Molecular Genetic Findings of Spirometra decipiens and S. ranarum in Korea

Hyeong-Kyu Jeon¹, Sun Huh², Woon-Mok Sohn³, Jong-Yil Chai⁴, Keeseon S. Eom¹*¹

¹Department of Parasitology, Parasite Research Center and Parasite Resource Bank, Chungbuk National University School of Medicine, Cheongju 28644, Korea; ²Department of Parasitology, College of Medicine, Hallym University, Chuncheon 24252, Korea; ³Department of Parasitology and Tropical Medicine, Institute of Health Sciences, Gyeongsang National University College of Medicine, Jinju 52727, Korea; ⁴Korea Association of Health Promotion, Seoul 07549, and Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, Seoul 03080, Korea

Abstract: The taxonomy of Spirometra species has been controversial despite the medical and veterinary importance. Currently, only a few Spirometra species are considered valid species in the genus Spirometra. In the present study, the distribution of Spirometra species obtained from animals in Korea were identified by molecular analysis of the mitochondrial cytochrome c oxidase I (cox1) gene. A total of 28 Spirometra species specimens were analyzed. These were all collected between 1973 and 2008 in the Republic of Korea. Mitochondrial cox1 sequences were examined for a total of 28 specimens comprising 14 S. decipiens and 14 S. ranarum. The difference in partial cox1 sequences (316 bp) between S. erinaceieuropaei (KJ599680) and S. ranarum (this study) was 9.3%, while that between S. decipiens (KJ599679) and S. ranarum (this study) was 2.2%. Genetic analyses identified 2 Spirometra species in animals such as cat, leopard cat, dog, duck and snake in Korea as S. decipiens and S. ranarum. S. decipiens and S. ranarum were present in Gyeongnam Province (P), Jeonnam P, Gangwon P, and Seoul. S. decipiens was found in tadpoles, snakes, ducks, cats, leopard cats and dogs, while S. ranarum was found in cats and dogs. The ratio of S. decipiens:S. ranarum calculated from the molecular data was 14:14 (or 1:1). These results indicate that S. decipiens and S. ranarum are sympatrically distributed in Korea.

Key words: Spirometra decipiens, S. ranarum, animals, sympatric distribution, molecular identification, Korea

INTRODUCTION

Species of the genus Spirometra belong to the family Diphyllobothriidae and includes intestinal parasites of cats and dogs. These parasites require 2 different intermediate hosts, larval forms of the first intermediate hosts are found in copepods (procercoid) and amphibians and reptiles (plerocercoid) as the second intermediate hosts. Sparganosis or human infection is a zoonotic disease caused by infection with the larval stages of Spirometra species.

The genus Spirometra has been described with morphological features of spirometrid species under the generic name Diphyllobothrium as found in China with complex life cycles and include S. mansoni, S. decipiens, S. mansonoides (Baer, 1927), S. ranarum (Gastaldi, 1854), S. mansoni (Cobbold, 1882) S. haughtoni (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 recognized as S. mansonioides (Mueller, 1935), which have a characteristic 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. mansonioides have been reported from wild fields in South America [3]. Four Spirometra species, S. erinaceieuropaei, S. pretoriensis (Baer, 1924), S. theleri (1924) and S. mansonioides have been acknowledged as valid species by Kamo [4].

The taxonomy of Spirometra species has been controversial despite the medical and veterinary importance. Currently, only a few Spirometra species are considered valid species in the genus Spirometra. The Spirometra species currently recognized by many researches worldwide are S. erinaceieuropaei, S. decipiens, S. mansoni, S. ranarum and S. mansonioides [1-4]. Additionally, sparganum proliferum is still an unnamed taxon [5]. A recent report has suggested that there are at least 2 Spirometra species in South America that differ from S. erinaceieuropaei and sparganum proliferum [5]. Unidentified mitochondrial genotypes of Spirometra
species were reported from South Sudan and Ethiopia, in which 37 cases of human sparganosis differed from Asian and South American cases by analysis of mitochondrial DNA sequence data [6,7]. The molecular data of Spirometra species showed that at least 4 Spirometra species such as *S. erinaceieuropaei*, *S. decipiens*, *S. mansonioides* and *sparganum proliferum* are distributed in Asian, South American and African countries [3-6].

The most recent studies reported identification of *S. ranarum* from frogs (*Hyllobatrachus rugulosus*; syn: *Rana rugulosa*) in Myanmar by morphological and genetic analyses [8]. Another report demonstrated the distribution of *S. ranarum* from lions in Tanzania by analysis of 2 complete mitochondrial genes and morphological observations (to be published). *S. ranarum* was first reported by Gastaldi (1854) from *Rana esculenta* (syn: *Pelophylax esculenta*) in Italy, and Meggitt (1925) described it as *S. ranarum* from a dog fed spargana isolated from the same frog host by Gastaldi (1854) in Myanmar [9,10]. Following this, Joyeux et al. [11] and Faust et al. [1] described *S. ranarum*. Wardle and McLeod (1952) recognized *S. ranarum* as a valid species [12]. This Spirometra species has not been reported since 1929. Currently, mitochondrial DNA sequence evidence combined with examination of morphological features strongly supports the distinctiveness of Spirometra species, thus the resurrection of *S. ranarum* has been proposed in recent reports of Spirometra species collected from Myanmar and Tanzania (to be published).

The *Spirometra* species in the 50 cases of human sparganosis were identified as *S. erinaceieuropaei* and *S. decipiens* by molecular and morphological features [3]. Another study identified *S. decipiens* plerocercoids (n = 904) in terrestrial snakes from Korea and China [13]. A report concerning the examination of *Spirometra* species from a stray cat identified multiple infections of *S. decipiens* [15]. The recent studies suggested that *S. erinaceieuropaei* is not the only species inducing human sparganosis but that *S. decipiens* is another cause of human sparganosis in Korea [3,13,14].

In the present study, *Spirometra* species obtained from animals in Korea were identified by molecular analysis of the mitochondrial cytochrome c oxidase I (*cox1*) gene and phylogenetic analysis of mitochondrial DNA sequence data.

**MATERIALS AND METHODS**

**Specimens**

A total of 28 *Spirometra* species were analyzed in this study (Table 1). These specimens were collected between 1973 and 2008 in the Republic of Korea. All specimens originated from Korea and obtained from the Department of Parasitology, Gyeongsang National University, Hallym University and Seoul National University. Eight specimens from Gyeongsang National University were collected from a snake (*Rhabdophis tigrinus tigrinus*), tadpole and duck were used to infect cats for maintaining the complete life cycle of *Spirometra* species in the laboratory. Twelve specimens from Seoul National University were collected from naturally infected cats. Seven specimens from Hallym University were collected from naturally infected dogs. One specimen was collected from leopard cat (*Prionailurus bengalensis*), which was donated from the Parasite Resource Bank. Twenty specimens were preserved in 10% neutral buffered formalin, and 8 specimens were kept in 70% ethanol for experimental use.

**PCR and DNA sequencing**

Total genomic DNA extraction and PCR reactions were employed as previously described by Jeon et al. [3]. The partial

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**Table 1. Spirometra species from animals analyzed in this study (1973-2008)**

| Code     | Locality (Korea) | Host   | Year | Molecular identification |
|----------|------------------|--------|------|--------------------------|
| G1246    | Jinju            | cat    | 2001 | *S. decipiens*           |
| G1247    | Jinju            | cat    | 2001 | *S. decipiens*           |
| G1248    | Jinju            | cat    | 2001 | *S. decipiens*           |
| G1250    | Jinju            | snake  | 2001 | *S. decipiens*           |
| G1251    | Jinju            | snake  | 2001 | *S. decipiens*           |
| G1252    | Jinju            | tadpole| 2001 | *S. decipiens*           |
| G1272    | Jinju            | cat    | 2001 | *S. decipiens*           |
| G1273    | Jinju            | duck   | 2001 | *S. decipiens*           |
| G1341    | Seoul            | cat    | 1973 | *S. decipiens*           |
| G1339    | Seoul            | cat    | 1987 | *S. decipiens*           |
| G1539    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1540    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1541    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1542    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1543    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1544    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1546    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1547    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1548    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1549    | Shinan-gun       | cat    | 2004 | *S. ranarum*             |
| G1556    | Chuncheon        | cat    | 1988 | *S. decipiens*           |
| G1563    | Chuncheon        | dog    | 2005 | *S. decipiens*           |
| G1564    | Chuncheon        | dog    | 2002 | *S. decipiens*           |
| G1565    | Chuncheon        | dog    | 2002 | *S. decipiens*           |
| G1569    | Chuncheon        | dog    | 1995 | *S. ranarum*             |
| G1571    | Chuncheon        | dog    | 1999 | *S. ranarum*             |
| G1573    | Chuncheon        | dog    | 2000 | *S. decipiens*           |
| G1681    | Seoul            | leopard| 2008 | *S. decipiens*           |

*Prionailurus bengalensis.*
**RESULTS**

Sequence divergences

The mitochondrial **cox1** sequences obtained from Korean isolates of *Spirometra* species were compared with the reference **cox1** sequences of *S. erinaceieuropaei*, *S. decipiens* and *S. ranarum* which were deposited in GenBank (accession numbers KJ599680, KJ599679 and MH298843). The mitochondrial **cox1** sequences for a total of 28 specimens were identified as 14 *S. decipiens* and 14 *S. ranarum*. The difference in partial **cox1** sequences (316 bp) between *S. erinaceieuropaei* (KJ599680) and *S. ranarum* (this study) was 9.3%, while that of *S. decipiens* (KJ599679) and *S. ranarum* (this study) was 2.2%. The sequence identities determined of *Spirometra* specimens in this study were 99.8% (*S. ranarum*, MH298843), 89.7% (*S. erinaceieuropaei*), and 89.7% (*S. decipiens*). The similarity to other Diphyllobothrium species was 84.1% (*D. nihonkaiense*) and 83.1% (*D. latum*). The similarity of mitochondrial large subunit RNA sequences (987 bp) from Korean isolates to the references sequences was 98.2% (*S. erinaceieuropaei*), 83.1% (*D. latum*) and 80.0% (*D. nihonkaiense*) (Table 2).

Phylogenetic relationships

Phylogenetic analyses of *Spirometra* species were performed using the Bayesian inference and maximum likelihood methods based on partial mitochondrial **cox1** sequences of *S. erinaceieuropaei*, *S. decipiens*, *S. ranarum*, *D. nihonkaiense* and *D. latum*. The partial **cox1** sequences (316 bp) revealed 34 polymorphic sites with 34 synonymous and 0 non-synonymous substitutions among *S. erinaceieuropaei*, *S. decipiens* and *S. ranarum* (GenBank no. MH298843). Phylogenetic analysis of the mitochondrial **cox1** sequences for a total of 28 specimens identified

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**Table 2.** Percentage pairwise sequence homologies of the mitochondrial **cox1** gene and large subunit ribosomal RNAs between *Spirometra* sp. of Korea and various *Spirometra* species, *Diphyllobothrium latum* and *D. nihonkaiense*

| Species                  | S. ranarum | S. decipiens | S. erinaceieuropaei | D. latum | D. nihonkaiense |
|--------------------------|------------|--------------|---------------------|----------|-----------------|
| GenBank No.              | (MH298843) | (KJ599679)   | (KJ599680)          | (DQ985706) | (EF420138)       |
| Genes                    | **cox1**rRNA | **cox1**rRNA | **cox1**rRNA        | **cox1**rRNA | **cox1**rRNA     |
| Spirometra sp. (Korea)   | 99.7/100   | 89.7/98.2    | 89.7/89.4           | 83.1/79.5 | 84.1/80.0        |
Spirometra species as basal to the D. nihonkaiense and D. latum clade. Phylogenetic tree topologies generated using the Bayesian inference and maximum likelihood methods were identical and showed a high level of confidence values for the 3 major branches of the 3 Spirometra species such as S. erinaceieuropaei, S. decipiens and S. ranarum in the cox1 gene (Fig. 1).

Species composition

Genetic analyses identified 2 Spirometra species in wild animals from Korea as S. decipiens and S. ranarum. S. decipiens and S. ranarum were presented in Gyeongsang, Jeonnam, Gangwon, Chungbuk, and Seoul. S. decipiens was found in tadpoles, snakes, ducks, cats, leopard cats and dogs while S. ranarum was found in cats and dogs (Table 1). The species ratio of S. decipiens: S. ranarum calculated from the molecular data was 14:14 (or 1:1) (Fig. 1).

DISCUSSION

In the present study, we first report S. ranarum from natural infections of cats and dogs in Korea using mitochondrial cox1 gene sequence analysis. S. ranarum (under the name Ligular ranarum) was first described by Gastaldi (1854) from Rana esculenta (syn: Pelophylax esculentus) from Italy. Meggitt (1924) reported the presence of spargana in the stomach wall of frogs (Rana tigrina) from Yangon, Myanmar. The frogs were found to contain large numbers of a larval tapeworm. These spargana were fed to a young dog and then eight adult tapeworms were recovered 58 days after infection, which the author described as S. ranarum (under name the Ligular ranarum) [9]. Meggitt (1925) described this species and detailed the following features: being up to 1,130 mm in length by 5 mm in breadth, scolex 1.4-1.7 mm in length and 0.37-0.41 mm in breadth, all the segments either broader than long or square, male genital aperture almost at the anterior border of the segment and median, female aperture slightly lateral to it, testes in 2 bands, 100-1 10 in each band, 3 to 5 uterine coils, uterus extending laterally to the genital apertures, a terminal uterine enlargement, eggs 58-67 by 34-36 μm [10]. Meggitt et al. [10] studied the complete life cycle of this species through the intermediate hosts found and showed it to be suitable for final hosts. Faust et al. [1] studied S. ranarum (under the name D. ranarum) from natural infections of cats and dogs in Beijing, Xiamen, Canton and by experimental feeding of spargana obtained from dogs in Fujian.

Spirometra species have been reported sporadically by many authors in the Republic of Korea. Helminth infections such as Clonorchis sinensis, Paragonimus sp., Hydatigera taeniaeformis, Spirometra sp. and Toxocara cati were examined from 41 cats in Gyeongsangnam-do (Province) [18]. Seven helminth species, T. cati, Anisakis simplex larvae, C. sinensis, Pharyngostomum condatum, S. erinaceieuropaei and H. taeniaeformis were reported from 41 cats in Seoul [19]. Four helminth species including T. cati, Diphyllolothrium latum, S. erinaceieuropaei and H. taeniaeformis were
detected from 133 cats in Jeollanam-do (Province) [20]. More than 29 helminth species were reported from feral cats purchased from a market in Busan, and 23 trematodes, 5 cestodes and 4 nematodes species in cats were reported in Korea [21,22]. Currently, S. erinaceieuropaei and S. decipiens are recognized as being Spirometra species in Korea [3]. The first case of human sparganosis in Korea was reported by Uemura [23]. Snakes and frogs were identified as second intermediate hosts from reports of 63 human sparganosis cases during the years between 1924 and 1974 [24]. An additional 56 human sparganosis cases were reviewed during the years between 1975 and 1989 [25].

In this study, we found 2 genotypes in our sequence variation analyses of the cox1 gene from 28 Spirometra specimens obtained from 6 kinds of animals. The sequence difference in the cox1 gene between 14 Spirometra specimens and S. ranarum (GenBank no. MH298843) was 0.1%, while that for the rest of the 14 specimens was 2.2% with S. decipiens and 9.5% with S. erinaceieuropaei. These results indicated that the examined Spirometra specimens in this study were identified as S. decipiens and S. ranarum by mitochondrial DNA sequence divergence. These reports have provoked many questions with respect to the epidemiological discrepancy between humans and animals. In a previous study, human sparganosis cases were identified as S. erinaceieuropaei and S. decipiens, and no cases of S. ranarum were not found in that study. Therefore, although many studies have examined Spirometra species in Korea, those previous studies may need reexamination using molecular techniques to better understand the epidemiological status of Spirometra species in Korea.

The morphological similarity of both adult and larva forms of Spirometra species have been studied to resolve species identification by use of molecular techniques along with an assessment of morphological variation. Molecular identification has played an important role in improving understanding of phylogenetic relationships, genetic variation and taxonomy. Mitochondrial DNA sequences have been utilized for phylogenetic reconstruction, taxonomic identification, population genetics and epidemiological investigations [26]. In an effort to delineate the phylogenetic relationships and genetic variation of Spirometra species, DNA sequence analysis of small (18S) and large (28S) subunit ribosomal RNA, ribosomal internal transcribed spacer 1, ribosomal internal transcribed 2, and mitochondrial genes such as cytochrome c oxidase subunit 1 (cox1) and 3 (cox3) and NADH dehydrogenase subunit 1 (nad1), 3 (nad3) and 4 (nad4) have been studied and reported [27-32]. Mitochondrial DNA sequence variation of Spirometra species ranged from 0.0-3.5% in China, Myanmar, Thailand and Lao PDR [33]. DNA sequence variation of the Spirometra spp. cox1 gene ranges from 0.0-2.6% in Japan, India and Indonesia [34]. The degree of mtDNA sequence divergence of the cytochrome b (cob) gene between sister or congeneric species and con-familial genera was greater than 2% in amphibian, reptilian, avian, and mammalian species [35]. The closely related species of vertebrates showed more than 2% sequence divergence in the cox1 gene [36]. Regarding these previous studies, it was assumed that at least 2 Spirometra species were distributed in those endemic areas.

In conclusion, S. decipiens and S. ranarum were identified from natural infections of cats and dogs, with overall results showing 14 S. decipiens and 14 S. ranarum. These results indicate that 2 Spirometra species are sympatrically distributed in Korea.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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