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▣ ORIGINAL ARTICLE

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

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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, Chungbuk 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. erinaceieuropaei* (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 recognized as *S. mansonoides* (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. mansonoides* have been reported from wild fields in South America [3]. Four *Spirometra* species, *S. erinaceieuropaei, S. pretoriensis* (Baer, 1924), *S. theileri* (1924) and *S. mansonoides* 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. mansonoides* [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*

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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. mansonoides* 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 (*Hoplobatrachus 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 esculentus*) 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.

Table 1. *Spirometra* specimens 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. ranarum*
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. ranarum*
G1569	Chuncheon	dog	1995	*S. ranarum*
G1571	Chuncheon	dog	1999	*S. ranarum*
G1573	Chuncheon	dog	2000	*S. decipiens*
G1681	Seoul	leopard cat*	2008	*S. decipiens*

**Prionailurus bengalensis.*

PCR and DNA sequencing

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

cox1 gene was amplified and sequenced by PCR and cycle sequencing. The partial sequence of the mitochondrial *cox1* gene was amplified using forward primer p1f, 5′-TGG TTT TTT GGA CAT CCT GAA -3′, and reverse primer p1r, 5′-ATC ACA TAA TGA AAG TGA GCC-3′, which amplified a 440-bp product. A second set of PCR primers was used for cycle sequencing of the internal forward primer p1f1, 5′-GTG TTG ATT TTG CCT GGG TTT-3′, and internal reverse primer p1r1, 5′-TAC AAA CCA AGT ATC ATG TAA-3′, which yielded a 390-bp product. These primers were designed from the complete sequence of *S. erinaceieuropaei* (KJ599680) and *S. decipiens* (KJ599679) mitochondrial genomes to amplify a partial sequence of the *cox1* gene corresponding to the region between base pair positions 707 and 1,146. The mitochondrial large subunit RNA was amplified using forward primer rRNA F, 5′GAT TTT GTA AAT CAG GGG GTA-3′, and reverse primer rRNA R, 5′-AAT TTA TGC GAT TCA CCT TAA-3′ which amplified a 987 bp product. DNA sequencing was performed using a Big-Dye Terminator kit (version 3.1, Applied Biosystems, Foster City, California, USA) and reaction products were sequenced directly using a DNA sequencer (ABI3730XL, Applied Biosystems).

DNA sequence analyses

The DNA sequence of 28 partial *cox1* gene sequences were assembled using the Geneious 9.0 program (Biometer, Auckland, New Zealand) and then aligned using MAFFT methods in the Geneious 9.0 program by comparison with sequences of *S. erinaceieuropaei* and *S. decipiens* in the GenBank database. Phylogenetic relationships were reconstructed using Bayesian inference (BI) and maximum-likelihood (ML) using partial mitochondrial *cox1* (390 bp) sequences of *S. erinaceieuropaei* (KJ599680), *S. decipiens* (KJ599679) and *S. ranarum* (MH298843). BI analyses were conducted using MrBayes 3.2 and running 4 simultaneous Monte Carlo Markov chains (MCMC) for 10 million generations, sampling every, 1,000 generations and discarding the first 25% generations as burn-in [15]. BI analysis was evaluated as posterior probability (PP). ML analyses of *cox1* used RAxML v. 7.3.1 [16] after TRN+G+I substitution model sampling was chosen according to the Modeltest using the program Partition Finder [17]. Phylogenetic trees were constructed using Bayesian inference (BI) and maximum likelihood (ML) with *Diphyllobothrium nihonkaiense* (EF420138) and *D. latum* (DQ985706) as outgroups.

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 number 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. decipiens*), 89.4% (*S. erinaceieuropaei*), 79.5% (*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

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

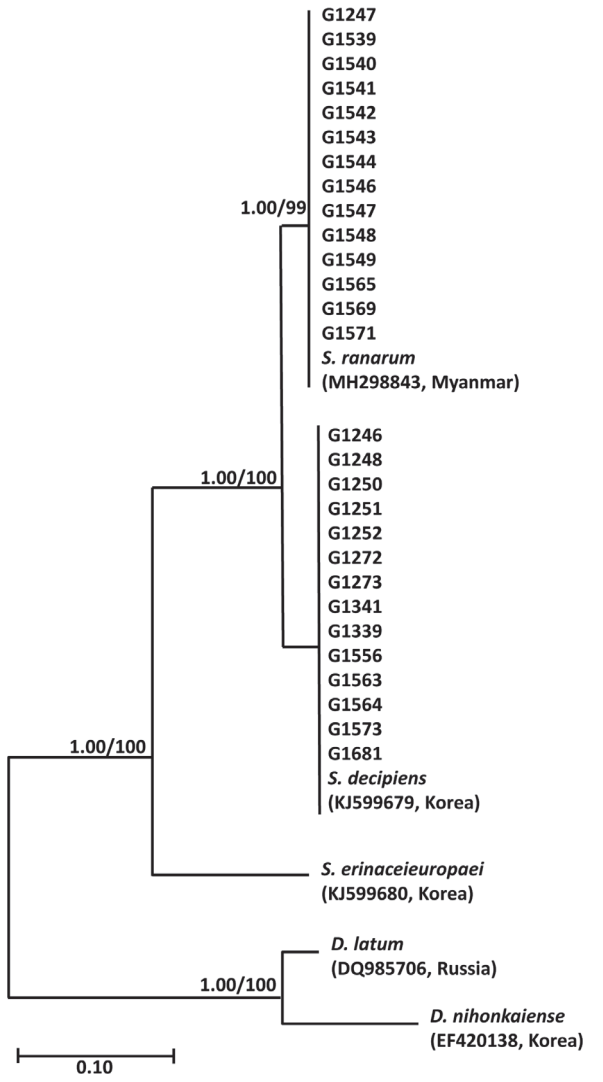

Fig. 1. Phylogenetic tree of *Spirometra* species based on partial *cox1* sequences. Numbers above the branches represent bootstrap values for maximum likelihood (ML) and the support values of Bayesian inference (BI) are indicated by the posterior probabilities. *S. decipiens* and *S. ranarum* were presented 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. The species ratio of *S. decipiens*: *S. ranarum* calculated from the molecular data was 14: 14 (or 1: 1).

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, 100-110 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 cordatum, S. erinaceieuropaei* and *H. taeniaeformis* were reported from 41 cats in Seoul [19]. Four helminth species including *T. cati, Diphyllobothrium 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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