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Genetic variability among *Schistosoma japonicum* isolates from different endemic regions in China revealed by sequences of three mitochondrial DNA genes

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ABSTRACT

The present study examined sequence variation in three mitochondrial DNA (mtDNA) regions, namely cytochrome *c* oxidase subunit 3 (*cox*3), NADH dehydrogenase subunits 4 and 5 (*nad*4 and *nad*5), among *Schistosoma japonicum* isolates from different endemic regions in China, and their phylogenetic relationships were re-constructed. A portion of the *cox*3 gene (p*cox*3), a portion of the *nad*4 and *nad*5 genes (p*nad*4 and p*nad*5) were amplified separately from individual trematodes by polymerase chain reaction (PCR) and the amplicons were subjected to direct sequencing. In the mountainous areas, sequence variations between parasites from Yunnan and those from Sichuan were 0.3% for p*cox*3, 0.0–0.1% for p*nad*4, and 0.0–0.2% for p*nad*5. In the lake/marshland areas, sequence variations between male and female parasites among different geographical locations were 0.0–0.3% for p*cox*3, 0.0–0.7% for p*nad*4, and 0.0–1.6% for p*nad*5. Sequence variations between *S. japonicum* from mountainous areas and those from lake/marshland areas were 0.0–0.5% for p*cox*3, 0.0–0.7% for p*nad*4, and 0.0–1.6% for p*nad*5. Phylogenetic analyses based on the combined sequences of p*cox*3, p*nad*4 and p*nad*5 revealed that *S. japonicum* isolates from mountainous areas (Yunnan and Sichuan provinces) clustered together. For isolates from the lake/marshland areas, isolates from Anhui and Jiangsu provinces clustered together and was sister to samples from Jiangxi province, while isolates from Hubei and Zhejiang province clustered together. However, isolates from different geographical locations in Hunan province were in different clades. These findings demonstrated the usefulness and attributes of the three mtDNA sequences for population genetic studies of *S. japonicum*, and have implications for studying population biology, molecular epidemiology, and genetic structure of *S. japonicum*, as well as for the effective control of schistosomiasis.

1. Introduction

Mitochondria are involved in respiratory metabolism in most eukaryotes which rarely enter or persist in the zygote, and thus mitochondrial (mt) DNA is generally inherited maternally in almost all metazoans (Le et al., 2000a,b, 2002). The mt genome is considered to be clonal and rarely or never undergoes recombination. Sequences generated from the mt genome provide excellent molecular markers for defining population groups, for tracing the genetic history of an individual or a particular group of related individuals, and for re-constructing deep-branch

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taxonomic phylogenies (Avise et al., 1987; Wolstenholme, 1992; Boore et al., 1995; Avise and Wollenberg, 1997; Rollinson et al., 1997; Boore, 1999; Le et al., 2000a,b). However, there is a paucity of information on the genetic variation in populations of some important parasite groups, such as *Schistosoma japonicum*, which is one of the etiological agents for human and animal schistosomiasis.

Schistosomiasis is a global disease that remains a major public health problem in many countries of the developing world (Li et al., 2000; Gryseels et al., 2006). Of the three major schistosome species infecting humans, Asian or Oriental schistosomiasis, caused by *S. japonicum*, is recognized as the most difficult to control because of its zoonotic nature. In the mid-1950s, schistosomiasis was endemic in China's 12 southern provinces, approximately 12 million people were infected, and millions of them got killed (Zhou et al., 2005). After approximately 50 years of continued control efforts, schistosomiasis has been eliminated in five of the 12 previously endemic provinces, and the prevalence of schistosomiasis also dropped significantly in the remaining seven endemic provinces (Utzinger et al., 2005; Zhou et al., 2005). However, since the end of the World Bank Loan Project (WBLP), the number of human cases has been increasing again, up to approximately 850,000 in 2003 (Zhou et al., 2005). A new national survey carried out in 2004 confirmed the re-emergence of schistosomiasis in China (Engels et al., 2005). Hence, in 2004, China re-defined schistosomiasis control as one of three highest priorities in communicable disease control-together with the control of HIV/AIDS and tuberculosis (Zhou et al., 2008).

mtDNA provides valuable markers for studies on flatworms (Le et al., 2000c). Earlier studies using partial cytochrome *c* oxidase subunit 1 (p*cox*1) gene (Bowles et al., 1993) and NADH dehydrogenase 1 (p*nad*1) gene (Sørensen et al., 1998, 1999; Attwood et al., 2002) helped to establish the population and genetic relationship among *Schistosoma* species and subpopulation relationships within *S. japonicum*. However, the finding that biologically distinct geographical populations of *S. japonicum* showed very little variation in the mitochondrial p*nad*1 (Sørensen et al., 1998) and p*cox*1 gene (Bowles et al., 1993), and computer simulation analysis indicated that *cox*1 gives high support values and correct topology, but has a low level (31%) of phylogenetically informative characters (Zarowiecki et al., 2007), so p*cox*1 and p*nad*1 would not be the ideal marker for either species identification (bar coding) or population studies within *Schistosoma* species. Instead, for accurate identification (sufficient variability) and a consistent phylogenetic signal (sufficient number of phylogenetically informative characters per total length), cytochrome *c* oxidase subunit 3 gene (*cox*3) is the preferred gene, with high variability in characters, but not in length, and NADH dehydrogenase subunits 4 and 5 genes (*nad*4 and *nad*5) also have more characters of phylogentic information and variability (Zarowiecki et al., 2007). Therefore, they would provide better markers for both phylogenetic and population studies.

The objectives of the present study were to examine sequence variability in three mitochondrial DNA (mtDNA) regions, namely *cox*3, *nad*4 and *nad*5, among *S. japonicum* isolates from different endemic regions in mainland China. Based on the combined sequences of these three mtDNA regions, phylogenetic relationships of *S. japonicum* in mainland China were also re-constructed.

2. Materials and methods

2.1. Parasites and isolation of genomic DNA

S. japoanicum isolates were collected from different geographical locations in mainland China (Fig. 1), and sample codes, gender and GenBank™ accession number are listed in Table 1. All the adult parasites were collected from rabbits experimentally infected with cercariae from infected snails *Oncomelania hupensis*. The male and female adult parasites were stored in 70% molecular grade ethanol, and stored at −20 °C before extraction of genomic DNA. Total genomic DNA was extracted from individual samples by SDS/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega) and eluted into 60 μl H_2O according to the manufacturer's recommendations.

2.2. Enzymatic amplification and sequencing

A portion of the *cox*3 gene (p*cox*3) was amplified with primers Sjcou1 and Sjcod1, part of the *nad*4 gene (p*nad*4) with primers Zghnd4u and Zghna4d, and part of the *nad*5 gene (p*nad*5) with primers p1F and p1R (Zarowiecki et al., 2007) (Table 2). PCR reactions (25 μl) were performed in 2 mM of $MgCl_2$, 2.5 μM of each primer, 2.5 μl 10× 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 °C for 5 min, then 94 °C for 30 s (denaturation); 48 °C (for p*cox*3) or 51 °C (for p*nad*4 and p*nad*5) 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

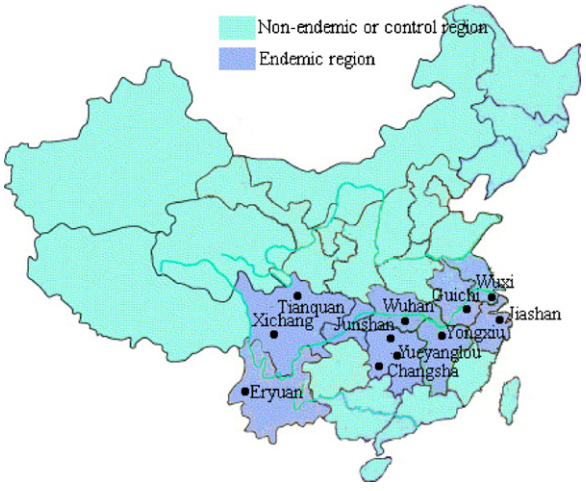

Fig. 1. The sampling locations for *Schistosoma japoniucm* isolates in different regions of China.