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The divergence pattern of *Trichinella spiralis* isolates from China revealed by mitochondrial and ribosomal markers

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Abstract. *Trichinella spiralis* is the main etiological agent of human trichinellosis. In China, trichinellosis remains a serious food-borne parasitic zoonosis and poses a serious threat to human health. However, the genetic structure of Chinese *T. spiralis* population is still little known. In this study, we used three molecular markers to analyze phylogeographic structure of the Chinese *T. spiralis* population. A total of 11 *T. spiralis* isolates were collected from 10 geographical locations in mainland China. The cytochrome *c*-oxidase gene (*COI*), large subunit ribosomal DNA (mt-lsrDNA) and 5S ribosomal DNA intergenic spacer region (5S ISR) genes of each isolate was amplified and sequenced. Only four haplotypes were found in these concatenated sequences. Both multimodal frequency distributions of mismatch analysis and the Bayesian skyline plot analysis rejected a possible population expansion of Chinese *T. spiralis* population. The phylogenetic inference based on neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and the Bayesian estimation of divergence times under the uncorrelated log-normal relaxed molecular-clock model suggested that the Chinese *T. spiralis* isolates started radiating in the late Miocene.

INTRODUCTION

Trichinella spiralis (Owen, 1835) is the main etiological agent of human trichinellosis caused by eating raw or undercooked meat infected with its larvae (Murrell & Pozio, 2011). Domestic and wild pigs are the most important reservoir for this parasite (Pozio *et al.*, 2009; Wang *et al.*, 2015). *T. spiralis* is widespread throughout Europe, Egypt, North America, South America, New Zealand, and the core distribution area extends to China, Laos, Myanmar, South Korea, Thailand and Vietnam (Pozio & Zarlenga, 2013; Liu *et al.*, 2015). In endemic areas of trichinellosis, the number of infected domestic and wild animals tends to coincide with the integrity of swine animal production and wildlife management (Burke *et al.*, 2008; Blaga *et al.*, 2009; Pozio & Zarlenga, 2013). Therefore, knowledge regarding the phylogeographic structure of this species is valuable for illustrating determinants to zoonotic risk (Zarlenga *et al.*, 2009).

Molecular phylogeny is a well-established approach and played an indispensable role in study on modern animal evolution and systematic over the last decade (Zhang *et al.*, 2014). Using this method, some pioneer scientists concentrated their studies on the phylogeny of *Trichinella* and an ocean of excellent advances have been made (Zarlenga *et al.*, 2006; Rosenthal *et al.*, 2008; Zarlenga *et al.*, 2009; Krivokapich *et al.*, 2012; La Rosa *et al.*, 2012; Wang *et al.*, 2012; Mohandas *et al.*, 2014). These studies provided comprehensive evaluation on the taxonomy, phylogeny, systematic, population genetics and evolution of the genus *Trichinella*, nevertheless, few of them focused their research on the genetic diversity of *T. spiralis* isolates from China

(Rosenthal *et al.*, 2008; La Rosa *et al.*, 2012).

In China, trichinellosis remains a serious food-borne parasitic zoonosis, with 1387 cases reported in 15 outbreaks of human trichinellosis during 2004–2009 (Cui *et al.*, 2011). Pork is the most important source of human infection in China, and all swine *Trichinella* isolates from different geographical regions of China have been identified as *T. spiralis* (Wang *et al.*, 2007; Fu *et al.*, 2009; Wang *et al.*, 2012; Cui *et al.*, 2013). Unfortunately, despite the medical significance of *T. spiralis*, the genetic diversity of this species has not been well investigated across its wide geographical distribution in China, and such information may provide insight into the epidemiology of *T. spiralis* and the development of its control measures.

The purpose of this study was to explore the divergence pattern of *T. spiralis* isolates from eight provinces in mainland China by using two mitochondrial genes (cytochrome *c*-oxidase, *COI*; large subunit ribosomal DNA, mt-lsrDNA) and a nuclear ribosomal genes (5S ribosomal RNA intergenic spacer region, 5S ISR). These molecular markers were selected since they were suitable for inferring genetic diversity and phylogenetic analysis of *Trichinella* species (La Rosa *et al.*, 2001; Pozio *et al.*, 2002; Yang *et al.*, 2008; Wang *et al.*, 2012).

MATERIAL AND METHODS

Sample collection

The information about geographical origins and hosts of *T. spiralis* isolates used in this study are shown in Table 1. All *T. spiralis* isolates were maintained by serial passages in 6-week-old, specific pathogen-free male BALB/c mice at 6–8 month intervals. Muscle larvae of all isolates were obtained by the conventional artificial digestion of infected mouse carcasses (Gamble *et al.*, 2000; Li *et al.*, 2010).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from individual muscle larvae of *T. spiralis* using the EasyPure Genomic DNA Kit (Transgen, China) following the manufacturer's protocol. Three target genes, viz. *COI*, mt-lsrDNA and 5S ISR, were amplified by PCR using the primer combinations listed in the Supplementary Table S1. PCR (50µl) was performed in 4 mM $MgCl_2$, 5µM of each primer, 5µl 10×rTaq buffer, 1mM of each deoxyribonucleoside triphosphate (dNTP), 2.5U of rTaq DNA polymerase (Takara, China), and 2µl of DNA sample in a thermocycler under the following conditions: after an initial denaturation at 94°C for 5 min, then 94°C for 30 s (denaturation); 48°C

Table 1. Geographical origins (different locations in China) of *Trichinella spiralis* isolates used in this study, as well as their GenBank accession numbers for sequences of *COI*, mt-lsrDNA and 5S ISR

Sample codes	Host	Geographical origin	Accession number		
			COI	mt-lsrDNA	5S ISR
GX-td	*Paguma larvata*	Tiandong, Guangxi	KT894076	KT894087	KT894065
HB-xf	*Sus scrofa*	Xiangfan, Hubei	KT894077	KT894088	KT894066
HLJ-hb1	*Sus scrofa*	Harbin, Heilongjiang	KT894078	KT894089	KT894067
HLJ-hb2	*Canis familiaris*	Harbin, Heilongjiang	KT894079	KT894090	KT894068
HLJ-tj	*Sus scrofa*	Tongjiang, Heilongjiang	KT894080	KT894091	KT894069
HN-ay	*Sus scrofa*	Anyang, Henan	KT894081	KT894092	KT894070
HN-ny	*Sus scrofa*	Nanyang, Henan	KT894082	KT894093	KT894071
SX-xa	*Sus scrofa*	Xian, Shaanxi	KT894083	KT894094	KT894072
TJ	*Sus scrofa*	Tianjin	KT894084	KT894095	KT894073
Tibet-lz	*Sus scrofa*	Linzhi, Tibet	KT894085	KT894096	KT894074
YN-dl	*Sus scrofa*	Dali, Yunnan	KT894086	KT894097	KT894075

(for *COI* and 5S ISR), 50ºC (for mt-lsrDNA) for 30 s (annealing); 72ºC for 1 min (extension) for 35 cycles, followed by a final extension at 72ºC for 5 min. These optimized amplification conditions for the specific and efficient amplification of individual DNA fragments were obtained after varying annealing and extension temperatures. PCR products were purified using the EasyPure PCR Purification Kit (Transgen, 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

Sequences were aligned with default settings in the program Clustal X v2.0 (Larkin *et al.*, 2007). The nucleotide composition, conserved sites, variable sites, parsimony-informative sites, singleton sites, and genetic divergence were estimated using MEGA v5.0 (Tamura *et al.*, 2011). The number of haplotypes, calculation of haplotype diversity (Hd) and nucleotide diversity (Pi) were performed in DnaSP v5.10.01 (Librado & Rozas, 2009).

Phylogenetic inference

The phylogenetic relationship among *T. spiralis* isolates was estimated through three methods of neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML), respectively. NJ analysis was performed in MEGA v5.0 (Tamura *et al.*, 2011) using the HKY sequence evolution model selected by jModelTest v0.1 (Posada, 2008) under the Akaike information criterion. MP analysis also performed in MEGA v5.0 (Tamura *et al.*, 2011) using heuristic searches. Confidence in each node was assessed by boot-strapping (2000 pseudo-replicates). ML analysis was performed in PhyML v3.0 (Guindon & Gascuel, 2003) with model selected by jModelTest v0.1 (Posada, 2008). The support of each internal branch of the phylogeny was estimated using non-parametric bootstrap (1000 replicates). *Trichinella pseudospiralis*, a supposed sister species to *T. spiralis* (Zarlenga *et al.*, 2006), was included as the outgroup taxon to root the resulting trees. We also used Network v4.5 (Bandelt *et al.*, 1999) to draw a median-joining network to analyze the relationships among detected haplotypes.

Molecular dating

The approximate divergence times were estimated for the lineages of *T. spiralis* using the software BEAST v1.6.1 (Drummond & Rambaut, 2007). The concatenated sequence alignment was analyzed using a relaxed molecular-clock model. Sequence variation was partitioned into three subsets according to different genes. Gene-specific nucleotide substitution model parameters were used, with each gene allowed to evolve at a different rate (Zhang & Zhou, 2013). Rate variation among branches was modeled using uncorrelated lognormal relaxed clocks. A Yule process was used for the tree prior. Posterior distributions of the parameters, including the tree, were estimated via Markov chain Monte Carlo (MCMC) sampling. Two replicate MCMC runs were performed, with the tree and parameter values sampled every 1 000 steps over a total of 3×10^8 steps. The molecular evolutionary rate were fixed to 0.01 substitutions per site per million year (Myr) for mtDNA (*COI*) and 0.0004 substitutions per site per Myr for the nuclear ribosomal gene (5S ISR) according to Zarlenga *et al.* (2006).

Demographic history analysis

Demographic analysis was assessed through mismatch distribution using Arlequin v3.5 (Excoffier & Lischer, 2010) with the number of bootstrap replicates set to 5000. The validity of the expansion model was tested by using the sum of squared deviations (SSD) and Harpending's raggedness index (Rag) between observed and expected mismatches. The neutrality tests using Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) were also applied as an assessment of possible population expansion through Arlequin v3.5 (Excoffier & Lischer, 2010). The Bayesian skyline plot (BSP) was used to estimate the demographic history of *T. spiralis* population using the program BEAST v1.6.1 (Drummond & Rambaut, 2007). A piecewise-constant skyline model was selected, and a relaxed uncorrelated log-

normal molecular clock was used with the mutation rate of each marker as described above.

RESULTS

Nucleotide polymorphism

All amplifications were successful, with fragments 381 bp for *COI*, 407 bp for mt-lsrDNA and 710 bp for 5S ISR. A total of 11 variable sites were all parsimony-informative. These polymorphic sites identified 4 haplotypes within eleven isolates from ten localities. The haplotype diversity was high (0.764 ± 0.099), accompanied by low nucleotide diversity (0.00328 ± 0.00062). The genetic divergence of *COI* and 5S ISR sequences of Chinese *T. spiralis* isolates ranged from 0 to 0.3% and 0 to 1.3% respectively. However, all mt-lsrDNA sequences were identical, so this data set was excluded in the next analyses (Supplementary Tables S2–S4).

Phylogenetic pattern

Three tree-building methods based on the concatenated sequences (*COI* + 5S ISR) yielded consistent and complete phylogenetic resolution among all 11 *T. spiralis* isolates (Figure 1A). The tree topology suggested that the earliest diversifications among Chinese isolates gave rise to Henan isolates (HN-ay and HN-ny), then to Tibet isolate and Yunnan isolate. The next diversification event would have separated

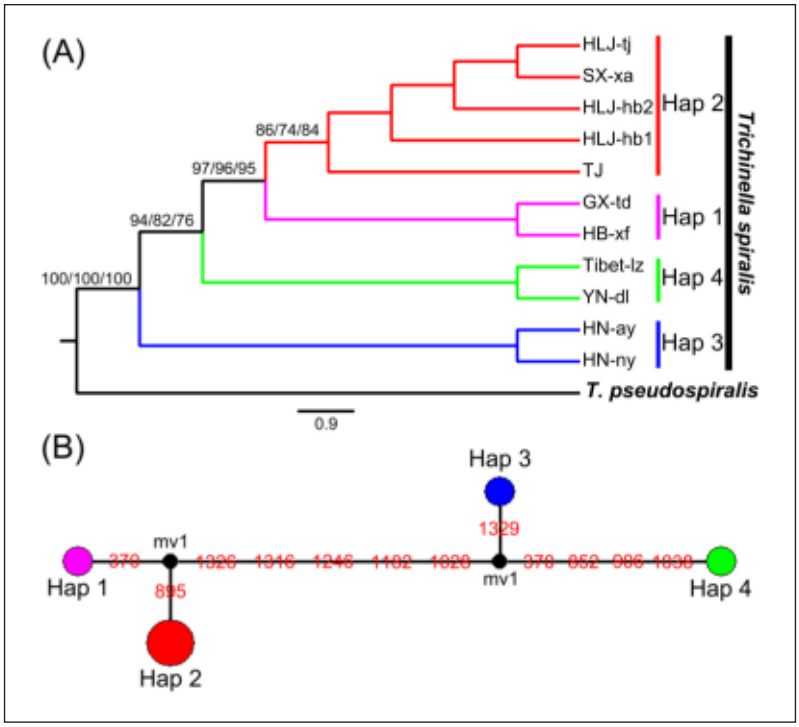

Figure 1. (A) Phylogenetic relationship among isolates of *Trichinella spiralis* from China inferred by neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses based on combined dataset (*COI* + 5S ISR), using *Trichinella pseudospiralis* as outgroup; numbers along branches indicate bootstrap values resulting from different analyses in the order: NJ/MP/ML. (B) Median-joining network of haplotypes of *T. spiralis* isolates; Each haplotype is represented by a circle, with the area of the circle proportional to its frequency; Numbers represent the mutational site; Median vector (mv1 and mv2) is indicated by black circle.

the remaining isolates (HB-xf, GX-td, SX-xa, TJ, HLJ-tj, HLJ-hb1 and HLJ-hb2). This relationship was supported with high bootstrap values (97, 96 and 95, respectively). The median-joining network analysis showing Hap2 was the most prominent haplotype which shared by samples from 4 geographical locations: Xian of Shaanxi province, Tianjin, Tongjiang and Harbin of Heilongjiang province (Figure 1B).

Age estimation

The results of the relaxed molecular clock analysis with concatenated data (Figure 2) suggested that the Chinese *T. spiralis* isolates started radiating in the late Miocene epoch (7.68Myr). The earliest divergence between the Tibet + Yunnan clade and the remaining isolates was estimated at about 6.07 Myr with a 95% highest posterior density (HPD) of 1.99–11.52 Myr. The early branching of the clade HB-xf + GX-td + TJ + SX-xa + HLJ-tj + HLJ-hb started at about 2.24 Myr (late Pliocene) with a 95% HPD of 0.48–5.18 Myr. And the divergence time between *T. spiralis* and *T. pseudospiralis* was estimated at the late Miocene (10.03 Myr, with a 95% HPD of 3.62–19.96 Myr).

Demographic history

Neutrality tests of Tajima's D and Fu's F_S revealed non-significant positive values for concatenated sequences of pooled samples, rejecting possible population expansion (Table 2).

We also calculated the sum of squared deviation and raggedness index under the demographic expansion model for the *T. spiralis* population and low and non-significant values were found (Table 2).

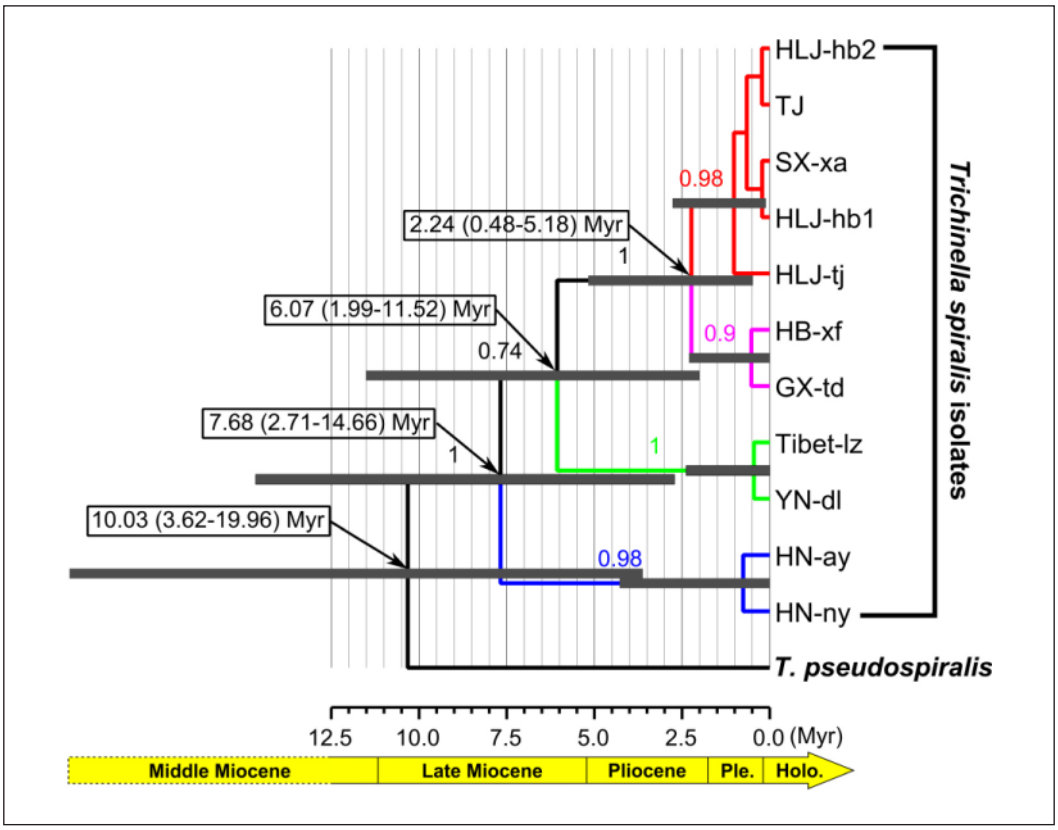

Figure 2. BEAST chronogram of *Trichinella spiralis* isolates from China; Grey bars at each node show 95% highest posterior density interval for the main nodes; Numbers above branches represent the Bayesian posterior probabilities, only posterior probabilities above 0.6 are shown; Numbers in the square frames indicate the estimated age and 95% confidence intervals (shown in parenthesis).

Table 2. Mismatch and neutrality tests results of *Trichinella spiralis* isolates from China

Phylogroups	Neutrality Tests		Mismatch	
	Fu's F_S	Tajima's D	SSD	Rag
Total population	3.26955 (0.93)	1.32162 (0.93)	0.10499 (0.08)	0.30182 (0.06)

SSD = Sum of Squared deviation, Rag = Harpending's Raggedness index. Significant level = 0.05. Number in parentheses is *P* value.

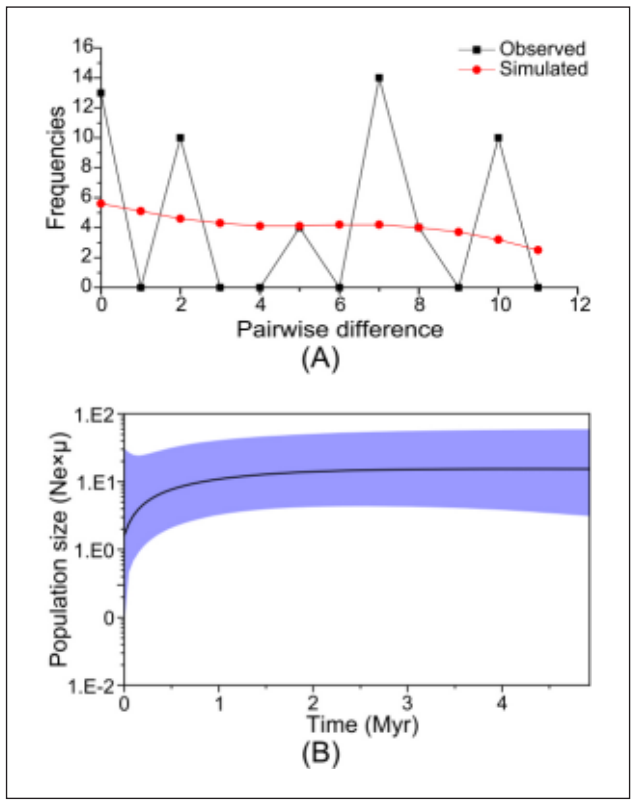

Figure 3. (A) Mismatch distribution analysis, the line charts represent the observed frequencies of pairwise differences among haplotypes. (B) A Bayesian skyline plot derived from *T. spiralis* isolates; The X-axis is in units of million years in the past and the Y-axis is Ne×ì (effective population size × mutation rate per site per generation), the median estimates are shown as thick solid lines, and the 95% highest posterior density (HPD) limits are shown by the blue areas.

Besides, the mismatch distribution analysis showed multimodal frequency distributions (Figure 3A). The Bayesian skyline plot analysis not only unsupported a sudden population expansion but also revealing a population decline after about 0.5 Myr ago (Figure 3B).

DISCUSSION

The nucleotide diversity value of 11 *T. spiralis* isolates from ten geographical locations of mainland China was below 0.01, indicating that there was low genetic variation among these isolates (Neigel &

Avise, 1993; Zhang *et al.*, 2015a). No apparent genetic structure was revealed in the median-joining network analysis, suggesting that *T. spiralis* isolates from China should be a single population (Forster *et al.*, 2001). Under the assumption of neutrality, a population expansion produces a negative value of Tajima's *D* and Fu's F_S tests (Fu, 1997; Tajima, 1989), however, non-significant positive values got in this study. Meanwhile, both multimodal frequency distributions of mismatch analysis and the Bayesian skyline plot analysis rejected a possible population expansion of Chinese *T. spiralis* population (Drummond *et al.*, 2005; Zhang *et al.*, 2015b).

The phylogenetic hypothesis and chronogram generated herein affords us an opportunity to explore the preliminary divergence pattern of *T. spiralis* isolates from China. Firstly, the Chinese *T. spiralis* isolates started radiating from the Henan province in the late Miocene (7.68 Myr), and then to the Yunnan province and Tibet 1.61 million years later (6.07 Myr). Next, the parasite diverged into two clades: one clade transmitted to Guangxi province and Hubei province at about late Pliocene (2.24 Myr), the other group spread to Shaanxi, Tianjin and Heilongjiang provinces. Nevertheless, the two mitochondrial genes (*COI* and mt-lsrDNA) used in this study seems too conserved to explore the phylogenetic diversity of *T. spiralis* population in China (The genetic divergence of *COI* only ranged from 0 to 0.3%, and all mt-lsrDNA genes were identical). Hence, in order to evaluate the *T. spiralis* genetic structure more accurately, more nuclear genes should be included, especially the microsatellite markers, which have been verified more suitable for inferring genetic differentiation of the *Trichinella* genus (Rosenthal *et al.*, 2008; La Rosa *et al.*, 2012).

In conclusion, a low genetic diversity of *T. spiralis* isolates was revealed by the DNA polymorphism analysis. Both mismatch analysis and the Bayesian skyline plot analysis rejected a possible population expansion of Chinese *T. spiralis* population. The phylogenetic pattern and BEAST analysis suggested that the Chinese *T. spiralis* isolates started radiating in the late Miocene.

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SUPPLEMENTARY MATERIALS

Table S1. Primers used to amplify the sequences studied.
Table S2. Uncorrected pairwise *p*-distance among the *COI* sequences of the *Trichinella spiralis* group.
Table S3. Uncorrected pairwise *p*-distance among the mt-lsrDNA sequences of the *Trichinella spiralis* group.
Table S4. Uncorrected pairwise *p*-distance among the 5S ISR sequences of the *Trichinella spiralis* group.

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