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The phylogenetic diversity of *Spirometra erinaceieuropaei* isolates from southwest China revealed by multi genes

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ABSTRACT

The larval plerocercoid of *Spirometra erinaceieuropaei* can parasitize humans, causing a serious food borne parasitic zoonosis known as sparganosis. Sparganosis have increased in China in recent years. In this study, the prevalence of sparganum infection in wild frogs in 9 geographical areas in southwest China was firstly investigated. Of 276 caught frogs, 55 frogs were found to be infected with sparganum. Then, the population genetic structure of these sparganum isolates was explored based on four molecular markers (*cytb*, *cox1*, *rrnS* and 28S rDNA D1). Highly genetic diversity and the genetic differentiation among sparganum isolates from different sites were revealed in the DNA polymorphism analyses. Both the phylogenetic inference and the analysis of the median-joining network supported two clades in the southwest *S. erinaceieuropaei* population. However, none demographic population expansion of the southwest *S. erinaceieuropaei* population was observed in the neutrality test, mismatch distribution analysis and Bayesian skyline plot analysis. Finally, the phylogenetic diversity of *S. erinaceieuropaei* from eastern, central, southern and southwest China was analyzed, the result suggested that Chinese *S. erinaceieuropaei* population should be divided into two groups (Group I and Group II), and they started to divergence in the middle Pliocene.

1. Introduction

The plerocercoid larvae (spargana) of diphyllobothriid tapeworms can parasitize in humans and cause serious parasitic zoonosis. *Spirometra erinaceieuropaei* plerocercoid is the main etiological agent of human sparganosis and results in blindness, epilepsy, paralysis, and even death (Shirakawa et al., 2010). China has the largest number of cases with sparganosis in the world (Liu et al., 2015). Sparganum infection in human is caused mainly by ingesting raw or undercooked meat of second intermediate hosts, such as frogs and snakes (Liu et al., 2015). A number of inhabitants in rural villages of China have the habit of eating frog meat and believe that frog meat or tadpoles has a medicinal role for skin diseases and eye inflammations (Cui et al., 2011). Investigation of sparganum infection in frogs is therefore valuable for food safety and the prevention and control of human sparganosis. Sparganum infection in frogs have been reported in several areas of China (Liu et al., 2010; Wang et al., 2011; Wang et al., 2014; Zhang et al., 2014; Wei et al., 2015). However, the prevalence of frog sparganum infection in southwest China is not clear, where the ethnic minorities have the habit of eating raw meat and believe that frog meat is a delicious and nutritious food (Wang et al., 2014). Additionally, the local cases with sparganosis in southwest China have increased in recent years (Zhou et al., 2005; Chen, 2011; Huang et al., 2014) and sparganosis has been considered as an emerging enzootic disease in several districts of China (Cui et al., 2011). Therefore, it is urgent to investigate the prevalence of sparganum infection in frogs to assess the risks of eating frog meat for human sparganum infection.

In addition, it is important to analyze the genetic characteristics of sparganum population in relation to local environmental conditions, so that we can get valuable clues about the genetic variation and population changes for the prevention and control of sparganosis (Glenn et al., 2013). In the past three years, we have explored the phylogenetic diversity of *S. erinaceieuropaei* isolates collected from central, eastern and southern China by using two mitochondrial genes: cytochrome *b* (*cytb*) and cytochrome *c* oxidase subunit I (*cox1*) (Zhang et al., 2015a; Zhang et al., 2015b). To get a preliminary perspective of the genetic structure of *S. erinaceieuropaei* from China, the genetic variance between *S. erinaceieuropaei* sparganum isolates from southwestern and other regions of China was compared in this study by using additional two molecular

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markers: the mitochondrial small subunit of ribosomal RNA gene (*rrnS*) and the D1 region of nuclear ribosomal large subunit RNA gene (28S rDNA D1).

The main aims of the present study was to investigate: (1) the prevalence of *S. erinaceieuropaei* sparganum infection in wild frogs collected from southwest China; (2) the genetic diversity of these *S. erinaceieuropaei* isolates; and (3) the phylogenetic pattern of Chinese *S. erinaceieuropaei* isolates.

2. Materials and methods

2.1. Ethical approval

The performance of this study was strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China, and our protocol was approved by the Life Science Ethics Committee of Zhengzhou University (Permission No. SYXK 2014-0011).

2.2. Sparganum collection

Wild frogs were collected from a field site in nine geographical locations of southwest China during May 2013 to September 2014 (Supplementary Table S1). The presence of spargana was examined according to the methods of Wei et al. (2015).

2.3. Sequencing of target genes and data collection

Total genomic DNA was extracted from individual plerocercoid using the EasyPure Genomic DNA Kit (Transgen, China) follow the manufacturer's protocol. Four molecular markers (*cytb*, *cox1*, *rrnS* and 28S rDNA D1) were amplified by PCR using the primer combinations listed in the Supplementary Table S2. PCR products were purified using the EasyPure PCR Purification Kit (Transgen, China) and sequenced in both directions at the Genwiz Company (Beijing, China). All sequences were deposited in the GenBank database under accession numbers KT376546-KT376597 (*cytb*), KT376494-KT376545 (*cox1*), KT376650-KT376701 (*rrnS*) and KT376598-KT376649 (28S rDNA D1). All available complete sequences of *cytb* and *cox1* of *S. erinaceieuropaei* in the GenBank database were also included in this study to make a comparison. A total of 402 sequences representing 201 sparganum isolates collected from 34 geographical locations of China were included (Supplementary Table S1).

2.4. Sequences alignment

The sequences for *cytb* and *cox1* were initially aligned using the default settings in the program Clustal X v.2.0 (Larkin et al., 2007) and adjusted in MEGA v.5.0 (Tamura et al., 2011) according to their amino acid sequences. For the *rrnS* and 28S rDNA D1 sequences, secondary structure was inferred and used as a guide for manual sequence alignment in MEGA v.5.0. Nucleotide saturation was analyzed by plotting number of transitions (Ti) and transversions (Tv) against corrected genetic distance values in the software DAMBE v.5.2 (Xia and Xie, 2001). The nucleotide composition, conserved sites, variable sites, parsimony-informative sites, and singleton sites were estimated using MEGA v.5.0.

2.5. Genetic structure of S. erinaceieuropaei isolates from southwest China

Haplotypes of southwest *S. erinaceieuropaei* isolates based on the concatenated sequences of *cytb*, *cox1*, *rrnS* and 28S rDNA D1 genes were inferred in DnaSP v.5.10 (Librado and Rozas, 2009), and the same program was used to calculate values of genetic diversity per population. Haplotype network reconstructions were performed in the software Network v.4.5 (Bandelt et al., 1999). A partitioned multi-gene (four partitions: *cytb*, *cox1*, *rrnS* and 28S rDNA D1) phylogenetic analysis of southwest *S. erinaceieuropaei* isolates was performed in MrBayes v.3.1 (Ronquist and Huelsenbeck, 2003), after determining the appropriate substitution models for each partition by applying the Akaike information criterion (AIC) in jModelTest 2 (Darriba et al., 2012). The analysis consisted of two runs, each with four MCMC chains running for 5,000,000 generations, and sampling every 100th generation. Effective sample size (ESS), mixing and convergence were checked using Tracer v.1.5 (Rambaut and Drummond, 2009). Stationarity was also reassessed using a convergence diagnostic. The consensus tree was drawn after removing the first 10 000 trees (20%) as the burn-in phase.

The approximate divergence time of the southwest sparganum isolates wase estimated based on the concatenated sequence of *cytb* and *cox1* using an uncorrelated log-normal relaxed molecular-clock model in the software BEAST v. 1.6.1 (Drummond and Rambaut, 2007). The substitution models were assigned following model selection by jModelTest 2. Gene-specific nucleotide substitution model parameters were used, with each gene allowed to evolve at a different rate (Zhang and Zhou, 2013). For the earlier tree, a basic coalescent model assuming a constant population size over the time period considered was chosen. Two replicate MCMC runs were performed, with the tree and parameter values sampled every 1000 steps over a total of 1×10^8 steps. The molecular evolutionary rate was fixed at 0.0195 and 0.0225 substitutions per site per million years (Myr) for *cytb* and *cox1*, respectively (Hoberg et al., 2001; Michelet et al., 2010).

Analyses of demographic change were estimated using mismatch distributions based on the concatenated sequences in the software Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). The validity of the expansion model was tested by using the sum of squared deviations (SSD) and raggedness index (RI) between observed and expected mismatches, as well as Fu's F_S test (Fu, 1997) and Tajima's D (Tajima, 1989). Analysis of molecular variance (AMOVA) was computed in Arlequin v.3.5.1.2 to detect the partitions of genetic diversity within and among populations. Population pairwise genetic distances were also estimated in the Arlequin v.3.5.1.2 to explore levels of genetic differentiation among the populations. To estimate the change in population size through time, and the time to the most recent common ancestor (tMRCA) of each *S. erinaceieuropaei* isolate, we performed a Bayesian Skyline Plot analysis (BSP) implemented in BEAST v.1.6.1. Two independent analyses were performed with the concatenated sequence of *cytb* and *cox1*. In both cases, a piecewise-constant skyline model was selected, and a relaxed uncorrelated log-normal molecular clock was used. The molecular evolutionary rate for *cytb* and *cox1* were selected as described above.

2.6. Phylogenetic analysis

The phylogenetic pattern of *S. erinaceieuropaei* isolates collected from eastern, central, southern and southwest China (Supplementary Table S1) was estimated using the concatenated sequences of *cytb* and *cox1* through maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods, respectively. MP analyses were performed in MEGA v.5.0. Confidence in each node was assessed by boot-strapping (1000 pseudo-replicates). ML analysis was performed in PhyML v.3.0 (Guindon and Gascuel, 2003) using models selected by jModelTest 2 (Darriba et al., 2012) under the Akaike information criterion. The support of each internal branch of the phylogeny was estimated using nonparametric bootstrapping (1000 replicates). BI analyses were performed in MrBayes v.3.1 with 10,000,000 generations, sampling trees every 100 gen-