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Original Article

Phylogenetic Location of the *Spirometra* Sparganum Isolates from China, Based on Sequences of 28S rDNA D1

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Abstract

Background: The phylogenetic location of Chinese *Spirometra* sparganum isolates remains unclear. The aim of this study was to explore the phylogenetic location of the *Spirometra* sparganum isolates from China.

Methods: The 28S ribosomal DNA (rDNA) D1 sequences of 14 *Spirometra* sparganum isolates collected from thirteen locations in China were analyzed by using Neighbor-Joining (NJ), maximum parsimony (MP) and Bayesian inference (BI), respectively. To investigate the deep variance of 28S rDNA D1 region among included species, the secondary structure of 28S rDNA D1 region was also calculated using the program RNA structure.

Results: The genus *Spirometra* as a monophyletic group was evidenced by two inference methods (MP and BI). All sequences within the genus *Spirometra* had a bulge of a cytosine residue (Bulge C) in the stem 13 of the secondary structure model of 28S rRNA D1 region. Varietal sites in sequences from all thirteen Chinese isolates were appeared in loops. In loops, adenine was the most abundant base (averagely 41.9%) followed by guanine (averagely 30.0%), and cytosine (averagely 15.1%). In stems, the average percentage of G + C (58.3%) was higher than the percentage of A + T (41.7%).

Conclusion: The 'Bulge C' in the stem 13 of the 28S rDNA D1 secondary structure could be as a suitable mark to identify the *Spirometra* species.

Introduction

Tapeworms of the genus *Spirometra* belong to the class Cestoda, order Pseudophyllidea, family Diphyllbothriidae. The plerocercoid larvae (spargana)

of *Spirometra* can lodge in the subcutaneous tissues and sometimes invades the abdominal cavity, eye, and central nervous system of human causing a serious parasitic zoonosis:

sparganosis; human infection results mainly from ingesting raw flesh of frogs and snakes infected with plerocercoids, drinking raw water contaminated with cyclops harboring pro-cercoids, or placing frog or snake flesh on open wound for treatment of skin ulcers or eye inflammations (1-6). Sparganosis poses a serious threat to human health and also cause significant economic losses (7). Phylogenetic studies to such an important parasite group is therefore of great significance to the prevention and control of human sparganosis as well as to understand the genetic structure of parasites. However, the knowledge about the phylogenetic position of the genus *Spirometra*, and its affiliation to other Diphylobothriid species (such as the genera *Diphylobothrium*, *Digramma*, *Duthiersia* and *Schistocephalus*) are still fragmentary. Therefore, there is a pressing requirement to investigate the phylogenetic relationships of *Spirometra*, *Diphylobothrium* and other important species within the family Diphylobothriidae.

The nuclear rDNA gene repeat unit harbors different regions that evolve at varying rates, thus adds useful and often significant resolution to molecular systematic estimates of phylogeny at a number of different taxonomic levels (8, 9). The large subunit RNA gene (18S rDNA or 28S rDNA) has been extensively utilized in estimation of the relationships existing within and among the Cestoda (9-12). In the phylogenetic study, the secondary structures of the transcribed rRNA are more conserved than the primary sequences due to the compensatory or semi-compensatory mutations, and some changes of a certain helix could be specific to a taxon to help a lot in species identification (13-15).

So, the secondary structures have drawn lots of attention from phylogenetic scientists (15-17). However, until now, few researchers have been concentrated their studies on the phylogeny of *Spirometra* with the 28S rDNA sequences, even more considered the secondary structures.

The main aim of this study was to explore the phylogenetic location of the *Spirometra* sparganum isolates from China based on the primary and corresponding secondary structures of partial 28S rDNA D1 sequences. In addition, the relationships of species among *Spirometra*, *Diphylobothrium* and other important genera within the family Diphylobothriidae were established using the molecular data obtained.

Materials and Methods

Taxon selection and sampling

The plerocercoids (spargana) of *Spirometra* were collected from subcutaneous tissue and muscles of the naturally infected wild frogs (*Rana nigromaculata*, *R. rugulosa*, *R. temporaria*, *R. limmochari*) and snakes (*Enhydria chinensis*) at thirteen locations of China (Table 1). Spargana dissected from frogs and snakes were wrinkled, whitish, and ribbon-shaped worms, which continuously crept in normal saline.

These spargana were 1–13 cm long and 1–2.5 mm wide. To study the phylogenetic relationships among diphylobothroid cestodes, other members of the genera *Spirometra*, *Digramma*, *Diphylobothrium*, *Duthiersia* and *Schistocephalus* within the family Diphylobothriidae were considered in the present study (Table 1), with two species of the family Taeniidae (*Taenia saginata* AF096224 and *T. taeniaeformis* AF004721) as out-group to root the resulting trees.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from individual plerocercoid sample using the Tiangen DNeasy Blood and Tissue Kit (Tiangen, China) following the manufacturer's protocol. The 28S rDNA D1 region was amplified by PCR using the primer combination of Lee et al. 2007 (9): forward primer (JB10, 5'-GATTACCCGCTGAACTTAAGCATA-3') and reverse primer (JB9, 5'-GCTGCATTCACAAACACCCCGACTC-3').

Table 1: Geographical origins (different locations in China) of *Spirometra* sparganum isolates and related taxa of the family Diphyllbothriidae used in this study, as well as their GenBank accession numbers for sequences of 28S rDNA D1 region. Asterisks indicate sequences newly reported in this study (N/a=Not available)

Genus	Species	Sample codes	Location	Host	GenBank No.
<i>Spirometra</i>	<i>S. erinaceieuropaei</i>	GX-NN1	Nanning, Guangxi	<i>Rana rugulosa</i>	KF874629*
	<i>S. erinaceieuropaei</i>	GX-NN2	Nanning, Guangxi	<i>R. rugulosa</i>	KF874630*
	<i>S. erinaceieuropaei</i>	GX-NN-Yn1	Yongning, Guangxi	<i>Enhydryis chinensis</i>	KF874631*
	<i>S. erinaceieuropaei</i>	GX-NN-Yn2	Yongning, Guangxi	<i>E. chinensis</i>	KF874632*
	<i>S. erinaceieuropaei</i>	GX-NN-Xxt1	Xixiangtang, Guangxi	<i>E. chinensis</i>	KF874633*
	<i>S. erinaceieuropaei</i>	GX-NN-Xxt2	Xixiangtang, Guangxi	<i>E. chinensis</i>	KF874634*
	<i>S. erinaceieuropaei</i>	GX-NN-Xxt3	Xixiangtang, Guangxi	<i>R. temporaria</i>	KF874635*
	<i>S. erinaceieuropaei</i>	GX-NN-Xxt4	Xixiangtang, Guangxi	<i>R. temporaria</i>	KF874636*
	<i>S. erinaceieuropaei</i>	GX-YL-Lc1	Luchuan, Guangxi	<i>R. rugulosa</i>	KF874637*
	<i>S. erinaceieuropaei</i>	GX-YL-Lc2	Luchuan, Guangxi	<i>R. rugulosa</i>	KF874638*
	<i>S. erinaceieuropaei</i>	GX-GL1	Guilin, Guangxi	<i>R. rugulosa</i>	KF874639*
	<i>S. erinaceieuropaei</i>	GX-GL2	Guilin, Guangxi	<i>R. rugulosa</i>	KF874640*
	<i>S. erinaceieuropaei</i>	GX-LG-Lt1	Lingui, Guangxi	<i>R. rugulosa</i>	KF874641*
	<i>S. erinaceieuropaei</i>	GX-LG-Lt2	Lingui, Guangxi	<i>R. rugulosa</i>	KF874642*
	<i>S. erinaceieuropaei</i>	GX-LG-Wt1	Lingui, Guangxi	<i>R. temporaria</i>	KF874643*
	<i>S. erinaceieuropaei</i>	GX-LG-Wt2	Lingui, Guangxi	<i>R. temporaria</i>	KF874644*
	<i>S. erinaceieuropaei</i>	HeN-KF1	Kaifeng, Henan	<i>R. temporaria</i>	KF874645*
	<i>S. erinaceieuropaei</i>	HeN-KF2	Kaifeng, Henan	<i>R. temporaria</i>	KF874646*
	<i>S. erinaceieuropaei</i>	HeN-KF-Tx1	Tongxu, Henan	<i>R. limnochari</i>	KF874647*
	<i>S. erinaceieuropaei</i>	HeN-KF-Tx2	Tongxu, Henan	<i>R. limnochari</i>	KF874648*
	<i>S. erinaceieuropaei</i>	HeN-LH1	Luohe, Henan	<i>R. limnochari</i>	KF874649*
	<i>S. erinaceieuropaei</i>	HeN-LH2	Luohe, Henan	<i>R. limnochari</i>	KF874650*
	<i>S. erinaceieuropaei</i>	HeN-XX1	Xinxiang, Henan	<i>R. limnochari</i>	KF874651*
	<i>S. erinaceieuropaei</i>	HeN-XX2	Xinxiang, Henan	<i>R. limnochari</i>	KF874652*
	<i>S. erinaceieuropaei</i>	HeN-ZK-Fg1	Fugou, Henan	<i>R. limnochari</i>	KF874653*
	<i>S. erinaceieuropaei</i>	HeN-ZK-Fg2	Fugou, Henan	<i>R. limnochari</i>	KF874654*
	<i>S. erinaceieuropaei</i>	HeN-ZZ1	Zhengzhou, Henan	<i>R. nigromaculata</i>	KF874655*
	<i>S. erinaceieuropaei</i>	HeN-ZZ2	Zhengzhou, Henan	<i>R. nigromaculata</i>	KF874656*
	<i>S. erinaceieuropaei</i>	N/a	N/a	N/a	AF004717
	<i>S. mansonioides</i>	N/a	N/a	N/a	AF004718
<i>Diphyllbothrium</i>	<i>D. latum</i>	N/a	N/a	N/a	AF004719
	<i>D. latum</i>	N/a	Russia	<i>Gymnocephalus cer-nuus</i>	DQ925326
	<i>D. latum</i>	N/a	Finland	N/a	AB302387
	<i>D. nibonkaiense</i>	N/a	Japan	N/a	AB302388
	<i>D. pacificum</i>	N/a	Peru	<i>Homo sapiens</i>	DQ925327
	<i>D. stemmacephalum</i>	N/a	USA	<i>Lagenorhynchus acutus</i>	AF286943
<i>Digramma</i>	<i>D. interrupta</i>	N/a	Russia	<i>Hemiculter lucidus</i>	DQ925325
<i>Duthiersia</i>	<i>D. fimbriata</i>	N/a	Ghana	<i>Varanus exanthematicus</i>	DQ925328
<i>Schistocephalus</i>	<i>S. solidus</i>	N/a	USA	<i>Gasterosteus aculatus</i>	AF286944

Polymerase chain reactions (PCRs) (25µl) were performed in 2mM MgCl₂, 2.5µM of each

primer, 2.5µl 10× rTaq buffer, 0.5mM of each deoxyribonucleoside triphosphate (dNTP),

1.25U of *rTaq* DNA polymerase (Takara, China), and 1 μ l of DNA (5–10 ng) sample. The amplification profile for 28S rDNA D1 region consisted of an initial denaturation for 2 min at 94 °C, 40 cycles of: 20 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C; followed by a 6 min final extension at 72 °C. PCR products were purified using the High Pure PCR Product Purification Kit (Takara, China) and sequenced in both directions using an automated sequencer (ABI Prism 3730 XL DNA Analyzer; ABI Prism, Foster City, CA) at the Genwiz Company (Beijing, China).

Sequence analysis

The 28S rDNA D1 sequences were aligned using the computer program Clustal X 2.0 (18). Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (19) was employed to analyze the nucleotide composition, conserved sites, variable sites, parsim-info sites and singleton sites, as well as to compute uncorrected pairwise divergence. The program DAMBE 5.0 (20) was used to measure the nucleotide substitution saturation using the method of Xia et al., 2003 (21) as the substitution saturation masked the phylogenetic signal.

Phylogenetic analysis

The aligned 28S rDNA D1 sequences were analyzed by using three methods of Neighbor-Joining (NJ), maximum parsimony (MP) and Bayesian inference (BI), respectively. NJ analysis was performed in PAUP*4b10 (22) using the Kimura two-parameter distance selected by Modeltest 3.7 (23) under the Akaike information criterion and the “heuristics” search option with the “simple” addition sequence and TBR (tree bisection-reconnection) swapping. MP analysis was also performed in PAUP*4b10 (22) using heuristic searches with TBR branch swapping and 2,000 random addition sequences. Confidence in each node was assessed by bootstrapping (2000 pseudo-replicates, heuristic search of 20 random addition replicates with TBR option). BI analyses were performed in MrBayes v3.1 (24) with

5,000,000 generations, sampling trees every 100 generations. Stationarity was assessed using a convergence diagnostic. An average standard deviation of the split frequencies (ASDSF) < 0.03 were used as criteria of convergence between both runs.

Predication of secondary structure

The thermodynamic secondary structure of 28S rDNA D1 region was calculated using the program RNA structure 5.6 (25). We recognized standard Watson-Crick base pairs and noncanonical G: U interactions in all putative stem positions, because G : U can replace a Watson-Crick pair to maintain a helical region and may even have a special function in some instances (26). Information on compensatory and/or semi-compensatory base substitutions was also used to confirm local secondary structures. The RNAviz 2.0 (27) was used to produce the figures of the structures.

Results

The 28S rDNA D1 fragments of the *Spirometra sparganum* isolates from thirteen locations of China were successfully amplified, and sequenced to be 298 bp in eleven isolates (isolates from Luchuan, Yongning, Xixiangtang, Nanning, Guilin, Liutang and Wutong of Guangxi Zhuang Autonomous Region; and Kaifeng, Tongxu, Xinxiang and Zhengzhou of Henan province), 297 bp in two Henan isolates from Luohe and Fugou. The alignment of the fourteen 28S rDNA D1 sequences obtained in the present study and the eleven sequences of the family Diphylobothriidae obtained from the GenBank resulted in a total of 302 characters including gaps. The MEGA analysis presented 97.32% (290/298) sequence identity, 8 variable sites, and 2 singletons in the fourteen sparganum isolates from China. The average contents of A, C, G and T in these sparganum isolates were 24.8, 22.8, 31.6, and 20.8%, respectively. The nucleotide frequencies were a little biased toward G + C, averaging 54.4 %. The genetic divergence of

28S rDNA D1 sequences of species within the family Diphylobothriidae ranged from 0 to 6.1%. However, when only took account of Chinese isolates, the upper limits would decline to 0.4%.

The test of substitution saturation showed that the observed index of substitution saturation (*I_{ss}*) for 28S rDNA D1-alignments was 0.0461, and the corresponding critical index substitution saturation (*I_{ss.c}*) was 0.6149 for a symmetrical tree and 0.3805 for an extreme asymmetrical tree. The index of *I_{ss}* was significantly lower than the corresponding *I_{ss.c}* (*p* = 0.0000), indicating that there was little saturation in our sequences. The most suitable substitution model was K2 for 28S rDNA D1 region following model selection by Modeltest.

In order to explore the phylogenetic location of the *Spirometra* sparganum isolates from China and the relationships of important spe-

cies within the family Diphylobothriidae, phylogenetic trees were reconstructed based on partial 28S rDNA D1 sequences under NJ, MP, BI three inference methods, respectively (Fig. 1). As shown in Fig. 1, the monophyly of the family Diphylobothriidae was supported by all three methods with high support values (100/100/1). Within Diphylobothriidae, the genus *Duthiersia* was in the basal of the family, the genera *Schistocephalus*, *Digramma*, *Diphylobothrium* and *Spirometra* made up a monophyletic group (60/69/0.72). The clade including all isolates from China and two species (*Spirometra erinaceieuropaei* AF004717 and *Spirometra mansonioides* AF004718) obtained from the GenBank was supported by MP and BI methods (69/0.99). The genera *Schistocephalus*, *Digramma* and *Diphylobothrium* were recovered as a single clade but with very weak support.

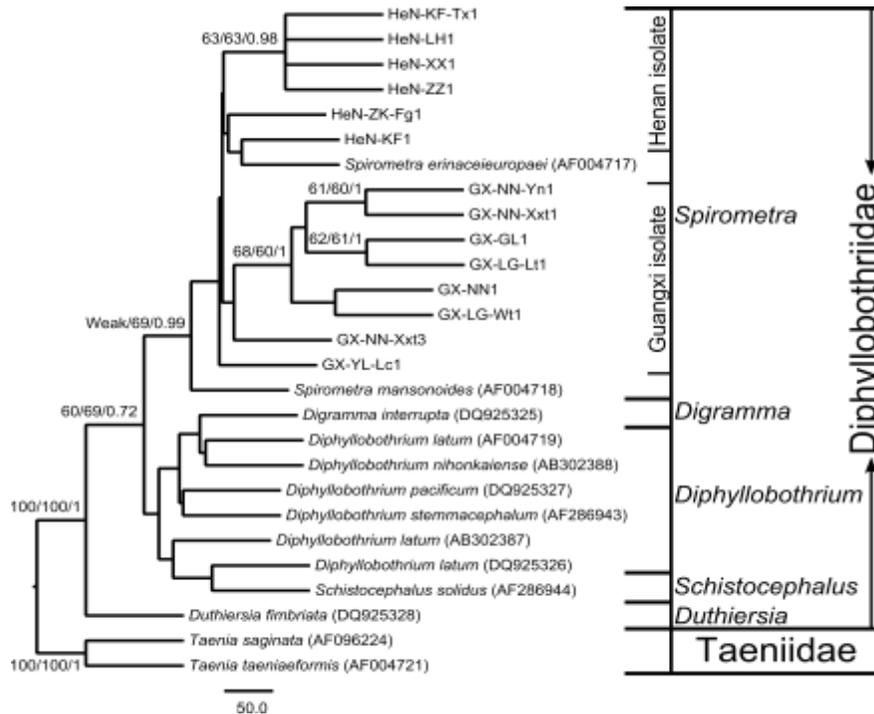


Fig. 1: Phylogenetic relationship among the examined *Spirometra erinaceieuropaei* sparganum isolates from China and other Diphylobothriid species inferred by Neighbor-Joining (NJ), maximum parsimony (MP) and Bayesian inference (BI) analyses based on 28S rRNA D1 sequences. The numbers along branches indicate bootstrap values and posterior probabilities resulting from different analyses in the order: NJ/MP/BI. The bootstrap values lower than 60 and the posterior probabilities lower than 0.6 are given as ‘Weak’

Our core secondary structure model of 28S rRNA D1 region based on the *Spirometra* isolate from Nanning of China is shown in Fig. 2. We recognized totally fifteen stems, which were numbered 1–15. Positions within stems were indicated by numbers after dashes: 1-1 indicates the first [5'-side] base in stem 1, paired with its complement. Two of the fifteen stems were supported by positional covariance among the 25 Diphyllbothriid sequences included in this analysis. One was position 9-3 in stem 9 of *Diphyllbothrium nihonkaiense*, *D. pacificum*, *D. stemmacephalum* and *Digramma interrupta*, respectively; the other was position 10-4 in stem 10 of *Duthiersia fimbriata* and *Spirometra mansonoides* (Fig. 3). All sequences within the genus *Spirometra* had a

bulge of a cytosine residue (Bulge C in Fig. 2) in the stem 13, but the bulge structure was absent in the genera *Diphyllbothrium*, *Digramma*, *Duthiersia* and *Schistocephalus* (Fig. 3). Total and varietal sites, and nucleotide percentages for Diphyllbothriid 28S rRNA D1 stems and loops are given in Table 2. Varietal sites in sequences from all Chinese isolates were appeared in loops. However, these sites were more likely to reveal in stems of *Diphyllbothrium*, *Digramma*, *Duthiersia* and *Schistocephalus*. In loops, adenine is the most abundant base (averagely 41.9%) followed by guanine (averagely 30.0%), and cytosine (averagely 15.1%). In stems, the average percentage of G + C (58.3%) was higher than the percentage of A + T (41.7%).

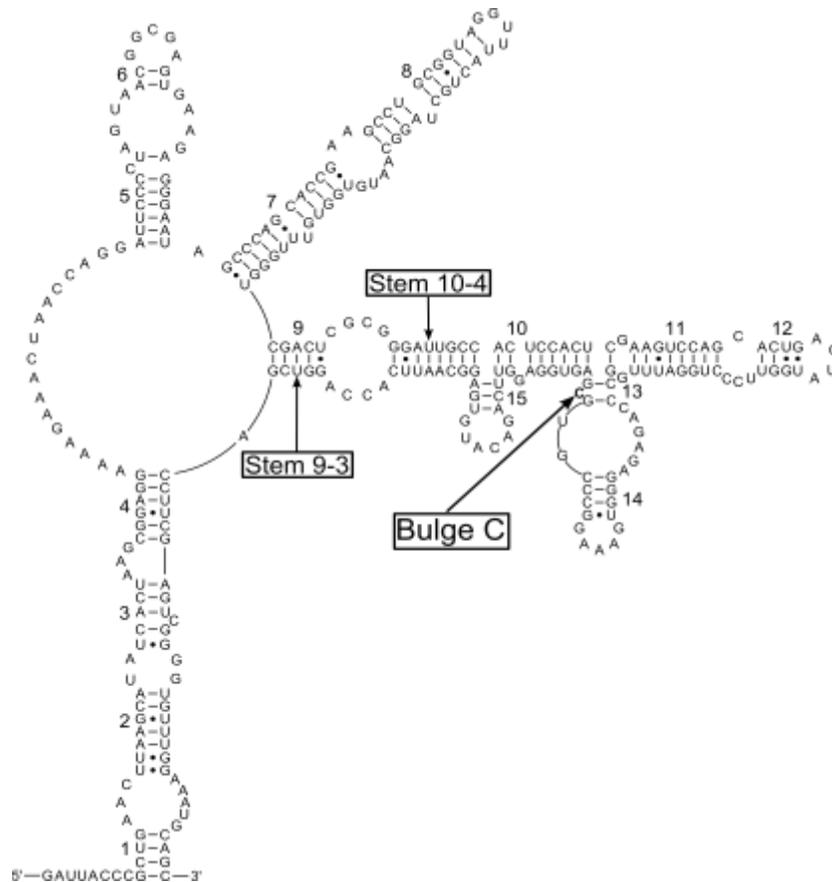


Fig. 2: A Core secondary structure model for the Diphyllbothriid 28S rRNA D1 region illustrated using a *Spirometra erinacei europaei* sparganum isolate from Nanning of China (GenBank Accession No. KF874629). Base pairing is indicated as follows: standard canonical pairs by lines (G–C, A–U), wobble G:U pairs by dots (G•U)

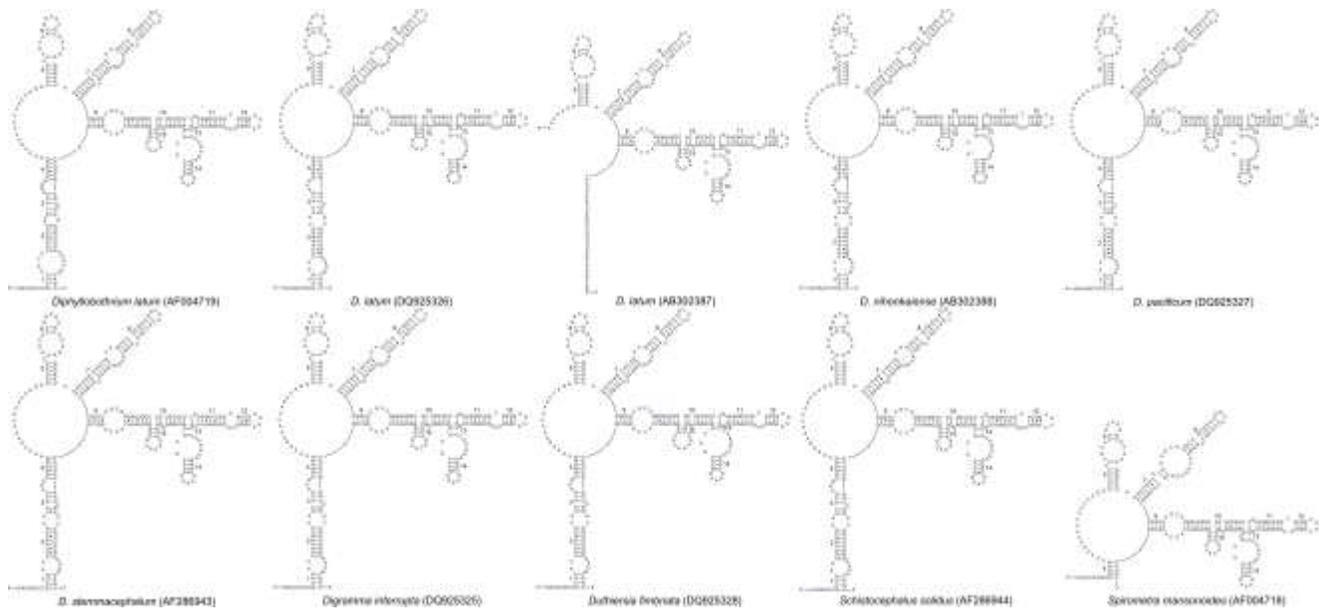


Fig. 3: Secondary structure models for the 28S rRNA D1 region of species within genera *Diphyllobothrium*, *Digamma*, *Duthiersia*, *Schistocephalus* and *Spirometra mansonoides*. Base pairing is indicated as follows: standard canonical pairs by lines (G–C, A–U), wobble G:U pairs by dots (G•U)

Table 2: Molecular properties of Diphyllobothriid 28S rRNA D1 stems and loops

Sequences	Length (bp)	Total sites		Varietal sites		Loops				Stems			
		Loop	Stem	Loop	Stem	%A	%C	%G	%T	%A	%C	%G	%T
<i>Spirometra</i>													
GX-NN1	298	91	190	0	0	42.9	15.4	29.7	12.0	16.8	24.7	33.2	25.3
GX-NN-Yn1	298	91	190	1	0	41.8	15.4	30.8	12.0	16.8	24.7	33.2	25.3
GX-NN-Xxt1	298	91	190	1	0	41.8	15.4	30.8	12.0	16.8	24.7	33.2	25.3
GX-NN-Xxt3	298	91	190	1	0	41.8	16.5	29.7	12.0	16.8	24.7	33.2	25.3
GX-YL-Lc1	298	91	190	2	0	42.9	15.4	30.8	10.9	16.8	24.7	33.2	25.3
GX-GL1	298	91	190	2	0	41.8	14.3	30.8	13.1	16.8	24.7	33.2	25.3
GX-LG-Lt1	298	91	190	3	0	40.7	14.3	30.8	14.2	16.8	24.7	33.2	25.3
GX-LG-Wt1	298	91	190	1	0	42.9	14.3	29.7	13.1	16.8	24.7	33.2	25.3
HeN-KF1	298	90	190	1	0	42.2	15.6	30.0	12.2	16.8	24.7	33.2	25.3
HeN-KF-Tx1	298	91	190	2	0	40.7	15.4	31.9	12.0	16.8	24.7	33.2	25.3
HeN-LH1	297	90	190	2	0	41.1	15.6	31.1	12.2	16.8	24.7	33.2	25.3
HeN-XX1	298	91	190	2	0	40.7	15.4	31.9	12.0	16.8	24.7	33.2	25.3
HeN-ZK-Fg1	297	90	190	1	0	42.2	15.6	30.0	12.2	16.8	24.7	33.2	25.3
HeN-ZZ1	298	91	190	3	0	40.7	15.4	30.8	13.1	16.8	24.7	33.2	25.3
<i>S. erinaceiropaei</i>	302	92	192	1	2	42.4	15.2	29.3	13.1	17.2	24.5	32.8	25.5
<i>S. mansonoides</i>	251	84	152	3	4	39.3	14.3	34.5	11.9	17.1	25.7	32.9	24.3
<i>Diphyllobothrium</i>													
<i>D. latum</i> (AF004719)	299	93	188	3	0	40.9	15.1	30.1	13.9	17.0	25.0	33.0	25.0
<i>D. latum</i> (DQ925326)	297	89	192	2	3	42.7	14.6	28.1	14.6	16.1	25.0	33.9	25.0
<i>D. latum</i> (AB302387)	249	69	146	1	1	37.7	17.4	31.9	13.0	16.4	26.0	33.6	24.0
<i>D. nibonkaiense</i>	297	89	192	2	4	43.8	14.6	28.1	13.5	16.1	25.0	33.9	25.0
<i>D. pacificum</i>	297	89	192	2	4	43.8	14.6	27.0	14.6	16.1	25.5	33.9	24.5
<i>D. stemmacephalum</i>	297	89	192	2	4	42.7	14.6	28.1	14.6	16.1	25.5	33.9	24.5
<i>Digamma interrupta</i>													
<i>Digamma interrupta</i>	297	89	192	2	4	42.7	14.6	28.1	14.6	16.1	25.0	33.9	25.0
<i>Duthiersia fimbriata</i>													
<i>Duthiersia fimbriata</i>	297	93	188	4	8	45.2	15.1	25.8	13.9	14.4	24.5	35.6	25.5
<i>Schistocephalus solidus</i>													
<i>Schistocephalus solidus</i>	296	89	192	1	3	42.7	14.6	29.2	13.5	16.1	25.0	33.4	25.5
Average	294.0	89.4	187.1	1.8	1.5	41.9	15.1	30.0	13.0	16.6	24.9	33.4	25.1

Discussion

In this study, the phylogenetic position of the *Spirometra* sparganum isolates from China was explored based on the 28S rDNA D1 sequence, and the secondary structure of 28S rDNA D1 region was inferred. The phylogeny reveals that all thirteen Chinese sparganum isolates combined with *Spirometra erinaceiropaei* and *S. mansonioides* species make up a monophyletic group. The secondary structure of 28S rDNA D1 sequences within the genus *Spirometra* (*S. erinaceiropaei* and *S. mansonioides*) has a bulge of a cytosine residue (Bulge C) in the stem 13.

Unlike the nucleotide frequencies of Diphyllbothriid mitochondrial sequences which are more biased toward A + T (28, 29), our analysis of the 28S rDNA D1 region shows that the G + C nucleotide frequencies are higher than A + T, which is consistent with the study of Lee et al. 2007 (9). This may indicate that the Diphyllbothriid nuclear ribosomal DNA sequence is generally GC rich. In the phylogenetic trees, a high posterior probability (0.99) supports the monophyly of the genus *Spirometra*. Inside the clade of *Spirometra*, the species *Spirometra mansonioides* was in the basal, all isolates from China and the species *Spirometra erinaceiropaei* made up a sub-clade. As the species *Spirometra erinaceiropaei* is mainly distributed in Asia, and *S. mansonioides* is the most common in North America (30, 31), the sparganum isolates in China should be represent *S. erinaceiropaei*. The genera *Schistocephalus*, *Digamma* and *Diphyllbothrium* were revealed as a monophyletic group, which is consistent with the conclusion of Brabec et al. 2006 (11) based on both small and large subunit nuclear ribosomal RNA genes. And the close relationship between *Schistocephalus* and *Diphyllbothrium* is also supported by the analysis of Olson et al. 2001 (10). In our analysis, the sister-group relationship between *Spirometra* and *Schistocephalus* + *Digamma* + *Diphyllbothrium* was moderate supported by NJ, MP and BI inference methods, this tree topology indicat-

ed that the two important species groups (*Spirometra* and *Diphyllbothrium*) of the family Diphyllbothriidae might be sibling relationship. Certainly, the confirmed relationship between *Spirometra* and *Diphyllbothrium* should wait for an even more comprehensive phylogenetic study with broader sampling of the more terminal representative taxa and more other effective molecular markers as well as morphological data.

In the secondary structure of 28S rDNA D1 region, a higher percentage of adenine in unpaired regions and higher nucleotide frequencies of G + C are consistent with results of previous studies (32, 33). As insertion or deletion, the multiple sequence alignments sometimes seem less reliable during the phylogenetic research, but the secondary structures could help to make a more reliable assignment of nucleotide homology in this situation (14). What's more, some changes, such as expansions and deletions, of a certain helix could be specific to a taxon to help a lot in species identification (15).

Conclusion

According to the results of phylogenetic analyses, the thirteen sparganum isolates from China represent *S. erinaceiropaei*. The 'Bulge C' in the stem 13 of the 28S rDNA D1 secondary structure could be used as a suitable mark to identify the *Spirometra* species.

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