SHORT COMMUNICATION

A cleaved amplified polymorphic sequence (CAPS) method for the identification of geographical isolates of *Schistosoma japonicum* in China

Schistosomiasis japonica, caused by the blood fluke *Schistosoma japonicum*, is a severe parasitic zoonosis, causing significant morbidity, mortality and considerable economic losses in south-east Asia, particularly in mainland China (Zhou *et al.*, 2005; Bergquist *et al.*, 2008; Zhou *et al.*, 2008; Li *et al.*, 2010). Although there have been significant efforts to control schistosomiasis in China, this disease appears to be re-emerging due to climate changes and human activities (Wang *et al.*, 2009). Thus far, schistosomiasis is still endemic in seven provinces (Yunnan, Sichuan, Anhui, Hubei, Jiangxi, Jiangsu and Hunan provinces) of China (Ross *et al.*, 2001), and the impact of this communicable disease in China is almost equal to that of HIV/AIDS and tuberculosis (Engels *et al.*, 2005).

In previous studies, genetic variation among *S. japonicum* populations from different endemic provinces in China has been detected using a variety of genetic markers (Bøgh *et al.*, 1999; Sørensen *et al.*, 1999; Zhu *et al.*, 1999; Shrivastava *et al.*, 2005; Zhao *et al.*, 2009a; Zhao *et al.*, 2009b; Zhao *et al.*, 2009c). However, these markers and the detected genetic variability have not been utilized for the practical identification and differentiation of *S. japonicum* geographical isolates in China. The economic reform and the deterioration of ecological environment, flash floods, landslides and the mobility of human population appear to be having an impact on the transmission of *S. japonicum* (Li *et al.*, 2000; Seto *et al.*, 2008). Consequently, it is important to be able to identify different genetic variants of *S. japonicum* in China.

Various methods for the analysis of genetic variation have been developed, including single-strand conformation polymorphism (Simões *et al.*, 2007), denaturing high-performance liquid chromatography (Nickerson *et al.*, 2000), gene chips (Schmalzing *et al.*, 2000), TaqMan probe (Shi *et al.*, 1999) and pyrosequencing technology (Ahmadian *et al.*, 2000). However, such techniques can be time-consuming and/or require detection instruments or labelled oligonucleotides. In contrast, the cleaved amplified polymorphism sequence (CAPS) technique is a simple and effective method to allow the rapid detection of single nucleotide polymorphisms (SNPs) (Neff *et al.*, 1998; Komori and Nitta, 2005).

CAPS is based on the PCR-based amplification of targeted genes and subsequent digestion of amplicons using restriction enzymes. The digested PCR products are separated by agarose gel electrophoresis. CAPS generates the same type of data as traditional restriction fragment length polymorphism analysis, but significantly reduces the amount of purified DNA required for analysis and time-consuming steps. This method can detect both homozygous and heterozygous individuals (Weiland and Yu, 2003; Komori and Nitta, 2005). CAPS has been successfully used to study genetic diversity in plants, such as *Arabidopsis thaliana* (Hardtke *et al.*, 1996; Barth *et al.*, 2002), *Cryptomeria japonica* (Tsumura and Tomaru, 1999; Tsumura *et al.*, 1999) and *Pisum sativum* L. (Konovalov *et al.*, 2009), but has seldom been used to study variation in parasites (Gandhi *et al.*, 2009). The aims of the present study were to identify SNP sites specific for geographical isolates of *S. japonicum* based on

results of previous studies. Using these SNPs, the CAPS technique was developed and used to identify and differentiate *S. japonicum* isolates from Yunnan province and those from other endemic provinces.

MATERIALS AND METHODS

Isolates of *S. japonicum* were obtained from eight endemic provinces in mainland China (Anhui, Hubei, Jiangxi, Jiangsu, Zhejiang,

Hunan, Sichuan and Yunnan provinces). A total of 291 samples representing adult *S. japonicum* from different geographical origins were collected (Table). Individual *S. japonicum* samples (except those samples from Eryuan county (I), Yunnan province) were raised in rabbits infected with cercariae shedding from snails *Oncomelania hupensis* naturally infected with *S. japonicum*. These snails were collected from the field in a village per province (Zhao *et al.*, 2009c). For parasites from Eryuan county (I), Yunnan

TABLE. *Schistosoma japonicum* samples used in the present study

Endemic province	Geographical origin	Sample codes	Gender of worm
Yunnan	Eryuan county (I)	SJYEIM1-5*, SJYEIM6*, SJYEIM8*, SJYEIM9*, SJYEIM10*	Male
		SJYEIF1-9*	Female
	Eryuan county (II)	SJYEIIM1, SJYEIIM2, SJYEIIM3, SJYEIIM5, SJYEIIM9, SJYEIIM50-59	Male
		SJYEIIF2, SJYEIIF3, SJYEIIF5, SJYEIIF6, SJYEIIF7, SJYEIIF9, SJYEIIF50-59	Female
	Eryuan county (III)	SJYEIIM1-10	Male
		SJYEIIF1-10	Female
Sichuan	Weishan county	SJYWM1, SJYWM2, SJYWM3, SJYWM4	Male
	Xichang county	SJSXM4, SJSXM6, SJSXM7, SJSXM10, SJSXM51-60	Male
		SJSXF51-60	Female
	Tianquan county	SJSTM1, SJSTM3, SJSTM5, SJSTM6, SJSTM50-69	Male
Anhui	Maoshan county	SJSMM1, SJSMM2, SJSMM4, SJSMM6	Male
	Guichi county	SJAGM7, SJAGM21, SJAGM51-60	Male
Hubei	Wuhan city	SJAGF7, SJAGF21, SJAGF51-60	Female
		SJHWM3, SJHWM4, SJHWM51-60	Male
Jiangxi	Yongxiu county	SJHWF2, SJHWF4, SJHWF51-60	Female
		SJJYM1, SJJYM2, SJJYM51-60	Male
Jiangsu	Wuxi city	SJJYF1, SJJYF3, SJJYF51-60	Female
		SJJWM1, SJJWM2, SJJWM51-60	Male
Zhejiang	Jiashan county	SJJWF2, SJJWF4, SJJWF51-60	Female
		SJZJM3, SJZJM4, SJZJM51-60	Male
Hunan	Junshan county	SJZJF3, SJZJF4, SJZJF51-60	Female
		SJHYM2, SJHYM5, SJHYM51-60	Male
	Yueyanglou district	SJHYF1, SJHYF3, SJHYF51-60	Female
		SJHLM2, SJHLM3, SJHLM50-59	Male
	Changsha city	SJHLF2, SJHLF3, SJHLF50-59	Female
		SJHCM2, SJHCM3, SJHCM50, SJHCM51, SJHCM52, SJHCM53, SJHCF1, SJHCF4	Male
			Female

*These samples were adult worms from naturally infected cattle. All of the other samples were harvested from individual rabbits infected with *Schistosoma japonicum* cercariae shedding from snails *Oncomelania hupensis* collected from different geographical locations, and these snails were naturally infected with *S. japonicum*.

province, adult parasites were obtained from naturally infected cattle. The male and female adult parasites were stored in 70% ethanol at -20°C . Total genomic DNA was extracted from individual samples by SDS/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System; Promega, Madison, WI, USA), eluted into 60 μl H_2O and RNAase treated. The quality of genomic DNA was confirmed by amplification of the first internal transcribed spacer of ribosomal DNA (van Herwerden *et al.*, 1998).

The sequence of part of the mitochondrial cytochrome *c* oxidase subunit 3 gene (*pcox3*) has proven a suitable marker for population genetic study of *S. japonicum* (Zhao *et al.*, 2009c). In the present study, *pcox3* sequences available in GenBank (accession number NC_002544, AF215860) and the *pcox3* sequences reported by Zhao *et al.* (2009c) were aligned to define SNPs for the differentiation of geographical *S. japonicum* isolates. Then, the CAPS technique was established to identify and delineate geographical isolates from Yunnan province using restriction endonuclease sites identified using the MapDraw procedure within the program DNASTar 5.0 (Burland, 2000).

The *pcox3* region was amplified from 55 *S. japonicum* samples (Table) representing two ecological zones (western mountainous and eastern lake/marshland types) from eight endemic provinces of mainland China using primers described previously (Zhao *et al.*, 2009c). The PCR conditions were optimized by varying the amount of DNA template, annealing temperatures and Mg^{2+} concentrations. PCR reactions (in a volume of 25 μl) were performed in 2.5 μl $10\times$ rTaq buffer, 0.2 mM of dNTPs, 1.5–2.5 mM of MgCl_2 , 2.5 μM of each primer, 1.25 unit of rTaq polymerase (TaKaRa) and different amounts (40, 20, 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.1 and 0.08 ng) of genomic DNA in a thermocycler (Biometra, Goettingen, Germany) under the following conditions: 94°C for 5 minutes (initial denaturation), followed by 35 cycles

of 94°C for 30 seconds (denaturation), $45\text{--}55^{\circ}\text{C}$ for 30 seconds (annealing), 72°C for 30 seconds (extension) and a final extension of 72°C for 10 minutes. Samples with rabbit DNA or without genomic DNA were included in each PCR run as 'negative' controls. An aliquot (5 μl) of each amplicon was examined on 1% agarose-TBE (65 mM Tris-HCl, 22.5 mM boric acid and 1.25 mM EDTA, pH 9.0) gels, stained with ethidium bromide and photographed using a gel documentation system (UVItect, Cambridge, UK). The DL2000 marker (TaKaRa, Dalian, China) was used to estimate amplicon size.

Then, the CAPS analyses were performed. The *pcox3* PCR products were digested using *Nde* I (TaKaRa). The reaction (10 μl) was performed in 1 μl $10\times$ H buffer, 1.0 U of restriction enzyme and 2 μl PCR products, and incubated at 37°C for 1 hour. An aliquot (5 μl) of each digest was examined by agarose gel electrophoresis, and profiles were detected upon ultraviolet transillumination and photographed. The sensitivity of CAPS reaction was evaluated by titrating the amount of restriction enzyme from 1.5 to 0.1 U, and using digestion times ranging from 5 minutes to 1 hour. The limit of detection was defined as the least amount of DNA amplifiable and the least amount of restriction enzyme and time used to achieve complete digestion. After this optimization, CAPS was applied to 58 *S. japonicum* samples from Yunnan province and 178 samples from other endemic provinces in China (Table) to validate its utility to distinguish genetically distinct geographical isolates.

RESULTS AND DISCUSSION

Sequence alignment and comparison showed that there were six SNPs among 30 *pcox3* sequences from the *S. japonicum* isolates collected from 12 endemic locations in eight provinces. Among these SNPs, one

FIG. 1. Representative agarose gel electrophoresis of *Nde* I-digested PCR products amplified from *Schistosoma japonicum* samples. Lanes 1–15 represent samples SJSXM4, SJSTM3, SJSMM1, SJAGF7, SJHWM3, SJJYF1, SJJWF4, SJZJM3, SJHYM2, SJHLF3 and SJHCF4 from other provinces, and samples SJYEIM1, SJYEIIF3, SJYEIIF2 and SJYWM1 from Yunnan province (*cf.* Table), respectively. M represents a DNA size marker 2000 (ordinate values in bp).

transversion mutation (T<→C) at sequence position 244 could differentiate between *S. japonicum* isolates from Yunnan province and those from other provinces. This mutation resulted in an amino acid change (UAU<→CAU). This SNP was linked to a recognition site (CATATG) for the endonuclease *Nde* I for *S. japonicum* from Sichuan, Hunan, Hubei, Anhui, Jiangxi, Jiangsu and Zhejiang provinces, whereas no such *Nde* I recognition site was available for any isolates from Yunnan province.

Based on the presence/absence of the *Nde* I recognition site in the *cox3* sequences, CAPS could be used to differentiate *S. japonicum* isolates from Yunnan province and those from other provinces by digestion of the amplified *pcox3* PCR products using endonuclease *Nde* I. Genomic DNA was prepared from 55 representative adult *S. japonicum* (including male and female) from western mountainous or eastern lake/marshland regions in China. The optimal annealing temperature was 50°C and the optimal Mg²⁺ concentration was 2.0 mM. The smallest amount of DNA amplifiable was 0.16 ng. Under the optimized conditions, *pcox3* amplicons (~640 bp) were amplified and subjected to agarose gel electrophoresis. No size variation was detected on agarose gel among any of the amplicons examined (not shown).

Then, all the PCR products were digested using *Nde* I. Although digestion of *pcox3* amplicons from *S. japonicum* isolates from other endemic provinces produced two bands of ~380 and 260 bp, respectively,

samples from Yunnan province remained undigested because of the absence of *Nde* I restriction site due to the presence of T/C SNP at nucleotide position 244 in the *pcox3* sequence (Fig. 1). Optimization experiments showed that two rapid and economic protocols could be used: either digestion with 0.4 U *Nde* I for 10 minutes, or digestion with 0.2 U *Nde* I for 20 minutes. The entire CAPS procedure (from PCR to gel detection) could be completed in 3 hours, indicating that this method is convenient, time-effective and cost-effective for the specific identification/delineation of *S. japonicum* geographical isolates, consistent with previous studies in plants (Weiland and Yu, 2003; Komori and Nitta, 2005).

To further validate the efficiency, specificity and applicability of CAPS, 236 *S. japonicum* isolates collected from 15 geographical origins from endemic provinces (Table) were subjected to analysis. As expected, *S. japonicum* isolates from four endemic locations in Yunnan province did not have an *Nde* I site, whereas isolates from the 11 other geographical origins did (not shown), indicating good reproducibility and applicability of the technique.

Current evidence indicates that multiple ecological zones of *S. japonicum* exist in mainland China (Jiang *et al.*, 2002; Shrivastava *et al.*, 2005; Zhao *et al.*, 2009c). Yunnan province is considered the most important endemic region for schistosomiasis in mountainous areas in China (Zou *et al.*, 2010). Epidemiological data show that in the 13 known schistosomendemic villages of Yunnan province, the

seroprevalence of schistosomiasis varied from 16.9 to 84.8%, and the highest prevalence is estimated for the plain between the Lakes Erhai and Eryuan (Steinmann *et al.*, 2007).

Interestingly, some *S. japonicum* infections occurred in villages that were considered as non-endemic in recent years (Steinmann *et al.*, 2007), which raises the question as to whether the infections were transmitted from endemic areas or whether they represent an emerging problem in non-endemic areas. In addition, environmental changes, which have resulted from the development of new water resources including the Three Gorges Dam across the Yangtze River, as well as the growth and migration of human populations might facilitate the spread of schistosomiasis and thus intensify the disease problem (Li *et al.*, 2000). Consequently, it is becoming more important to identify informative genetic markers for different geographical isolates to trace disease and infection sources linked to outbreaks of schistosomiasis. The CAPS technique should provide a useful tool also for future epidemiological studies.

In conclusion, the present study established a novel CAPS technique for the differentiation of *S. japonicum* isolates from Yunnan province and those from other endemic provinces in China. The CAPS assay is simple, rapid and inexpensive, and should have important implications for tracing the source of *S. japonicum* infections in humans and animals and for monitoring epidemiological trends of schistosomiasis in China.

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