## RESEARCH NOTE

# MITOCHONDRIAL GENE SEQUENCES AMONG DIFFERENT GEOGRAPHICAL ISOLATES OF *SCHISTOSOMA JAPONICUM* IN YUNNAN PROVINCE, CHINA

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**Abstract.** In order to evaluate differentiate genetic differences among *Schistosoma japonicum* isolates from Dali Ancient City, Xizhou and Yongsheng County, Yunnan Province, China, mitochondrial *co1, cytb*, *nd1*, *nd6*, and *nd4l* were PCR amplified and sequenced, revealing nucleotide difference(s) among these strains of 8, 1, 5, 4, and 0, respectively. Phylogenetic analysis showed that *S. japonicum* from the three different geographical locations of Yunnan Province were clustered genetically together and were more similar to *S. malayensis* and *S. mekongi* than *S. haematobium* or *S. mansoni*. For intra-species differentiation purposes, *Schistosoma* mitochondrial *co1, nd1*, and *nd6* are better genetic markers than *cytb* and *nd4l*.

**Keywords:** *Schistosoma japonicum*, mitochondrial gene, phylogenetic tree, Yunnan Province, China

## INTRODUCTION

Schistosomiasis, caused by Schistosoma, is one of the most important tropical diseases in the world in terms of public health impact, second only to malaria (Eleanor *et al*, 2015). Of the six schistosome species infecting humans (Williams, 2008) *Schistosoma japonicum* is mainly epidemic in China, eastern Indonesia and Philippines, with China accounting two-third of the endemic areas in Asia (Lier, 2006). *S. japonicum* infection is an important disease that threatens people's health and hinders economic and social development in epidemic areas (Zeng *et al*, 2009; Zheng, 2009).

Over the years research on *S. japonicum* genetic markers have indicated that on mainland China it is not a single strain, but consists of four strains, namely, Anhui-Hubei, Guangxi, Sichuan and Yunnan. So far, it is unclear whether all four strains exist in every China mainland geographical endemic region. In addition, techniques to distinguish among the strains is still in the exploratory stage. With the development of molecular genetic technology and its application in parasitology, a number of genetic markers have been used to study the genetic variations of *S. japonicum* populations (Zheng and Li, 2014). Mitochondrial genomes of different organisms

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show great diversity in number of genes and gene arrangements, and as the rate of evolution of mtDNA is 5-10 times faster than that of nuclear DNA, mtDNA has become an important source for studying the origin and evolution of species (Xia and Wang, 1998; Zhang and Mi, 1998; Gou and Wang, 1999; Saccone *et al*, 1999). *S. japonicum* mitochondrial (mt)genes encoding cytochrome c oxidase subunit 1 (CO1), cytochrome c oxidase subunit 2 (CO2), cytochrome c oxidase subunit 3 (CO3), NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2 (ND2), NADH dehydrogenase subunit 3 (ND3), NADH dehydrogenase subunit 4 (ND4), NADH dehydrogenase subunit 4L (ND4L) and adenosine triphosphatase 6 (ATP6) have been used to study genetic variation (Yin and Hu, 2007). However, less is known regarding differences in strains of parasites from various endemic geographical regions with their unique ecological environments within the same province.

This study employed mitochondrial gene sequences of *S. japonicum* obtained from different geographical regions of Yunnan Province, a mountainous endemic area, which is the focus of schistosomiasis prevention and control in China, to explore intra-species variations.

## MATERIALS AND METHODS

### Acquisition of natural isolates of adult *S. japonicum*

Naturally *S. japonicum*-infected snails collected from Dali Ancient City, Xizhou and Yongsheng County (schistosomiasis epidemic areas), Yunnan Province, China were placed (3 from each location) into glass tubes filled with chlorinated water and kept in a sunny environment at 25°C. Shedding of *S. japonicum* cercariae was observed under a light microscope (252x magnification) over a period of 2-3 hours. Then, rabbits were infected each with 200-250 cercariae (3 animals per region of snail collection). Adult *S. japonicum* parasites were isolated 42 days later as previously described (Li *et al*, 2005), washed in normal saline, separated into males and females, and stored at -80°C until used.

### PCR

Genomic DNA was extracted using TaKaRa MiniBest universal genomic DNA extraction kit (TaKaRa, Kyoto, Japan) and stored at -80°C until used. Five primer pairs (Table 1) were designed using GeneTool software (Genebio, Geneva, Switzerland ) based on the sequence of reference *S. japonicum* (GenBank accession no. NC_002544) and synthesized by Shanghai Sheng Gong Biological (Shanghai, China). PCR (25 μl) contained 17.5 μl of distilled $H_2O$, 2.5 μl of 10× PCR buffer (Shanghai Sheng Gong Biological, Shanghai, China), 1.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 0.8 μl of 0.02 mM primer pairs, 2 μl of template DNA, and 0.2 μl of 5U *Taq* polymerase (TaKaRa, Kyoto, Japan). Thermocycling, conducted in PCR amplification instrument (Type 2720, Applied Biosystem, Foster City, CA), was performed as follows: 94°C for 5 minutes; followed by 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 1 minute; with a final step at 72°C for 10 minutes. Amplicons were analyzed by 1% agarose gel-electrophoresis, stained with ethidium bromide (Aresco, Solon, OH) and observed under UV illumination. Samples were stored at -80°C until used.

### Nucleotide sequence determination and phylogenetic tree construction

Amplicons were extracted and purified from agarose gel using DNA purification kit (TaKaRa, Kyoto, Japan) according to the manufacturer's protocol.

Table 1
PCR primers used in amplification of *S. japonicum* mitochondrial genes.

| Gene | Primer (5′ - 3′) |
|---|---|
| *co1* | F: CGGTTACGTTGGTGAATAGAGG |
| | R: ATCATAAGCCATTCGGGAACTAG |
| *nd1* | F: GAATCGGAGTTTGTCAGGCTTTAG |
| | R: TCTCGGCTAAATATAACAACAAGTCA |
| *nd6* | F: TGGTGTCTTECGTCGGTTATTG |
| | R: GCCGATTAAACTTCAACCTACACA |
| *nd4l* | F: GGGGTTGTCATGCGGAGTATC |
| | R: ACGCCCACCATTACCATAGAAC |
| *cytb* | F:GCCAGGTGTGATGTGCATATAGA |
| | R: TGAAAACAACTTGACAATCCTGAA |

F, forward; R, reverse.

Table 2
GenBank accession numbers of *S. japonicum* mitochondrial gene fragments.

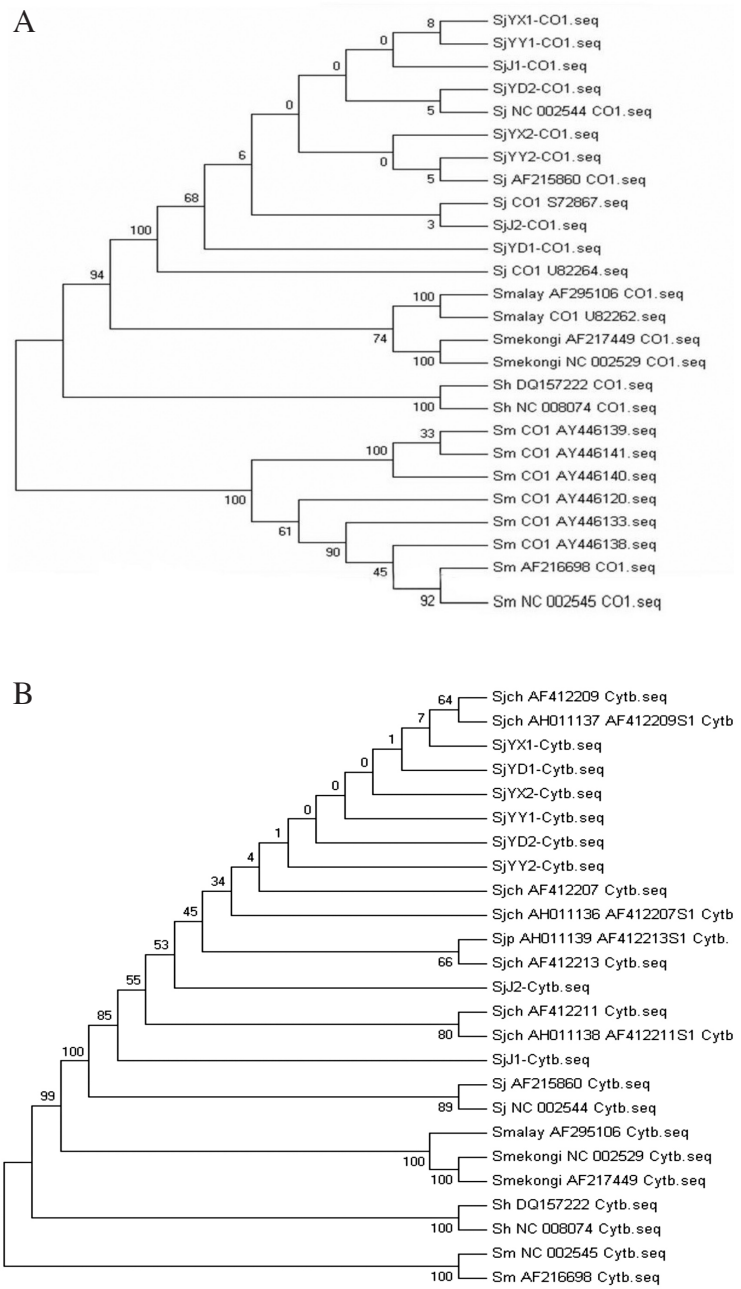
| Location in Yunnan Province, China | Gene/Accession number | | | | |
|---|---|---|---|---|---|
| | *co1* | *cytb* | *nd1* | *nd6* | *nd4l* |
| Dali Ancient City | EU340357 | EU325886 | EU325882 | EU136136 | EU199446 |
| | EU325879 | EU325887 | EU325885 | | |
| | EU325878 | | EU325880 | | |
| | | | EU325881 | | |
| Xizhou | EU340358 | EU325888 | EU325883 | | |
| | EU325891 | EU325888 | EU325884 | | |
| | | EU325890 | EU340361 | | |
| Yongsheng County | EU340353 | EU340354 | EU340349 | EU325894 | |

Nucleotide sequencing was conducted using Applied Biosystems 3130 Genetic Analyzer (Foster City, CA). Sequences were aligned and compared with those in GenBank database using DNAstar software (DNASTAR, Madison, WI) and phylogenetic tree constructed using Mega 3.1 software.

## RESULTS

Amplicons of *S. japonicum* (from Dali Ancient City, Xizhou and Yongsheng County, schistosomiasis epidemic areas of Yunnan Province, China) mitochondrial gene encoding CO1, CYTb, ND1, ND6 and ND4L were of the expected size of 1,810, 1,457, 1,146, 653, and 526 bp, respectively (data not shown). The nucleotide sequences of these amplicons from the three geographical regions were submitted to GenBank and their respective accession numbers are shown in Table 2. Sequences of the same mitochondrial gene fragment from the three regions were 99-100% identical. There were 8 different nucleotides among *co1* fragments, 1 among *cytb*, 5 among *nd1*, and 4 among *nd6* but no differences among *nd4l* in

strains from the three regions (data not shown). Phylogenetic trees constructed from the mitochondrial gene fragments of this study and those deposited in GenBank revealed that at the inter-species level the five mitochondrial gene of *S. japonicum* from Yunnan Province differed significantly from those of *S. haematobium* and *S. mansoni,* but genetically closer to *S. malayensis*, and *S. mekongi*; and at the intra-species level, the five *S. japonicum* mitochondrial gene from the three regions



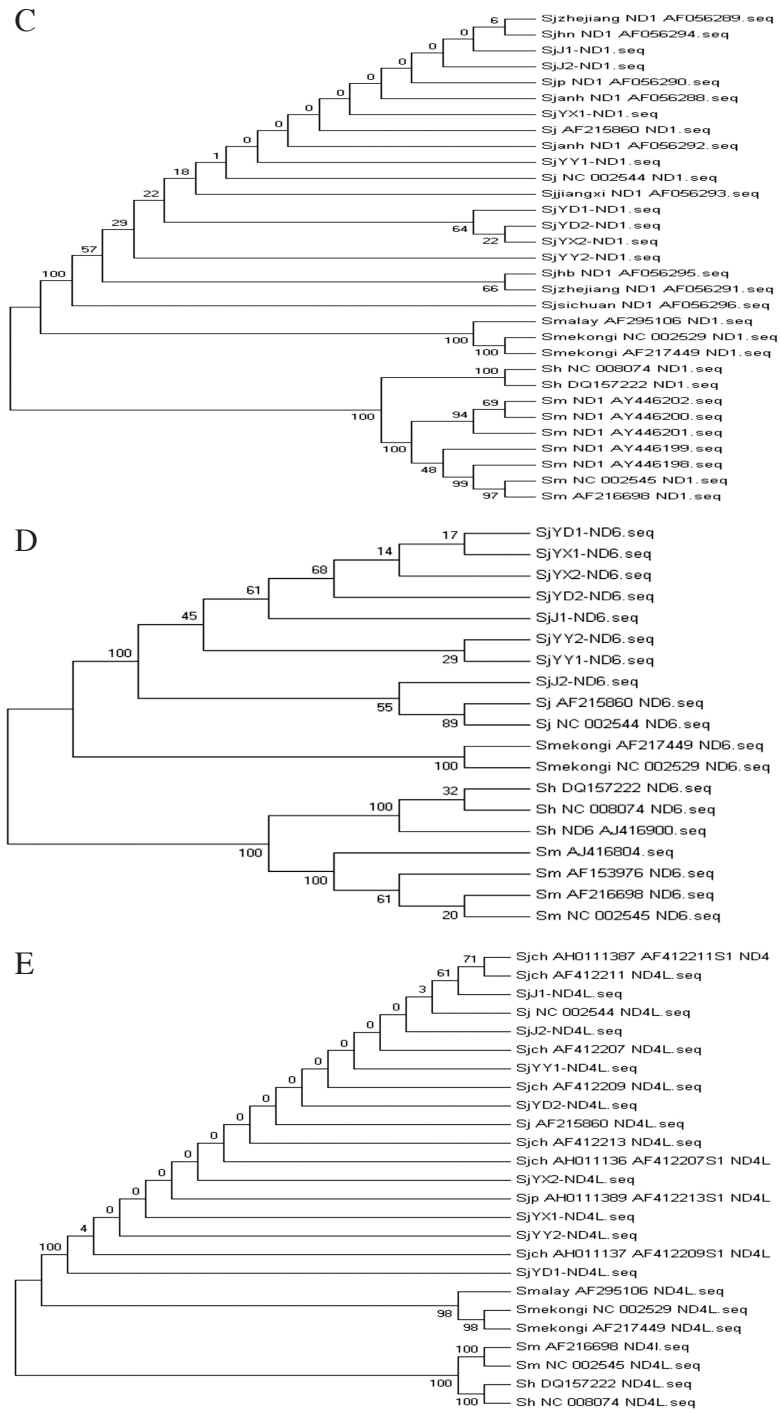



Fig 1–Phylogenetic tree of *Schistosoma* spp based on mitochondrial *co1*(A), *cytb*(B), *nd1*(C), *nd6*(D), *nd4l*(E) sequences obtained from this study and from GenBank. The trees were constructed by using Neighbor-Joining method performed by Mega 3.1 software. Numbers at each branch show bootstrap value.

of Yunnan were clustered together and did not form independent branches (Fig 1).

## DISCUSSION

Variations in mitochondrial *co1* sequences have been found among *S. japonicum* isolates from seven different provinces in China (Bogh *et al*, 1999), as well as between strains from mainland China and Taiwan (Guo and Niu, 2004a). *S. japonicum* strains on mainland China can be divided into two groups, namely, strains from mountainous regions and those from lakes and marshlands (Guo and Niu, 2004a,b,c).

However, there has been, to date, no study in China of genetic variation of *S. japonicum* strains from different locations within the same province. Yunnan Province is located in southwest China and contains lofty mountains and steep hills, with diverse geographical environment. Schistosomiasis endemic areas in Yunnan Province are distributed as isolated localities. The current study explored sequences of five mitochondrial gene fragments (*co1, cytb, nd1, nd6,* and *nd4l*) from DaLi Ancient City, Xizhou and Yongsheng. These five genes from the three locations had high homologies, which is understandable for strains from DaLi Ancient City and Xizhou as these two localities are in Dali Prefecture, with an altitude of 1,973 m and 1,956 m above sea level, respectively and separated by 10 km; but Yongsheng is in Lijiang Prefecture, at an altitude of 1,900 m and located 200 km from Dali Prefecture. The reasons for this observation need to be explored further.

Sequence variations of *co1* and *nd1* were more significant among *S. japonicum* strains from the three locations in Yunnan Province than those of other three mitochondrial gene. These results were in accordance with previous reports (Bogh *et al*, 1999; Qiu *et al*, 2002; Guo and Niu, 2004a), but the sequence variations in *co1* and *nd1* found in the current study were less than those in the previous reports. It is worth noting that in the previous studies, *S. japonicum* came from different geographical regions of China. This indicates that *co1* and *nd1* are more suitable for intraspecific classification of *S. japonicum* from relatively distant geographic isolates. However, further studies are needed to confirm this suggestion. The very limited sequence variations of *nd4l* and *cytb* suggest that these two mitochondrial gene may not be suitable for *S. japonicum* intraspecific classification.

Phylogenetic analysis based on *S. japonicum co1, cytb, nd1, nd6,* and *nd4l* sequences revealed closer genetic relationship with *S. malayensis* and *S. mekongi*, being of Southeast Asian origin, than *S. haematobium* and *S. mansoni*, being of African origin. *S. japonicum* strains from DaLi Ancient City, Xizhou and Yongsheng were clustered together genetically. This may be due to the similar ecological environment in these three locations being conducive to the growth and dispersal of the intermediate snail host (*Oncomelania hupensis*), which live along the mountain streams, walls and bottoms of irrigation channels, soggy grass and terrace terrains. In addition, mountains form natural barriers against migration of snails and people. Another possible explanation for the genetic similarity of *S. japonicum* collected from these three regions of Yunnan Province is the lack of sufficient genetic diversities in the mitochondrial gene used in the study. Future studies involving analysis of other *S. japonicum* mitochondrial gene need to be conducted, as well as comparison with other regions of Yunnan Province.

In summary, *S. japonicum* from three different geographical locations of Yunnan Province were clustered genetically together and were more similar to *S. malayensis* and *S. mekongi* than *S. haematobium* or *S. mansoni*. For intra-species differentiation purposes, *Schistosoma* mitochondrial *co1*, *nd1*, and *nd6* are better genetic markers than *cytb* and *nd4l*.

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