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▣ BRIEF COMMUNICATION

Complete Sequence of the Mitochondrial Genome of *Spirometra ranarum*: Comparison with *S. erinaceieuropaei* and *S. decipiens*

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Abstract: This study was undertaken to determine the complete mitochondrial DNA sequence and structure of the mitochondrial genome of *Spirometra ranarum*, and to compare it with those of *S. erinaceieuropaei* and *S. decipiens*. The aim of this study was to provide information of the species level taxonomy of *Spirometra* spp. using the mitochondrial genomes of 3 *Spirometra* tapeworms. The *S. ranarum* isolate originated from Myanmar. The mitochondrial genome sequence of *S. ranarum* was compared with that of *S. erinaceieuropaei* (GenBank no. KJ599680) and *S. decipiens* (GenBank no. KJ599679). The complete mtDNA sequence of *S. ranarum* comprised 13,644 bp. The *S. ranarum* mt genome contained 36 genes comprising 12 protein-coding genes, 22 tRNAs and 2 rRNAs. The mt genome lacked the *atp8* gene, as found for other cestodes. All genes in the *S. ranarum* mitochondrial genome are transcribed in the same direction and arranged in the same relative position with respect to gene loci as found for *S. erinaceieuropaei* and *S. decipiens* mt genomes. The overall nucleotide sequence divergence of 12 protein-coding genes between *S. ranarum* and *S. decipiens* differed by 1.5%, and 100% sequence similarity was found in the *cox2* and *nad6* genes, while the DNA sequence divergence of the *cox1, nad1,* and *nad4* genes of *S. ranarum* and *S. decipiens* was 2.2%, 2.1%, and 2.6%, respectively.

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

Cestodes of the genus *Spirometra* (Mueller, 1934) are intestinal parasites of cats and dogs that use the fresh water copepod as the first intermediate host and reptiles or amphibians as the second intermediate host. *Spirometra* species have been reported from felids and canids worldwide under the generic name *Diphyllobothrium* since 19th century. *Spirometra* species were described from the morphological identification of 6 *Spirometra* species, comprising *S. erinaceieuropaei* (Rudolphi, 1819), *S. decipiens* (Diesing, 1850), *S. ranarum* (Gastaldi, 1854), *S. mansoni* (Cobbold, 1882), *S. houghtoni* (Syn. *S. mansoni*), and *S. okumurai* by Faust et al. [1]. Fifteen *Spirometra* species were reviewed as valid species and divided into 2 groups by Wardle and McLeod [2]. A *Spirometra* species in North America was reported as *S. mansonoides* (McIntosh, 1935) [3]. Five *Spirometra* species have been reported from wild felids in South America such as *S. decipiens, S. mansoni, S. longicollis* (Parodi and Widakowich, 1917), *S. gracilis* (Baer, 1927), and *S. mansonoides* [4]. *S. pretoriensis* and *S. theileri* (Baer, 1924) have been reported from a bush cat (*Leptailurus serval*) and a tiger cat (*Felis lybica cafra*) [5]. Currently, *Spirometra* species causing human sparganosis have been identified as *S. erinaceieuropaei* and *S. decipiens* by morphological and genetic analyses in Korea [6]. The most recent reports include identification of *S. ranarum* from frogs (*Hoplobatrachus rugulosus*) in Myanmar and from lions (*Panthera leo*) in Tanzania by analysis of mitochondrial genes and morphological observation [7,8]. *S. ranarum* obtained from cats and dogs were identified by molecular and phylogenetic analysis of DNA sequence data of the mitochondrial cytochrome *c* oxidase (*cox1*) gene in Korea [9].

A species level taxonomy of *Spirometra* species was employed based on morphological characteristics combined with molecular analysis. Mitochondrial DNA is considered to provide a useful molecular marker for taxonomic identification, inferences of phylogenetic relationships, population genetics and

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epidemiological investigation [10]. Mitochondrial DNA sequences have been studied for genetic variation, taxonomy, and phylogenetic relationships using *cox1*, *cox3*, *nad1*, *nad3*, and *nad4* [10-14]. The complete mitochondrial genomes of order Diphyllobothridea have been published for *D. latum*, *D. nihonkaiense*, *S. erinaceieuropaei*, and *S. decipiens* [15-17]. This study was undertaken to determine the complete mitochondrial DNA sequence and structure of the mitochondrial genome of *S. ranarum*, and to compare it with those of *S. erinaceieuropaei* and *S. decipiens*. The aim of this study was to provide information of the species level taxonomy of *Spirometra* spp., by analysis of mitochondrial genomes of 3 *Spirometra* tapeworms.

The *S. ranarum* isolate originated from Myanmar. This *Spirometra* tapeworm was identified using morphological and molecular characteristics as well as animal inoculation experiments [7]. Specific identity was confirmed using sequences of the mitochondrial *cox1* and *nad1* genes previously reported for *S. ranarum* (GenBank No. MH298843, MH2998844). Mitochondria from a single specimen were isolated using a Qproteome Mitochondria Isolation Kit (Qiagen, Hilden, Germany). Mitochondrial DNA (mtDNA) was extracted from mitochondria using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA). Whole genome amplification (WGA) of extracted mtDNA was performed using an REPLI-g Mitochondrial DNA Kit (Qiagen, Hilden, Germany) combined with Exonuclease-Resistant Random Primer: 5′NpNpNpNpNpSNpSN-3′ (Thermo Scientific, Hudson, New Hampshire, USA). Amplified mtDNA was sequenced using an Illumina sequencing genome analyzer (Macrogen, Seoul, Korea). The mitogenome was reconstructed using the Geneious 9.0 software program (Biomatters Ltd, Auckland, New Zealand) and employing the partial *cox1* gene of *S. ranarum* as initial bait. The complete mitochondrial genome was annotated and recovered using MITOS and Dogma [18,19]. The mitochondrial genome sequence of *S. ranarum* was compared with that of *S. erinaceieuropaei* (GenBank no. KJ599680) and *S. decipiens* (GenBank no. KJ599679). The 12 protein-encoding genes in the mtDNA was confirmed by comparison with mitochondrial gene sequences of other cestodes available in the GenBank database. Platyhelminth mitochondrial genetic code was used to translate the mitochondrial protein-coding genes. Twenty-two putative tRNA genes were identified using tRNAscan-SE.2.1 [20] and anticodon sequences. The putative stem-loop structures of non-coding regions were inferred using the RNAdraw program [21].

The complete mtDNA sequence of *S. ranarum* comprised 13,644 bp. The *S. ranarum* mt genome contained 36 genes comprising 12 protein-coding genes, 22 tRNAs and 2 rRNAs. The mt genome lacked the *atp8* gene as with other cestodes. All genes in the *S. ranarum* mitochondrial genome are transcribed in the same direction and arranged in the same relative position with respect to gene loci as found for *S. erinaceieuropaei* and *S. decipiens* mt genomes (Table 1). The nucleotide composition of the entire mt genome of *S. ranarum* comprised 20.4% A, 11.2% C, 22.6% G, and 45.8% T (66.2% A+T). An A+T richness was observed in *Spirometra* species *S. erinaceieuropaei* (65.7% A+T) and *S. decipiens* (66.3% A+T) as with other cestodes. Some genes were found to overlap in the mitochondrial genome of *S. ranarum*: *cox1*/*trnT* (10 bp), *nad4L*/*nad4* (39 bp), *trnQ*/*trnF* (3 bp), and *trnF*/*trnM* (3 bp) (Table 1).

Accounting for some 74% of the entire mitochondrial genomes of *Spirometra* species are protein-coding genes. All putative open reading frames (ORFs) of the 12 protein-coding genes of *S. ranarum* mtDNA started and ended with complete codons. The ATG initiation codon was used in 11 genes (*atp6*, *cob*, *cox1*, *cox2*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, and *nad6*), while the GTG initiation codon was used only in the *cox3* gene in *S. ranarum*, *S. decipiens* and *S. erinaceieuropaei*. The TAG stop codon was used in 4 genes (*cox1*, *nad2*, *nad4*, and *nad4L*), while the TAA stop codon was used in 6 genes (*atp6*, *cob*, *cox2*, *nad1*, *nad5*, and *nad6*) in *S. ranarum* and *S. decipiens*. The TAG codon was used in the *cox2* gene in *S. erinaceieuropaei*. In *cox3* and *nad3*, the abbreviated stop codon U was confirmed in *S. ranarum* and *S. decipiens*. The use of TA termination was found in *cox3* for *S. erinaceieuropaei* mtDNAs. The most commonly used codons were for leucine, phenylalanine, valine and serine in mitochondrial proteins of 3 *Spirometra* species. The mitochondrial genome of *Spirometra* tapeworms appear to use the flatworm mitochondrial code, namely TTR and CTN for leucine, TTY for phenylalanine, GTN for valine, and AGN and TCN for serine.

Twenty-two tRNA genes were identified as putative secondary structures comprising a typical cloverleaf shape, with length ranging from 56 to 69 bp in *S. ranarum* and *S. decipiens*, respectively, and 56 to 70 bp in *S. erinaceieuropaei*. The inferred secondary structure of 19 tRNA exhibited a typical cloverleaf shape with 4 arms comprising aminoacyl acceptor arms, a DHU arm, anticodon stems and TΨC arms. *trnR*, *trnS1*, and *trnS2* were replaced with 7-12 bp of unpaired loop in the DHU arms. The aminoacyl acceptor arms comprising 7 nt such as *trnA*, *trnI*, *trnM*, *trnQ*, *trnR*, *trnS2*, *trnT*, and *trnV* contained 1 or 3 non-canonical base pairs. The anticodon stems comprising 5 nt as

Table 1. Position and characteristics of the protein-coding and non-coding sequences in the mt genome of *Spirometra ranarum*, *S. decipiens* and *S. erinaceieuropaei*

Genes	Length of gene and sequence						Codon used for					
	Nucleotide			Amino acid			Initiation			Termination		
	Sr	Sd	Se	Sr	Sd	Se	Sr	Sd	Se	Sr	Sd	Se
trnG	67	67	67									
*cox3**	643	643	644	214	214	214	GTG	GTG	GTG	T	T	TA
trnH	69	69	70									
cob	1,110	1,110	1,110	370	370	370	ATG	ATG	ATG	TAA	TAA	TAA
nad4L	261	261	261	87	87	87	ATG	ATG	ATG	TAG	TAG	TAG
nad4	1,254	1,254	1,254	418	418	418	ATG	ATG	ATG	TAG	TAG	TAG
trnQ	64	64	64									
trnF	64	64	64									
trnM	68	68	68									
atp6	516	516	516	172	172	172	ATG	ATG	ATG	TAA	TAA	TAA
nad2	873	873	873	291	291	291	ATG	ATG	ATG	TAG	TAG	TAG
trnV	65	65	65									
trnA	61	61	61									
trnD	64	64	66									
nad1	891	891	891	297	297	297	ATG	ATG	ATG	TAA	TAA	TAA
trnN	66	66	66									
trnP	65	65	65									
trnI	66	64	64									
trnK	63	63	63									
*nad3**	346	346	346	115	115	115	ATG	ATG	ATG	T	T	T
trnS1 (*AGN*)	59	59	59									
trnW	66	66	65									
cox1	1,566	1,566	1,566	522	522	522	ATG	ATG	ATG	TAG	TAG	TAG
trnT	70	70	69									
rrnL	972	973	967									
trnC	65	65	65									
rrnS	730	730	733									
cox2	570	570	570	190	190	190	ATG	ATG	ATG	TAA	TAA	TAG
trnE	65	65	65									
nad6	468	468	465	156	156	155	ATG	ATG	ATG	TAA	TAA	TAA
trnY	68	68	68									
NR1	204	204	201									
trnL1 (*CUN*)	67	67	67									
trnS2 (*UGN*)	65	66	66									
trnL2 (*UUN*)	65	65	65									
trnR	57	57	56									
nad5	1,569	1,569	1,569	523	523	523	ATG	ATG	ATG	TAA	TAA	TAA
NR2	175	175	185									

Sr: *Spirometra ranarum*; Sd: *S. decipiens*; Se: *S. erinaceieuropaei*; NR1: non-coding region 1; NR2: long non-coding region 2.
*TAA stop codon is completed by the addition of 3' A residues to the mRNA.

with typical stem structures. The TΨC arms comprised a 2-5 nt stem with a 3-9 nt loop. The variable loop between the anticodon and the TΨC stems comprised 3-5 nt in 3 *Spirometra* species mtDNA. These 22 tRNAs had the same structure in the mitochondrial genome of other parasitic platyhelminths. Two mitochondrial ribosomal subunit genes *rrnL* and *rrnS* in the 3 *Spirometra* tapeworms were separated by *trnC*. The putative 16S rRNA and 12S rRNA genes in *S. ranarum* were 972 and 730 nt long, respectively. These sizes are similar to those of rRNA genes in *S. decipiens* and *S. erinaceieuropaei*, which range from 967 to 973 nt for 16S rRNA, and 730 to 733 nt for 12S rRNA (Table 1). The nucleotide content of the 16S rRNA and 12S

Table 2. Divergences of nucleotides and amino acids of the protein-coding genes

cox1	Sr	Sd	Se	*cox2*	Sr	Sd	Se	*cox3*	Sr	Sd	Se	*cob*	Sr	Sd	Se
Sr	-	2.2	8.8		-	0.0	10.4		-	1.4	12.7		-	2.4	11.2
Sd	0.4	-	9.4		0.0	-	10.4		0.9	-	12.0		1.1	-	10.9
Se	2.9	2.9	-		3.2	3.2	-		5.1	5.6	-		3.8	4.1	-
atp6				*nad1*				*nad2*				*nad3*			
Sr	-	1.9	14.1		-	2.1	10.0		-	1.7	14.0		-	1.7	13.6
Sd	1.2	-	13.4		0.7	-	9.8		1.0	-	13.7		0.9	-	13.0
Se	8.2	8.2	-		5.4	6.1	-		7.6	8.6	-		7.8	7.0	-
nad4				*nad4L*				*nad5*				*nad6*			
Sr	-	2.6	13.7		-	1.2	11.5		-	1.4	18.1		-	0.0	18.8
Sd	1.4	-	14.0		0.2	-	11.9		0.8	-	18.1		0.0	-	18.8
Se	9.1	9.4	-		2.3	2.3	-		12.1	11.9	-		14.8	14.8	-

Percentage pairwise divergences of nucleotides (above diagonal) and amino acids (below diagonal) of the 12 protein-coding genes of the *Spirometra* tapeworms (Sr: *Spirometra ranarum* [present study], Sd: *S. decipiens* [KJ599679], Se: *S. erinaceieuropaei* [KJ599680]).

rRNA genes in *S. ranarum* was 25.0% A, 12.5% C, 24.3% G and 38.1% T (63.1% A+T), whereas the A+T content was 63.1% in *S. decipiens* and 63.6% in *S. erinaceieuropaei*. Two major non-coding regions present in 3 *Spirometra* species mtDNA were predicted between *trnY* and *trnL1*, and between *trnR* and *nad5*. Non-coding region 1 (NR1) between *trnY* and *trnL1*, was 204 nt (*S. ranarum*), 204 nt (*S. decipiens*) and 201 nt (*S. erinaceieuropaei*) in length, while non-coding region 2 (NR2) between *trnR* and *nad5* was 175 nt (*S. ranarum*), 175 nt (*S. decipiens*) and 185 nt (*S. erinaceieuropaei*) in length.

A percentage pairwise comparison of sequence divergence of the 12 protein-coding genes among *S. ranarum*, *S. decipiens*, and *S. erinaceieuropaei* is shown in Table 2. The 12 protein-coding genes constituted 10,067 bp and 3,355 codons (*S. ranarum*), 10,067 bp and 3,355 codons (*S. decipiens*), and 10,065 bp and 3,355 codons (*S. erinaceieuropaei*) of their respective mitochondrial genomes. The overall nucleotide sequence divergence of 12 protein-coding genes of *S. ranarum* and *S. decipiens* differed by 1.5%, while that of *S. ranarum* and *S. erinaceieuropaei* differed by 13.0%. The divergence of amino acid sequences of 12 protein-coding genes of *S. ranarum* and *S. decipiens* ranged from as low as 0.0% (*cox2* and *nad6*) to as high as 1.4% (*nad4*) (Table 2). The nucleotide sequence divergence of the *nad4* gene (the most variable gene), and the *cox2* and *nad6* (the most highly conserved gene) genes was 2.6% and 0.0% between *S. ranarum* and *S. decipiens*, respectively. The ribosomal RNA genes of *S. ranarum* and *S. decipiens* differed by 1.6% (16S rRNA) and 1% (12S rRNA). The percent of nucleotide sequence difference was greater than that of the amino acid difference for the *cox1*, *cox3*, *cob*, *atp6*, *nad1*, *nad2*, *nad3*, *nad4*, and *nad4L* genes of *S. ranarum* and *S. decipiens*, suggesting the existence of synonymous substitutions.

The degree of divergence in mtDNA sequences between sister species or congeneric species was estimated using the genetic distance of the *cob* gene among mammalian groups such as amphibians, reptiles and avian birds. The sequence divergence of the sister or congeneric species *cob* gene among mammalian groups is greater than 2% [22]. Another study reported that *cox1* divergence among 13,320 species in 11 animal phyla ranged from as low as 0.0% to as high as 53.7%, while 79% of those species showed greater than 8% sequence divergence at the species taxonomy level [23]. The aforementioned studies have established that 98% of congeneric animal species showed greater than 2% sequence divergence in the *cox1* gene [22,23].

Mitochondrial *cox1* sequence variation of *Spirometra* species has been reported to range from 0.0-3.5% in China, Myanmar, Thailand, and Lao PDR, [24] and range from 0.0-2.6% in Japan, India and Indonesia [25]. In the present study, the complete mitochondrial genome of *S. ranarum* was sequenced and characterized, and comparison with that of *S. erinaceieuropaei* and *S. decipiens* showed that overall nucleotide sequence divergence of 12 protein-coding genes of *S. ranarum* and *S. decipiens* differed by 1.5%, with 100% sequence similarity in the *cox2* and *nad6* genes, while the DNA sequence divergence of *cox1*, *nad1*, and *nad4* genes of *S. ranarum* and *S. decipiens* was 2.2%, 2.1% and 2.6%, respectively. These *cox1*, *nad1*, and *nad4* genes

have been used as genetic markers for taxonomic identification and phylogenetic reconstruction by many researchers [4,6-9,11-14,24,25]. The degree of sequence divergence of the *cox1*, *cob*, *nad1*, and *nad4* genes of *S. ranarum* and *S. decipiens* was greater than 2%, which indicates that species are independent species. However, sequence differences of the *cox3*, *atp6*, *nad2*, *nad3*, *nad4L*, and *nad5* genes of *S. ranarum* and *S. decipiens* was less than 2%, while that of the *cox2* and *nad6* genes was 0%, despite these 2 *Spirometra* species having morphological differences.

Although mitochondrial DNA sequence divergence has been useful as a genetic marker for species identification, including identification of sister or congeneric species, improving the reliability of this method requires the accumulation of DNA sequence data and criteria pertaining to morphological identification. A consensus has yet to be reached regarding criteria of mitochondrial DNA sequence divergence for species level taxonomy of *Spirometra* tapeworms, even though identified *Spirometra* species have been analyzed using mitochondrial DNA sequence divergence based on morphological characteristics. Further studies are needed to clarify species identification and to delineate the precise genetic variation of these *Spirometra* species.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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