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Genetic variation among *Clonorchis sinensis* isolates from different geographic regions in China revealed by sequence analyses of four mitochondrial genes

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Abstract

The present study examined sequence variation in four mitochondrial (mt) genes, namely cytochrome *c* oxidase subunits 1 (*cox1*) and 2 (*cox2*), and NADH dehydrogenase subunits 1 and 2 (*nad1* and *nad2*) among *Clonorchis sinensis* isolates from different endemic regions in China, and their phylogenetic relationships with other zoonotic trematodes were reconstructed. A portion of the *cox1* and *cox2* genes (p*cox1* and p*cox2*), and *nad1* and *nad2* genes (p*nad1* and p*nad2*) were amplified separately from individual liver flukes by polymerase chain reaction (PCR) and the amplicons were subjected to sequencing from both directions. The intra-specific sequence variations within *C. sinensis* were 0–1.6% for p*cox1*, 0–1.4% for p*cox2*, 0–0.9% for p*nad1* and 0–1.0% for p*nad2*. Phylogenetic analyses based on the combined sequences of p*cox1*, p*cox2*, p*nad1* and p*nad2* revealed that all the *C. sinensis* isolates grouped together and were closely related to *Opisthorchis felineus*. These findings revealed the existence of intra-specific variation in mitochondrial DNA (mtDNA) sequences among *C. sinensis* isolates from different geographic regions, and demonstrated that mtDNA sequences provide reliable genetic markers for phylogenetic studies of zoonotic trematodes.

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Introduction

Mitochondrial DNA (mtDNA) sequences have been used extensively for studies of population or ecological genetics, and also for phylogenetic and evolutionary analyses at various taxonomic levels of different organisms, including nematodes, trematodes, cestodes and protozoans (M.W. Li *et al.*, 2008; Zhao *et al.*, 2009; Shekhovtsov *et al.*, 2010; Lin *et al.*, 2011; Liu *et al.*, 2011; Dai *et al.*, 2012), due to the maternal inheritance, rapid evolutionary rate and lack of recombination of mtDNA. However, there is limited information on the genetic variation in populations of some important parasite groups from China, such as the liver fluke *Clonorchis sinensis* infecting animals and humans.

Clonorchis sinensis is an important liver fluke parasite of the family Opisthorchiidae and it parasitizes the bile duct of many mammalian hosts, including humans. Clonorchiasis caused by *C. sinensis* is endemic in South-East Asia, including China, Vietnam, Japan and Korea (Lun *et al.*, 2005). It is estimated that approximately 35 million people globally are infected with *C. sinensis*, of whom about 15 million are in China (Lun *et al.*, 2005). *Clonorchis sinensis* has been considered to be carcinogenic to humans (Shin *et al.*, 2010).

Genetic variation is common in parasite populations, and it is a valuable resource for studying population biology, epidemiology and genetic structure of parasites (Gasser & Newton, 2000; Li *et al.*, 2008; Cerutti *et al.*, 2010). Recently, sequences of nuclear ribosomal DNA (rDNA) and mtDNA have been used to study genetic variations in helminths of human and animal health significance (M.W. Li *et al.*, 2008; Zhao *et al.*, 2009; J. Li *et al.*, 2010; Ai *et al.*, 2011). Although intra- and inter-specific variations have recently been studied using nuclear rDNA and mtDNA sequences among *C. sinensis* from different geographic regions (Park, 2007), there is a paucity of information on genetic variation among populations of *C. sinensis* from China, where *C. sinensis* infection remains an important human and animal health problem.

The objectives of the present study were to examine sequence variability in four mtDNA regions, namely cytochrome *c* oxidase subunits 1 (*cox1*) and 2 (*cox2*), and NADH dehydrogenase subunits 1 and 2 (*nad1* and *nad2*), among *C. sinensis* isolates from different endemic regions in China. Based on the combined sequences of these four mtDNA regions, phylogenetic relationships of *C. sinensis* in China with other zoonotic trematodes were also reconstructed.

Materials and methods

Parasites and isolation of genomic DNA

All adult worms of *C. sinensis* were obtained from naturally infected cats and dogs from different geographic locations in China. These *C. sinensis* were washed in physiological saline, identified preliminarily to species based on host preference, morphological characters and predilection sites (Kaewkes, 2003), fixed in 70% (v/v) ethanol and stored at −20°C until use. Their sample codes, gender and GenBank accession numbers are listed in table 1.

Enzymatic amplification and sequencing

A portion of the *cox1*, *cox2*, *nad1* and *nad2* were amplified by polymerase chain reaction (PCR) with primers listed in table 2. PCR reactions (25 ml) were performed in 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 200 mM each of deoxynucleoside triphosphate (dNTP), 50 pmol of each primer and 2 U *Taq* 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); 50°C (for p*cox1*) or 52°C (for p*cox2*) or 58°C (for p*nad1*) or 61°C (for p*nad2*) for 30 s (annealing); 72°C for 30 s (extension) for 35 cycles, followed by a final extension at 72°C for 10 min. These optimized cycling conditions for the specific and efficient amplification of individual mtDNA fragments were obtained after varying annealing temperatures. Samples without genomic DNA (no-DNA controls) and host genomic DNA (host-DNA controls) were included in each amplification run, and in no case were amplicons detected in the no-DNA and host-DNA controls (not shown). Each amplicon (5 μl) was examined by agarose gel (1%) electrophoresis to validate amplification efficiency. PCR products were sent to Sangon Company (Shanghai, China) for sequencing from both directions.

Sequence analysis and reconstruction of phylogenetic relationships

Sequences of the four mt 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 *C. sinensis* isolates 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).

Representative samples whose four gene sequences were available in this study were used for phylogenetic analyses. Three inference methods, namely, Bayesian analysis (Bayes), neighbour joining (NJ) and maximum likelihood (ML), were used for phylogenetic reconstructions. Bayesian analyses were conducted with four independent Markov chains run for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 1000 generations in MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003). The first 250 trees were omitted as burn-in and the remaining trees were used to calculate Bayesian posterior probabilities (PP). NJ analyses were carried out using the PAUP 4.0 Beta 10 program (Swofford, 2002), and ML analyses were performed using PhyML 3.0 (Guindon & Gascuel, 2003), and the general time reversible (GTR) model with its parameter for the concatenated dataset was determined for the ML analysis using JModeltest (Posada, 2008) based on the Akaike information criterion (AIC). Bootstrap support for ML trees was calculated using 100 bootstrap replicates. To study the phylogenetic relationships with other zoonotic trematode species, *C. sinensis* Russia isolate (FJ381664), *C. sinensis* Korea isolate (JF729304), *C. sinensis* China isolate (JF729303), *Schistosoma japonicum* (NC_002544), *S. mansoni* (NC_002545),

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Table 1. Geographic origins of *Clonorchis sinensis* samples used in the present study, as well as their GenBank accession numbers for partial sequences of mitochondrial *cox1* and *cox2* genes (p*cox1* and p*cox2*), and *nad1* and *nad2* genes (p*nad1* and p*nad2*).

Sample codes	Geographic origins	Hosts	Accession numbers			
			p*cox1*	p*cox2*	p*nad1*	p*nad2*
CSHP1	Guangxi (Hepu)	Cat	FJ965376	FJ965405	FJ965412	FJ965433
CSHP8	Guangxi (Hepu)	Cat	FJ965377	FJ965406	FJ965413	FJ965434
CSHP9	Guangxi (Hepu)	Cat	FJ965378	FJ965407	FJ965414	FJ965435
CSGXNN1	Guangxi (Nanning)	Dog	JN936214	JN936230	JN936246	JN936262
CSGXNN2	Guangxi (Nanning)	Dog	JN936215	JN936231	JN936247	JN936263
CSGXNN3	Guangxi (Nanning)	Dog	JN936216	JN936232	JN936248	JN936264
HJB	Heilongjiang (Jiamusi)	Cat	FJ965379	FJ965409	FJ965415	FJ965436
HJD	Heilongjiang (Jiamusi)	Cat	FJ965380	FJ965408	FJ965416	FJ965437
CSFSC17-1	Guangdong (Foshan)	Cat	FJ965389	FJ965400	FJ965425	FJ965445
CSFSC16-1	Guangdong (Foshan)	Dog	FJ965387	FJ965397	FJ965423	FJ965446
CSFSD10	Guangdong (Foshan)	Dog	FJ965388	FJ965398	FJ965422	FJ965447
CSXXC5-2	Guangdong (Xinxing)	Cat	FJ965391	FJ965402	FJ965427	FJ965438
CSXXC7-2	Guangdong (Xinxing)	Cat	FJ965390	FJ965401	FJ965426	FJ965439
CSXXD5-1	Guangdong (Xinxing)	Dog	FJ965392	FJ965403	FJ965428	FJ965440
CSXXD12-2	Guangdong (Xinxing)	Dog	FJ965393	FJ965404	FJ965429	FJ965441
CSSGD3-2	Guangdong (Shaoguan)	Dog	FJ965385	FJ965395	FJ965431	FJ965443
CSSGD12-1	Guangdong (Shaoguan)	Dog	FJ965384	FJ965394	FJ965430	FJ965442
CSSGD11-2	Guangdong (Shaoguan)	Dog	FJ965386	FJ965396	FJ965432	FJ965444
CSGDYC1	Guangdong (Yangchun)	Dog	JN936207	JN936223	JN936239	JN936255
CSGDYC2	Guangdong (Yangchun)	Dog	JN936208	JN936224	JN936240	JN936256
CSGDYC3	Guangdong (Yangchun)	Dog	JN936209	JN936225	JN936241	JN936257
CSGDSZ1	Guangdong (Shenzhen)	Dog	JN936210	JN936226	JN936242	JN936258
CSGDSZ2	Guangdong (Shenzhen)	Dog	JN936211	JN936227	JN936243	JN936259
CSGDSZ3	Guangdong (Shenzhen)	Dog	JN936212	JN936228	JN936244	JN936260
CSGDMZ1	Guangdong (Meizhou)	Dog	JN936213	JN936229	JN936245	JN936261
CSGDQY1	Guangdong (Qingyuan)	Dog	JN936217	JN936233	JN936249	JN936265
CSGDQY2	Guangdong (Qingyuan)	Dog	JN936218	JN936234	JN936250	JN936266
CSGDQY3	Guangdong (Qingyuan)	Dog	JN936219	JN936235	JN936251	JN936267
CSGDZQ1	Guangdong (Zhaoqing)	Dog	JN936220	JN936236	JN936252	JN936268
CSGDZQ2	Guangdong (Zhaoqing)	Dog	JN936221	JN936237	JN936253	JN936269
CSGDZQ3	Guangdong (Zhaoqing)	Dog	JN936222	JN936238	JN936254	JN936270

S. haematobium (DQ157222), *Opisthorchis felineus* (EU921260), *O. viverrini* (JF739555), *Fasciola hepatica* (NC_002546) and *Paragonimus westermani* (NC_002354) were included in the present study, with *Ascaris suum* (NC_001327) as the outgroup. Phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

Results and discussion

Genomic DNA was prepared from 31 individual adult *C. sinensis* isolates from three endemic provinces in mainland China. Amplicons of p*cox1*, p*cox2*, p*nad1* and p*nad2* (~420, 460, 480 and 580 bp, respectively) were amplified individually and subjected to agarose gel electrophoresis. For each mtDNA region, no size variation was detected on agarose gel among any of the amplicons examined (not shown).

To examine sequence variation in the four mtDNA regions among *C. sinensis* isolates from different endemic provinces, amplicons of p*cox1*, p*cox2*, p*nad1* and p*nad2* representing different endemic types were subjected to direct sequencing. The sequences of p*cox1*, p*cox2*, p*nad1* and p*nad2* were 381 bp, 420 bp, 432 bp and 531 bp in length, respectively. The A + T contents of the sequences were 57.74–58.53% (p*cox1*), 59.05–59.76% (p*cox2*), 60.65–61.34% (p*nad1*) and 63.16–63.74% (p*nad2*), respectively. The intra-specific sequence variations within populations of all *C. sinensis* isolates were 0–1.6% for p*cox1*, 0–1.4%

Table 2. Sequences of primers used to amplify a portion of the mitochondrial *cox1* and *cox2* genes (p*cox1* and p*cox2*), and *nad1* and *nad2* genes (p*nad1* and p*nad2*) from *Clonorchis sinensis* isolates in China. These primers were designed on mt genomes of *C. sinensis*.

Name of primer	Sequence (5′ to 3′)
For p*cox1*	
Cox1u1	TAATGAAAATGAGCAATAC
Cox1d1	CATCCTGAGGTTTATGTGT
For p*cox2*	
Cox2u1	GTGAGTTTTTTTAGTGCTT
Cox2d1	TAATACTACAGCTTTAGGC
For p*nad1*	
Nad1u1	ATTCGTAAGGGGCCTAATAAG
Nad1d1	CTCACAAAGCATACCAGCAAG
For p*nad2*	
Nad2u1	GCTCTTTCTTGAGTTGGCTTCCT
Nad2d1	AACTGCGGCACTCGTCTCCAT

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Table 3. Number and position of codon variations in a portion of the mitochondrial *cox1* and *cox2* genes (p*cox1* and p*cox2*), and *nad1* and *nad2* genes (p*nad1* and p*nad2*) among *Clonorchis sinensis* isolates in China.

		Variable nucleotide positions[a]			
mtDNA region	Length (bp)	Codon 1	Codon 2	Codon 3	Total no.
p*cox1*	381	3	1	12	16
p*cox2*	420	4	1	12	17
p*nad1*	432	4	0	11	15
p*nad2*	513	8	3	16	27

[a] The first codon position of each sequence was determined in relation to the complete mitochondrial DNA sequence of *C. sinensis* (Shekhovtsov *et al.*, 2010).

for p*cox2*, 0–0.9% for p*nad1* and 0–1.2% for p*nad2*. These results were consistent with that of previous studies for this parasite (Park, 2007; Lai *et al.*, 2008). Similarly, genetic variability has also been detected among populations of the other members of the Opisthorchiidae, such as *Opisthorchis viverrini* and *O. felineus*, by analysis of mtDNA sequences (Katokhin *et al.*, 2008; Saijuntha *et al.*, 2008).

For p*cox1*, p*cox2*, p*nad1* and p*nad2*, intra-specific nucleotide variation was related mainly to changes at the first and third codon positions, consistent with results of other helminths, including trematodes and cestodes (M.W. Li *et al.*, 2008; Zhao *et al.*, 2009; Dai *et al.*, 2012). For p*cox1* and p*cox2*, there was only one change at the second codon position. For p*nad1*, there was no change in the second codon (table 3). Intra-specific nucleotide variations represented transitions (A ↔ G or C ↔ T: $n = 13$ for p*cox1*, $n = 15$ for p*cox2*, $n = 13$ for p*nad1* and $n = 20$ for p*nad2*) and transversions (A ↔ C, A ↔ T, and/or T ↔ G: $n = 3$ for p*cox1*, $n = 2$ for p*cox2*, $n = 2$ for p*nad1*, $n = 7$ for p*nad2*) (table 3).

The combined sequences of p*cox1*, p*cox2*, p*nad1* and p*nad2* were aligned over a consensus length of 1764 bp. Topologies of all trees inferred by different methods (NJ, MP and ML) with different building strategies and/or different distance models were identical or similar, with only the small difference of bootstrap values (fig. 1). In this tree, the Schistosomatidae, Fasciolidae, Paragonimidae and Opisthorchiidae form monophyletic groups, respectively. Within the clade consisting of the Fasciolidae, Paragonimidae and Opisthorchiidae, all the isolates of *C. sinensis* were sister to *O. felineus*. These results were consistent with that of a previous study using internal transcribed spacer (ITS) and *cox1* sequences (Kang *et al.*, 2008), but were not consistent with that of a previous study (Cai *et al.*, 2012) using concatenated amino acid

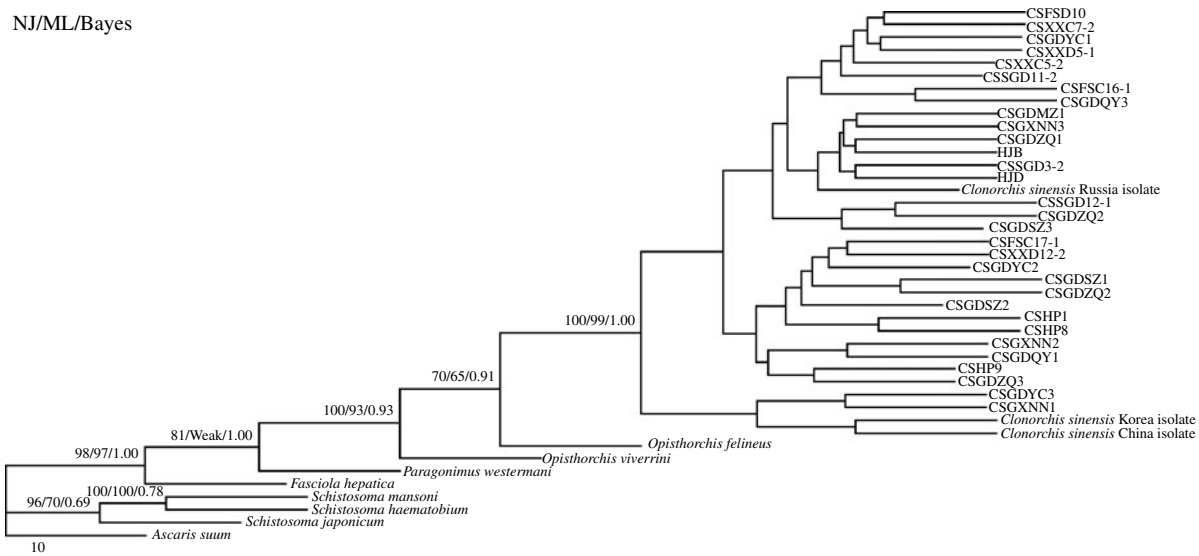

Fig. 1. Phylogenetic relationship of *Clonorchis sinensis* isolates from mainland China with other zoonotic trematodes inferred by Bayesian (Bayes), maximum likelihood (ML) and neighbour-joining (NJ) analyses using the combined dataset (*cox1* + *cox2* + *nad1* + *nad2*), with *Ascaris suum* as outgroup. Bootstrap values (in percentage) above 50% from 1000 pseudo–replicates are shown for the neighbour-joining (the first value), maximum likelihood (the second value) and Bayesian analyses (the third value). Weak = node resolved by method but very weak (<50%).

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sequences of the 12 protein-coding genes, which showed that *C. sinensis* was more closely related to *O. viverrini* than to *O. felineus*. Here, we will not discuss the phylogenetic relationships between *O. viverrini* and *C. sinensis* further, since phylogenetic relationships are not robustly supported. However, our results provided some useful information for future studies.

In conclusion, genetic variations among *C. sinensis* isolates from different geographic regions in China were revealed by sequence analyses of mt *cox1*, *cox2*, *nad1* and *nad2*, and demonstrated that mitochondrial DNA sequences provide reliable genetic markers for phylogenetic studies of zoonotic trematodes. For the four mt DNA genes, sequence variation was higher in p*cox1* than in p*cox2*, p*nad2* and p*nad1*.

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