as the template. The overlapping fragments of *S. decipiens* and *S. erinaceieuropaei* mtDNA were amplified using 16 oligonucleotide primers. The primers were used to amplify the *cob-nad2* region (Spiro-cob-F1: TTT YCA YTC TTA TTT TAC YAC TAA GAA and Spiro-nad2-R1: AYC ACA CAT ACT CCC ARC TTG GGC TAC, 3.1 kbp), *nad2-nad1* region (Spiro-nad2-F1: TTT GGG CST YKT GTT GYR TGT GTT ATT and Spiro-nad1-R1: CCA ACC RGC ACA CAA TAA AGC ATA ACT, 1.0 kbp), *nad1-cox1* region (Spiro-nad1-F1: TAT GCY GAG TCG GAG AGG GAG TTG GTT and Spiro-cox1-R1: AAC MCC AAT AAT CAT AGT YAC AGA ACT, 2.1 kbp), *cox1-rrnS* region (Spiro-cox1-F1: GAC TGG TAA GTT AAT TTA AAC TGT and Spiro-12S-R1: CAT CTA ACC CAA CCG TAA CAT A, 2.5 kbp), *rrnS-nad5* region (Spiro-12S-F1: GTA TTA ATA TTT AAG CTA AGT CTA TGT GCT and Spiro-nad5-R1: AAA CRC ACC AAG CAA TTT TAT TAC AGG TRG, 3.0 kbp) and *nad5-cob* region (Spiro-nad5-F1: CYA CCT GTA ATA AAA TTG CTT GGT GYG TTT and Spiro-cob-R1: YAA ACA AAC ATG AGC TGA AAA WAC ACG AAC, 2.5 kbp). PCR and DNA sequencing was performed as described previously [11].

Sequencing analysis

Sequences were assembled and aligned using Geneious 6.1.5 program (Biomatters Co., Auckland, New Zealand). The sequenced regions were identified using BLAST searches and compared with platyhelminth sequences in the GenBank database. Protein-coding genes were identified based on similarity of inferred amino acid sequences to those of other platyhelminth mtDNAs, as well as multiple comparisons with mitochondrial gene sequences in the GenBank database. The mitochondrial genetic code of platyhelminths was used to obtain putative translational products of the mitochondrial protein-coding sequences. Two ribosomal RNA genes (12S and 16S subunits) were determined by alignments with other known rRNA genes of platyhelminths. Twenty-two putative tRNA genes were identified using the tRNAscan-SE software [12] and anticodon sequences. The putative stem-loop structures of non-coding mitochondrial regions were inferred using the RNAdraw program [13].

Phylogenetic analysis was conducted using PAUP 4.0 [14]. The following mitochondrial genome sequences were used: *S. erinaceieuropaei* (KJ599680; this study), *S. decipiens* (KJ599679; this study), *Diphyllobothrium latum* (NC_008945), *D. nihonkaiense* (NC_009463), *Echinococcus granulosus* (NC_008075), *E. multilocularis* (NC_000928), *Hymenolepis diminuta* (NC_002767), *Taenia solium* (NC_004022), *T. saginata* (NC_009938), *T. asiatica* (NC_004826), and *T. crassiceps*

(NC_002647). Phylogenetic trees were constructed using the maximum likelihood (ML), maximum parsimony (MP), neighbor joining (NJ), and Bayesian inference [15], with *Fasciola hepatica* (NC_002546) and *Paragonimus westermani* (NC_002354) as outgroups. Confidence values for tree branches were determined by bootstrap analyses with 1,000 replicates.

RESULTS

Gene content and organization

The mitochondrial genomes of *Spirometra* tapeworms were 13,643 bp (*S. erinaceieuropaei*) and 13,641 bp (*S. decipiens*) in length. The genomes each contained 36 genes, of which 12 were protein-coding genes, 2 were ribosomal RNA (rRNAs, small and large subunit), and 22 were transfer RNAs (tRNAs) (Fig. 1). As with other cestodes, both genomes lacked the *atp8* gene. We assumed that all the genes were transcribed on 1 strand in the same direction and arranged in the same relative positions as gene loci in known cestode mitochondrial genomes. The arrangement of mitochondrial genes in *Spirometra* tapeworms was identical to that of other pseudophyllidean cestodes published to date, with the exception of *Hymenolepis diminuta*, in which the order of *trnL* and *trnS2* is reversed. The nucleotide compositions of the whole mitochondrial genomes are 19.8% A, 45.9% T, 23.5% G, and 10.9% C (*S. erinaceieuropaei*) and 20.3% A, 46.0% T, 22.7% G, and 11.0% C (*S. decipiens*). As in other cestodes, the genomes are A + T rich: *S. erinaceieuropaei*, 65.7% A + T; and *S. decipiens*, 66.3% A + T (Table 1). Some genes overlap at the boundaries: *cox3*/*trnH* (10 bp), *nad4L*/*nad4* (40 bp), *trnQ*/*trnF* (4 bp), *trnF*/*trnM* (4 bp), *nad3*/

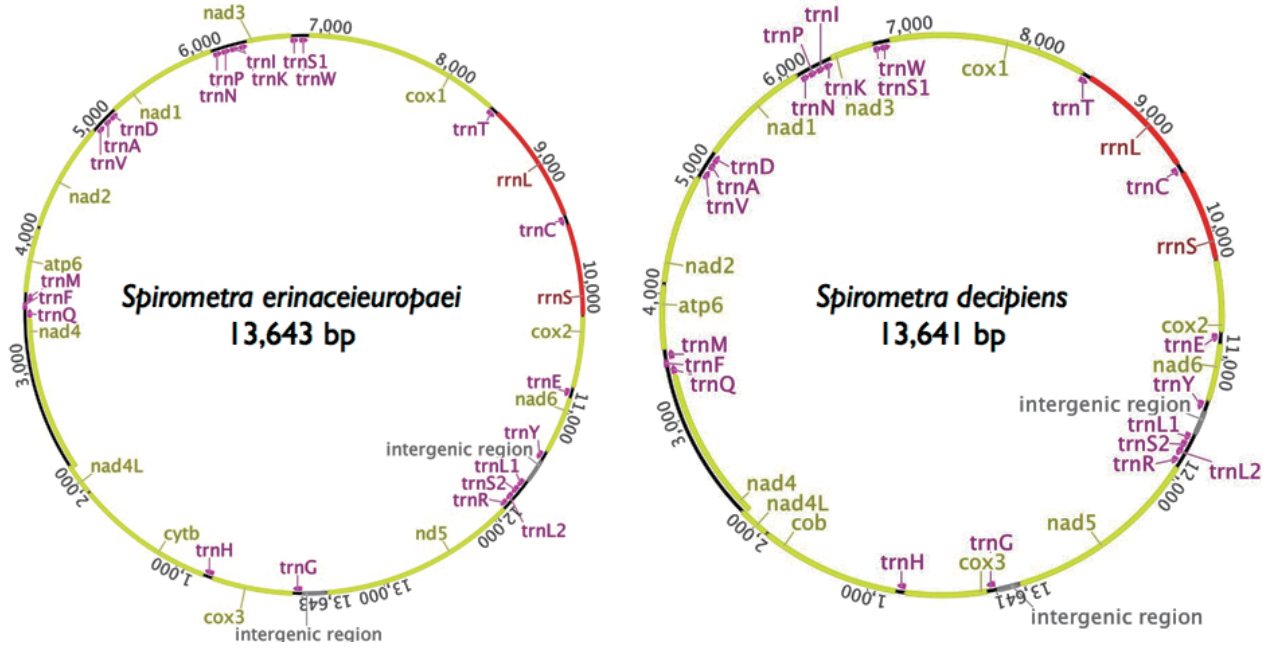

Fig. 1. Schematic representation of the mitochondrial genomes of *S. erinaceieuropaei* and *S. decipiens*.

Table 1. Nucleotide compositions of the complete mitochondrial genomes, protein-coding genes, and ribosomal RNA sequences of 2 *Spirometra* species

Species	Complete mtDNA sequence						Protein-coding sequence						rRNA sequence					
		T	C	A	G	T+A		T	C	A	G	T+A		T	C	A	G	T+A
	Length (bp)	%	%	%	%	%	Length (bp)	%	%	%	%	%	Length (bp)	%	%	%	%	%
Se[a]	13,643	45.9	10.9	19.8	23.5	65.7	10,083	48.3	10.6	17.5	23.5	65.8	1,700	38.7	12.2	24.9	24.2	63.6
Sd[b]	13,641	46.0	11.0	20.3	22.6	66.3	10,086	48.6	10.6	18.3	22.5	66.9	1,703	37.6	12.9	25.1	24.4	62.7

[a]*Spirometra erinaceieuropaei*.
[b]*S. decipiens*.

Table 2. Position and characteristics of the protein-coding and non-coding sequences in the mitochondrial genomes of *Spirometra erinaceieuropaei* and *S. decipiens*

Genes	Length of genes and sequences				Codon used for				Position in genome (5'-3')	
	Nucleotide		Amino acid		Initiation		Termination			
	Se[a]	Sd[b]	Se	Sd	Se	Sd	Se	Sd	Se	Sd
trnG	67	67							1-67	1-67
cox3	651	651	216	216	GTG	GTG	TAG	TAG	71-721	71-721
trnH	70	69							712-781	712-780
cob	1,110	1,110	369	369	ATG	ATG	TAA	TAA	785-1,894	784-1,893
nad4L	261	261	86	86	ATG	ATG	TAG	TAG	1,899-2,159	1,898-2,158
nad4	1,254	1,254	417	417	ATG	ATG	TAG	TAG	2,120-3,373	2,119-3,372
trnQ	64	64							3,374-3,437	3,373-3,436
trnF	64	64							3,434-3,497	3,433-3,496
trnM	68	68							3,494-3,561	3,493-3,560
atp6	516	516	171	171	ATG	ATG	TAA	TAA	3,565-4,080	3,564-4,079
nad2	873	873	290	290	ATG	ATG	TAG	TAG	4,092-4,964	4,087-4,959
trnV	65	66							4,969-5,033	4,970-5,035
trnA	61	61							5,051-5,111	5,052-5,112
trnD	66	64							5,116-5,181	5,118-5,181
nad1	891	891	296	296	ATG	ATG	TAA	TAA	5,182-6,072	5,182-6,072
trnN	66	66							6,078-6,143	6,078-6,143
trnP	65	65							6,150-6,214	6,150-6,214
trnI	64	64							6,220-6,283	6,220-6,283
trnK	63	63							6,291-6,353	6,290-6,352
nad3	357	357	118	118	ATG	ATG	TAG	TAG	6,359-6,715	6,356-6,712
trnS1$_{(AGN)}$	59	59							6,705-6,763	6,702-6,760
trnW	65	66							6,773-6,837	6,763-6,828
cox1	1,566	1,566	521	521	ATG	ATG	TAG	TAG	6,845-8,410	6,836-8,401
trnT	69	70							8,401-8,469	8,392-8,461
rrnL	967	973							8,470-9,436	8,462-9,434
trnC	65	65							9,437-9,501	9,435-9,499
rrnS	733	730							9,502-10,234	9,500-10,229
cox2	570	570	189	189	ATG	ATG	TAG	TAA	10,235-10,804	10,230-10,799
trnE	65	65							10,810-10,874	10,805-10,869
nad6	465	468	154	155	ATG	ATG	TAA	TAA	10,879-11,343	10,874-11,341
trnY	68	68							11,350-11,417	11,348-11,415
NR1[c]	201	204							11,418-11,618	11,416-11,619
trnL1$_{(CUN)}$	67	67							11,619-11,685	11,620-11,686
trnS2$_{(UGN)}$	66	66							11,688-11,753	11,689-11,754
trnL2$_{(UUN)}$	65	65							11,757-11,821	11,759-11,823
trnR	56	57							11,831-11,886	11,839-11,895
nad5	1,570	1,569	522	522	ATG	ATG	TAA	TAA	11,890-13,458	11,899-13,467
NR2[c]	184	174							13,459-13,643	13,468-13,641

[a]*Spirometra erinaceieuropaei*.
[b]*S. decipiens*.
[c]Non-coding region.

trnS1 (11 bp), and *cox1*/*trnT* (10 bp), respectively (Table 2).

Protein-coding genes

Approximately 65% of the mitochondrial genomes of *Spirometra* tapeworms consisted of protein-encoding genes, similar to the values reported for other cestodes. The 12 protein-coding genes constituted 10,083 bp (*S. erinaceieuropaei*) and 10,086 bp (*S. decipiens*) of their respective mitochondrial genomes (Table 1). All of the putative open reading frames (ORFs) of 12 protein-coding genes in both species start and end with complete codons. The ATG initiation codon was used in 11 genes (*atp6*, *cob*, *cox1*, *cox2*, *nad1*, *nad2*, *nad3*, *nad4*,

Table 3. Codon usage in the 12 protein-coding genes of the mitochondrial genomes of *Spirometra* species

NC[c]	AA[d]	Se[a]		Sd[b]		NC	AA	Se		Sd	
		No.[e]	%	No.	%			No.	%	No.	%
TTT	Phe	399	11.9	386	11.5	TAT	Tyr	158	4.7	179	5.3
TTC	Phe	24	0.7	34	1.0	TAC	Tyr	42	1.2	25	0.7
TTA	Leu	187	5.6	209	6.2	TAA	*[f]	5	0.1	6	0.2
TTG	Leu	225	6.7	190	5.7	TAG	*	7	0.2	6	0.2
CTT	Leu	58	1.7	73	2.2	CAT	His	44	1.3	38	1.1
CTC	Leu	8	0.2	4	0.1	CAC	His	10	0.3	12	0.4
CTA	Leu	19	0.6	25	0.7	CAA	Gln	5	0.1	4	0.1
CTG	Leu	28	0.8	26	0.8	CAG	Gln	18	0.5	18	0.5
ATT	Ile	142	4.2	142	4.2	AAT	Asn	51	1.5	54	1.6
ATC	Ile	16	0.5	14	0.4	AAC	Asn	10	0.3	7	0.2
ATA	Ile	64	1.9	74	2.2	AAA	Asn	30	0.9	36	1.1
ATG	Met	79	2.4	80	2.4	AAG	Lys	47	1.4	46	1.4
GTT	Val	179	5.3	192	5.7	GAT	Asp	64	1.9	54	1.6
GTC	Val	22	0.7	18	0.5	GAC	Asp	5	0.1	14	0.4
GTA	Val	55	1.6	46	1.4	GAA	Glu	12	0.4	18	0.5
GTG	Val	107	3.2	98	2.9	GAG	Glu	50	1.5	49	1.5
TCT	Ser	122	3.6	120	3.6	TGT	Cys	122	3.6	125	3.7
TCC	Ser	13	0.4	18	0.5	TGC	Cys	13	0.4	9	0.3
TCA	Ser	35	1.0	40	1.2	TGA	Trp	24	0.7	38	1.1
TCG	Ser	18	0.5	16	0.5	TGG	Trp	75	2.2	57	1.7
CCT	Pro	39	1.2	47	1.4	CGT	Arg	44	1.3	47	1.4
CCC	Pro	25	0.7	19	0.6	CGC	Arg	2	<0.1	2	<0.1
CCA	Pro	11	0.3	12	0.4	CGA	Arg	4	0.1	2	<0.1
CCG	Pro	10	0.3	6	0.2	CGG	Arg	6	0.2	7	0.2
ACT	Thr	67	2.0	71	2.1	AGT	Ser	80	2.4	96	2.9
ACC	Thr	13	0.4	16	0.5	AGC	Ser	18	0.5	9	0.3
ACA	Thr	12	0.4	10	0.3	AGA	Ser	23	0.7	15	0.4
ACG	Thr	19	0.6	17	0.5	AGG	Ser	27	0.8	18	0.5
GCT	Ala	70	2.1	65	1.9	GGT	Gly	151	4.5	144	4.3
GCC	Ala	16	0.5	21	0.6	GGC	Gly	15	0.4	10	0.3
GCA	Ala	7	0.2	12	0.4	GGA	Gly	20	0.6	26	0.8
GCG	Ala	11	0.3	7	0.2	GGG	Gly	79	2.4	83	2.5

Putative initiation (ATG and GTG) and termination (TAA and TAG) codons are underlined.
[a]*Spirometra erinaceieuropaei*; 3,361 codons were used to encode 3,349 amino acids and 12 stop codons.
[b]*S. decipiens*, 3362 codons were used to encode 3,350 amino acids and 12 stop codons.
[c]Nucleotide codons.
[d]Amino acid.
[e]Number of codons.
[f]Termination codons.

nad4L, and *nad6*), whereas the GTG initiation codon was used only in the *cox3* gene. The TAG stop codon was used in 7 genes (*cox1*, *cox2*, *cox3*, *nad2*, *nad3*, *nad4*, and *nad4L*) in *S. erinaceieuropaei* and 6 genes (*cox1*, *cox3*, *nad2*, *nad3*, *nad4*, and *nad4L*) in *S. decipiens*, whereas the TAA termination codon was used in the remaining 5 genes (*atp6*, *cob*, *nad1*, *nad5*, and *nad6*) in *S. erinaceieuropaei* and 6 genes (*atp6*, *cob*, *cox2*, *nad1*, *nad5*, and *nad6*) in *S. decipiens* (Table 2). Codon usage is shown in Table 3. The 4 most commonly used codons were Leu (TTR and CTN; 15.6%), Phe (TTY; 12.6%), Val (GTN; 10.8%) and Ser (AGN and TCN; 9.9%) in *S. erinaceieuropaei*, and Leu (TTR and CTN; 15.7%), Phe (TTY; 12.5%), Val (GTN; 10.7%), and Ser (AGN and TCN; 9.9%) in *S. decipiens*.

Transfer RNA and ribosomal RNA genes

We identified a total of 22 tRNA genes in the *S. erinaceieuropaei* and *S. decipiens* mitochondrial genomes. The tRNA genes, ranging in length from 56 bp to 70 bp, were identified based on putative secondary structures such as the typical cloverleaf shape (Fig. 2). The inferred secondary structure of 19 tRNA ex-

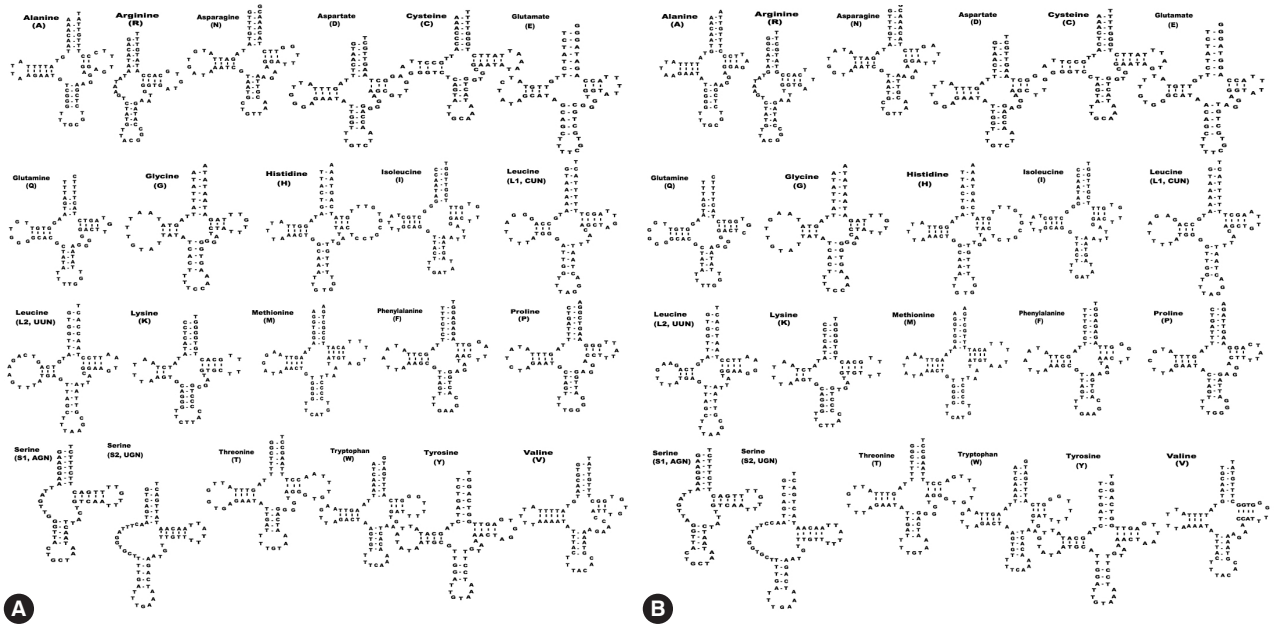

Fig. 2. Inferred secondary structures of the 22 mitochondrial tRNA from *S. erinaceieuropaei* (A) and *S. decipiens* (B).

Table 4. Divergences of nucleotides and amino acids of the protein-coding genes

		Spirometra erinaceieuropaei													
		cox1	*cox2*	*cox3*	*cob*	*atp6*	*nad1*	*nad2*	*nad3*	*nad4*	*nad4L*	*nad5*	*nad6*	*rrnL*	*rrnS*
S. decipiens	NC[a]	9.3	10.4	12.1	10.9	13.4	9.8	13.7	12.6	14	11.9	18.1	18.8	11.2	6.9
	AA[b]	1.9	3.2	5.6	4.1	8.2	6.1	8.6	6.8	9.4	2.3	11.9	14.8		

Percentage pairwise divergences of nucleotides and amino acids of the 12 protein-coding genes of *Spirometra erinaceieuropaei* and *S. decipiens*.
[a]Nucleotide codons.
[b]Amino acids.

hibited the typical cloverleaf shape, with paired DHU arms; in the remaining 3 tRNAs (*trnR*, *trnS1*, and *trnS2*), this arm was replaced with a 7-12 nt unpaired loop. The aminoacyl acceptor arms consisted of 7 bp, and *trnA*, *trnI*, *trnM*, *trnQ*, *trnR*, *trnS2*, *trnT*, and *trnV* contained 1 or 3 non-canonical base pairs. The anticodon stems of all 22 tRNAs contained of 5 bp, as in typical stem structures. The predicted secondary structures of the 22 tRNAs in *S. erinaceieuropaei* and *S. decipiens* had paired TΨC arms, consisting of a 2-5 bp stem with a 3-9 bp loop. The variable loop between the anticodon and the TΨC stems consisted of 3-5 nt, except in *trnR*, *trnC*, *trnS1*, and *trnS2* (Fig. 2). In both species, the ribosomal RNA genes *rrnL* and *rrnS* were separated by *trnC*. The *rrnL* and *rrnS* genes were 967 bp and 733 bp long, respectively, in *S. erinaceieuropaei*, and 973 bp and 730 bp long in *S. decipiens*. The nucleotide content of the rrn genes was 63.6% (A+T) in *S. erinaceieuropaei* and 62.7% (A+T) in *S. decipiens* (Table 1).

Non-coding regions

A total of 23 intergenic sequences, varying from 1 to 204 bp in size, were interspersed in the *S. erinaceieuropaei* (total, 504 bp) and *S. decipiens* (total, 496 bp) mtDNA. Two major non-coding regions present in the mtDNA were predicted to form hairpin structures. Non-coding region 1 (NR1), between *trnY* and *trnL1*, is 201 bp (*S. erinaceieuropaei*) or 204 bp (*S. decipiens*) in length, and non-coding region 2, between *nad5* and *trnG* was 185 bp (*S. erinaceieuroapei*) or 174 bp (*S. decipiens*) in length. The non-coding regions in these 2 species had A + T content of 70.9% (*S. erinaceieuropaei*) and 67.7% (*S. decipiens*) and contained stem-loop structures. The nucleotide contents of the NR1 and NR2 were 32.6% A, 9.3% C, 38.3% T, and 19.7% G in *S. erinaceieuropaei* mtDNA, while those of *S. decipiens* were 32.3% A, 11.9% C, 35.4% T, and 20.4% G in *S. decipiens* mtDNA.

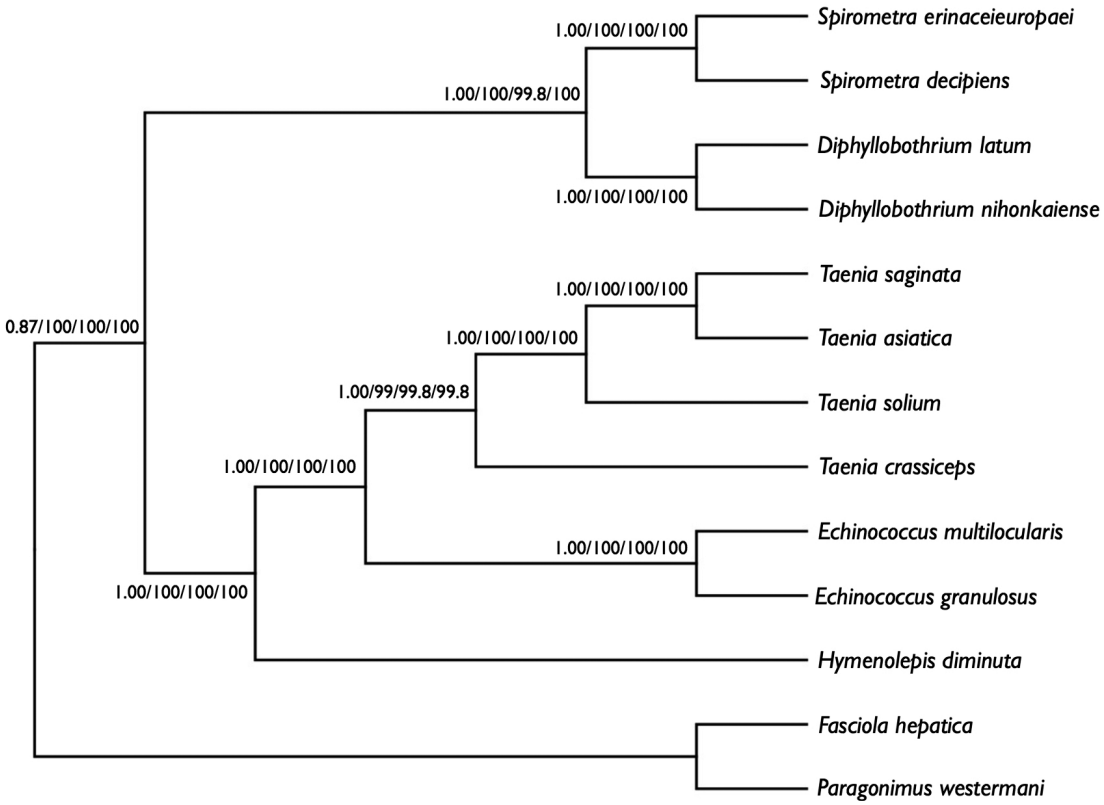

Fig. 3. Phylogenetic relationship among eucestode species based on inferred amino acid sequence data selected from 12 mitochondrial protein-coding gene loci for 13 platyhelminthes. The numbers above the branches represent bootstrap values for Bayesian inference, maximum likelihood (ML), neighbor joining (NJ), and maximum parsimony (MP), respectively.

Mitochondrial sequence divergence between *S. erinaceieuropaei* and *S. decipiens*

A pairwise comparison of sequence divergence of the 12 protein-coding genes and 2 ribosomal RNA genes of *S. erinaceieuropaei* and *S. decipiens* is shown in Table 4. The protein-coding sequences of *S. erinaceieuroapei* contained 10,083 bp and 3,361 codons, whereas those of *S. decipiens* contained 10,086 bp and 3,362 codons. The 12 protein-coding genes of *S. erinaceieuropaei* and *S. decipiens* differed by 12.4%, whereas the full mtDNA sequences differed by 12.9%. Divergences in protein-coding genes between *S. erinaceieuropaei* and *S. decipiens* ranged from a low of 9.3% to a high of 13.7% (Table 4). Amino acid sequence divergences of *cox1* (the most highly conserved gene) and *nad6* (the most variable gene) were 1.9% and 14.8%, respectively. The ribosomal RNA genes of *S. erinaceieuropaei* differed by 11.2% (*rrnL*) and 6.9% (*rrnS*) relative to those of *S. decipiens*.

Phylogenetic relationships of pseudophyllidean cestode among the eucestodes

We performed phylogenetic analyses of *S. decipiens* and *S. erinaceieuropaei* using 4 methods (Bayesian inference, ML, NJ, and MP), based on concatenated amino acid sequences of 12 protein genes from 11 cestodes and 2 trematodes. To this end, we used an alignment set of 10,394 bp including all 12 mitochondrial protein-coding gene loci. Of the 3,141 (30.3%) homologous positions and 66.1% pairwise identity showed in the set of those mtDNA sequences from maximum likelihood analysis. A concatenated alignment set of 3,389 homologous amino acid positions and 1,425 variable sites were phylogenetically informative under MP criterion. Phylogenetic relationships among the eucestodes determined using the 4 analytic approaches exhibited identical tree topologies. In the consensus tree, order Cyclophyllidea, including family Taeniidae (*Taenia* and *Echinococcus*) formed a well-supported monophyletic group. Family Hymenolepididae is a sister taxon to the Taeniidae. Within the Pseudophyllidea clade, *Diphyllobothrium*

and *Spirometra* formed a monophyletic group, and sister genera are well supported (Fig. 3).

DISCUSSION

In the present study, we sequenced and analyzed whole mitochondrial genomes of *S. erinaceieuropaei* and *S. decipiens* based on the morphological analysis described in detail recently by the present authors [16]. The full mtDNA sequences of *S. erinaceieuropaei* and *S. decipiens* differ by 12.9% which means *S. erinaceieuropaei* and *S. decipiens* are valid species that can be distinguished from each other by comparison of mitochondrial DNA sequences and morphological data as well.

Morphologically, *S. erinaceieuropaei* can be clearly distinguished from *S. decipiens* by its spirally coiled uterus [2]. The uterus of *S. erinaceieuropaei* consists of 5-7 complete turns, whereas that of *S. decipiens* consists of 4–4½ coils. The lateral margins of the subterminal uterine coil of *S. erinaceieuropaei* are parallel, while those of *S. decipiens* are the broadest as subspherical in contour. In *S. erinaceieuropaei*, the uterine pore lies in the midline, a small distance behind the anterior margin of the uterine terminal ball, whereas in *S. decipiens*, it is a conspicuous sphincter in a ventral position under the bulge of the terminal uterine coil. The vaginal pore is a broad crescent slit behind the male genital pore in *S. erinaceieuropaei*, whereas in *S. decipiens*, it is crescent-shaped and elliptical. The cirrus is strongly muscular and elongated in shape in *S. decipiens*, whereas in *S. erinaceieuropaei*, it is smaller than in other related species [2].

The molecular characteristics of the mitochondrial genome of *S. erinaceieuropaei* and *S. decipiens* that we identified in this study, gene arrangement, nucleotide composition, genetic code, and secondary structure of tRNA, were similar to those of other cestodes. The genetic distance between *S. erinaceieuropaei* and *S. decipiens* was determined by a percentage pairwise comparison of the nucleotide and amino acid compositions of the mitochondrial genomes. The 12 protein-coding genes of *S. erinaceieuropaei* and *S. decipiens* differed by 12.4%, whereas the sequence differences for the whole mitochondrial sequences were 12.9%. Divergences of amino acid sequences between *S. erinaceieuopaei* and *S. decipiens* ranged from a low of 1.9% (*cox1*) to a high of 14.8% (*nad6*). Therefore, these 2 parasitic organisms represent distinct species within a same genus.

These species have not been clearly identified in terms of which specifically infect humans. Conventionally, *S. erinaceieuropaei* larval stages were considered to be the main reason for human infections through eating raw or undercooked snakes but actually the spargana were not seen at all naturally in the snakes. Rather, *S. decipiens* has been found from snakes (unpublished data). Natural infections with *S. decipiens* has been mostly observed in carnivorous animals such as cats and dogs, but no such natural infections have been observed for *S. erinaceieuropaei*. Thus, *S. decipiens* may only be found in dogs and cats (unpublished data). In this and the previous study [16], adult *S. erinaceieuropaei* worms were obtained by feeding dogs the larvae collected from humans or the muscle fascia of the hedgehog (*Erinaceus dealbatsu*); after the initial infection, adults can be harvested in about 4 weeks. The only known second intermediate host of *S. erinaceieuropaei* is the Chinese hedgehog. Therefore, in the context of human sparganosis, the infection route and relevant intermediate host of *S. erinaceieuropaei* remain unclear, and there is an epidemiological discrepancy between eating habits and distributions of *Spirometra* species in animals.

The information derived from the complete sequence of the *S. erinaceieuropaei* and *S. decipiens* mitochondrial genome will add to the available mitochondrial sequence data of parasitic cestodes, and provide a resource for comparative mitochondrial genome analyses of pseudophyllidean tapeworms. Our results show that *S. decipiens* is a valid species that can be distinguished from *S. erinaceieuropaei* by comparison of mitochondrial DNA sequence as well as morphological data in the previous study [16].

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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