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Comparative Analysis of 18S and 28S rDNA Sequences of *Schistosoma japonicum* from Mainland China, the Philippines and Japan

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Abstract: In the present study, a portion of the 18S and 28S ribosomal DNA (rDNA) sequences of 35 *Schistosoma japonicum* isolates representing three geographical strains from mainland China, the Philippines and Japan were amplified and compared and phylogenetic relationships were also reconstructed by Unweighted Pair-Group Method with Arithmetic averages (UPGMA) using combined 18S and 28S rDNA sequences as well as the corresponding sequences of other species belonging to the Schistosoma genus available in the public database. The results indicated that the partial 18S and 28S rDNA sequences of all *S. japonicum* isolates were 745 and 618 bp, respectively and displayed low genetic variation among *S. japonicum* strains and isolates. Phylogenetic analysis revealed that the combined 18S and 28S rDNA sequences were not able to distinguish *S. japonicum* isolates from three geographical origins but provided an effective molecular marker for the inter-species phylogenetic analysis and differential identification of different *Schistosoma* species.

Key words: *Schistosoma japonicum*, 18S rDNA, 28S rDNA, phylogenetic analysis, mainland China, Philippines, Japan

INTRODUCTION

Schistosomiasis caused by trematodes of the genus *Schistosoma* is a parasitic disease of medical and veterinary importance in a number of countries with an estimated approximately 200 million people being infected and approximately 200,000 deaths per year (Chitsulo *et al*., 2004; Taylor, 2008; Huyse *et al*., 2009). Among six human schistosomes (*Schistosoma japonicum*, *Schistosoma haematobium*, *Schistosoma mansoni* (*Schistosoma intercalatum* = *S. guineensis*), *Schistosoma mekongi* and *Schistosoma malayensis*), three main species (*S. japonicum*, *S. haematobium* and *S. mansoni*) are reported to have significant human health and social-economic impact (Zhao *et al*., 2009a). *Schistosomiasis japonica*, one of the most serious schistosomiasis caused by *S. japonicum* was mainly distributed in China, Japan, the Philippines and parts of Indonesia and caused various degrees of morbidity and mortality (Zhou *et al*., 2005; Zhao *et al*., 2009a, b). In addition to the six human schistosomes, other 13 *Schistosoma* species have been identified as parasites in animals. *S. hippopotami* and *S. edwardiense* are found in hippopotamus (Morgan *et al*., 2003), *S. mattheei*, *S. bovis*, *S. leiperi*, *S. margrebowiei*, *S. curassoni*, *S. indicum*, *S. spindale* and *S. nasale* found in ruminant animals such as cattle, buffalo, sheep, goat and lechwe (De Bont *et al*., 1994; Vercruysse *et al*., 2003; Singh *et al*., 2004; Vercruysse and Gabriel, 2005; Littlewood *et al*., 2006; Sato *et al*., 2008) and *S. rodhaini*, *S. incognitum*, *S. sinensium* mainly inhabited in rodents and carnivores (Bunnag *et al*., 1983; Walker *et al*., 1989; Zhang *et al*., 2001).

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Ribosomal DNA (rDNA) forms a tandem array of repeat sequences and each repeat includes 18S, 5.8S and 28S subunits separated by spacers (Zhao *et al*., 2011). The 18S and 28S rDNA sequences have been identified as good genetic markers for identification and phylogenetic studies of members of the genus *Schistosoma* (Johnston *et al*., 1993; Barker and Blair, 1996; Attwood *et al*., 2002; Lockyer *et al*., 2003; Webster *et al*., 2006). Recent studies also have demonstrated that different *S. japonicum* strains from mainland China and the Philippines has extensive homology in 18S rDNA sequences and 28S rDNA-D2 domain was also relatively stable among species (Yu *et al*., 2000; Li *et al*., 2008). But sequence variation in 18 and 28S rDNA among *S. japonicum* strains from mainland China, the Philippines and Japan have not been investigated comprehensively.

The objectives of the present study were to examine sequence variation in the 18S and 28S rDNA among *S. japonicum* isolates from different endemic regions in mainland China, the Philippines and Japan and to reconstruct the phylogenetic relationships among members of the Schistosoma genus using the combined partial 18S and 28S rDNA sequences.

MATERIALS AND METHODS

Parasites and isolation of genomic DNA: The 35 *S. japonicum* isolates were collected from the endemic areas in mainland China, the Philippines and Japan with sample codes, geographical origin and gender shown in Table 1. The male and female adult parasites were fixed in 70% molecular grade ethanol and stored at -20°C before extraction of genomic DNA. Total genomic DNA was extracted from individual parasites 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.

Enzymatic amplification and sequencing: A portion of the 18S (p18S) and 28S rDNA were amplified with primers 18Su and 18Sd, 28Su and 28Sd, respectively (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 1 min (denaturation); 45°C for 30 sec (annealing); 72°C for 1 min (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 both rDNA fragments were obtained after adjusting annealing temperatures. Each amplicon (4 μL) was examined by agarose (1%) gel electrophoresis to validate amplification efficiency. The p18S and p28S amplicons of 35 samples were sequenced by BGI-Guangzhou company from both directions using the same primers as used in primary amplification.

Sequences alignment and analysis: Sequences of the p18S and p28S rDNA were separately aligned using the computer program Clustal X 1.81 (Thompson *et al*., 1997). Meanwhile, Megalign procedure within the DNAStar 5.0 (Burland, 2000) was also used to analyze sequence similarity and to calculate transition and transversion.

Table 1: Information of Schistosoma samples used in the present study

				GenBank accession number	
Species/sample codes	Geographical strains	Geographical origin	Gender	p18S rDNA	p28S rDNA
Schistosoma japonicum/SjYeF55	Mainland China	Yunnan (Eryuan)	Femal	JF721330	JF721363
S. japonicum/SjYeM55	Mainland China	Yunnan (Eryuan)	Male	JF721331	JF721364
S. japonicum/SjHyF54	Mainland China	Hunan (Yueyang)	Femal	JF721328	JF721361
S. japonicum/SjHyM54	Mainland China	Hunan (Yueyang)	Male	JF721329	JF721362
S. japonicum/SjZjF60	Mainland China	Zhejiang	Femal	JF721326	JF721365
S. japonicum/SjZjM60	Mainland China	Zhejiang	Male	JF721327	JF721366
S. japonicum/SjLeF1	The Philippines	Letye	Femal	JF721338	JF721373
S. japonicum/SjLeF2	The Philippines	Letye	Femal	JF721339	JF721374
S. japonicum/SjLeF4	The Philippines	Letye	Femal	JF721340	JF721375
S. japonicum/SjLeM1	The Philippines	Letye	Male	JF721341	JF721376
S. japonicum/SjLeM2	The Philippines	Letye	Male	JF721342	JF721377
S. japonicum/SjLeM4	The Philippines	Letye	Male	JF721343	JF721378
S. japonicum/SjMiF1	The Philippines	Mindoro	Femal	JF721349	JF721384
S. japonicum/SjMiF2	The Philippines	Mindoro	Femal	JF721350	JF721385
S. japonicum/SjMiF4	The Philippines	Mindoro	Femal	JF721351	JF721386
S. japonicum/SjMiM1	The Philippines	Mindoro	Male	JF721352	JF721387
S. japonicum/SjMiM2	The Philippines	Mindoro	Male	JF721353	JF721388
S. japonicum/SjMiM3	The Philippines	Mindoro	Male	JF721354	JF721389

Table 1: Continue

Species/sample codes	Geographical strains	Geographical origin	Gender	GenBank accession number	
				p18S rDNA	p28S rDNA
S. japonicum/SjSoF2	The Philippines	Sorsogor	Femal	JF721344	JF721379
S. japonicum/SjSoF3	The Philippines	Sorsogor	Femal	JF721345	JF721380
S. japonicum/SjSoM1	The Philippines	Sorsogor	Male	JF721346	JF721381
S. japonicum/SjSoM3	The Philippines	Sorsogor	Male	JF721347	JF721382
S. japonicum/SjSoM4	The Philippines	Sorsogor	Male	JF721348	JF721383
S. japonicum/SjAsF1	The Philippines	Asuncium	Femal	JF721332	JF721367
S. japonicum/SjAsF3	The Philippines	Asuncium	Femal	JF721333	JF721368
S. japonicum/SjAsF4	The Philippines	Asuncium	Femal	JF721334	JF721369
S. japonicum/SjAsM1	The Philippines	Asuncium	Male	JF721335	JF721370
S. japonicum/SjAsM2	The Philippines	Asuncium	Male	JF721336	JF721371
S. japonicum/SjAsM4	The Philippines	Asuncium	Male	JF721337	JF721372
S. japonicum/SjYYF1	Japan	Yamanashi	Femal	JF721355	JF721390
S. japonicum/SjYYF2	Japan	Yamanashi	Femal	JF721356	JF721391
S. japonicum/SjYYF4	Japan	Yamanashi	Femal	JF721357	JF721392
S. japonicum/SjYYM2	Japan	Yamanashi	Male	JF721358	JF721393
S. japonicum/SjYYM3	Japan	Yamanashi	Male	JF721359	JF721394
S. japonicum/SjYYM4	Japan	Yamanashi	Male	JF721360	JF721395
S. intercalatum	-	-	-	AY157235	AY157262
S. rodhaini	-	-	-	AY157230	AY157256
S. spindale	-	-	-	Z11979	Z46505
S. bovis -	-	-	AY157238	AY157266	
S. edwardiense	-	-	-	AY197344	AY197344
S. haematobium	-	-	-	Z11976	Z46521
S. hippopotam	-	-	-	AY197343	AY197343
S. incognitum	-	-	-	AY157229	AY157255
S. japonicum	-	-	-	AY157226	Z46504
S. leiperi -	-	-	AY157234	AY157261	
S. malayensis	-	-	-	AY157227	AY157252
S. mattheei	-	-	-	AY157237	AY157265
S. mekongi -	-	-	AY157228	AY157253	
S. nasale -	-	-	AY157232	AY157259	
S. sinensium	-	-	-	AY157225	AY157251
S. mansoni	-	-	-	M62652	Z46503
S. indicum -	-	-	AY157231	AY157258	

Table 2: Sequences of primers used to amplify a portion of the 18S and 28S ribosomal DNA from *Schistosoma japonicum* isolates from mainland China, the Philippines and Japan

Name of primer	Sequence (5'-3')	Product length
For 18S rDNA	-	745 bp
18Su (forward)	CTTATGCTGTGCCTGTTACATT	-
18Sd (reverse)	TTACTTCGGATCCGAAAACCAAC	-
For 28S rDNA	-	618 bp
28Su1 (forward)	GGGTATGTGTAGACGTTCTTAT	-
28Sd (reverse)	AACACAAGGTCGCATGTCTACGT	-

These sequences were used for phylogenetic analyses. The sequences of the two *rRNA* genes were concatenated into single alignments. The Unweighted Pair-Group Method based on Arithmetic averages (UPGMA) (Sneath and Sokal, 1973) in MEGA v. 4.0 (Tamura *et al*., 2007) was carried out to examine the genetic relationship, starting from a distance matrix based on the Kimura 2-parameter index (Kimura, 1980). The consensus tree was obtained after bootstrap analysis with 1, 000 replications. The pairwise comparisons were made of the level of sequence differences (Chilton *et al*., 1995). To study the phylogenetic relationships of Schistosoma, the corresponding sequences of other *Schistosoma* species/isolates namely; *S. intercalatum*, *S. rodhaini*, *S. spindale*, *S. bovis*, *S. edwardiense*, *S. haematobium*, *S. hippopotam*, *S. incognitum*, *S. indicum*, *S. japonicum*, *S. leiperi*, *S. malayensis*, *S. mattheei*, *S. mekongi*, *S. nasale*, *S. sinensium* and *S. mansoni* obtained from GenBank were also used for phylogenetic analyses. The phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

RESULTS AND DISCUSSION

Genomic DNA was prepared from 35 individual adult trematodes (including male and female *S. japonicum*) representing three geographical strains from mainland China, the Philippines and Japan. Amplicons of p18S and p28S rDNA (~750 and 620 bp, respectively) were amplified. For each rDNA fragment, no size variation was detected on 1% agarose gel among any of the amplicons examined in dicating that the PCR primers and reactions of this study had good specificity (Fig. 1).

To examine sequence variations in the two rDNA fragments among three geographical strains, amplicons of p18S and p28S rDNA were subjected to direct sequencing. The sequences of p18S and p28S rDNA were 745 and 618 bp in length, respectively. One variable nucleotide position was identified in the sequences of p18S and three in p28S rDNA with intra-specific variation of 0.14% (1/704) and 0.51% (3/586) for p18S and p28S

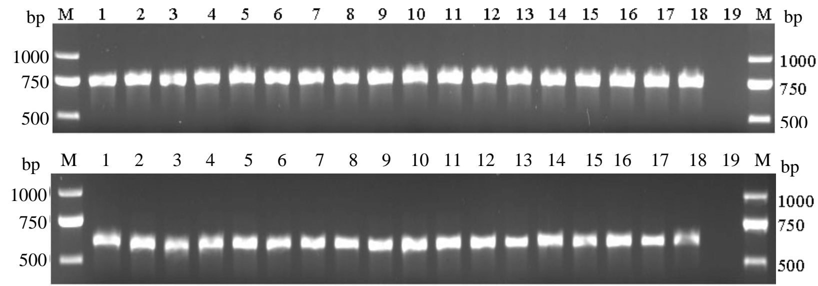

Fig. 1: Representative PCR products for a portion of the 18S (upper) and 28S (bottom) ribosomal DNA of *Schistosoma japonicum* isolates in mainland China, the Philippines and Japan. Lanes 1-18 represent samples SjYeM55, SjHyM54, SjZjF60, SjLeM1, SjLeM4, SjLeF1, SjMiM1, SjMiF1, SjMiF4, SjSoM3, SjSoM4, SjSoF2, SjAsM1, SjAsM2, SjAsF3, SjYYM2, SjYYM3, SjYYF1, respectively (cf. Table 1). Lane 19 represents no-DNA control. M represents a DNA size marker (ordinate values in bp)

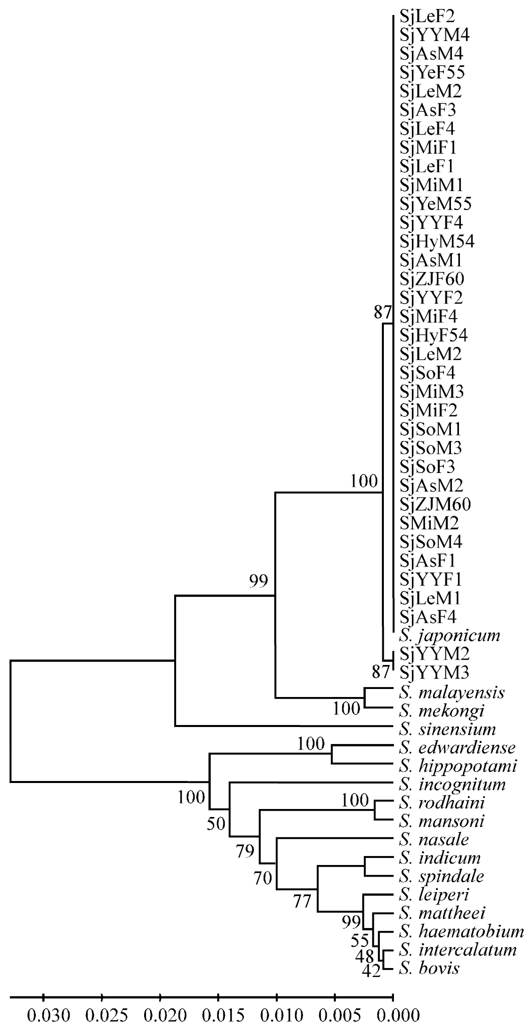

Fig. 2: Phylogenetic relationships of *Schistosoma* species/isolates inferred by UPGMA analysis using the combined sequences of partial 18S and p28S ribosomal DNA. Numbers at nodes indicate bootstrap values (%) resulting from UPGMA tree

rDNA, respectively. There were one transitions (A<->G) with intra-specific variation for p18S and two (A<->G) and one (C<->T) transitions for p28S rDNA. It appeared that the main transition was A<->G and there was no transversion within these two fragments. The low variations between the p18S and p28S rDNA were consistent with previous reports (Yu *et al*., 2000; Li *et al*., 2008).

The combined sequences of p18S and p28S rDNA were aligned over a consensus length of 1,290 bp. The phylogenetic relationships among the individual *S. japonicum* isolates were constructed by UPGMA analyses (Fig. 2). The phylogenetic tree consisted of two large clades.

All of the *S. japonicum* isolates from mainland China, the Philippines and Japan clustered in the *S. japonicum* clade, grouped with *S. malayensis* and *S. mekongi* and sistered to *S. sinensium*. All of the other *Schistosoma* species grouped in the other large clade with high bootstrap values.

The combined sequences of p18S and p28S rDNA allowed the unequivocal differentiation of all the *Schistosoma* species examined in the present study which is consistent with previous studies (Johnston *et al*., 1993; Littlewood and Johnston, 1995; Attwood *et al*., 2002; Morgan *et al*., 2003; Webster *et al*., 2006).

CONCLUSION

The present study revealed low level variation in p18S and p28S rDNA sequences among 35 *S. japonicum* isolates from mainland China, the Philippines and Japan. The combined p18S and p28S rDNA sequences were highly conserved and could not distinguish *S. japonicum* isolates from three geographical origins by phylogenetic analysis but could differentiate species in the *Schistosoma* genus suggesting that the p18S and p28S rDNA sequences were not suitable markers for studying population relationships among *S. japonicum* isolates but

is an effective genetic marker for inter-species phylogenetic analysis of Schistosoma and identification of schistosomes.

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