The Thai Journal of Veterinary Medicine

Volume 43
Issue 2 *June, 2013*

Article 18

6-1-2013

Genetic Diversity of Spirometra erinaceieuropaei from Dogs in Hunan province, China Based on Analyses of Two Mitochondrial Sequences

Liang Liang

Jian Liang

Hui Shen

Xiao Lei Yao

Short Communication

Genetic Diversity of *Spirometra erinaceieuropaei* from Dogs in Hunan province, China Based on Analyses of Two Mitochondrial Sequences

Liang Liang* Jian Liang Hui-Shen Xiao-Lei Yao

Abstract

Sequence variability in two mitochondrial DNA (mtDNA) regions, namely NADH dehydrogenase subunit 5 (*nad*5) and small subunit of ribosomal RNA (*rrn*S) in *Spirometra erinaceieuropaei* from dogs in Hunan province in China was examined. A portion of the *nad*5 (p*nad*5) and *rrn*S (p*rrn*S) genes were amplified separately from individual *S. erinaceieuropaei* by polymerase chain reaction (PCR). Representative amplicons were subjected to sequencing in order to estimate sequence variability. The sequences of p*nad*5 and p*rrn*S were 531 and 328 bp in size, respectively. The intra-specific sequence variations within each of the *S. erinaceieuropaei* were 0-3.5% for p*nad*5 and 0-1.4% for p*rrn*S, while the inter-specific sequence variations within each of the *S. erinaceieuropaei* were 12.2-35.7% for p*nad*5 and 10.4-11.8% for p*rrn*S. Phylogenetic analysis using neighbour joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) methods, indicated that all the isolates in Hunan province represented *S. erinaceieuropaei*. These findings demonstrated clearly the usefulness of the three mtDNA sequences for population genetics studies of *S. einaceieuropaei* in human and animal health significance.

Keywords: genetic variation, mitochondrial DNA (mtDNA), hylogenetic analysis, *Spirometra erinaceieuropaei*

Department of Plastic and Aesthetic, Affiliated Ruikang Hospital of Guangxi Traditional Chinese Medicine University, Nanning 530011, Guangxi Zhuang Autonomous Region, China
**Correspondence author E-mail: 404877308@qq.com (LL)*

บทคัดย่อ

ความหลากหลายทางพันธุกรรมของ *Spirometra erinaceieuropaei* จากสุนัขในจังหวัดหูหนาน ประเทศจีน โดยวิเคราะห์จากลำดับพันธุกรรมของไมโตคอนเดรีย

Liang Liang Jian Liang Hui-Shen Xiao-Lei Yao*

การศึกษาครั้งนี้ศึกษาความแปรปรวนของลำดับพันธุกรรมของไมโตคอนเดรีย (mtDNA) ที่ NADH dehydrogenase subunit 5 (nad5) และหน่วยย่อยของ ribosomal RNA (rrnS) ของ *Spirometra erinaceieuropaei* จากสุนัขในจังหวัดหูหนาน ประเทศจีน โดยส่วนหนึ่งของยีน *nad5* (p*nad5*) และ *rrn*S (p*rrn*S) ถูกเพิ่มจำนวนแยกต่างหากจาก *S. erinaceieuropaei* ด้วยวิธี Polymerase chain reaction (PCR) โดยผลผลิต amplicons ที่ได้นำไปวิเคราะห์ลำดับพันธุกรรมเพื่อประเมินหาความแปรปรวนของลำดับพันธุกรรม โดย p*nad5* และ p*rrn*S มีขนาดเท่ากับ 531 และ 328 bp ตามลำดับ พบว่าความแตกต่างภายในลำดับพันธุกรรมของแต่ละตัวอย่างของ *S. erinaceieuropaei* มีค่าเท่ากับ 0-3.5% สำหรับ p*nad5* และ 0-1.4% สำหรับ p*rrnS* ในขณะที่ความแตกต่างระหว่างลำดับพันธุกรรมของแต่ละตัวอย่างของ *S. erinaceieuropaei* มีค่าเท่ากับ 12.2-35.7% สำหรับ p*nad5* และ 10.4-11.8% สำหรับ p*rrn*S การวิเคราะห์ phylogenetic ด้วยวิธี neighbour joining (NJ) maximum likelihood (ML) และ maximum parsimony (MP) ชี้ให้เห็นว่าเชื้อที่แยกได้จากจังหวัดหูหนานทั้งหมดเป็นตัวแทนของ *S. erinaceieuropaei* ผลการศึกษานี้แสดงให้เห็นอย่างชัดเจนถึงประโยชน์ของลำดับพันธุกรรมของไมโตคอนเดรีย ในการศึกษาด้านพันธุศาสตร์ประชากรของ *S. erinaceieuropaei* ในด้านวิทยาศาสตร์สุขภาพของคนและสัตว์

คำสำคัญ: ความแปรปรวนของลำดับพันธุกรรม ลำดับพันธุกรรมของไมโตคอนเดรีย การวิเคราะห์ phylogenetic *Spirometra erinaceieuropaei*

Department of Plastic and Aesthetic, Affiliated Ruikang Hospital of Guangxi Traditional Chinese Medicine University, Nanning 530011, Guangxi Zhuang Autonomous Region, China
***ผู้รับผิดชอบบทความ E-mail:** 404877308@qq.com (LL)*

Introduction

Sparganosis is an important worldwide parasitic disease caused by infection with spargana, the plerocercoid larvae of various diphyllobothroid tapeworms belonging to the genus Spirometra (Ooi et al., 2000; Pampiglione et al., 2003; Wiwanitkit et al., 2005). The spargana invade mainly the brain, spinal cord, eye, subcutaneous tissues and abdominal cavity, and can cause blindness, and even death (Li et al., 2009). This disease is most frequently found in Asia countries (including China), and causes significant public health problem in humans and major economic impact in animals (Cui et al., 2011).

Molecular approaches for studying the taxonomy, systematics, and population genetics of animals are available recently. Mitochondrial DNA (mtDNA) sequences have been proven useful and reliable genetic markers due to their maternal inheritance, fast rate of evolutionary change, and relatively conserved genome structures than nuclear ribosomal genome (McManus and Bowles, 1996; Blouin, 2002). Previous studies have shown that the cytochrome c oxidase subunit 3 gene (cox3) is the preferred gene for genetic variation and phylogentic analyses (Zarowiecki et al., 2007), and NADH dehydrogenase subunits 1 and 4 genes (nad1 and nad4) also have more characters of phylogentic information and variability (Gasser et al., 1999; Zhao et al., 2009). Therefore, these mt genes can provide markers for both phylogenetic and population studies. In the *S. erinaceieuropaei*, recent studies have shown that the cytochrome c oxidase subunit 1 gene (cox3) and NADH dehydrogenase subunits 1 and 4 genes (nad1 and nad4) are the preferred gene for genetic variation and phylogentic analyses (Liu et al., 2002[a]), but there is a paucity of information on the genetic variation of nad4 and small subunit of ribosomal RNA (rrnS) genes.

The objectives of the present study were to examine sequence variability in mitochondrial nad5 and rrnS regions, among *S. erinaceieuropaei* isolates from dogs in Hunan province in China. Based on the pnad5 and prrnS sequences, the phylogenetic relationships in *S. erinaceieuropaei* 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

(WizardTM DNA Clean-Up, Promega) and eluted into 50 µL H2O according to the manufacturer's recommendations.

Enzymatic amplification: The primer sets for nad5 and rrnS genes were designed by author based on sequences well-conserved in many distantly related taxa (Table 2). These primers were synthesied on a Biosearch Model 8700 DNA synthesizer (Shanghai, China). PCR reactions (25 µl) were performed in 2 mM of MgCl2, 2.5 µM of each primer, 2.5 µl 10 x rTaq buffer, 0.2 mM of each dNTPs, 1.25 U of rTaq DNA polymerase (Takara), and 1 µl of DNA sample in a thermocycler (Biometra) under the following conditions: after an initial denaturation at 94^0C for 5 min, then 94^0C for 30 sec (denaturation), 55^0C (for pnad5 and prrnS) for 30 sec (annealing), 72^0C for 30 sec (extension) for 38 cycles, followed by a final extension at 72^0C 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 were 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.

Sequences 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 (Chiltonet et al., 1995).

The sequences of two mitochondrial genes available in this study were used for phylogenetic analyses. Three methods, namely neighbour joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) were used for phylogenetic re-constructions. Standard unweighted MP was performed using 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 and Haeseler, 1996). The consensus tree was obtained after bootstrap analysis, with 1000 replications for NJ and MP trees, and 100 for ML tree, with values above 50% reported. To study the genetic relationships among cestodes, other cestodes were considered into the present study (*Diphyllobothrium nihonkaiense* NC_009463; *D. latum* AB269325; *Spirometra erinaceieuropaei* NC_011037; *Taenia asiatica* NC_004826; *T. crassiceps* NC_002547; *T. saginata* NC_009938; *T. solium* NC_004022; *Echinococcus multilocularis* AB018440; *E. granulosus* NC_008075; *Hymenolepis diminuta* AF314223), with *Ascaris suum* (GenBank accession number HQ704901) as the outgroup. Phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

Results and Discussion

Genomic DNA was extracted from 30 individual cestodes representing 11 geographical locations in Hunan province, China (Table 1). Pnad5 and prrnS (~580 and 380 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 two mtDNA regions within and between isolates, amplicons of

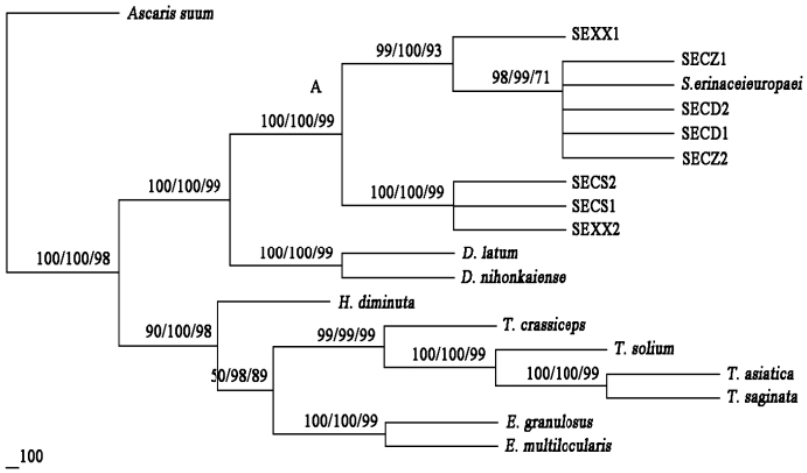

Figure 1 Phylogenetic relationship among examined cestode species inferred by maximum parsimony (MP), maximum likelihood (ML) and neighbour joining (NJ) analyses based on combined mitochondrial dataset (p*nad*5+p*rrn*S) sequences, using one nematode species (*Ascaris suum*) as outgroup. Numbers along branches indicate bootstrap values resulting from different analyses in the order: MP/ML /NJ.

pnad5 and prrnS from samples representing different isolates were selected and then subjected to sequencing. The sequences of pnad5 and prrnS were 531 and 328 bp in size, respectively. The intra-specific sequence variations within each of the *S. erinaceieuropaei* were 0-3.5% for pnad5 and 0-1.4% for prrnS; while the inter-specific sequence variations within each of the *S. erinaceieuropaei* were 12.2-35.7% for pnad5 and 10.4-11.8% for prrnS, consistent with those recently reported (Liu et al., 2012[a]).

For the pnad4 and prrnS, 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, consistent with results of other organisms (Li et al., 2008; Zhao et al., 2009). The combined sequences of pnad5 and prrnS representing different isolates were aligned over a consensus length of 859 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 1). These results indicate that all the 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 recent classifications based on complete mitochondrial genome datasets (Liu et al., 2011; Liu et al., 2012b).

Table 1 Geographical origins (different locations in Hunan province) of *Sparganum erinaceieuropaei* samples used in the present study, as well as their GenBank accession numbers for sequences of partial mitochondrial NADH dehydrogenase subunits 5 (*pnad5*) and small subunit of ribosomal RNA (*prrnS*) genes

Sample codes	Geographical origin	GenBank accession number	
		P*nad*1	P*nad*4
SECS1	Changsha	GU946413	GU946433
SECS2	Changsha	GU946414	GU946434
SECS3	Changsha	GU946415	GU946435
SECS4	Changsha	GU946416	GU946436
SECS5	Changsha	GU946417	GU946437
SECZ1	Chenzhou	GU946418	GU946438
SECZ2	Chenzhou	GU946419	GU946439
SECZ3	Chenzhou	GU946420	GU946440
SECZ4	Chenzhou	GU946421	GU946441
SECZ5	Chenzhou	GU946422	GU946442
SECD1	Changde	GU946423	GU946443
SECD2	Changde	GU946424	GU946444
SECD3	Changde	GU946425	GU946445
SECD4	Changde	GU946426	GU946446
SECD5	Changde	GU946427	GU946447
SEXX1	Xiangxi	GU946428	GU946448
SEXX2	Xiangxi	GU946429	GU946449
SEXX3	Xiangxi	GU946430	GU946450
SEXX4	Xiangxi	GU946431	GU946451
SEXX5	Xiangxi	GU946432	GU946452

In conclusion, the genetic variability among *S. erinaceieuropaei* isolates from China could be revealed by sequences of two mitochondrial DNA genes. For the two mt DNA genes, genetic variation of pnad5 was higher than *prrnS*. The results of the present study also have implications for the diagnosis and control of *S. erinaceieuropaei* infections in animal and human health significance.

Table 2 Sequences of primers used to amplify a portion of the mitochondrial NADH dehydrogenase subunits 5 (*pnad5*) and small subunit of ribosomal RNA (*prrnS*) genes from *Sparganum erinaceieuropae*i in the present study.

Name of primer	Sequence (5′ to 3′)
For p*nad*5	
Se*nad*5F	TCATACTGGGTCTATCAGGTGTT
Se*nad*5R	ACAGCAAAGTTAGGGGGTAATAGGT
For p*rrn*S	
Se*rrn*SF	TAGTTTGGCAGTGAGTTATTCCG
Se*rrn*SR	GGCTACCTTGTTACGACTTACCTCA

References

Blouin MS 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Int J Parasitol. 32: 527-531.

Chilton NB, Gasser RB and Beveridge I 1995. Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the Hypodontus macropi complex (Nematoda: Strongyloidea). Int J Parasitol. 25: 647-651.

Cui J, Lin XM, Zhang HW, Xu BL and Wang ZQ 2011. Sparganosis, Henan Province, central China. Emerg Infect Dis. 17: 146-147.

Felsenstein J 1995. PHYLIP (Phylogeny Inference Package), version 3.57c, Department of Genetics, University of Washington, Seattle, WA.

Gasser RB, Zhu X and McManus DP 1999. NADH dehydrogenase subunit 1 and cytochrome c oxidase subunit I sequences compared for members of the genus Taenia (Cestoda). Int J Parasitol. 29: 1965-1970.

Li MW, Lin RQ, Song HQ, Sani RA, Wu XY and Zhu XQ. 2008. Electrophoretic analysis of sequence variability in three mitochondrial DNA regions for ascaridoid parasites of human and animal health significance. Electrophoresis. 29: 2912-2917.

Li MW, Lin HY, Xie WT, Gao MJ, Huang ZW, Wu JP, Li C, Lin RQ and Zhu XQ. 2009. Enzootic sparganosis in Guangdong, China. Emerg Infect Dis. 15: 1317-1318.

Liu GH, Lin RQ, Li MW, Liu W, Liu Y, Yuan ZG, Song HQ, Zhao GH, Zhang KX and Zhu XQ. 2011. The complete mitochondrial genomes of three cestode species of Taenia infecting animals and humans. Mol Biol Rep. 38: 2249-2256.

Liu W, Liu GH, Li F, He DS, Wang T, Sheng XF, Zeng DL, Yang FF and Liu Y. 2012[a]. Sequence variability in three mitochondrial DNA regions of Spirometra erinaceieuropaei spargana of human and animal health significance. J Helminthol, 86: 271-275.

Liu GH, Li C, Li JY, Zhou DH, Xiong RC, Lin RQ, Zou FC and Zhu XQ. 2012[b]. Characterization of the complete mitochondrial genome sequence of Spirometra erinaceieuropaei (Cestoda:

Diphyllobothriidae) from China. Int J Biol Sci. 8: 640-649.

McManus DP and Bowles J 1996. Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. Int J Parasitol. 26: 687-704.

Ooi HK, Chang SL, Huang CC, Kawakami Y and Uchida A 2000. Survey of Spirometra erinaceieuropaei in frogs in Taiwan and its experimental infection in cats. J Helminthol. 74: 173-176.

Page RD 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comput Appl Biosci. 12: 357-358.

Pampiglione S, Fioravanti ML and Rivasi F 2003. Human sparganosis in Italy. Case report and review of the European cases. APMIS: Acta Pathologica, Microbiol Immunol. 111: 349-354.

Strimmer K and Haeseler AV 1996. Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topologies. Mol Biol Evolution. 13: 964-969.

Tamura K, Dudley J, Nei M and Kumar S 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 24: 1596-1599.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgin DG 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality Analysis tools. Nucleic Acids Res. 24: 4876-4882.

Wiwanitkit V 2005. A review of human sparganosis in Thailand. Int Infect Dis. 9: 312-316.

Zarowiecki MZ, Huyse T and Littlewood DT 2007. Making the most of mitochondrial genomes--markers for phylogeny, molecular ecology and barcodes in Schistosoma (Platyhelminthes: Digenea). Int J Parasitol. 37: 1401-1418.

Zhao GH, Mo XH, Zou FC, Weng YB, Lin RQ, Xia CM and Zhu XQ 2009. Genetic variability among *Schistosoma japonicum* isolates from different endemic regions in China revealed by sequences of three mitochondrial DNA genes. Vet Parasitol. 162: 67-74.