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Variability in intron sequences of housekeeping and antigen-coding genes among *Schistosoma japonicum* isolates in mainland China

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ABSTRACT

The accurate characterization of *Schistosoma japonicum* has important implications for analyzing genetic variation and would provide basic data for disease control. Previous studies using proteins, coding sequences, and especially antigen-coding genes showed lower genetic variation among *S. japonicum* isolates from mainland China. Therefore, the present study focused on variations in intron sequences of housekeeping and antigen-coding genes, which may be more informative for genetic analysis. We compared sequence variation between introns of two housekeeping genes and two antigen-coding genes. All 4 genes were polymorphic among all the *S. japonicum* isolates in mainland China, with 103, 158, 47, and 19 polymorphic (segregating) sites per kilobase in intron sequences of Actin, FBPA, 22.6 kDa antigen and GST-26, respectively. Introns of housekeeping genes were slightly more polymorphic than coding and non-coding regions of antigen-coding genes examined in the present study within or among lake/marshland and mountainous types. Phylogenetic analysis based on sequences of single gene or combined sequences of multiple genes showed no specific clustering comprising parasites from single geographical or endemic regions. These results demonstrated that introns of housekeeping and antigen-coding genes were polymorphic, but the intron sequences examined in the present study were not suitable markers for examining genetic relationship among different isolates from endemic regions in mainland China.

1. Introduction

Schistosomiasis japonica has been documented for more than two millennia and most probably was known as a specific disease in historic times in China [1]. 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 [1–3], which were divided into two main endemic types (mountainous and lake/marshland types) based on geographical, water-snail distribution, epidemiological information and some molecular markers [3–6].

The accurate characterization of parasites at different taxonomic levels has important implications for analyzing genetic variation and would provide basic data for disease control. Previous studies using some portions of mitochondrial DNA (mtDNA) [3,6,7] and coding regions of antigen-coding genes [8] showed limited genetic variation among *Schistosoma japonicum* isolates from different countries and between or within two endemic types.

Many eukaryotic genes have their coding regions interrupted by intervening sequences or introns [9]. The presence, absence, and mutation of these introns present us with an opportunity to examine intron transfer events, and the evolutionary history of introns and species or subspecies within the same or different genera. Intron sequences have been successfully used to study the genetic variation and phylogenetic relationships of *Toxoplasma gondii* [10,11], *Metarhizium* [12], chicken and turkey [13], non-human primates [14], the family Cyprinidae [15], and some plants [9,16].

In the present study, we compared sequence variability in introns of two housekeeping genes (Actin and fructose-1,6-bisphosphate aldolase genes) and in non-coding regions of two antigen-coding genes (22.6-kDa tegument membrane-associated antigen and 26 kDa glutathiones-

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transferase genes) of *S. japonicum* isolates from different geographical origins in mainland China. The results showed that all the intron sequences of four selected representative genes showed variability, and introns of housekeeping genes were slightly more polymorphic than non-coding and coding regions of the examined antigen genes, but the variations were neither associated with geographical origins, nor related to the two classical types of endemicity.

2. Materials and methods

2.1. Parasites and isolation of genomic DNA

A total of 32 *S. japonicum* samples (including male and female parasites), collected from 10 geographical origins in eight endemic provinces in mainland China (Table 1), was used. All the *S. japonicum* samples were prepared as previously described [3,5,6,17], except for *S. japonicum* samples from Hunan and Yunnan provinces. Briefly, *Oncomelania hupensis* snails infected with *S. japonicum* were collected from one village per province, and transported to the laboratory. For each isolate, 50 infected snails were shed under light for 3 h, cercariae were pooled, and each of two male adult rabbits was infected percutaneously with 1,000 cercariae and housed in separate cages. After 45 days, adult schistosomes were perfused from the mesenteric veins of infected rabbits using 0.15 mM NaCl and 25 mM sodium citrate, washed extensively in physiological saline, fixed in 90% molecular grade ethanol, and stored at −20 °C. For isolates from Yunnan (Eryuan) province and Hunan (Changsha city, Junshan county, and Yueyanglou district) province, three infected snails were collected, and two male adult rabbits were infected as mentioned above. After 60 days, adult schistosomes were obtained and fixed in 70% molecular grade ethanol and stored at −20 °C.

Total genomic DNA was extracted from a single adult parasite (male or female representing each of the *S. japonicum* isolates (Table 1) by SDS/proteinase K treatment. Then the genomic DNA was column-purified by the Wizard® SV Genomic DNA Purification System (Promega) and eluted into 60 μl H_2O according to the manufacturer's recommendations [3].

2.2. Genetic markers

Four single-copy genes were chosen for sequencing (Tables 1 and 2; Fig. 1), including two housekeeping genes and two antigen-coding genes. The group named "housekeeping genes" encodes proteins that are unlikely to play a role in protective immunity [11] and were selected on the basis of the presence of an intron at least 250 bp long. It includes actin [18], and fructose-1, 6-bisphosphate aldolase (FBPA) [18,19] genes. FBPA is a ubiquitous enzyme essential for glycolysis, gluconeogenesis and the Calvin cycle, which has been demonstrated to induce immune responses and to be useful in the immunodiagnosis of schistosomiasis japanica of water buffaloes [19] and humans [20]. The group named "antigen-coding genes" encodes proteins that probably plays a role in protective immunity, which includes 22.6 kDa tegumental membrane-associated antigen (22.6 kDa antigen) [21] and 26 kDa glutathione S-transferase (GST-26) [22], whose intron at least 400 bp long was selected. All unique sequences were submitted to GenBank (accession numbers: GU567821–GU567948).

2.3. Enzymatic amplification and sequencing

External and internal sets of primers (Table 2) were designed for each gene, except Actin gene, on the basis of the published sequences and *S. japonicum* genome contigs. PCR reactions (25 μl) were performed in 2 mM of $MgCl_2$, 0.4 μM of each primer, 2.5 μl 10 × r*Taq* buffer, 0.2 mM of each dNTPs, 1.25 U of r*Taq* 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

Table 1
Schistosoma japonicum samples used in the present study.

Endemic type	Province	Geographical origin	Sample codes	Gender	GenBank accession number			
					Actin	FBPA	22.6 kDa	GST-26
Lake/marshland type	Hunan	Changsha city	SJHCM4	Male	GU567917	GU567885	GU567821	GU567853
			SJHCM33		GU567918	GU567886	GU567822	GU567854
			SJHCM34		GU567919	GU567887	GU567823	GU567855
			SJHCF25	Female	GU567920	GU567888	GU567824	GU567856
		Junshan county	SJHYM23	Male	GU567921	GU567889	GU567825	GU567857
			SJHYM24		GU567922	GU567890	GU567826	GU567858
			SJHYF23	Female	GU567923	GU567891	GU567827	GU567859
		Yueyanglou district	SJHLM23	Male	GU567924	GU567892	GU567828	GU567860
			SJHLM25		GU567925	GU567893	GU567829	GU567861
			SJHLF2	Female	GU567926	GU567894	GU567830	GU567862
			SJHLF3		GU567927	GU567895	GU567831	GU567863
	Hubei	Wuhan city	SJHWM31	Male	GU567928	GU567896	GU567832	GU567864
			SJHWF5	Female	GU567929	GU567897	GU567833	GU567865
			SJHWF7		GU567930	GU567898	GU567834	GU567866
	Anhui	Guichi county	SJAGM1	Male	GU567931	GU567899	GU567835	GU567867
			SJAGF24	Female	GU567932	GU567900	GU567836	GU567868
	Jiangxi	Yongxiu county	SJJYM5	Male	GU567933	GU567901	GU567837	GU567869
			SJJYM24		GU567934	GU567902	GU567838	GU567870
			SJJYF21	Female	GU567935	GU567903	GU567839	GU567871
			SJJYF43		GU567936	GU567904	GU567840	GU567872
	Jiangsu	Wuxi city	SJJWM25	Male	GU567937	GU567905	GU567841	GU567873
			SJJWM35		GU567938	GU567906	GU567842	GU567874
	Zhejiang	Jiashan county	SJZJM21	Male	GU567946	GU567914	GU567850	GU567882
			SJZJM25		GU567947	GU567915	GU567851	GU567883
			SJZJF1	Female	GU567948	GU567916	GU567852	GU567884
Mountainous type	Sichuan	Tianquan county	SJSTM7	Male	GU567939	GU567907	GU567843	GU567875
			SJSTM10		GU567940	GU567908	GU567844	GU567876
			SJSTM11		GU567941	GU567909	GU567845	GU567877
	Yunnan	Eryuan county	SJYEIIM57	Male	GU567942	GU567910	GU567846	GU567878
			SJYEIIM58		GU567943	GU567911	GU567847	GU567879
			SJYEIIM59		GU567944	GU567912	GU567848	GU567880
			SJYEIIF57	Female	GU567945	GU567913	GU567849	GU567881