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Research Article

Taxonomic Suggestion for Spirometra Species (Cestoda: Diphyllobothriidae) Tapeworms with Molecular Characteristics Obtained from Several Origins of Worm Samples

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Abstract

The species validity of the genus *Spirometra tapeworm is still controversial.* Here, we aimed to investigate molecular characteristics with a total of 11 *Spirometra* samples collected from Asia and USA. They were all examined based on mitochondrial cytochrome c oxidase subunit 1 (*CO1*) and NADH dehydrogenase subunit 1 (*ND 1*) gene sequences. The *CO1* sequences of 7 *Spirometra* samples from Korea (n=3), China (n=2), and USA (n=2) were presumptively designated into *S. mansoni* (99.1-99.9% identities), but they were hardly differentiated to *S. decipiens* (99.0-99.8% identities) with the only one base pair-difference. The *ND1* sequences of above 7 samples showed 100% identity with *S. decipiens*, but we could not compare them with those of *S. mansoni* due to lacking database. The *CO1* sequences obtained from 3 Myanmar and 1 Cambodia samples were supposed to be new *Spirometra* species. The *ND1* sequences of 2 Myanmar samples revealed 100% identity with *S. ranarum* (SP1803) and *S. decipiens* (SP1804), respectively. Seven samples of *S. mansoni* from Korea, China and USA were co-clustered without the exact differentiation among *S. decipiens* or *S. erinaceieuropaei.* Three Myanmar samples were relatively close to each other, but quite different to a Cambodian *Spirometra* species. We found that several *CO1* sequences of *S. mansoni, S. decipiens*, *S. erinaceieuropaei* deposited in GenBank were quite similar. Our data showed that it is not easy to differentiate between *S. mansoni, S. decipiens*, and *S. erinaceieuropaei* even with *CO1* sequences and the expansion of *ND1* sequences may be further needed for their differentiation.

Keywords: *Spirometra,* molecular characteristics; Mitochondrial cytochrome c oxidase subunit 1; NADH dehydrogenase subunit 1*, Asia, USA*

Introduction

Genus *Spirometra* species (Cestoda: Diphyllobothriidae) tapeworms mainly inhabit in the small intestines of canine and feline hosts worldwide and their plerocercoid larva, sparganum (pl. spargana), causes sparganosis in humans [1]. Humans are accidental hosts and acquire the parasite by ingesting water contaminated with copepods carrying procercoids, by ingesting uncooked or undercooked meat of the second intermediate and/or paratenic hosts with plerocercoids, or by using the frog and snake flesh as a poultice in traditional medicine [2]. In humans, the ingested sparganum via the gastrointestinal track usually migrates to subcutaneous tissues and muscles of the abdominal wall and limbs, where it forms a nodule and presents as a creeping eruption [3]. *Spirometra* spp. tapeworms are found worldwide but human infections with larval and adult worms are most often reported in Asian countries, typically China, Republic of Korea (Korea), Japan and Thailand [4-6]. Until now, nearly 64 nominal species have been described in the genus *Spirometra, but only 4 are considered valid* [7-9]. The life

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cycle of *Spirometra* species have been studied and their genus name were validated by several researchers. Joyeux and Houdemer (1928) infected dogs with spargana obtained from various animals in India and recovered specimens, naming them *Diphyllobothrium mansoni* [10]. Faust et al. (1929) infected dogs and cats with spargana detected in Chinese hedgehogs (*Erinaceus dealbatus*) and named the recovered specimens as *Diphyllobothrium erinacei* [11]. On the other hand, Iwata (1934) referred to the species distributed in the Far East as *D. erinacei*, Joyeux et al. (1934) named those found in Southeast Asia as *D. mansoni* [12], Mueller (1935) named those distributed in North America as *Diphyllobothrium mansonoides*, and Brumpt (1936) listed those found in Europe as *D. erinaceieuropaei* [13, 14]. In Korea, Lee et al. (1990) conducted laboratory experiments to complete the life cycle of *Spirometra* species distributed in Korea and based on their biological and morphological characteristics, they named the species distributed in Korea as *S. erinacei* [15].

Adult worms of *Spirometra* species is traditionally identified by examination of the reproductive system, especially the number and shape of uterine coils, but it is not enough to distinguish some of *Spirometra* based on morphological characteristics only. The word "sparganum" is a generic name for a plerocercoid, a tissue-migrating larva of tapeworms of the *Diphyllobothrium* and *Spirometra* species, when their adult worm is unknown [4]. The recent progress in genetic evidence by DNA barcoding methods, along with the analysis of morphological features, allows for the support of the distinctiveness of *Spirometra* species [9]. Recently, mitochondrial genome sequences from sparganа have been extensively reported and mitochondrial genome sequences of *Spirometra erinaceieuropaei, Spirometra decipiens* and *Spirometra ranarum* have been addressed [16-18]. Some researchers reported that *S. decipiens, S. ranarum* and *S. mansoni* might be regarded as synonyms of *S. erinaceieuropaei*, however this remains to be verified also [19]. Herein, we investigate molecular characteristics based on mitochondrial cytochrome c oxidase subunit 1 (*CO1*) and NADH dehydrogenase subunit 1 (*ND 1*) gene sequences among several spargana specimens collected from several Asian countries and USA.

A total of 11 samples, including 10 spargana and 1 adult *Spirometra*, were collected in the naturally infected snakes and frogs, and experimentally infected tadpoles, mice and cat between 2002 to 2019 (Supplemental Table 1). All specimens were kept in -80℃ deepfreezer or were preserved in 70% ethanol for experimental use. Total genomic DNA was extracted from each samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol, and used as a template for PCR. The two kind of primer sets were used; Spi-CO1F (5′- GACTAAGTGTTTTCAAAACACTAAGTG -3′) and Spi-CO1R (5′- CACCCTACCCCTGATTTACAAAAT -3′) for *CO*1 gene, and Spi-ND1F (5´- GGAGAATATTGGTTTGTCTAACCA -3´) and Spi-ND1R (5´- CCTTCTTAACGTTAACAGCATTACGAT -3´) for *ND1* gene [16]. The PCR products were sent to Macrogen (Seoul, Korea) for direct sequencing using the PCR primers listed above. The chromatograms of both sequences were trimmed manually and assembled using SeqMan software (DNASTAR, Madison, Wisconsin, USA). The assembled sequences were compared with *Spirometra* species sequences deposited in GenBank using blast searches. The phylogenetic relationships among the sequences were inferred using neighbor-joining (NJ) analysis with Geneious software (11.1.3) based on Tamura-Nei genetic distance model. Bootstrap analysis was performed with 1,000 replication. The *CO1* sequences of the *Spirometra* samples were compared with the reference *CO1* sequences of *S. mansoni, S. decipiens*, *S. erinaceieuropaei*, and *S. ranarum* deposited in

Table 1: Matched base pairs (%) of the *CO1* and *ND1* sequences in 11 *Spirometra* samples obtained from Asia and USA in this study.

Sample	Identity to the *CO1* sequences of various *Spirometra* species (Accession no./Country)				Identity to the *ND1* sequences of various *Spirometra* species (Accession no./Country)			Final ID[a]	Accession No.
	S. mansoni (LC328896/ Japan)	*S. decipiens* (MW031094/ Korea)	*S. erinaceieuropaei* (KX528074/ China)	*S. ranarum* (MH298843/ Myanmar)	*S. decipiens* (MT274581/ Korea)	*S. erinaceieuropaei* (MN432159/ China)	*S. ranarum* (MH298844/ Myanmar)		
SP0301	1520/1521 (99.9%)	1519/1521 (99.8%)	1515/1521 (99.6%)	-	878/878 (100%)	623/636 (97.9%)	860/878 (97.9%)	*S. mansoni*	OR028864
SP0501	1530/1531 (99.9%)	1529/1531 (99.8%)	1525/1531 (99.6%)	-	891/891 (100%)	623/636 (97.9%)	873/891 (97.9%)	*S. mansoni*	OR028865
SP1801	1536/1537 (99.9%)	1535/1537 (99.8%)	1531/1537 (99.6%)	-	891/891 (100%)	623/636 (97.9%)	873/891 (97.9%)	*S. mansoni*	OR028866
SP0201	1510/1523 (99.1%)	1509/1523 (99.0%)	1506/1523 (98.8%)	-	891/891 (100%)	623/636 (97.9%)	873/891 (97.9%)	*S. mansoni*	OR028867
SP0401	1530/1531 (99.9%)	1529/1531 (99.8%)	1525/1531 (99.6%)	-	891/891 (100%)	623/636 (97.9%)	873/891 (97.9%)	*S. mansoni*	OR028868
SP0202	1515/1523 (99.4%)	1514/1523 (99.4%)	1510/1523 (99.1%)	-	886/886 (100%)	623/636 (97.9%)	868/886 (97.9%)	*S. mansoni*	OR028869
SP0203	1554/1555 (99.9%)	1553/1555 (99.8%)	1549/1555 (99.6%)	-	891/891 (100%)	623/636 (97.9%)	873/891 (97.9%)	*S. mansoni*	OR028870
SP1802	1514/1530 (98.9%)	1513/1530 (98.8%)	1509/1530 (98.6%)	1497/1530 (97.8%)	-	-	-	*Spirometra* species	OR026041
SP1803	1536/1564 (98.2%)	1535/1564 (98.1%)	1531/1564 (97.8%)	1537/1564 (98.2%)	868/886 (97.9%)	628/636 (98.7%)	886/886 (100%)	*Spirometra* species	OR026042
SP1804	1503/1532 (98.1%)	1502/1532 (98.0%)	1498/1532 (97.7%)	1499/1532 (97.8%)	891/891 (100%)	623/636 (97.9%)	858/876 (97.9%)	*Spirometra* species	OR026043
SP1901	1493/1536 (97.2%)	1492/1536 (97.1%)	1489/1536 (96.9%)	1515/1536 (98.6%)	858/873 (98.2%)	629/636 (98.9%)	860/873 (98.5%)	*Spirometra* species	OR026044

[a]Identification.

GenBank (accession number LC328896, MW031094, KX528074, MH298843) summarized in Table 1. The *CO1* sequences obtained from 3 Korean isolates (SP0301, SP0501, SP1801) of *Spirometra* species ranging 1,521-1,537 bp showed 99.9% identity with *S. mansoni* and 99.8% identity with *S. decipiens*, but 99.6% identity with *S. erinaceieuropaei*. The *CO1* sequences obtained from 2 Chinese samples (SP0201, SP0401) ranging 1,523-1,531 bp showed 99.1-99.9% identity with *S. mansoni* and 99.0-99.8% identity with *S. decipiens*, but 98.8%-99.6% identity with *S. erinaceieuropaei*. The *CO1* sequences of 2 samples (SP0202, SP0203) obtained from USA ranging 1,523-1,555 bp showed 99.4-99.9% identity with *S. mansoni* and 99.4-99.8% identity with *S. decipiens*, but 98.1%-99.6% identity with *S. erinaceieuropaei*. Overall, above 7 *Spirometra* species samples from Korea, China and USA were presumptively designated into *S. mansoni,* although they were hardly differentiated to *S. decipiens* with the only one base pair-difference. The *CO1* sequences obtained from 3 Myanmar samples (SP1802, SP1803, SP1804) ranging 1,503-1,564 bp showed 98.1-98.9% identity with *S. mansoni* and 98.0-98.8% identity with *S. decipiens*, 97.7%-98.6% identity with *S. erinaceieuropaei*, and 97.8-98.2% identity with *S. ranarum*, respectively. A Cambodian sample (SP1901) with 1,536 bp showed 98.6% identity with *S. ranarum,* but 97.2% identity with *S. mansoni*, 97.1% identity with *S. decipiens*, and 96.9% identity with *S. erinaceieuropaei*, respectively. Overall, the exact species level could not be designated to above 4 samples from Myanmar and Cambodia by *CO1* sequences, suggesting that they were supposed to be new *Spirometra* species. The *ND1* sequences of the 11 samples of *Spirometra* species were compared with the reference *ND1* sequences of *S. decipiens*, *S. erinaceieuropaei*, and *S. ranarum* which were deposited in GenBank (accession number MW031094, MN432159 and MH298844). We could not compare with the *ND1* sequences of *S. mansoni,* due to lacking enrolled *ND1* sequences of *S. mansoni* deposited in GenBank yet. The *ND1* sequences of 8 samples from Korea (n=3), China (n=2), USA (n=2), and Myanmar (n=1) showed 100% identity with *S. decipiens*, but 97.7% identity with *S. erinaceieuropaei* or *S. ranarum*. One *Spirometra* sample from Myanmar (SP1803) showed 100% identity with *S. ranarum*, but 97.9% identity with *S. decipiens* and 98.7% identity with *S. erinaceieuropaei*. ACambodian sample (SP1901) showed 98.2%, 98.9%, and 98.5% identities with *S. decipiens*, *S. erinaceieuropaei*, and *S. ranarum*, respectively. The phylogenic tree showed that 7 *Spirometra* samples from Korea, China and USA presumptively designated into *S. mansoni* were co-clustered without the exact differentiation among *S. mansoni, S. decipiens*, and *S. erinaceieuropaei* (Figure 1). Three Myanmar samples were relatively close to each other, but quite different to a Cambodian *Spirometra* species. We found that several *CO1* sequences of *S. decipiens*, *S. erinaceieuropaei* deposited in GenBank were quiet similar and they could not well differentiated each other.

Yamasaki et al. performed haplotype analysis based on mitochondrial *CO1* gene sequences and noticed two distinct *Spirometra* species, Type I and Type II, were present in Asia and neither of which is close to likely European species, *S. erinaceieuropaei* [3]. In addition, they have pointed out that 2 species, *S. decipiens* and *S. ranarum* reported in Asia were conspecific with Type I, and Type I was probably conspecific with *S. mansoni*, and undescribed Type II species. Similar with this notion, our study also showed that all *Spirometra* species collected from Korea, China, and USA were the mostly close to *S. mansoni.* Furthermore, 3 Myanmar and a Cambodian *Spirometra* species may be rather new ones, relatively close to *S. ranarum*. In spite of that, we could not completely determine their species names without proper matching results at Genbank database. We found that several *CO1* sequences of *S. mansoni, S. decipiens*, *S. erinaceieuropaei* deposited in GenBank were quite similar and they could not well differentiate each other. Despite notable attempts to clarify the taxonomy, host specificity, and geographical distribution of *Spirometra*, it remains still obscure. Collectively, our data showed that it is not easy to differentiate between *S. mansoni, S. decipiens*, and *S. erinaceieuropaei* even with *CO1* sequences and the expansion of *ND1* sequences may be further needed for the species identification of *Spirometra* species tapeworms.

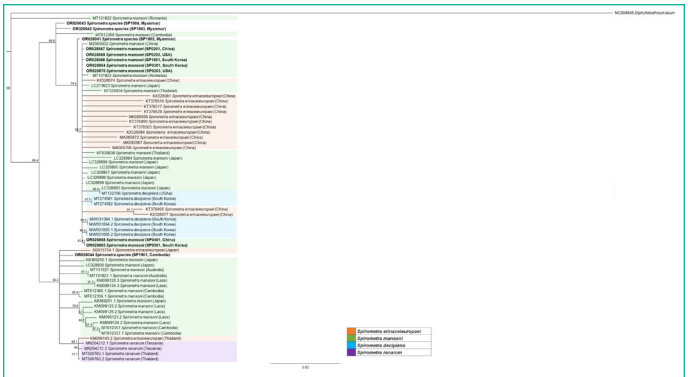

Figure 1: Phylogenic tree constructed based on *CO1* sequences from *Spirometra* samples collected in this study and several *Spirometra* species from sequences deposited in GenBank. The bootstrap values for the clade with consensus support (%) were displayed on the tree to the left of the most recent common ancestral node for that clade. The scale bar at the bottom of the tree indicated the length of the branches of the tree.

Molecular identification has played an important role in improving understanding of phylogenetic relationships, genetic variation and taxonomy. With uncertainty in morphology, however, unlabeled or mislabeled molecular data from several cases of sparganosis leaded to many misidentifications and further confusion [8]. It may be not easy to completely differentiate them even with molecular evidence. Several factors such as the quality and quantity (reading length) of sequences, the quality of database, and mitochondrial DNA sequence variation of *Spirometra* species which might be existed even in the same *Spirometra* spp. should be considered in their interpretation. Previously, partial mitochondrial DNA sequence variations of *S. erinaceieuropaei* isolates from China, Japan and Indonesia ranged from 0.0-8.4% for *CO1* [20]. According to the latest taxonomy, members of *Spirometra* have been classified into at least 6 separate species, *S. erinaceieuropaei*, *S. folium*, *S. mansoni*, *Spirometra* sp. 1, and the tentative assignment of *S. decipiens* complex 1 and 2 [9]. This work described the molecular characteristics based on *CO1* and *ND 1* gene sequences among several spargana specimens collected from several Asian countries and USA. Our data support above taxonomy with notion that several Korean isolates previously reported as "*S. decipiens*" might be rather conspecific with *S. mansoni.* It should be cautious to read *CO1* sequences of *Spirometra* spp. considering that *S. mansoni, S. decipiens*, *S. erinaceieuropaei* deposited in GenBank might be quite similar, harboring less discriminative power.

Author Statements

Declaration of Competing Interest

The authors declare no conflicts of interest related to this study.

Data Availability

No data was used for the research described in the article.

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Author Contributions

Conceptualization: Sohn WM, Won EJ; Data curation: Lee YJ; Formal analysis: Lee YJ; Funding acquisition: Won EJ; Investigation: Lee YJ, Won EJ; Methodology: Lee YJ, Won EJ; Project administration: Won EJ; Supervision: Sohn WM; Writing – original draft: Lee YJ, Won EJ; Writing – review & editing: Lee YJ, Won EJ, Sohn WM.

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