

# Human Infections with *Spirometra decipiens* Plerocercoids Identified by Morphologic and Genetic Analyses in Korea

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**Abstract:** Tapeworms of the genus *Spirometra* are pseudophyllidean cestodes endemic in Korea. At present, it is unclear which *Spirometra* species are responsible for causing human infections, and little information is available on the epidemiological profiles of *Spirometra* species infecting humans in Korea. Between 1979 and 2009, a total of 50 spargana from human patients and 2 adult specimens obtained from experimentally infected carnivorous animals were analyzed according to genetic and taxonomic criteria and classified as *Spirometra erinacei* or *Spirometra decipiens* depending on the morphology. Morphologically, *S. erinacei* and *S. decipiens* are different in that the spirally coiled uterus in *S. erinacei* has 5-7 complete coils, while in *S. decipiens* it has only 4.5 coils. In addition, there is a 9.3% (146/1,566) sequence difference between *S. erinacei* and *S. decipiens* in the *cox1* gene. Partial *cox1* sequences (390 bp) from 35 Korean isolates showed 99.4% (388/390) similarity with the reference sequence of *S. erinacei* from Korea (G1724; GenBank KJ599680) and an additional 15 Korean isolates revealed 99.2% (387/390) similarity with the reference sequences of *S. decipiens* from Korea (G1657; GenBank KJ599679). Based on morphologic and molecular databases, the estimated population ratio of *S. erinacei* to *S. decipiens* was 35:15. Our results indicate that both *S. erinacei* and *S. decipiens* found in Korea infect humans, with *S. erinacei* being 2 times more prevalent than *S. decipiens*. This study is the first to report human sparganosis caused by *S. decipiens* in humans in Korea.

**Key words:** *Spirometra erinacei*, *Spirometra decipiens*, mitochondrial gene, molecular identification, morphology

## INTRODUCTION

Tapeworms of the genus *Spirometra* are pseudophyllidean cestodes. The genus was established under the name *Spirometra* by Mueller [1] in 1937, although this name had been used earlier by Faust et al. [2] in 1929. There have been some controversies surrounding this genus because of its species classification; however, it has been recognized as independent and separate to *Diphyllobothrium* at the generic level. In 1952, Wardle and McLeod [3] recognized *Spirometra mansoni* Joyeux and Houdemer, 1927 and *Spirometra erinacei* Faust, Campbell and

Kellogg, 1929 as independent species, with *S. mansoni* as the type species. In 1959, Yamaguti [4] classified *S. mansoni* and *S. erinacei* as the same species and put under the name *S. erinacei* (hyphen has been omitted later by other authors). In 1972, Iwata [5] insisted that all species in the genus *Spirometra* are synonyms of *S. erinacei* Faust, Campbell and Kellogg, 1929. However, in 1974, Mueller [6] distinguished *S. mansonioides* distributed in North America from *S. mansoni* distributed in Asian region.

The species of the genus *Spirometra*, including *S. erinacei* (*S. erinacei*), *S. decipiens*, *S. mansoni*, *S. ranarum*, and *S. mansonioides* are all intestinal parasites of canine and feline hosts [1-6]. The main characters of species differentiation are the spirally coiled uterus of *S. erinacei* (5-7 coils) and *S. decipiens* (4-4½ coils) [2]. The life cycle of these parasites require 2 different intermediate hosts; the fresh water cyclops as the first intermediate host, and vertebrates such as amphibians

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and reptiles as the second intermediate hosts [6,7]. The procercoids develop in cyclops, and the plerocercoids (spargana) develop in frogs or snakes and cause sparganosis in humans.

Human sparganosis has been reported sporadically worldwide. The first case in Korea was reported under the name *Sparganum mansoni* [8]. Between 1924 and 1974, snakes and frogs were identified as the second intermediate host causing human sparganosis in 63 patients [9]. Between 1975 and 1989, an additional 56 cases of human sparganosis were reviewed [10]. Further studies identified adult worm infections with *S. erinaceiropaei* (under the name *S. erinacei*) in 2 human patients [7]. The experimental complete life cycle was established in the laboratory [11].

However, the cases of human sparganosis have not been clearly identified in the aspect of what kind(s) of *Spirometra* species were involved in human infections. Accurate identification of *Spirometra* spp. in Korea had been difficult because of lack of morphologic and molecular characterizing features. Therefore, a reliable taxonomic criterion based on morphologic characteristics and molecular data is needed. The present study aimed to provide information for classification and identification of *Spirometra* spp. in Korea by focusing on morphologic differences as well as nucleotide sequencing of mitochondrial genes of *S. erinaceiropaei* and *S. decipiens*.

## MATERIALS AND METHODS

### Specimens and experimental infection to animals

A total of 50 human-infected plerocercoids (1979-2009) and 2 adult tapeworms recovered from experimentally infected cat and dog were analyzed (Table 1). The plerocercoids from humans were obtained from hospitals and researchers in different parts of the country. The procedures for obtaining 2 adult tapeworms were as follows: In April 2008, a plerocercoid of *S. decipiens* was collected from a snake (*Rhabdophis tigrinus tigrinus*) and used to infect a cat. In May 2009, a plerocercoid of *S. erinaceiropaei* was collected from a 58-year-old man; a small part of the larva was cut for molecular studies and the remainder was used to infect a dog. After 8 weeks of infection, the cat and the dog were sacrificed and the adult tapeworms were recovered from the intestine. The adult worms were pressed and fixed in alcohol-formalin-acetic acid (AFA) for carmine staining. The vaginal opening, uterus, uterine pore, cirrus, genital pore, testes, and vitellaria of mature and gravid proglottids were observed, and longitudinal sections of gravid

proglottids were stained with hematoxylin and eosin (H&E) to observe their relationships. The morphologic data were compared to those of Faust et al. [2]. Some of the gravid proglottids were preserved in a -70°C deep freezer and in 70% ethanol for further use. Experimental animals were used following the ethical guidelines in commission of laboratory animals in Chungbuk National University, Cheongju, Korea (2008). Parasite materials used in this study were provided by the Parasite Resource Bank of Korea National Research Resource Center, Republic of Korea.

The 2 adult tapeworms, code numbers G1724 (*S. erinaceiropaei*) and G1622 (*S. decipiens*), were used as the reference criteria for morphologic and genetic observations using mitochondrial *cox1* sequences.

### DNA extraction, PCR, and nucleotide sequencing

A single proglottid was chopped into small pieces and the total genomic DNA was extracted using a DNeasy tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Thirty-eight specimens were fixed in formalin fixative and the rest of them were preserved in a -70°C deep freezer and in 70% ethanol. Genomic DNA of specimens preserved in formalin was extracted using the protocols of Jeon et al. [12]. The partial mitochondrial *cox1* gene was amplified by PCR.

PCR were performed in a 50 µl reaction mixture with 0.01 µg/µl of genomic DNA, 10× PCR buffer (20 mM Mg<sup>+</sup>), 10 mM dNTP mixture, 10 pmoles of each primer, and 2.5 U/µl *Taq* DNA polymerase (High Fidelity PCR system, Roche, Mannheim, Germany). PCRs were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Langen, Germany). The PCR primers used were p1f (5'-TGGTTTTTGGACATCCTGAA-3') and p1r (5'-ATCACATAATGAAAGTGAGCC-3'), which amplified a 440 bp product corresponding to the positions 707-1,146 bp of the *cox1* gene. The second set of PCR primers, used for sequencing was p1f1 (5'-GTGTTGATTTTGCCTGGGTTT-3') and p1r1 (5'-TACAAACCAAGTATCATGTAA-3'), which yielded a 390 bp product corresponding to positions 732-1,122 bp of the *cox1* gene. These were designed from *S. erinaceiropaei* (KJ599680) and *S. decipiens* (KJ599679). The PCR reaction was performed as follows: 1 cycle of initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 20 sec, 46°C for 40 sec, 72°C for 1 min, and incubation at 72°C for 4 min. This resulted in 390 bp DNA fragments, which were isolated on a 1.0% agarose gel, excised under long-wave UV light, and extracted using a QIAquick PCR

**Table 1.** Spargana specimens analyzed in this study (1979-2009)

Code	Locality	Sex/Age	Year	Molecular identification	Genbank <sup>a</sup>
G1249	Cheongju (Chungbuk)	M/49	2001	<i>S. erinaceiropaei</i>	
G1254	Goesan (Chungbuk)	M/60	1996	"	
G1286	Chungbuk	M/50	1997	"	
G1295	Cheongwon (Chungbuk)	F/78	1998	"	
G1300	Jeju	-	2002	<i>S. decipiens</i>	
G1301	Jeju	-	2002	"	
G1302	Jeju	-	2002	"	
G1326	Sancheong (Gyoungnam)	M/64	2002	<i>S. erinaceiropaei</i>	
G1327	Sancheong (Gyoungnam)	M/64	2002	"	
G1328	Sancheong (Gyoungnam)	M/64	2002	<i>S. decipiens</i>	
G1343	Seoul	-	1979	"	
G1344	-	F/59	-	<i>S. erinaceiropaei</i>	
G1345	Seoul	-	-	"	
G1346	Seoul	-	-	"	
G1347	Seoul	-	-	"	
G1348	-	F/51	1987	"	
G1353	Seoul	F/66	2003	"	
G1354	Seoul	M/79	2003	"	
G1356	Cheongju (Chungbuk)	M/37	2000	"	
G1357	-	M/41	1999	"	
G1393	Seoul	F/42	2003	"	
G1394	Seoul	-	2003	"	
G1395	Seoul	F/49	2003	"	
G1396	Seoul	-	2003	"	
G1398	Cheonan (Chungnam)	F/44	2003	"	
G1401	Seoul	F/59	2003	"	
G1402	Cheonan (Chungnam)	F/49	2003	"	
G1404	Busan	-	1990	"	
G1405	Busan	-	1990	"	
G1406	Busan	F/23	1990	<i>S. decipiens</i>	
G1407	Busan	F/47	1994	"	
G1409	Busan	M/65	1995	<i>S. erinaceiropaei</i>	
G1410	Busan	M/65	1995	"	
G1411	Busan	F/55	2001	"	
G1412	Busan	M	2003	<i>S. decipiens</i>	
G1413	Busan	M/71	2003	"	
G1476	Busan	M/71	2003	<i>S. erinaceiropaei</i>	
G1530	Busan	M/72	2003	"	
G1574	Gangwon	F/68	2002	<i>S. decipiens</i>	
G1576	Gangwon	F/57	2005	<i>S. erinaceiropaei</i>	
G1578	Jinju (Gyeongnam)	M/42	2005	"	
G1622 <sup>b</sup>	Chungbuk			<i>S. decipiens</i>	KJ599679
G1625	Seoul	F/52	2006	"	
G1628	Gangwon	F/57	2002	"	
G1657	Cheongju (Chungbuk)	M/59	2008	"	
G1681	Cheongju (Chungbuk)	F/46	2008	<i>S. erinaceiropaei</i>	
G1718	Cheongju (Chungbuk)	F/42	1994	"	
G1719	Chungbuk	M/45	2008	<i>S. decipiens</i>	
G1720	Chungbuk	F/67	2009	<i>S. erinaceiropaei</i>	
G1721	Jinju (Gyeongnam)	-	2002	<i>S. decipiens</i>	
G1724 <sup>c</sup>	Cheongju (Chungbuk)	M/58	2009	<i>S. erinaceiropaei</i>	KJ599680

<sup>a</sup>Accession numbers in GenBank, National Center for Biotechnology Information, USA.

<sup>b</sup>The sparganum was obtained from a snake and a *Spirometra decipiens* adult was recovered after experimental infection of a cat.

<sup>c</sup>The sparganum was obtained from a patient (molecular study was done cutting a small part of this worm) and a *S. erinaceiropaei* adult was recovered from experimental infection of a dog.

**Table 2.** Morphologic features of *Spirometra erinaceiueuropaei* and *S. decipiens*

Organs	Morphologic features	<i>S. erinaceiueuropaei</i>		<i>S. decipiens</i>		Others
		Size (mm)		Size (mm)		
Scolex	Spatulate	0.2		0.5		Diameter
		1		1.5		Length
Gravid proglottids	Trapezoid	3.1-4.2		6.8-10.8		Width
		2.1-3.2		1.1-2.0		Length
Uterus	Coiling	5-7		4-4½		Loops
Eggs	Operculate	0.057-0.061 × 0.033-0.036		0.056-0.060 × 0.034-0.036		Length width

purification kit (Qiagen).

The entire *cox1* gene was amplified by PCR. Primers were designed from the complete sequence of the *S. erinaceiueuropaei* and *S. decipiens* mitochondrial genome and used for amplifying the full *cox1* gene. Primers spcox1f (5'-GTA TTG AAG GAA TTA GIT AGG TTA-3') and spcox1r (5'-CAA CCC AAT TAA ATT AAG TTC CAC-3') were used to amplify and sequence the *cox1* gene. The PCR reaction was performed as follows: 1 cycle of initial denaturation at 94°C for 3 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and extension at 72°C for 10 min. Cyclic sequencing was done from 4 fragments using a Big-Dye terminator sequencing kit (version 3.2, Applied Biosystems, Foster City, California, USA), and the reaction products were directly sequenced using a DNA sequencer (3739XL model, Applied Biosystems).

#### DNA sequence analyses

The sequences were assembled and aligned using Geneious 6.1.5 (Biomatter, Auckland, New Zealand) and identified by BLAST searches and comparisons with the sequences of *S. erinaceiueuropaei* and *S. decipiens*, which had been deposited in GenBank database. The molecular identification of *Spirometra* tapeworm specimens was based on the similarity of the nucleotide sequences of the *cox1* gene and the phylogenetic relationship with those of *S. erinaceiueuropaei* (KJ599680) and *S. decipiens* (KJ599679). The phylogenetic analysis was conducted with PAUP 4.0 [13]. The phylogenetic trees were constructed using the maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods, using *D. nihonkaiense* as an outgroup.

## RESULTS

#### Morphologic features of *S. erinaceiueuropaei* and *S. decipiens*

The length of strobilae was 68 cm in *S. erinaceiueuropaei* and

47 cm in *S. decipiens*. The scolex was spatulate, measuring 0.2 mm in diameter by 1.0 mm length in *S. erinaceiueuropaei* and 0.5 mm diameter by 1.5 mm length in *S. decipiens*. The neck was immediately behind the scolex and measured 0.5 cm in *S. erinaceiueuropaei* and 1.0 cm in *S. decipiens* segmentation. The genital primordium measured 2.0-2.5 cm in *S. erinaceiueuropaei* and 2.5-3.0 cm in *S. decipiens*, and was located at the back of the scolex.

The mature proglottids with eggs were first visible almost 14 cm behind the scolex in both *S. erinaceiueuropaei* and *S. decipiens*. The width and length of gravid proglottids was 6.8-10.8 mm by 1.1-2.0 mm in *S. erinaceiueuropaei*, and 3.1-4.2 mm by 2.1-3.2 mm in *S. decipiens* (Table 2). The genital pore was situated ventrally on the midline in the anterior 1/5 of the segment in *S. erinaceiueuropaei* and *S. decipiens*. The uterine pores were on the midline behind the anterior margin of the terminal ball, 80-100 µm in *S. erinaceiueuropaei* and 80-110 µm in *S. decipiens*, respectively. The uterus consisted of 5-7 loops on each side in *S. erinaceiueuropaei* and 4-4.5 loops in *S. decipiens*. The dumbbell-shaped ovary was connected to the uterus and situated near the posterior margin of the segment in both *S. erinaceiueuropaei* and *S. decipiens* (Fig. 1A, B). Longitudinal sections of the cirrus sac showed an oval structure, 200-250 µm in length by 120-150 µm in width in *S. erinaceiueuropaei*, and 200-220 µm by 150-170 µm in *S. decipiens*. The seminal vesicle was elliptical and measured 200-250 µm in length by 100-150 µm in width in *S. erinaceiueuropaei*, and 240-280 µm by 100-150 µm in *S. decipiens*. The testes measured 70-80 µm in maximal diameter in both *S. erinaceiueuropaei* and *S. decipiens* (Fig. 1C, D). The eggs measured 57-61 µm by 33-36 µm in *S. erinaceiueuropaei* and 56-60 µm by 34-36 µm in *S. decipiens*.

#### Sequence divergence of mitochondrial *cox1* gene

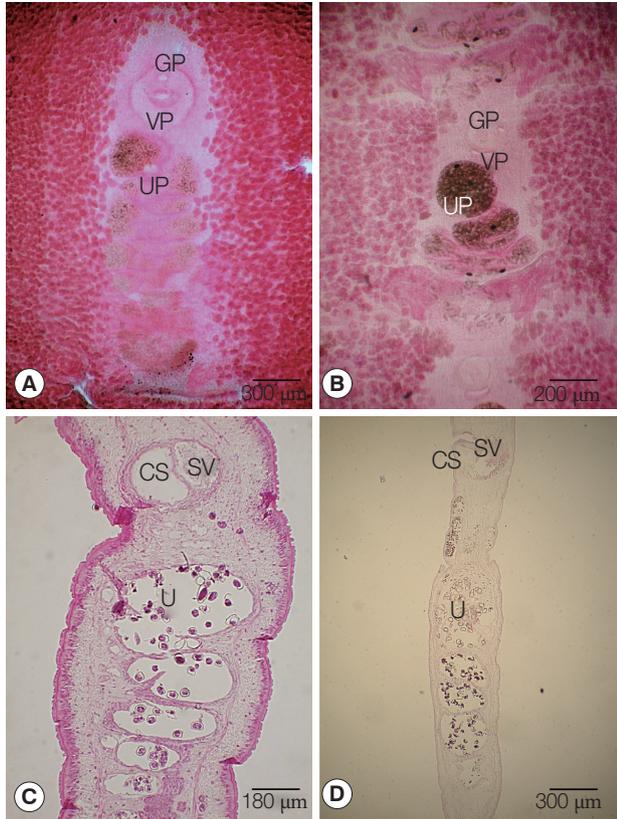
The entire *cox1* gene of both *S. erinaceiueuropaei* and *S. decipiens* was 1,566 bp in length and showed 146 polymorphic sites. The sequence difference between *S. erinaceiueuropaei* and *S. de-*

*decipiens* was 9.3% (146/1,566) in the *cox1* gene. Partial *cox1* sequences (390 bp) from 35 Korean isolates showed 99.4%

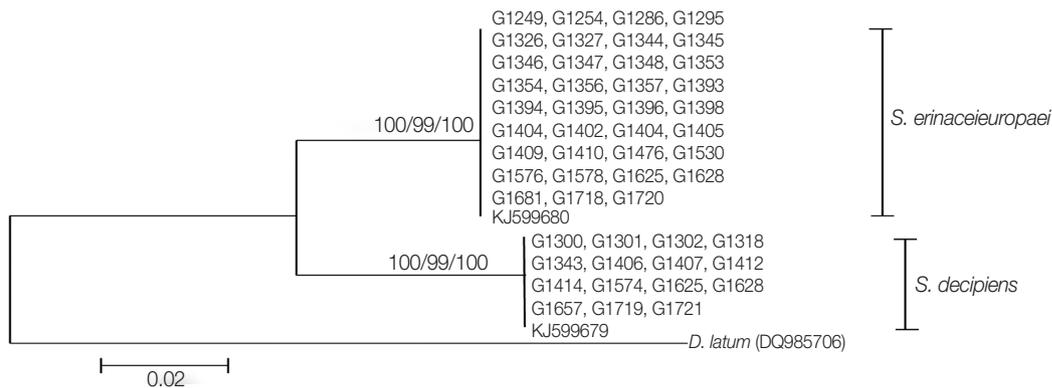
(388/390) similarity to references of the Korean *S. erinacei-europaei* (G1724; GenBank KJ599680), while the remaining 15 Korean isolates showed 99.2% (387/390) similarity with reference sequences of the Korean *S. decipiens* (G1657; GenBank KJ599679). The sequence difference in partial *cox1* gene between *S. erinacei-europaei* and *S. decipiens* was 9.2%. The ratio of *S. erinacei-europaei* and *S. decipiens* according to molecular data was 35:15. The phylogenetic relationship of the 50 isolates derived by different analytical methods (MP, NJ, and ML) is seen in Fig. 2, with *D. nihonkaiense* basal to the *S. erinacei-europaei*-*S. decipiens* clades.

### DISCUSSION

Diesing in 1854 used the terms sparganum, dubium, ligula, and plerocercoides for the larval spirometriid worms and assigned the name *Dubium erinacei-europaei* (Rudolphi, 1819) for the larval tapeworm encysted in the intercostal muscles of a European hedgehog (*Erinaceus europaeus*) [2,3]. In 1929, Faust et al. [2] reviewed spirometriid species (under the name *Diphyllobothrium*) with morphologic and biological studies; they reported *S. houghtoni*, *S. erinacei*, and *S. okumurai*. Later, the spirometriid (*Spirometra*) species were described to include *S. decipiens* Gedoelst, 1911, *S. reptans* Meggitt, 1924, *S. ranarum* Meggitt, 1925, *S. mansoni* Joyeux and Houdemer, 1927, *S. felis* Southwell, 1928, *S. houghtoni*, *S. erinacei*, and *S. okumurai* Faust, Campbell and Kellogg, 1929, *S. serpentis* Yamaguti, 1935, and *S. mansonioides* Mueller, 1935 [3]. The name *S. erinacei* was later substituted by *S. erinacei-europaei* by Yamaguti



**Fig. 1.** Gravid proglottids of *Spirometra erinacei-europaei* and *Spirometra decipiens*. (A, B) Whole mounted specimens of proglottids showing the uterus and cirrus sac ( $\times 40$ ), (C, D) Longitudinal section of a gravid proglottids showing the cirrus sac (CS), seminal vesicle (SV), and uterus (U) (H&E stain).



**Fig. 2.** A phylogenetic tree of spirometriid tapeworms based on partial *cox1* sequences inferred from neighbor-joining analysis. Numbers on branches indicate the bootstrap supporting values based on 1,000 replicates and bootstrap values by the maximum parsimony, maximum likelihood, and neighbor-joining methods are shown from left to right. The partial *cox1* sequences of *S. erinacei-europaei* and *S. decipiens* were divided into 2 clades, which differed by 29-32 nucleotides from each other. There were 390 bp corresponding to positions 732-1,122 bp of the *cox1* gene.

[4]. However, the name *S. erinacei* has been used repeatedly by various workers in Asia and Australia [7,14,15] and even at present by many authors. In the meantime, Schmidt [16] used the name *Diphyllobothrium erinacei* in 1986 (it should be *Spirometra erinacei*), and articles using *Spirometra erinacei* for this tapeworm began to appear from 1997 in PubMed Database (National Library of Medicine, Washington DC, USA).

In this study, the morphologic characteristics of *Spirometra* species were observed as in the above studies. Morphologic features of *S. erinacei* and *S. decipiens* are different in that the spirally coiled uterus of *S. erinacei* consists of 5-7 complete turns, while that of *S. decipiens* consist of only 4-4.5 coils [2]. This is the major differential features between the 2 species and our specimens completely fit to the criteria of those uterine morphology with some other features; the uterine pore lies in the midline, a little distance behind the anterior margin of the uterine terminal ball in *S. erinacei*, while in *S. decipiens* it is a conspicuous sphincter with 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. erinacei*, while in *S. decipiens* it is crescent shaped and elliptical. The cirrus is strongly muscular and elongated in shape in *S. decipiens*, while in *S. erinacei* it is smaller than in other related species. The morphologic characteristics of the male and female reproductive organs of *S. mansoni* overlap those of *S. decipiens* in terms of the number of rotations of the spirally coiled uterus, the shape of the terminal uterus, the vaginal opening, and the cirrus.

The taxonomic status of spirometrid tapeworms has been unsettled. It would be useful to determine which *Spirometra* species are present in Korea causing human sparganosis. The most common form of transmission to humans is by drinking water contaminated with proceroid larvae. In addition, transmission can also occur by eating raw or undercooked flesh of second intermediate hosts such as snakes and frogs, which is why the disease is more prevalent in adult males, who are the main consumers of these infection sources [9]. A previous study reported that snakes are an important source of human sparganosis; the cases had eaten raw snakes in about 50% of all cases in Korea [9]. However, another study revealed an epidemiological discrepancy between the eating habit and the ratio of *Spirometra* species distribution (unpublished observation). The mitochondrial genotype of sparganum infecting snakes has been shown to be different from that of human-in-

fecting sparganum (unpublished observation). Thus, additional studies are strongly needed to identify the correct intermediate hosts and the infection route. Natural infection with *S. decipiens* has been mostly observed in carnivorous animals such as cats and dogs. However, according to our data, no natural hosts and adult tapeworms of *S. erinacei* were available; therefore, the infection route of *S. erinacei* for humans still remains unclear.

In the present study, we first report human sparganosis caused by *S. decipiens*, which was identified from spargana collected between 1979 and 2010 in Korea. *Spirometra* infection was identified as *S. erinacei* in 35 cases and *S. decipiens* in 15 cases. This was done through sequence analysis of the mitochondrial *cox1* gene and by morphologic characteristics of the adult worms. Our results indicated that *S. erinacei* and *S. decipiens* are human-infecting *Spirometra* tapeworms in Korea, with *S. erinacei* infections accounting for 2/3 of all *Spirometra* infections.

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## CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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