



# Multiplex PCR for sexing *Schistosoma japonicum* cercariae and its utility

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## Abstract

Accurate discrimination of the *Schistosoma japonicum* cercariae gender is very important for establishing monosexual infection animal models and for standardizing the real intensity of infection. In this study, a multiplex PCR technique consisting of two pairs of primers, of which one amplifies a 185-bp band specific for the W chromosome and the other amplifies a 420-bp band for the Z chromosome, was established to sex the *S. japonicum* cercariae. For male cercariae (ZZ), a single 420-bp band is expected, and for female cercariae (ZW), two distinct 185-bp and 420-bp bands can be observed. There was no cross-reaction with *S. mansoni*, *S. haematobium*, *Clonorchis sinensis*, *Paragonimus westermani*, and *Trichinella spiralis*. After sexing the cercariae escaped from a single snail, mice in group A were infected with 60 male cercariae and mice of group B were infected with 40 female cercariae. Meanwhile, mice in group C were infected with 10 male and 10 female cercariae that were sexed by multiplex PCR. At 45 days postinfection, male and female adult worms were recovered to verify the accuracy of multiplex PCR for sexing *S. japonicum* cercariae and to calculate the male and female survival rate and paired worm ratio. Our results showed that the multiplex PCR technique could distinguish male cercariae with 100% accuracy. However, sometimes the discrimination results of multiplex PCR mis-scored mixed sexual cercariae as female cercariae. The mean male adult worm burden in mice of group C was  $10.7 \pm 2.4$ , and the mean female adult worm burden was  $7.7 \pm 2.5$ . There was a significant difference between the male worm burden and female worm burden in group C. The *P* value was 0.013. The real paired worm ratio of group C was 74.2% (95%CI 56.6–91.8%). These results demonstrated a male-biased sex ratio in the mice model with equilibrated sex ratio cercariae infection, as predicted by our multiplex PCR technique. In conclusion, our multiplex PCR technique is an effective tool for sexing *S. japonicum* cercariae, especially for distinguishing male cercariae, which is of great value for establishing monosexual cercariae infection mice models to harvest male adult worms for anti-schistosomal drug screening.

**Keywords** *Schistosoma japonicum* · Cercariae · Sex determination · Multiplex PCR

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Jing Xu and Chunxiang Li contributed equally to this work.

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## Introduction

*Schistosoma japonicum* has 8 pairs of chromosomes, of which the sex chromosomes are heterogametic in the female (ZW) and homogametic in the male (ZZ) (Liberatos and Short, 1983; Chevalier et al. 2016). There is a clear sexual dimorphism between adult male and female worms; however, there is no apparent morphological dimorphism at the larval stage (miracidia, sporocysts, and cercariae), and it is hard to discern sex with the unaided eye (Gasser, 1992; Boissier et al. 2001; Webster et al. 1989). The traditional propagation method for identifying

sex at the larval stage is time-consuming and laborious. This method requires collecting eggs from the liver, intestinal wall, or feces of a previously infected animal model to hatch miracidia. Snails are then attacked by a single miracidia, giving rise to thousands of monosexual cercariae. Thereafter, the monosexual cercaria is used to infect mice, which then develop into single-sex worms. The whole process takes at least 6 months to finally distinguish the gender of cercariae, and sometimes it is difficult to find worms in mice with single female cercariae infection (Mitchell et al. 1990). Liberatos and Short (1983) reported a C-band technique to detect the heterochromatic region of the W chromosome to sex the cercariae of *S. mansoni*. However, this method was not able to differentiate between nuclei of male and female cercariae in *S. japonicum* and *S. haematobium*. Hybridization techniques have been reported by several groups (Webster et al. 1989; Spotila et al. 1987; Walker et al. 1989; Drew and Brindley, 1995; Portela et al. 2010) and these techniques offer very effective means for sexing larval populations of *S. mansoni* but are usually not sufficiently sensitive to determine the sex of a single cercariae. Furthermore, these methods are also relatively labor intensive and time-consuming. In recent years, polymerase chain reaction (PCR) has provided an alternative technique for distinguishing the sex of *S. mansoni* cercaria based on amplification of female-specific DNA sequences (Chevalier et al. 2016; Gasser et al. 1991; Dias Neto et al., 1993; Grevelding et al. 1997; Boissier et al. 2001). However, until now, there have been very few reports on molecular methods for discriminating the gender of *S. japonicum* cercariae. Although some female-specific sequences have been identified by representational difference analysis (Yi and Ximei, 2000) and a random amplified polymorphic DNA technique (Youren et al., 2001), and the fragment was about 562 bp and 718 bp, respectively, they did not mention how to sex the cercariae of *S. japonicum*. Zhao et al. (2010) developed a sequence-characterized amplified region (SCAR)-PCR assay based on a female-specific sequence of 162 bp for identification and differentiation of female *S. japonicum*, and there was no cross reaction with male *S. japonicum*, *Fasciola hepatica*, *Clonorchis sinensis*, and *S. mansoni* (male and female parasites). Unfortunately, this report did not discuss the accuracy for sexing the larvae stages of *S. japonicum*.

In this study, a multiplex PCR technique was established for sexing the cercariae of *S. japonicum*. One pair of primers was based on the female-specific sequence on the *S. japonicum* W chromosome discovered by Zhao et al. (2010); another pair of primers targeting the Z chromosome not only allowed distinguishing male worms but also served as an internal control to confirm the presence of amplifiable

DNA within the sample. The objective of the present study was to evaluate the accuracy of the multiplex PCR technique for sexing *S. japonicum* cercariae and its utility for establishing special animal models and for assessing the real worm burden in mice models.

## Materials and methods

### Ethics statement

This study was performed in strict accordance with the recommendations of the Guidance for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments at Soochow University (Permit Number: 2007–13).

### Worms

The DNA of *Clonorchis sinensis*, *Paragonimus westermani*, *Trichinella spiralis*, *S. mansoni* (mixed sexual adult worm), and *S. haematobium* (egg) stored in our laboratory was used to test the specificity of the established multiplex PCR. The male and female adult worm DNA of *S. japonicum* was used as a positive control.

### Snails and cercariae

*S. japonicum*-infected snails (*Oncomelania hupensis*) were obtained from the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, China. Each living snail was placed in a U-shaped glass tube containing a 4/5 volume of dechlorinated water, and a light source was used to induce the shedding of *S. japonicum* cercariae (Chinese mainland strain).

### Extraction DNA of cercariae

Three samples containing one cercaria, two cercariae, and five cercariae were picked from a single snail; then, each sample was placed into a tube with 100  $\mu$ L of cercariae lysis buffer (50 mM Tris base, 5% Tween 20, 50  $\mu$ g/mL protease K, pH 7.6–8.0), incubated at 55  $^{\circ}$ C for 10 min and at 95  $^{\circ}$ C for 10 min to obtain cercariae DNA.

### Primers

The primers employed in this multiplex PCR technique were designed based on the previously reported primers (Tsexu2 and Tsexd2) (Zhao et al. 2010). The conventional PCR using Tsexu2 and Tsexd2 can amplify a 153-bp fragment, which is specific for female worms of *S. japonicum*; then, the specific

bands were sequenced and submitted to BLAST analysis (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The BLAST result showed the submitted sequence matched to the *S. japonicum* isolate Anhui clone SJC\_S000693 (GenBank Accession No. FN331663.1). Thereafter, we found that the female-specific sequence had a deletion of 26 bp compared with the male sequence. Based on this difference, we designed two pairs of primers to establish a multiplex PCR to distinguish the gender of *S. japonicum* cercariae (Table 1). The primers Pf5F and Pf8R were designed to step across the missing 26-bp bases to distinguish female cercariae, and the size of the expected product was 185 bp. Another pair of primers Pm0F and Pm8R was targeted on the Z chromosome with an expected 420-bp fragment.

### Multiplex PCR

Multiplex PCR was carried out in a total volume of 25  $\mu$ L containing 2.5  $\mu$ L of 10 $\times$  buffer (TaKaRa), 0.2 mM of dNTPs (TRANS), 2 mM of MgCl<sub>2</sub> (TaKaRa), 0.2  $\mu$ M of each primer, 1.25 U of EasyTaq DNA polymerase (TRANS), and 2  $\mu$ L of cercariae DNA in a thermocycler. The amplification program was as follows: 94  $^{\circ}$ C for 3 min (94  $^{\circ}$ C for 30 s, 52  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 30 s)  $\times$  35 cycles, 72  $^{\circ}$ C for 5 min. Five microliters of each amplicon was loaded on 2% agarose gels for electrophoresis, stained with GelRed (Biotium), and visualized under UV light. The 100-bp DNA Ladder (TRANS) was used to estimate the sizes of the amplicons. Two band patterns can be expected: a single 420-bp band for male cercariae (ZZ) and two distinct bands (185 bp and 420 bp) for female cercariae (ZW).

### Establishment of infection mice models

Thirty female ICR mice, weighing approximately 22–25 g, were randomly divided into 3 groups of 10 mice each. Each mouse in group A was percutaneously infected with  $60 \pm 5$  male cercariae identified by multiplex PCR. In group B, considering female worms alone can not get fully developed, each mouse was infected with  $40 \pm 2$  female cercariae identified by multiple PCR. In group C, each mouse was infected with  $10 \pm 1$  female cercariae and  $10 \pm 1$  male cercariae (Table 2).

**Table 1** Primers of multiplex PCR

Primer	Sequence of primers(5'—3')
Pm0F	GGTGAAACATCATAAGACTAGAATT
Pm8R	GCGCTTTCTGTGTACCAAACTTAG
Pf5F	CTCAGTGTGTAACATGCGTACTT
Pf8R	TTCGCTTTCTGTGTACTAAACTTA A

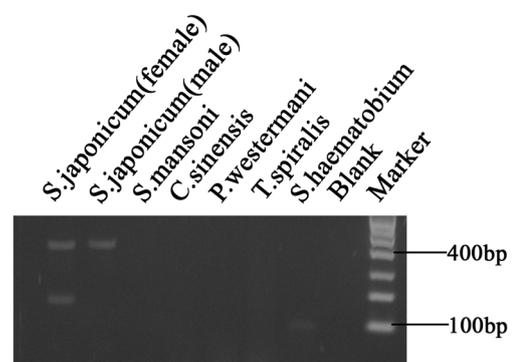
**Table 2** The predictions of infection in mice models identified by multiplex PCR

Number	Group A	Group B	Group C
1	64♂	40♀	11♂ + 11♀
2	61♂	40♀	10♂ + 10♀
3	60♂	40♀	11♂ + 11♀
4	63♂	41♀	10♂ + 10♀
5	60♂	41♀	11♂ + 11♀
6	60♂	40♀	11♂ + 11♀
7	61♂	40♀	11♂ + 10♀
8	60♂	41♀	11♂ + 10♀
9	60♂	40♀	11♂ + 11♀
10	62♂	42♀	10♂ + 10♀

Forty-five days postinfection, all mice of the three groups were sacrificed. Adult worms were recovered to verify the accuracy of multiplex PCR for identification of the gender of *S. japonicum* cercariae. Meanwhile, the real worm burden was examined in group C to evaluate the utility of gender discrimination by multiplex PCR.

### Statistics

All the data were analyzed by SPSS19.0 software. The male and female worm burden was expressed as the mean ( $\bar{x} \pm$  standard deviation) of the data, and difference between survival of males and females in group C was compared using a *t* test. Shapiro-Wilk test was used to verify the normal distribution of the data. *P* < 0.05 was used as a criterion for statistically significant differences.



**Fig. 1** Specificity of multiplex PCR for discrimination of the gender of *S. japonicum*. Lane Marker represents the 100-bp DNA ladder and lane Blank represents the blank control, double distilled water. Other lanes represent the DNA of *S. japonicum* female adult worms, *S. japonicum* male adult worms, *S. mansoni* mixed sexual adult worms, *C. sinensis*, *P. westermani*, *T. spiralis*, and the *S. haematobium* egg, respectively

## Results

### Specificity of multiplex PCR

As shown in Fig. 1, two distinct band patterns were observed: (i) a single band at 420 bp for the Z marker, and (ii) a double band at 185 bp for the W sequence and 420 bp for the Z marker. For female *S. japonicum* adult worms, it showed a double band at 420 bp (Z) and 185 bp (W), while for the male *S. japonicum* adult worms, only a 420-bp (ZZ) band was observed. No cross-reaction was found with *C. sinensis*, *P. westermani*, *T. spiralis*, *S. mansoni* (mixed sexual adult worm), and *S. haematobium* (egg).

### Sex determination using multiplex PCR

Cercariae that escaped from 10 positive individual snails were amplified by multiple PCR for sex discrimination. Three samples containing 1 cercaria, 2 cercariae, and 5 cercariae from each single snail were tested. If all three cercariae samples showed the two bands (185 bp and 420 bp) simultaneously, the gender of cercariae shedding from this single snail was considered as female, if all three samples displayed only a 420-bp band, the cercariae gender of this positive snail was male. Therefore, the cercariae that escaped from no. 1, no. 3, and no. 8 snails with two distinct bands were identified as female, and the cercariae shed from no. 4, no. 5, no. 7, and no. 10 snails with a single 420-bp band were considered as male. However, the amplification results of cercariae from no. 2, no. 6, and no. 9 snails displayed both single and double band patterns; thus, we regarded them as bisexual infected snails (Fig. 2), and these snails with mixed sexual infection were not used in this study.

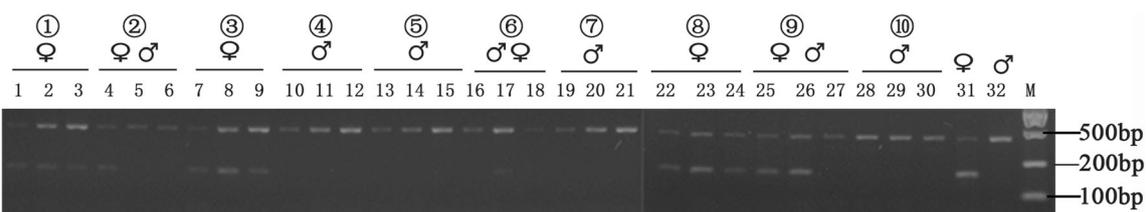
### The accuracy of multiplex PCR for sexing cercariae of *S. japonicum* and its utility

The accuracy of sex identified by multiplex PCR was confirmed by recovery of adult worms in a mice model. At 45 days postinfection, infection and perfusion of mice in group A showed that all the infective cercariae from no. 4, no. 5, no.

7, and no. 10 positive snails were male. The accuracy of multiplex PCR for discrimination of male cercariae was 100% (Table 3). While for discrimination of female cercariae, our results demonstrated that the multiplex PCR mis-scored mixed sexual cercariae as female cercariae. Male adult worms were recovered from no. 4, no. 6, no. 7, no. 8, no. 9, and no. 10 mice infected with only female cercariae predicted by multiplex PCR. Table 3 showed the real worm burden of the mice in group C infected with 10 paired male and female cercariae identified by multiplex PCR. In group C, the mean male and female adult worm burden was  $10.7 \pm 2.4$ ,  $7.7 \pm 2.5$ , respectively, and the paired ratio of group C was 74.2% (95%CI 56.6–91.8%). However, in the no. 4 mouse of group C, 13 male adult worms and 7 female adult worms were recovered, which was inconsistent with the number of previously infected male and female cercariae. The same situation was observed in no. 7 and no. 8 mice of group C in which more adult worms were recovered than previously infected male cercariae.

## Discussion

Schistosomes are dioecious trematodes, and the gender is determined by the sex chromosomes Z and W. The female is heterogametic (ZW) and the male is homogametic (ZZ) (Liberatos and Short, 1983; Chevalier et al. 2016; Gasser.1992; Boissier et al. 2001; Webster et al. 1989). Although there is a marked sexual dimorphism between adult male and female worms, the sexes are visually indistinguishable in the larval stages. Research on gender identification of *S. mansoni* has been carried out previously, and the existing methods have ranged from traditional propagation methods to molecular methods such as hybridization techniques (Spotila et al. 1987; Walker et al. 1989; Drew and Brindley, 1995; Portela et al. 2010), conventional PCR (Gasser et al.1991; Dias Neto et al., 1993; Grevelding et al.1997; Boissier et al. 2001), and real-time PCR (Chevalier et al. 2016). These methods, especially molecular methods, allow determination of the sex of cercariae rapidly and accurately, which is of great



**Fig. 2** Sex determination of *S. japonicum* cercariae shedding from infected snails using multiplex PCR. Three cercariae samples from a single positive snail were tested by multiplex PCR. All three samples from no. 1, no. 3, and no. 8 positive snails show two bands, 420 bp for the Z chromosome and 185 bp for the W chromosome. Samples from no.

4, no. 5, no. 7, and no. 10 positive snails displayed only a 420-bp band. Cercariae from no. 2, no. 6, and no. 9 positive snails demonstrated two band patterns: double bands at 420 bp (Z) and 185 bp (W), and a single band at 420 bp

**Table 3** The real status of mice infected with identified male and female cercariae

Mice	Group A		Group B			Group C			Total
	Male	Female	Male	Female	Pairs	Male*	Female*	Pairs	
No. 1	52♂	/	/	♀	/	11	11	11	22
No. 2	44♂	/	/	♀	/	11	7	7	18
No. 3	45♂	/	/	♀	/	10	9	9	19
No. 4	48♂	/	1	♀	1	13	7	7	20
No. 5	42♂	/	/	♀	/	11	11	11	22
No. 6	40♂	/	2	♀	2	10	7	7	17
No. 7	49♂	/	4	♀	4	14	7	7	21
No. 8	44♂	/	1	♀	1	12	4	4	16
No. 9	38♂	/	4	♀	4	5	4	4	9
No. 10	49♂	/	6	♀	6	10	10	10	20

\* The data of male adult worm survival number and female adult worm survival number in mice of group C were normally distributed. The statistics of Shapiro-Wilk test were 0.882 and 0.894, respectively, and the *P* values were 0.081 and 0.188, respectively

importance for studying unisexual infections in mice and for establishing genetic crosses.

Unfortunately, prior to the present study, there have been very few reports on molecular techniques for sexing *S. japonicum* cercariae. Although Zhao et al. (2010) discovered a female-specific sequence using SCAR-PCR, they did not mention the utility of this sequence for sexing the clonal cercariae population. In this study, on the basis of the female-specific sequence reported by Zhao et al. (2010), we designed two sets of primers to establish a multiplex PCR technique for distinguishing the gender of *S. japonicum* cercariae. It was noteworthy that the primers Pm8R and Pf8R had only a difference of a few bases, and this difference enabled the primer Pm8R to more likely bind to the Z chromosome, while the primer Pf8R was inclined to bind to the same position of the W chromosome. Our results revealed that the established multiplex PCR assay is a rapid method for sex determination, and the whole process is finished within 4 h, which allows performing experiments as soon as the first day of shedding. The detection results were unambiguous, a double band (185 bp and 420 bp) for females and a single band (420 bp) for males. No cross-reaction was observed with *C. sinensis*, *P. westermani*, *T. spiralis*, *S. mansoni* (mixed sexual adult worm), and *S. haematobium* (egg), suggesting this multiplex PCR was specific for sexing *S. japonicum*. The accuracy of multiplex PCR for discriminating male cercariae was 100%. For 4 snails (no. 4, no. 5, no. 7, and no. 10), all the 12 cercariae samples showed only a single 420-bp fragment (Fig. 2), and the infection and perfusion of mice in group A showed that all the snail infections were male (Table 3). This finding is of great value to establish monosexual infection to harvest male adult worms, which is the prerequisite for anti-schistosomal drug screening. However, sometimes, this method gave incorrect results for female cercariae discrimination. In mice

models with only female cercariae infection, male adult worms were recovered from No. 4, No. 6, No. 7, No. 8, No. 9 and No. 10 mice in group B, indicating that the multiplex PCR mis-scored mixed cercariae as female cercariae. In mice models with paired cercariae infection, more adult worms than predicted were recovered from 4 mice (no. 4 mouse in group B, no. 4, no. 7, and no. 8 mice of group C, Table 3), and this inconsistency could be explained by the inaccuracy of multiplex PCR for distinguishing female cercariae. As stated previously, the criterion of female cercariae was all the samples picked up from the single snails that showed two bands pattern (185 bp and 420 bp) simultaneously on an agarose gel. If the samples contained several male cercariae, the single 420-bp band would be covered by the two bands of the female cercariae. Hence, PCR amplification of 185-bp and 420-bp bands from mixed sexual cercariae led them to be incorrectly identified as females. To improve the accuracy of female cercariae determination, more samples containing 1 cercaria prepared for multiplex PCR amplification are needed.

In mice of group C with predicted paired cercariae infection, the male worm survival rate was higher than the female adult worm survival rate, and there was a significant difference between the survival of male worms and female worms (Table 4). These results were in accordance with findings of Liberatos (1987) and Mitchell et al. (1990), who have reported male-biased sex ratios in mice. The paired ratio of approximately 74% in the mice of group C demonstrated relatively little variation in the paired worm number. In other words, mice with paired male and female cercariae infection could minimize the variation in the sex ratio, which might influence the egg reproduction, and thus affect the pathogenicity of the mature eggs. This result was in accordance with Boissier and Moné (2000), who has reported that male and female cercarial infectivity was maximal when the sex ratio was equilibrated.

**Table 4** The statistics of the real worm burden in group C mice models

	Group C
Male survival number*	10.7 ± 2.4
Male survival rate	101.4% (95%CI 84.4~118.3%)
Female survival number*	7.7 ± 2.5
Female survival rate	74.2% (95%CI 56.6~91.8%)
Total worm survival rate	87.1% (95%CI 73.6~100%)
Pairs	7.7 ± 2.5
Paired ratio	74.2% (95%CI 56.6~91.8%)

\*Represents that there was significant difference between male survival number and female survival number in group C, the *t* value was 3.069, and the *P* value was 0.013

In sum, the multiplex PCR technique provides a convenient and rapid approach for determining the gender of cercariae, especially for male cercariae, which is of great value for establishing unisexual animal models to reserve male adult worms for anti-schistosomal drug screening. In addition, using equilibrated male and female cercariae to infect animal models can minimize the variation in the sex ratio, which is of great importance to standardize the infection intensity to control the progression of schistosomiasis. Ethically, using molecular methods for cercarial sexing may avoid the sacrifice of vertebrate hosts, which is presently necessary to discriminate cercariae sex.

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**Compliance with ethical standards** This study was performed in strict accordance with the recommendations of the Guidance for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments at Soochow University (Permit Number: 2007–13).

**Conflict of interest** The authors declare that they have no conflict of interest.

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