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Sequence variability in three mitochondrial DNA regions of *Spirometra erinaceieuropaei* spargana of human and animal health significance

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Abstract

Sequence variability in three mitochondrial DNA (mtDNA) regions, namely cytochrome *c* oxidase subunit 3 (*cox*3), NADH dehydrogenase subunits 1 and 4 (*nad*1 and *nad*4) in *Spirometra erinaceieuropaei* spargana from different geographical regions in China was examined. A portion of each of the *cox*3 (p*cox*3), *nad*1 (p*nad*1) and *nad*4 genes (p*nad*4) were amplified separately from individual *S. erinaceieuropaei* spargana by polymerase chain reaction (PCR). Representative amplicons were subjected to sequencing in order to estimate sequence variability. The sequences of p*cox*3, p*nad*1 and p*nad*4 were 541, 607 and 847 bp in length, respectively. The A + T contents of the sequences were 68.39–68.76% (p*cox*3), 63.76–64.91% (p*nad*1) and 67.18–67.77% (p*nad*4), respectively, while the intra-specific sequence variations within each of the *S. erinaceieuropaei* spargana were 0–1.5% for p*cox*3, 0–2.8% for p*nad*1 and 0–2.7% for p*nad*4. Phylogenetic analysis using neighbour joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) methods, indicated that all the spargana isolates in Hunan Province represented *S. erinaceieuropaei*. These findings demonstrated clearly the usefulness of the three mtDNA sequences for population genetics studies of *S. erinaceieuropaei* spargana of human and animal health significance.

Introduction

Sparganosis is an important neglected parasitic disease caused by invasion by spargana, plerocercoid larvae of various diphyllobothroid cestodes belonging to the genus *Spirometra*. Sparganosis is a zoonosis from amphibians, reptiles or mammals causing significant economic losses and also a public health problem in humans (Ooi *et al.*, 2000; Pampiglione *et al.*, 2003; Wiwanitkit, 2005). Although human sparganosis is a worldwide parasitic zoonosis, it is most frequently found in eastern and south-eastern Asia, including China (Cui *et al.*, 2011). The spargana invade mainly the brain, eye, spinal cord, subcutaneous tissues and abdominal cavity, and can cause blindness, paralysis and even death (Li *et al.*, 2009). Human infections are mainly acquired by eating raw or insufficiently cooked meat of frogs and snakes.

A traditional approach for the identification of *Spirometra* is by morphological observation, but this approach has limitations in the differentiation of *Spirometra erinaceieuropaei* from other similar species such as *Spirometra manson*, therefore causing confusion.

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However, accurate identification of parasites and their characterization at different taxonomic levels have important implications for the prevention and control of parasitic diseases. A range of studies has demonstrated that molecular markers are of particular utility for species-specific identification and detection of a number of parasite groups (Sun *et al.*, 2006, 2009).

Sequence variation is widespread in parasite populations, and the accurate analysis of genetic variation in parasites has important implications for the genetic structure of parasites, epidemiology and studying population biology. Previous studies have shown that the cytochrome *c* oxidase subunit 3 gene (*cox*3) is the preferred gene for genetic variation and phylogentic analyses, due to sufficient variability and a consistent phylogenetic signal (Zarowiecki *et al.*, 2007; Zhao *et al.*, 2009), and NADH dehydrogenase subunits 1 and 4 genes (*nad*1 and *nad*4) also have more characters of phylogenetic information and variability (Gasser *et al.*, 1999; Zhao *et al.*, 2009). Therefore, they provide better markers for both phylogenetic and population studies. However, there is a paucity of information on the genetic variation in populations of some important parasite groups from China, such as *S. erinaceieuropaei* spargana of human and animal health significance.

The objectives of the present study were to examine sequence variability in mitochondrial *cox*3, *nad*1 and *nad*4 regions, among *S. erinaceieuropaei* spargana isolates from different endemic regions in China. Based on the p*cox*3, p*nad*1 and p*nad*4 sequences, the phylogenetic relationships of *S. erinaceieuropaei* spargana were also reconstructed.

Materials and methods

Parasites and isolation of genomic DNA

The parasite species, with their sample codes, number of samples, host species and geographical origins are listed in table 1. Total genomic DNA was extracted from individual samples by sodium dodecyl sulphate/proteinase K treatment, column-purified (Wizard™ DNA Clean-Up, Promega, Madison, Wisconsin, USA) and eluted into 50 µl water according to the manufacturer's recommendations.

Table 1. Geographical origins (different locations in Hunan province) of *S. erinaceieuropaei* spargana samples used in the present study, as well as their GenBank™ accession numbers for sequences of partial mitochondrial cytochrome *c* oxidase subunit 3 gene (p*cox*3), NADH dehydrogenase subunits 1 and 4 genes (p*nad*1 and p*nad*4).

Sample codes	Geographical origin	GenBank™ accession number		
		p*cox*3	p*nad*1	p*nad*4
CS1	Changsha	HM475011	HM475044	HM475076
CS2	Changsha	HM475022	HM475054	HM475087
CS3	Changsha	HM475036	HM475065	HM475098
CZ1	Chenzhou	HM475012	HM475045	HM475077
CZ2	Chenzhou	HM475023	HM475055	HM475088
CZ3	Chenzhou	HM475033	HM475066	HM475099
HY1	Hengyang	HM475013	HM475046	HM475078
HY2	Hengyang	HM475024	HM475056	HM475089
HY3	Hengyang	HM475034	HM475067	HM475100
LD1	Loudi	HM475014	HM475047	HM475079
LD2	Loudi	HM475025	HM475057	HM475090
LD3	Loudi	HM475035	HM475068	HM475101
SY1	Shaoyang	HM475015	HM475048	HM475080
SY2	Shaoyang	HM475026	HM475058	HM475091
SY3	Shaoyang	HM475037	HM475069	HM475102
XP1	Xupu	HM475016	HM475049	HM475081
XP2	Xupu	HM475027	HM475059	HM475092
XP3	Xupu	HM475038	HM475070	HM475103
XT1	Xiangtan	HM475017	–	HM475082
XT2	Xiangtan	HM475028	HM475060	HM475093
XT3	Xiangtan	HM475039	HM475071	HM475104
YIY1	Yiyang	HM475018	HM475050	HM475083
YIY2	Yiyang	HM475029	HM475061	HM475094
YIY3	Yiyang	HM475040	HM475072	–
YY1	Yueyang	HM475019	HM475051	HM475084
YY2	Yueyang	HM475030	HM475062	HM475095
YY3	Yueyang	HM475041	HM475073	HM475105
ZJJ1	Zhangjiajie	HM475020	HM475052	HM475085
ZJJ2	Zhangjiajie	HM475031	HM475063	HM475096
ZJJ3	Zhangjiajie	HM475042	HM475074	HM475106
ZZX1	Zhuzhouxian	HM475021	HM475053	HM475086
ZZX2	Zhuzhouxian	HM475032	HM475064	HM475097
ZZX3	Zhuzhouxian	HM475043	HM475075	HM475107

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Table 2. Sequences of primers used to amplify a portion of the mitochondrial cytochrome *c* oxidase subunit 3 gene (p*cox*3), NADH dehydrogenase subunits 1 and 4 genes (p*nad*1 and p*nad*4) from *S. erinaceieuropaei* spargana in the present study.

Name of primer	Sequence (5′ to 3′)
For p*cox*3	
Secox3F	GGGTGTCATTTCTTCCTATTTTTAA
Secox3R	AAATGTCAATACCAAGTAACTAAAG
For p*nad*1	
Senad1F	ATAAGGTGGGGGTGATGGGGTTG
Senad1R	ATAAAAAATAAAAGATGAAAGGG
For p*nad*4	
Senad4F	TTTTTTCCTTGGGTTAAGATTAA
Senad4R	GCTACTACCCTCAAAAGACTCAC

Enzymatic amplification

The primer sets for *cox*3, *nad*1 and *nad*4 genes were designed by the authors, based on well-conserved sequences in many distantly related taxa (table 2). These primers were synthesized on a Biosearch Model 8700 DNA synthesizer (Shanghai, China). Polymerase chain reactions (PCRs) (25 µl) were performed in 2 mM $MgCl_2$, 2.5 µM of each primer, 2.5 µl 10 × r*Taq* buffer, 0.2 mM of each deoxyribonucleoside triphosphate (dNTP), 1.25 U of r*Taq* DNA polymerase (Takara, Dalian, China), and 1 µl of DNA sample in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: after an initial denaturation at 94°C for 5 min, then 94°C for 30 s (denaturation); 55°C (for p*cox*3, p*nad*1 and p*nad*4) for 30 s (annealing); 72°C for 30 s (extension) for 38 cycles, followed by a final extension at 72°C for 10 min. These optimized amplification conditions for the specific and efficient amplification of individual DNA fragments were obtained after varying annealing and extension temperatures. One microlitre (5–10 ng) of genomic DNA was added to each PCR reaction. Samples without genomic DNA (no-DNA controls) were included in each amplification run, and in no case were amplicons detected in the no-DNA controls (not shown). Five microlitres of each amplicon was examined by 0.8% (w/v) agarose gel electrophoresis to validate amplification efficiency. PCR products were sent to Sangon Company (Shanghai, China) for sequencing using a primer walking strategy.

Sequence analysis and phylogenetic reconstruction

Sequences of the three mitochondrial genes were separately aligned using the computer program Clustal X 1.83 (Thompson *et al.*, 1997). Pairwise comparisons were made of the level of sequence differences (D) among and within the species using the formula $D = 1 - (M/L)$, where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton *et al.*, 1995).

Phylogenetic analyses were based on the sequences of the three mitochondrial genes available in this study. Three methods, namely neighbour joining (NJ), maximum likelihood (ML) and maximum parsimony (MP), were used for phylogenetic reconstructions. Standard unweighted MP was performed using the package Phylip 3.67 (Felsenstein, 1995). NJ analysis was carried out using the Dayhoff matrix model implemented by MEGA 4.0 (Tamura *et al.*, 2007), and ML analysis was performed using PUZZLE 4.1 under the default setting (Strimmer & Haeseler, 1996). The consensus tree was obtained after bootstrap analysis, with 1000 replications for NJ and MP trees, and 100 for the ML tree, with values above 50% reported. To study the genetic relationships among diphyllobothroid cestodes, other members of the Diphyllobothriidae were considered in the present study (*Diphyllobothrium nihonkaiense* NC_009463; *D. latum* AB269325; *Spirometra erinaceieuropaei* NC_011037), with *Taenia solium* (GenBank™ accession number NC_004022) as the outgroup. Phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

Results and discussion

Genomic DNA was extracted from 33 individual spargana representing 11 geographical locations in Hunan Province, China (fig. 1). p*cox*3, p*nad*1 and p*nad*4 (~600, 650 and 900 bp, respectively) were amplified individually and subjected to agarose gel electrophoresis. The results showed that no size variation was detected on agarose gels among any of the amplicons examined for each mtDNA region. To assess sequence variation in these three mtDNA regions within and between isolates, amplicons of p*cox*3, p*nad*1 and p*nad*4 from samples representing different isolates were selected and then

Fig. 1. The sampling locations for *S. erinaceieuropaei* spargana isolates in different regions of Hunan, China.

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Table 3. Number and position of codon variations in a portion of the mitochondrial cytochrome *c* oxidase subunit 3 gene (p*cox*3) and NADH dehydrogenase subunits 1 and 4 genes (p*nad*1 and p*nad*4) among *S. erinaceieuropaei* spargana isolates in China.

mtDNA region	Length (bp)	Variable nucleotide positions[a]			
		Codon 1	Codon 2	Codon 3	Total no.
*cox*3	541	4	0	7	11
*nad*1	607	1	4	14	19
*nad*4	847	8	1	15	24

[a] The first codon position of each sequence was determined in relation to the complete mitochondrial DNA sequence of *S. erinaceieuropaei* (NC_011037).

subjected to sequencing. The sequences of p*cox*3, p*nad*1 and p*nad*4 were 541, 607 and 847 bp in length, respectively. The A + T contents of the sequences were 68.39–68.76% (p*cox*3), 63.76–64.91% (p*nad*1) and 67.18–67.77% (p*nad*4), respectively, while the intra-specific sequence variations within each of the *S. erinaceieuropaei* spargana were 0–1.5% for p*cox*3, 0–2.8% for p*nad*1 and 0–2.7% for p*nad*4, consistent with a recent report by Liu *et al.* (2010). For p*cox*3, no sequence variation was detected among Changsha, Hengyang, Xiangtan and Zhangjiajie isolates; while sequence variations of more than 0.5 were found among Xupu, Yiyang and Yueyang isolates. For p*nad*1, sequence variations of more than 3.0 were detected in four locations, including Chenzhou, Loudi, Xupu and Yeyang isolates. For p*nad*4, sequence variations of more than 2.0 were found among Xupu and Yueyang isolates.

For p*cox*3 and p*nad*4, intra-specific nucleotide variation was related mainly to changes at the third codon position, while fewer changes were detected at the first or second codon positions (table 3), consistent with results of other organisms (Li *et al.*, 2008; Zhao *et al.*, 2009). For example, the number of intra-specific variations were 4, 0, 7 for the first, second and third positions for *cox*1; 1, 4, 14 for *nad*1; and 8, 1, 15 for *nad*4, respectively. Intra–specific nucleotide variations represented transitions (A ↔ G or C ↔ T; $n = 10$ for p*cox*3, $n = 17$ for p*nad*1 and $n = 19$ for p*nad*4), transversions (A ↔ C, A ↔ T, C ↔ G, and/or T ↔ G; $n = 1$ for p*cox*3, $n = 2$ for p*nad*1, $n = 5$ for p*nad*4).

The combined sequences of p*cox*3, p*nad*1 and p*nad*4 representing different isolates were aligned over a consensus length of 1853 bp. Topologies of all trees constructed by different methods (NJ, MP and ML) with different building strategies and/or different distance models were identical or similar, with only small difference of bootstrap values (fig. 2). These results indicate that all the spargana isolates in Hunan Province represent *S. erinaceieuropaei*. From the phylogenetic tree: parasites of genus *Diphyllobothrium* were sister to the genus *Spirometra*, and *S. erinaceieuropaei* and *D. nihonkaiense* were more closely related to the other members of the *Diphyllobothrium* genus (*D. latum*), consistent with results of previous classifications based upon *cox*1 datasets (Liu *et al.*, 2011).

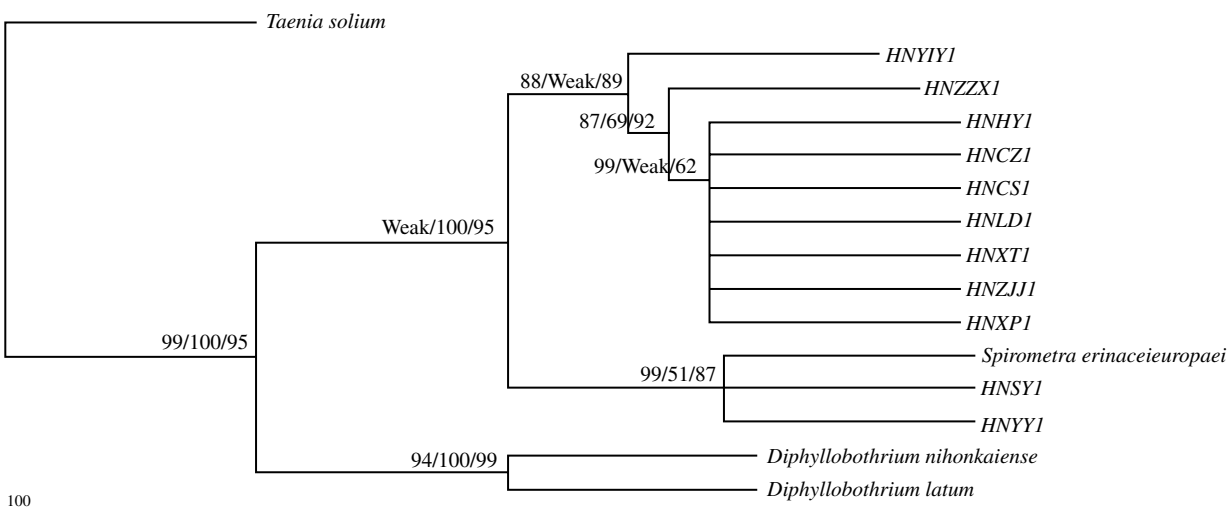

Fig. 2. Phylogenetic relationship among the examined cestode species inferred by maximum parsimony (MP), maximum likelihood (ML) and neighbour joining (NJ) analyses based on combined mitochondrial dataset (p*cox*3 + p*nad*1 + p*nad*4) sequences, using one *Taenia* species (*Taenia solium*) as outgroup. The numbers along branches indicate bootstrap values resulting from different analyses in the order: MP/ML /NJ. Values lower than 50 are given as 'Weak'.

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In conclusion, the genetic variability among *S. erinaceieuropaei* spargana isolates from different endemic regions in China could be revealed by sequences of three mitochondrial DNA genes. For the three mtDNA genes, genetic variation of p*nad*1 was higher than p*nad*4 and p*cox*3, and p*nad*4 was higher than p*cox*3. The results of the present study also have implications for the diagnosis and control of *S. erinaceieuropaei* spargana infections of animal and human health significance.

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