



Genetic and Morphologic Identification of *Spirometra ranarum* in Myanmar

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Abstract: In the present study, we identified a *Spirometra* species of Myanmar origin (plerocercoid) by molecular analysis using mitochondrial *cox1* and *nad1* genes, as well as by morphological observations of an adult tapeworm. Spargana specimens were collected from a paddy-field in Taik Kyi Township Tarkwa Village, Yangon, Myanmar in December 2017. A total of 5 spargana were obtained from 20 frogs *Hoplobatrachus rugulosus*; syn: *Rana rugulosa* (Wiegmann, 1834) or *R. tigrina* (Steindachner, 1867). The plerocercoids were used for experimental infection of a dog. After 4 weeks of infection, an adult tapeworm was recovered from the intestine of the dog. Morphologically, the distinct features of *Spirometra* sp. (Myanmar origin) relative to *S. erinacei-europaei* and *S. decipiens* include a uterine morphology comprising posterior uterine coils that larger than the terminal uterine ball and coiling of the uteri diagonally (swirling) rather than spirally. The *cox1* sequences (1,566 bp) of the Myanmar-origin *Spirometra* species showed 97.9% similarity to a reference sequence of *S. decipiens* (GenBank no. KJ599679) and 90.5% similarity to a reference sequence of *S. erinacei-europaei* (GenBank no. KJ599680). Phylogenetic tree topologies were identical and presented high confidence level of values for the 3 major branches of the 3 *Spirometra* species in *cox1* and *nad1* genes. These results indicated that Myanmar-origin *Spirometra* species coincided with those of *S. ranarum* and may be considered as a valid species.

Key words: *Spirometra ranarum*, spargana, molecular detection, *cox1*, *nad1*, Myanmar

INTRODUCTION

The genus *Spirometra* belongs to the family Diphylobothriidae and order Diphylobothriidea and includes intestinal parasites of cats and dogs. Human sparganosis is a zoonotic disease caused by infection with the larval forms (proceroid/plerocercoid) of *Spirometra* species. The genus *Spirometra* has been described with morphological features of spirometrid species under the generic name *Diphylobothrium* as found in China and including *S. erinacei-europaei* (Rudolphi, 1819), *S. decipiens* (Diesing, 1850), *S. ranarum* (Gastaldi, 1854), *S. mansoni* (Cobbold, 1882) *S. houghtoni* (Syn. *S. mansoni*, Faust et al., 1929) and *S. okumurai* (Faust et al., 1929) by Faust et al. [1]. *Spirometra* species in North America have been identified such as *S.*

mansonoides (Mueller, 1935) which possesses a characteristics C-shaped outer loop of the uterus [2]. Five *Spirometra* species, *S. decipiens*, *S. mansoni*, *S. gracilis* (Baer, 1927), *S. longicollis* (Parodi and Widakowich, 1917) and *S. mansonoides* have been reported from wild fields in South America [3].

Although human sparganosis is sporadically reported around the world, it is frequently reported in Asian countries. Among these species in the genus *Spirometra*, where *S. erinacei-europaei* is known as the only *Spirometra* species in Asian countries including China, Indonesia, Japan, Korea and Thailand [4,5]. Many investigations of the spargana in frogs and snakes have been reported in field studies covering 27 Provinces in China and are critical for the eventual implementation of measures for the prevention and control of human sparganosis as more than 1,000 cases of human sparganosis have been reported in China [6]. The nucleotide sequence variations of spargana in frogs (*Rana nigromaculata*) and snakes were 0-8.4% for *cox1*, 0-1.5% for *cox3*, 0-2.8% for *nad1*, and 0-2.7% for *nad4* in Hunan, Guangxi, and Henan Provinces, China, respectively [6-10]. In Thailand, 55 human sparganosis were report-

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ed as 53 non-proliferative and 2 proliferative. Among them, 12 cases were identified as infection with *S. erinaceiueuropaei* plerocercoids by PCR-based molecular identification of the worms [11]. Intraspecies variation of *cox1* gene of *S. erinaceiueuropaei* was reported from Japan, India and Indonesia by Okamoto et al. (<2.6%) [12]. Molecular identification of *Spirometra* species collected from animals has been studied using partial sequence analysis of the *cox1* gene of spargana isolated from frogs (*Hoplobatrachus rugulosus*) and snakes (*Ptyas korros*) from Lao PDR, Myanmar and Thailand [13]. The results revealed that nucleotide variation in the partial *cox1* sequence (429 bp) among the spargana ranged from 0-3.5%. Those molecular studies have indicated that 2 more genotypes were found in *Spirometra* species in those endemic regions revealing genetic variation of mitochondrial DNA sequences. As with the above studies, the most important causative agent of human sparganosis is *S. erinaceiueuropaei* in Asian countries. Two *Spirometra* species currently being considered as valid are *S. erinaceiueuropaei* and *S. decipiens* by morphological and molecular approaches using complete mitochondrial genome analysis [14]. Therefore, identification of *Spirometra* species requires the use of molecular and morphological techniques to better understand the epidemiological status of *Spirometra* spp. in its endemic regions including Asian countries.

One *Spirometra* species in Myanmar is known as *S. ranarum*, which was first reported by Gastaldi (1854) from *Rana esculenta* (syn: *Pelophylax esculentus*) in Italy, and Meggitt described it as *S. ranarum* from a dog fed spargana isolated from the same frog host by Gastaldi (1854) in Myanmar [15]. In the present study, we identified a *Spirometra* species of Myanmar origin (plerocercoid) by molecular analysis using 2 complete mitochondrial genes, cytochrome *c* oxidase I (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*), as well as by morphological observations of an adult tapeworm experimentally obtained from a dog fed with the plerocercoid.

MATERIALS AND METHODS

Specimens

Spargana specimens were collected from a paddy-field in Taik Kyi Township Tarkwa Village, Yangon, Myanmar in December 2017. A total of 5 spargana were obtained from 20 frogs (*Hoplobatrachus rugulosus*; syn: *Rana rugulosa* [Wiegmann, 1834] or *R. tigrina* [Steindachner, 1867], commonly known as Chinese edible frog, East Asian bullfrog or Taiwanese frog).

The 5 plerocercoids were then used to infect a dog. After 4 weeks of infection, an adult tapeworm was recovered from the intestine of the dog. The collected worm was fixed with 10% neutral buffered formalin before it was placed in hot water (90°C) for 5 min and microscopic observations were then performed following carmine staining. The vaginal opening, uterus, uterine pore, cirrus, genital pore, testes, and vitellaria were observed with carmine-stained mature and gravid proglottids according to the methods of Meggitt and Faust et al. [1,15]. Gravid proglottids in longitudinal section were stained with hematoxylin and eosin for observing genital organs. Some of the gravid proglottids were preserved in a -70°C deep freezer and in 70% ethanol for molecular studies. The experimental animal was treated according to the recommendations of the Guide for Institutional Animal Care and Use Committees (IACUC) of Chungbuk National University, Cheongju, Korea (Permission No. CBNUA-1090-18-02). Parasite materials used in this study were provided by the Parasite Resource Bank of the Korea National Research Resource Center, Republic of Korea (PRB000720).

PCR and DNA sequencing

Total genomic DNA was extracted from a single proglottid using a DNeasy tissue kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The primers were used to amplify the mitochondrial *cox1* gene (Spi-CO1F: 5'-GAC TAA GTG TTT TCA AAA CAC TAA GTG -3' and Spi-CO1R: 5'-CAC CCT ACC CCT GAT TTA CAA AAT-3') and *nad1* gene (Spi-ND1F: 5'-GGA GAA TAT TGG TTT GTC TAA CCA-3' and Spi-ND1R: 5'-CCT TCT TAA CGT TAA CAG CAT TAC GAT-3'). PCR was performed in a 50 µl of reaction volume containing 50 ng of genomic DNA, 10 pmol of each primer, 5 µl of 10X buffer, 10 mM dNTP mix, 25 mM MgCl₂, and 2.5 units *Taq* polymerase (High Fidelity PCR system, Roche, Mannheim, Germany). PCR was employed using a GeneAmp PCR system 9700 instrument (Applied Biosystems, Langen, Germany) under conditions comprising: 94°C for 3 min; 35 cycles of 94°C for 30 sec, 45°C for 40 sec, 72°C for 60 sec, and a final step at 72°C for 5 min. The *cox1* and *nad1* DNA fragments were extracted using a QIAquick PCR purification kit (Qiagen). DNA sequencing was performed by a private company (COSMOGENETECH, Daejeon, Korea). Cycle sequencing was performed using a Big-Dye terminator kit (version 3.1, Applied Biosystems, Foster City, California, USA). Reaction products were sequenced directly using a DNA sequencer (ABI3730XL,

Applied Biosystems).

DNA sequence analyses

DNA sequences were assembled and aligned using the Geneious 9.0 software program (Biometer, Auckland, New Zealand). These sequences were identified by comparison with the sequences of *S. erinaceiueuropaei* and *S. decipiens* in the GenBank database. Phylogenetic analyses were evaluated using Bayesian inference (BI) and maximum-likelihood (ML). ML analyses of *cox1* and *nad1* used RAxML v. 7.3.1 [16] after GTR+I+G substitution model sampling was chosen according to the Modeltest using the program Partition Finder [17]. BI analyses were used in MrBayes 3.2 and running four simultaneous Monte Carlo Markov chains (MCMC) for 10 million generations, sampling every 1,000 generations and discarding the first 25% generations as burn-in [18]. Phylogenetic trees were constructed using full mitochondrial *cox1* (1,566 bp) and *nad1* (891 bp) sequences of *S. erinaceiueuropaei* (KJ599680), *S. decipiens* (KJ599679), *D. nihonkaiense* (EF420138) and *D. latum* (DQ985706). Pairwise sequence differences based on the full mitochondrial *cox1* and *nad1* sequences were analyzed using the NJ method with the K2P substitution model.

RESULTS

Sequence similarity and phylogenetic relationships

The *cox1* sequences (1,566 bp) of the Myanmar-origin *Spirometra* species showed 97.9% similarity to reference sequences of *S. decipiens* (GenBank no. KJ599679) and 90.5% similarity to a reference sequence of *S. erinaceiueuropaei* (GenBank no. KJ599680). The similarity to other *Diphyllobothrium* species was 79.5% (*D. nihonkaiense*) and 80.1% (*D. latum*). The similarity of *nad1* sequences (891 bp) from Myanmar-origin *Spirometra* species to the reference sequence was 97.8% (*S. decipiens*), 89.1% (*S. erinaceiueuropaei*), 73.8% (*D. nihonkaiense*), and 73.3% (*D. latum*) (Table 1).

Phylogenetic analyses of *Spirometra* species were conducted using the 2 methods (BI and ML) based on the *cox1* and *nad1*

genes of *S. decipiens*, *S. erinaceiueuropaei*, *D. nihonkaiense*, and *D. latum*. The *cox1* sequences (1,566 bp) revealed 33 polymorphic sites, with 26 synonymous and 7 non-synonymous substitutions, while *nad1* sequences (891 bp) showed 19 polymorphic sites, with 16 synonymous and 3 non-synonymous substitutions between the Myanmar-origin *Spirometra* species and *S. decipiens* (GenBank no. KJ599679). Phylogenetic tree topologies generated using the 2 analytic methods were identical and presented high level of confidence values for the 3 major branches of the 3 *Spirometra* species in the *cox1* and *nad1* genes (Fig. 1).

Morphological characteristics

The length of the whole strobila was 237 cm. The mature proglottids with eggs were first visible almost 32 cm downward from the neck. The average width and length of the gravid proglottid was 15.0 mm by 2.0 mm (n = 10) (Table 2). The genital and vaginal pore were closely situated ventrally on the midline in the anterior 1/3 of the segment. The uterine pore was on the midline behind the anterior margin of the terminal ball 370-420 μm in diameter (n = 10). The uterus consisted of 3 loops with a diagonal direction of the second turn (Fig. 2A).

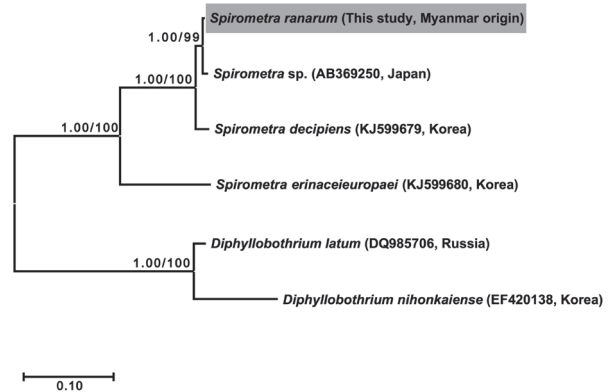


Fig. 1. Phylogenetic relationship of *Spirometra* species based on *cox1* and *nad1* gene sequences. Numbers on the branch represent bootstrap value for maximum likelihood (ML) and the support value of Bayesian inference (BI).

Table 1. Percentage pairwise sequence homologies of mitochondrial *cox1* and *nad1* genes of *Spirometra* species in Myanmar and various *Spirometra* species, *Diphyllobothrium latum* and *D. nihonkaiense*

Species genes	<i>S. ranarum</i>	<i>S. decipiens</i>	<i>S. erinaceiueuropaei</i>	<i>D. nihonkaiense</i>	<i>D. latum</i>
	<i>cox1/nad1</i>	<i>cox1/nad1</i>	<i>cox1/nad1</i>	<i>cox1/nad1</i>	<i>cox1/nad1</i>
<i>Spirometra</i> sp. (Myanmar origin)	100/100	97.9/97.8	90.5/89.1	79.5/73.8	80.1/73.3

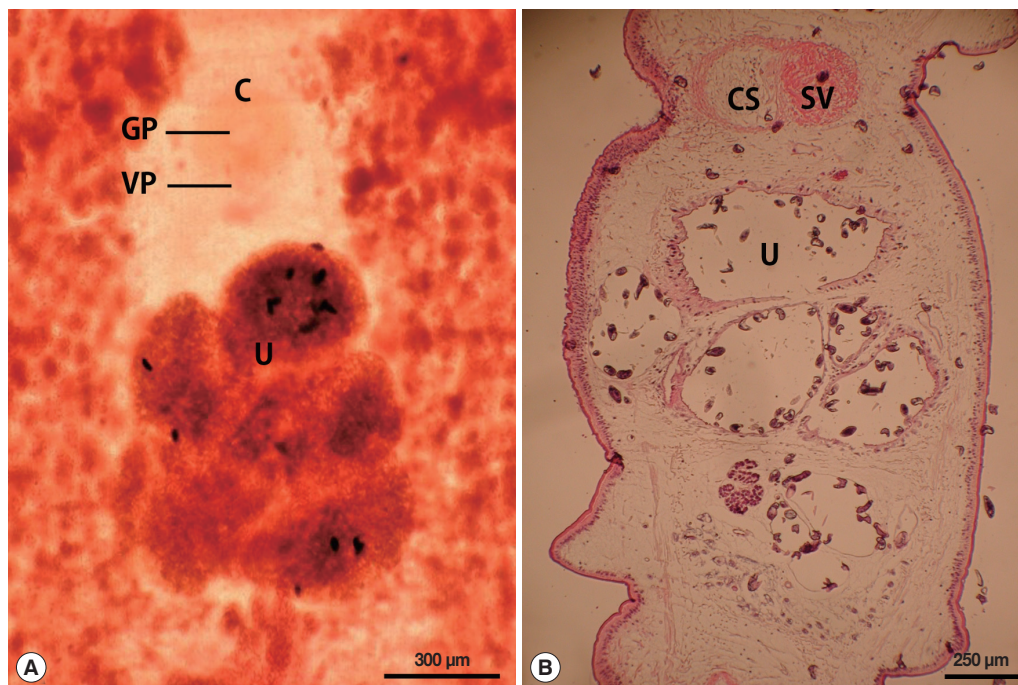


Fig. 2. Gravid proglottid of *Spirometra ranarum*. (A) Whole mounted specimen of proglottid showing the genital pore (GP), vaginal pore (VP), uterus (U), and cirrus pouch (C) ($\times 40$), (B) Longitudinal section of a gravid proglottid showing the cirrus sac (CS), seminal vesicle (SV), and uterus (U) (H&E stain).

Table 2. Morphological features of *Spirometra erinaceieuropaei*, *S. decipiens*, and *S. ranarum*

Organs	Morphological features	<i>S. erinaceieuropaei</i> ^a	<i>S. decipiens</i> ^b	<i>S. ranarum</i> ^c	<i>Spirometra</i> sp. ^d	Others
		Size (mm)	Size (mm)	Size (mm)	Size (mm)	
Scolex	Spatulate	0.2	0.5	0.3-0.4		Diameter
		1.0	1.5	1.4-1.7		Length
Gravid proglottids (n = 10)	Trapezoid	3.1-4.2	6.8-10.8	11.3	12.0	Width
		2.1-3.2	1.1-2.0	5.0	3.0	Length
Uterus (n = 10)	Coiling	5-7	4-4½	3-5	3	Loops
Eggs (n = 10)	Operculate	0.057-0.061	0.056-0.060	0.058-0.067	0.052-0.056	Length
		0.033-0.036	0.034-0.036	0.034-0.036	0.031-0.035	Width
Terminal ball (n = 10)	Oval	0.08-0.01	0.008-0.11		0.37-0.42	Diameter
Cirrus sac (n = 10)	Oval	0.2-0.25	0.2-0.22		0.17-0.25	Length
		0.12-0.15	0.15-0.17		0.17-0.25	Width
Seminal vesicle (n = 10)	Elliptical	0.2-0.25	0.24-0.28		0.19-0.22	Length
		0.1-0.15	0.1-0.15		0.19-0.22	Width
Testes (n = 10)	Polygonal	0.07-0.08	0.07-0.08		0.07-0.075	Width

^{a,b}Jeon et al. (2015) [14].

^cMeggitt (1925) [15].

^dThis study (Myanmar origin).

A longitudinal section of the cirrus sac showed an oval structure 175-250 μm in diameter (n = 10). The seminal vesicle was almost round in shape 197-222 μm in diameter. The testes (n = 10) measured an average 74 μm in width. The eggs measured 52-55 μm by 31-35 μm in transverse diameter (n = 10). The ovary was highly dendritic and was situated near the pos-

terior margin of the proglottids (Fig. 2B).

DISCUSSION

The larval stage (plerocercoid) of *S. ranarum* was first described by Gastaldi (1854) from *Rana esculenta* (syn: *Pelophylax*

esculentus) from Italy. In 1924, Meggitt reported spargana from frogs (*Rana tigrina*) from Yangon, Myanmar, and autopsies of dogs fed some of the spargana revealed the presence of adult spirometrids [15]. Meggitt, Joyeux and Baer (1927) described in detail this species with complete life cycle through intermediate hosts found and showed it to be suitable for final hosts [1]. Faust et al. (1929) found this species from natural infections of cats and dogs in Beijing, Xiamen, Canton and by experimental feeding of spargana obtained from dogs in Fujian [1]. The morphological characteristics of *S. ranarum* that have been detailed included the following features: 3-4½ uterine coils of which the posterior 2 are broader and more dilated than the terminal uterine ball, highly dendritic ovaries, the testes and vitellaria are closely positioned to the lateral margins of the lower 2 coils of the outer uterus, the vaginal and male genital pores are located in the median plane at small a distance anterior to the terminal uterine coil, and the eggs are similar in shape to those of *S. decipiens* [1].

The morphological features of the spirometrid tapeworm observed in the present study resembled that of previous studies by showing the genital and vaginal pore closely situated ventrally on the midline at the anterior 1/3 of the segment. The uterine pore was on the midline behind the anterior margin of the terminal uterine ball. The uterus consisted of 3 loops with a diagonal direction of the second turn and these 2 uterine coils were larger than the terminal uterine ball. The distinct features of *S. ranarum* relative to other *Spirometra* species include a uterine morphology comprising posterior uterine coils that are larger than the terminal uterine ball and coiling of the uteri diagonally (swirling) rather than spirally. These morphological characteristics of the *Spirometra* specimen (Myanmar origin) examined in this study are consistent with this *S. ranarum* specimen in terms of shape of the uterus.

Molecular approaches for taxonomy are usually based on genetic markers for identification and are applied to genetic variations especially among a group of morphologically similar parasites. The mitochondrial DNA sequences of *cox1*, *cox3*, *nad3*, and *nad4* genes have been used as genetic markers for phylogenetic reconstruction, taxonomic identification and epidemiological investigations [6-14]. Recent molecular data of *Spirometra* species reported from Asian, South American and African countries show that there are at least 4 *Spirometra* species in those endemic areas [3]. The nucleotide sequence variation of *S. erinaceiuroapai* ranged from 0.0-3.5% in China, Myanmar, Thailand and in Lao PDR as determined from frogs

and snakes. Some studies from Japan, India and Indonesia found 2.6% variation in the DNA sequence of *cox1* genes obtained from dogs [12]. The degree of mtDNA sequence divergence of cytochrome *b* (*cob*) gene between sister or congeneric species, and con-familial genera was greater than 2% in amphibian, reptilian, avian and mammalian species [19]. The closely related species of vertebrates showed more than 2% of sequence divergence in the *cox1* gene [20]. In this study, sequence differences in *cox1* and *nad1* genes between *Spirometra* species (Myanmar origin) and *S. decipiens* was 2.1%, while those of *S. erinaceiuroapai* were 9.5% (*cox1*) and 11.9% (*nad1*), respectively. Therefore, the results indicated that the examined *Spirometra* species from Myanmar matched *S. ranarum* in terms of morphological characteristics and mitochondrial sequence divergence. Some previous studies have reported that sequence divergence of the partial *cox1* gene (300-450 bp) in *Spirometra* species was greater than 3.0%, whereas sequence differences in the full mitochondrial *cox1* sequence was 2.1%. The results of this comparative study indicated that analysis of short sequences of the *cox1* gene may give rise to bias, and that analysis of the complete sequence data provides more reliable data.

Regardless of the molecular and phylogenetic results, the epidemiological status of *Spirometra* species should be considered in terms of species distribution and human sparganosis rather than assessment based primarily on GenBank DNA sequences. The Asian isolates of *Spirometra* species were revealed to be *S. decipiens* and *S. ranarum* in Myanmar, Thailand, Lao PDR, China, Korea and Japan, whereas only *S. erinaceiuroapai* human cases were found in Korea and Japan. These findings indicated that *S. erinaceiuroapai*, *S. decipiens*, and *S. ranarum* are a possible cause of human sparganosis. Further studies are needed to better understand the taxonomic and epidemiological status of *Spirometra* species for investigations of African and South American isolates.

This study has demonstrated the identification of Myanmar-origin *Spirometra* species by mitochondrial *cox1* and *nad1* sequence analysis and morphological examination of the adult worm. Furthermore, these results may indicate that *S. ranarum* is a valid species.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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