Recovery of primary sporocysts in vivo in the Schistosoma mansoni/Biomphalaria glabrata model using a simple fixation method suitable for extraction of genomic DNA and RNA.

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To cite this version:

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Abstract

Detailed studies of host/parasite interactions are currently limited because in-situ gene sequencing or monitoring of parasite gene expression is so far limited to genes presenting a high loci copy number in the Schistosome genome or a high level of expression. Indeed, how to investigate the host parasite molecular interplay when parasites are not directly accessible in vivo? Here we describe a method to circumvent this problem and to analyze DNA and RNA of *Schistosoma mansoni* during the interaction with its intermediate snail host *Biomphalaria glabrata*. We propose a technique for improved DNA and RNA extraction from the intra-molluscan stage of the parasite recovered after fixation of infected snails in Raillet-Henry solution. The extractions can be used for genetic analysis, transcription studies and microsatellite genotyping.

Keywords: *Schistosoma mansoni, Biomphalaria glabrata*, intramolluscan stage, DNA/RNA extraction.
1. Introduction

Schistosomiasis or bilharzia is a tropical parasitic disease affecting 200 million humans in 74 countries, causing 200,000 deaths annually (WHO, 2002). It is the second most important tropical disease in terms of morbidity after malaria. Schistosomiasis is caused by flatworms of the genus *Schistosoma* (Plathyhelminth, Digenea) (Chitsulo, et al., 2004, Gryseels, et al., 2006). The cycle of the parasite is complex. It requires humans as definitive hosts and freshwater snail species as intermediate hosts. Infection occurs in water by the free-living larval stages (cercaria for the definitive host and miracidia for the intermediate host). Significant attention has been paid to freshwater snails in the past because of both their medical and epidemiological importance as intermediate hosts for schistosome parasites. Moreover, the interaction between *Schistosoma mansoni* and the snail *Biomphalaria glabrata* provides a model of choice in evolutionary biology to investigate the host-parasite co-evolutionary dynamics but also invertebrate immune response (Baeza Garcia, et al., 2010, Lockyer, et al., 2008, Mone, et al., 2010, Roger, et al., 2008). While snail response to infection can be readily studied particularly at the molecular level (Adema, et al., 2010, Baeza Garcia, et al., 2010, Guillou, et al., 2007, Hanington, et al., 2010) it is not easy for the *S. mansoni* intramolluscan stage parasite that is not directly accessible. To avoid this problem, *in vitro* culture is often used in this model to obtain pure parasites and/or secretion products to identify the molecules involved in host-parasite interplay (Bender, et al., 2002, Coppin, et al., 2003, Guillou, et al., 2007, Roger, et al., 2008), to perform pharmacological investigations (Mattos, et al., 2006), or to study parasite development (Azzi, et al., 2009). However, miracidia to sporocyst *in vitro* transformation can be achieved only for a short period of time. For a longer period an artificial snail environment must be used involving sporocysts co-cultured with *Biomphalaria glabrata* embryonic cells (Bge cell line) (Coustau and Yoshino, 2000, Taft, et al., 2009, Vermeire, et al., 2004). These approaches has been used with great
success in the past, however, the *in-vivo* response of the parasite to the authentic host snail environment was never investigated.

Here we describe a new efficient method for improved DNA and RNA extraction from the *S. mansoni* intra molluscan stage. We show that the method can be used for PCR amplification, analysis of gene expression and microsatellite genotyping approaches.

2. Material and Methods

2.1. Biological material, Raillet-Henry fixation and parasite recovery

*Schistosoma mansoni* (Guadeloupean strain) was maintained in its sympatric *Biomphalaria glabrata* strain and in hamsters (*Mesocricetus auratus*), as described previously (Théron, et al., 1997). Miracidia were hatched from eggs recovered from 60-day-infected hamster livers. The livers were homogenized and the eggs were filtered out, washed, and transferred to spring water. The miracidia were allowed to hatch under exposure to artificial light. For each experiment, snails (7-9 mm in diameter) were exposed individually to 20 miracidia. For primary sporocysts (SpI) detection and recovery, the snails were fixed 15 days post-exposure, according to a modified method, previously described (Mone, et al., 2010, Theron and Gerard, 1994). In brief, each infected snail was relaxed in pond water containing an excess of crystalline menthol for 6 h (menthol relaxation help in shell removing and snail anatomic observation), the snail body was recovered and fixed in modified Raillet-Henry’s solution (0.6% NaCl; 2% acetic acid; 2% formalin). After 24 hours in fixative, a dissection of the head-foot zone was performed, and SpIs (Figure 1) were recovered, washed two times in Phosphate Buffer Saline (PBS) for 15 min on ice and kept at -80°C until use.

An alternative procedure can be used with a rapid fixation procedure when studying gene transcription. Infected snails were snap-frozen in liquid nitrogen and fixed directly with the
shell in modified Raillet-Henry’s solution (0.6% NaCl; 2% acetic acid; 2% formalin). After
24 hours in fixative, the shell is removed and SpI recovery was performed as above.

2.2. DNA and RNA extraction and reverse transcription

Genomic DNA (gDNA) was extracted from single Raillet-Henry fixed *S. mansoni* sporocysts
according to the following protocol. Sixty µl of TE (Tris 10mM; EDTA 1mM; ph 8)
containing 1.67mg/ml of Proteinase K (Merck) was added to the SpI. Samples were put 3
hours at 55°C and vortexed each 15 min. Then samples were heated 10 min at 100°C for
proteinase K inactivation. gDNA were kept at -20°C until use.

Total RNA was isolated from single or pool of 5 SpIs using the High Pure FFPE RNA
microkit (Roche) according to manufacturer recommendations. Reverse transcription was
performed using random hexamer primer and the RevertAid H minus First Strand cDNA
Synthesis kit (Fermentas) following the manufacturer’s protocol. cDNA were kept at -80°C
until use. It as been largely documented that these new extraction methods have been now
optimized for RNA extraction from formalin-fixed tissue to achieve high quality microarray
and qRT-PCR downstream applications (April, et al., 2009, Ribeiro-Silva, et al., 2007,
Serinsoz, et al., 2005).

2.3. PCR amplifications of gDNA and cDNA

gDNA PCR amplifications of fixed *S. mansoni* sporocyst were performed with the Advantage
2 PCR Enzyme System (Clontech). To test PCR on gDNA as template, the SmPoMuc loci
(Roger, et al., 2008, Roger, et al., 2008, Roger, et al., 2008) were amplified using specific
primers designed to amplify specific fragment lengths (see Table 1 for primer sequences and
PCR cycling conditions). PCR conditions were: 0.5µM primers; 1X final Advantage 2 PCR
buffer (2mM MgCl2); 0.4mM dNTPs; 1X final Advantage 2 polymerase and 5µl of gDNA in a total volume of 20µl of RNase, DNase free water.

cDNA PCR amplification of fixed *S. mansoni* sporocyst was performed with the GoTaq hotstart Enzyme (Promega). Five genes were tested (see Table 2 for gene name, primer sequences and PCR cycling conditions). PCR mix was: 0.4µM primers; 1.5mM MgCl2; 0.2mM dNTPs, 1 unit GoTaq hot-start and 1µl of cDNA in a total volume of 20µl of RNase, DNase free water. All PCR products were separated by electrophoresis through 1% agarose gels, and visualized by staining with ethidium bromide.

2.4. PCR amplification of microsatellite loci

Fifteen microsatellite markers, SmC1, SmDO11, SmDA28 (Curtis, et al., 2001), R95529, SmD57, SmD28, SmD25, SCMSMOXII, L46951 (Durand, et al., 2000), SmBR16, SmBR10, SmBR13 (Rodrigues, et al., 2007), SmS7-1 (Blair, et al., 2001), SmBR1, SmBR6 (Rodrigues, et al., 2002) were used in this study (Table 3). The relevant DNA fragments were amplified using PCR. Details on microsatellite sequences, primers and PCR conditions are available in Table 3. To maximise efficiency and minimize costs, these PCRs were performed in three multiplex reactions using the QIAGEN multiplex kit. The PCR amplifications of loci: R95529, SmC1, SmDO11, SmBR16 and SmD57 were grouped in the multiplex 1 (M1); loci: SmDA28, SmBR1, SmS7-1, SmD28, SCMSMOXII were grouped in the multiplex 2 (M2); and loci: SmD25, L46951, SmBR6, SmBR10 and SmBR13 were grouped in the multiplex 3 (M3). These multiplex reactions were carried out according to the manufacturer’s standard microsatellite amplification protocol in a final volume of 10µL and with 57°C as annealing temperature. PCR products were diluted in Sample Loading Solution (Beckman Coulter) with red-labelled size standard (CEQ™ DNA size standard kit, 400, Beckman Coulter) and electrophoresis was done on an automatic sequencer (CEQ™ 8000, Beckman Coulter).
Microsatellite sizes were determined using the fragment analyzer package of Beckman Coulter. To confirm that the fixation procedure did not modify the microsatellite length and thus did not affect negatively microsatellite analyses, we used a clonal strain of *S. mansoni* (Brazilian strain selected for locus homozygosis) to compare the microsatellite profile between fixed and non-fixed material. These samples were processed as described above.

2.5. Ethical Statements

Our laboratory has received the permit # A66040 for experiments on animals from both the French Ministry of Agriculture and Fishing and the French Ministry of National Education, Research and Technology. Housing, breeding and animal care of the hamster followed the ethical requirements of the French government. The experimenter possesses the official certificate for animal experimentation delivered by both ministries (Décret # 87–848 du 19 octobre 1987).

3. Results

3.1. Sporocysts detection and dissection.

After 24 hours in Raillet-Henry fixation, SpIs are readily observable as translucent white bodies within an opaque grey tissue background (Figure 1A). At higher magnifications we could see the spherical aspect of SpI (Figure 1B and C). Figure 1 shows a snail at 15 DPI. SpIs were at their optimal development (filled with secondary sporocysts (SpIIs)), the biggest reached 1mm in diameter and could be recovered by careful dissection of the snail head-foot zone under a binocular microscope. Dissecting SpIs before 6 DPI remains very difficult. It is important to recover the SpI without taking snail tissue in order to enrich the parasite signal for genomic DNA or transcript RNA extractions.
3.2. S. mansoni sporocysts fixation and genomic DNA amplification

We tested the possibility to amplify gDNA of single SpI after Raillet-Henry fixation. PCR products of up to 2 kb in length could be amplified from gDNA (columns 1, 2, 3; Figure 2). At 2.5 and 3 kb amplification did not work anymore or only occasionally for some individuals (see individual of column 3 at 2.5kb, Figure 2). The results were compared to a positive control (classical gDNA extraction) for which PCR amplifications worked for up to 3kb (column 4; Figure 2). To achieve this fragment length specific amplification, we design primers for SmPoMuc genes for Schistosoma mansoni polymorphic mucins. These mucins were specific of S. mansoni and no related genes were present in the snail host B. glabrata, thus no cross-amplifications did occur (data not showed).

3.3. S. mansoni sporocysts fixation and microsatellite amplification

Microsatellite PCR on DNA extracted from Raillet-Henry fixed SpIs shown good results (Figure 3). All microsatellite loci were amplified and fragment lengths were in agreement with the expected sizes (see Figure 3 and Table 3) based on previous studies done on DNA extracted from adults of the same parasite strain (Bech, et al., 2010, Durand, et al., 2000). In addition using a clonal strain of S. mansoni without microsatellite diversity we showed that microsatellite profiles were the same for fixed and non-fixed material (Table 4). The fixation procedure did not modify the microsatellite length and thus did not affect negatively microsatellite analyses.

3.4. S. mansoni sporocysts fixation and reverse-transcription PCR.

Five genes were selected to study the sensitivity of transcript amplification, (i) alpha tubulin is a highly expressed gene involved in microtubules (one of the main components of the
cytoskeleton) bio-synthesis, (ii) two genes involved in different metabolic pathways, the superoxide dismutase (SOD) that is part of the antioxidant defence against reactive oxygen species and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) involved mainly in glycolysis pathway; (iii) calreticulin is a multifunctional calcium binding protein encoded by a single copy gene in *S. mansoni* genome (Berriman, et al., 2009). Finally (iv) the *S. mansoni* antigen 10-3, a developmentally regulated surface antigen known to be expressed specifically in cercaria, male and female worms, was tested. Transcript amplifications were tested for these five genes after menthol anaesthesia and Raillet-Henry fixation or after snail were snap-frozen in liquid nitrogen and Raillet-Henry fixation and the same results were obtained for both techniques. For the four first tested genes, PCR products could be obtained when 5 SpI were used for cDNA preparation (Column 1, Figure 4). cDNA extracted from a single fixed SpI gave only an amplification for alpha-tubulin (Column 2, Figure 4) probably due to the greater amount of transcript for this highly expressed gene. PCR amplifications were highly specific as no cross amplifications with the intermediate host *B. glabrata* cDNA could be observed (Column 4, Figure 4). All those four genes were known to be expressed in miracidia and sporocysts of *S. mansoni*. Antigen 10-3 known not to be expressed was also tested as an internal control and as expected gave no amplifications (Figure 4).

4. Discussion

Identification of *S. mansoni* sporocyst intramolluscan stage using Raillet-Henry fixation is a classical technique used mainly to investigate prevalence and intensity or intramolluscan development of the larval stages in the *S. mansoni* / *B. glabrata* model (Sire, et al., 1998, Théron, et al., 1997, Theron, et al., 1998). However, recovery of these intramolluscan fixed parasite stages for extraction of DNA or RNA was never described. Indeed, study of genetic information in formalin-fixed tissues is often hampered by the impossibility to amplify the
desired DNA or RNA as a consequence of nucleic acid damage (Zimmermann, et al., 2008). It is thus important to dissect snails and to recover the parasites after a short period of fixation (less than 24 hours). Studying genomic or transcriptomic information of these fixed intramolluscan stages will be of interest in many fields of research including the molecular dialogue between parasite and the host’s immune system, distribution of infrapopulation genotypes or gene expression during development and maturation from SpIs to cercariae. The present work describes an improved technique for DNA and RNA extraction from such samples and delivers proof for their use for the study of genomic DNA, transcript expression or microsatellite genotyping.

Raillet-Henry fixation permits to recover SpIs that are more than 6 days old. To isolate SpIs before 6 DPI was quite impossible due to the minute size of these parasites at these steps. The use of this technique to study post-miracidial stage or SpI within the first days of infection remains therefore difficult. This constitutes the main limitation of this approach. However recovery of SpIIs or cercariae is feasible (data not shown). Our extraction protocols are efficient on SpIs but also on all other intramolluscan parasite stages (data not shown). However for gDNA a limited size of PCR amplification exists. Apparently, Raillet-Henry fixation results in gDNA breaks at around 2kb because amplification of fragment of 2.5 kb was difficult and of 3kb was impossible. Fragmentations in DNA extracted from formalin preserved samples has been described before and is based on nicks and double-strand breaks (Zimmermann, et al., 2008). Amplifications of microsatellite fragments of less than 500 nucleotides worked very well using our protocols. There is no difference in microsatellite amplification between fixed and non-fixed material. Raillet-Henry fixation did not affect negatively the microsatellite analyses. The technique will allow for genotyping of single parasite intramolluscan stages and thus could permit reconstruction of the population structure of sporocyst infra-populations inside the snail. Finally, RNA extraction and RT-PCR showed
good results, cDNA transcript amplification from Raillet-Henry fixed material with or without nitrogen freezing works well. In our model this will facilitate the study of gene expression for all intra-molluscan parasite developmental stages at 6 DPI and older. We hope that the technique developed herein paves the way to a better understanding of the host/parasite molecular dialogue by taking into account more easily an often neglected partner in this interaction, the parasite.

Acknowledgments

We are grateful to Bernard Dejean and Anne Rognon for technical assistance. We thank Julien Portela for helping with picture taking. We gratefully acknowledge Dr. C. Grunau for correcting the English version of this paper and for his critical reading of the manuscript. The work received funding from the BiomGenIm (ANR-07-BLAN-0214-03) programs of the French National Agency for Research, CNRS, and UPVD. The funding agency had no role in the study design, data collection, data analysis, the decision to publish, or the manuscript preparation.
References


**Table 1:** Primers used for genomic DNA PCR

<table>
<thead>
<tr>
<th>Fragment length</th>
<th>Forward primer name</th>
<th>Forward primer sequence</th>
<th>Reverse primer name</th>
<th>Reverse primer sequence</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1010 bp</td>
<td>Exon6F</td>
<td>TGAAGCTCAACTCAGTAAGCTGAAC</td>
<td>Exon5R</td>
<td>CTTGTATCGCCTTCGATTCCAATTC</td>
<td>Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles</td>
</tr>
<tr>
<td>1422 bp</td>
<td>10483ex13.F2</td>
<td>ACGAGGATTAATGATTACAATAATGC</td>
<td>Exon11R</td>
<td>TAGATAATGTACTGGCCACTTTGTG</td>
<td>Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles</td>
</tr>
<tr>
<td>1999 bp</td>
<td>InTron14/15.F</td>
<td>CACTTGTTCAAAACACGTGTCTTC</td>
<td>Exon11R</td>
<td>TAGATAATGTACTGCCCACCTTTGTG</td>
<td>Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles</td>
</tr>
<tr>
<td>2517 bp</td>
<td>Exon11F</td>
<td>ATTTCTTCTAGAATGTCTGAG</td>
<td>InTron6/7.R</td>
<td>TAAAGGTGGAATATGCCAAACTCAC</td>
<td>Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles</td>
</tr>
<tr>
<td>3052 bp</td>
<td>InT3/4-3.3.F</td>
<td>CTATGGACACTATGAACATATTGC</td>
<td>InTron6/7.R</td>
<td>TAAAGGTGGAATATGCCAAACTCAC</td>
<td>Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles</td>
</tr>
</tbody>
</table>

bp: base pairs
Ta: Primers annealing temperature in centigrade; elong: temperature in centigrade and duration of elongation in minutes.
### Table 2: Primers used for cDNA PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession No.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha tubulin</td>
<td>SCMSAT1A</td>
<td>AGCAGTTAAGCGTTGCAAAATCA</td>
<td>TGACGAGGTCACATTTCCACCATT</td>
<td>Ta: 53°C - elong: 72°C, 30sec - 40 cycles</td>
</tr>
<tr>
<td>Superoxide dismutase Cu/Zn (SOD)</td>
<td>XM_002580438</td>
<td>AGTGGACTCAAGGCTG</td>
<td>CCACGCGCTAAATCAT</td>
<td>Ta: 54°C - elong: 72°C, 30sec - 40 cycles</td>
</tr>
<tr>
<td>Glyceraldehydes-3-phosphate dehydrogenase (GAPDH)</td>
<td>XM_002576947</td>
<td>GCGAGGTTTCGACTGA</td>
<td>AACAACGAACATGGGTG</td>
<td>Ta: 55°C - elong: 72°C, 30sec - 40 cycles</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>XM_002574439</td>
<td>ATACGCTCTGGGACAT</td>
<td>CCTTGCTTCGGCATTAA</td>
<td>Ta: 54°C - elong: 72°C, 30sec - 40 cycles</td>
</tr>
<tr>
<td>S.mansoni antigen 10-3 (Ag10-3)</td>
<td>M22346.1</td>
<td>CACAAAGGTCTACTGTAACGGA</td>
<td>CCTTTAACATGGAATTATCAGTCTGG</td>
<td>Ta: 54°C - elong: 72°C, 30sec - 40 cycles</td>
</tr>
</tbody>
</table>

Ta: Primer annealing temperature in centigrade; elong: temperature in centigrade and duration of elongation in seconds. GenBank Accession numbers (No.) are indicated.
### Table 3: Primers used for Microsatellite PCR

<table>
<thead>
<tr>
<th>Multiplex</th>
<th>Microsatellite name</th>
<th>Accession No.</th>
<th>Range size (nt)</th>
<th>Repeat motif</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>R95529</td>
<td>R95529</td>
<td>228/275</td>
<td>(CAT)10</td>
<td>GTGATTGGGTTGATAAAAG</td>
<td>CATGTTTCTTCAGTGTCG</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
</tr>
<tr>
<td>SmC1</td>
<td>AF325694</td>
<td>287-296</td>
<td>(AAT)6-16</td>
<td>TGACGAGGTTGACATAATTCTAC</td>
<td>AACACAGATAAGAGCGTACGG</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>SmD57</td>
<td>AF202967</td>
<td>276-300</td>
<td>(TA)22(GA)9</td>
<td>TCCTTGATTCACCTGGTG</td>
<td>GCAGTAATCCGAAAGATTAG</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>SmBR16</td>
<td>L04480</td>
<td>337-341</td>
<td>(TA)10</td>
<td>TGTAACCTGGATGACACTGA</td>
<td>GCCTGTACAAAATCTCCGA</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>SmDO11</td>
<td>AF325698</td>
<td>303-367</td>
<td>(GATA)20-37</td>
<td>GTTCTACAGGTGTGTCGTCG</td>
<td>ACCCTGCAAGCTTACGTG</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>SmDA28</td>
<td>AF325695</td>
<td>91-115</td>
<td>(GATA)7-14</td>
<td>CATGATTTAGCTCAGAGACC</td>
<td>AGCCAGTATAGCGGATGTCATC</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
</tr>
<tr>
<td>SmBR1</td>
<td>L81235</td>
<td>154</td>
<td>(AC)9</td>
<td>GAGTATACGCTTCTTGGGA</td>
<td>CGGAACGACAAGAAATCAT</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>SmS7-1</td>
<td>AF330105</td>
<td>184</td>
<td>(AC)17</td>
<td>TCTCCCTCTCTATTTTCTCTTGT</td>
<td>ATTACGATTGCAACAGATACTTTTTC</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>SmD28</td>
<td>AF202966</td>
<td>240-244</td>
<td>(CAA)5</td>
<td>CATCACCATAATACACTC</td>
<td>TATTTAGTTGACGCG</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>SCMSMOXII</td>
<td>M85305</td>
<td>283-295</td>
<td>(CAT)9CGT(CAT)6</td>
<td>TTCTACAAATAATACCACAC</td>
<td>TTTTTTCTACTCATATACAC</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>SmBR10</td>
<td>DQ448293</td>
<td>109-133</td>
<td>(GATA)10</td>
<td>CATGATTTAGCTCAGAGACC</td>
<td>GTACATTTATTGTACAGTTAGCC</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
</tr>
<tr>
<td>L46951</td>
<td>L46951</td>
<td>168-174</td>
<td>(GAA)7</td>
<td>CAAACATAATACATTTGACAG</td>
<td>TGAATTGATGAATGATTGAG</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
<tr>
<td>SmBR13</td>
<td>DQ137790</td>
<td>205-225</td>
<td>(CTAT)16</td>
<td>GTCAGACATACGTGACAGCCTG</td>
<td>ACTCCCCAGCAAATTGTTCC</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
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</tr>
<tr>
<td>SmBR6</td>
<td>AF090659</td>
<td>272-278</td>
<td>(CTT)10</td>
<td>CTAAACAGAACATACACGC</td>
<td>GAATACAGCTATAATCTACA</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
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</tr>
<tr>
<td>SmD25</td>
<td>AF202965</td>
<td>268-274</td>
<td>(CA)10</td>
<td>GATTCGACCAATTAAGGCC</td>
<td>GCCATTAGATAATGACGTG</td>
<td>Ta: 57°C - elong: 72°C, 1min - 35 cycles</td>
<td></td>
</tr>
</tbody>
</table>

Characteristics of *Schistosoma mansoni* strain Guadeloupe (GUA) microsatellite loci, including locus name, GenBank Accession number (No.), size of PCR products in nucleotides (nt), nature of repeated motifs, forward and reverse primer sequences, annealing temperature in centigrade (Ta); elong: temperature in centigrade and duration of elongation in minutes.
Table 4: Comparison of microsatellite sizes for fixed and non-fixed materials.

<table>
<thead>
<tr>
<th>Multiplex</th>
<th>Microsatellite name</th>
<th>Size (bp) for fixed material</th>
<th>Size (bp) for non-fixed material</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>R95529</td>
<td>275</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td>SmC1</td>
<td>290</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>SmD57</td>
<td>296</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td>SmBR16</td>
<td>337</td>
<td>337</td>
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Microsatellite locus sizes of *Schistosoma mansoni* Brazilian clonal strain.
Legends to figures

Figure 1. Visualisation of implanted SpI in snail tissue at 15DPI after Raillet-Henry fixation. The SpI were readily observable as translucent white bodies within an opaque tissue background (A). Higher magnification showing the spherical aspect of SpI (B, C). Biomphalaria glabrata was exposed to 20 miracidia of Schistosoma mansoni.

Figure 2. PCR amplification of genomic DNA recovered from Raillet-Henry fixed sporocysts. Columns 1, 2 and 3 correspond to individual sporocysts fixed in Raillet-Henry. Column 4 is a PCR positive control using gDNA extracted from non fixed miracidia and column 5 correspond to the negative PCR control. Specific fragment lengths were amplified using specific primer couples designed on SmPoMuc gene (Roger, et al., 2008, Roger, et al., 2008, Roger, et al., 2008). See Table 1 for primer sequences and PCR conditions. MW: molecular weight in kilo bases.

Figure 3. Multiplex microsatellite fragments profiles visualised after PCR amplifications and sequencing using the CEQ 8000 fragment analyzer package (Beckman Coulter). For each multiplex (M1, M2, M3) the name and size position of microsatellites were indicated (see Table 3 for primer sequences and PCR conditions). For SCMSMOXII, as the dye signal is faint compared to the others, we showed a higher magnification of this microsatellite in the upper right corner of the M2 picture. nt: nucleotide; RFU: relative fluorescent units.

Figure 4. PCR amplification of cDNA transcripts recovered from Raillet-Henry fixed sporocysts. Column 1 corresponds to the pool of cDNA originating from 5 sporocysts fixed in
Raillet-Henry. Column 2 corresponds to the cDNA of 1 sporocyst fixed in Raillet-Henry. Column 3 corresponds to a positive control using cDNA originating from non fixed miracidia. Column 4 corresponds to *Biomphalaria glabrata* cDNA. Column 5 corresponds to retro-transcription negative control. Column 6 corresponds to PCR negative control. See Table 2 for primer sequences and PCR conditions.

MW: molecular weight in base pairs.