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## Cheap, rapid and efficient DNA extraction method to perform multilocus microsatellite genotyping on all *Schistosoma mansoni* stages

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3 Running headline: *Schistosoma mansoni* DNA extraction method

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5 Cheap, rapid and efficient DNA extraction method to perform multilocus  
6 microsatellite genotyping on all *Schistosoma mansoni* stages

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21 **Abstract**

22 Schistosomes are endoparasites causing a serious human disease called schistosomiasis.  
23 The quantification of parasite genetic diversity is an essential component to understand  
24 the schistosome epidemiology and disease transmission patterns. In this paper, we  
25 propose a novel assay for a rapid, low costly and efficient DNA extraction method of  
26 egg, larval and adult stages of *Schistosoma mansoni*. One euro makes possible to  
27 perform 60 000 DNA extraction reactions at top speed (only 15 minutes of incubation  
28 and 5 handling steps).

29

30 **Keywords :** *Schistosoma mansoni* ; DNA extraction ; Microsatellites

31 Schistosomes (Platyhelminth, Digenea) are endoparasites causing a serious  
32 human disease called schistosomiasis. Schistosomiasis ranks second only to malaria in  
33 terms of parasite-induced human morbidity and mortality, with more than 200 million  
34 people infected (Chitsulo et al. 2000; Crompton 1999). The quantification of parasite  
35 genetic diversity is an essential component to understand schistosome epidemiology and  
36 disease transmission patterns. This genetic diversity could be assessed either at the adult  
37 stage (Theron et al. 2004) or, more recently, at the larval stage (Shrivastava et al. 2005;  
38 Sorensen et al. 2006). The use of adult worms to quantify the genetic diversity in the  
39 definitive host is only relevant when worms can be directly recovered from naturally  
40 infected rodents (Theron et al. 2004). The quantification of parasite genetic diversity  
41 from intra-human (Brouwer et al. 2001; Curtis et al. 2002; Stohler et al. 2004) or intra-  
42 molluskan stages (Dabo et al. 1997; Eppert et al. 2002; Sire et al. 2001) requires a long  
43 time for a passaging through experimental hosts. However, mollusk or vertebrate  
44 experimental host may induce a bias due to this host selective pressure. Indeed, such  
45 laboratory passage may be predicted to result in genetic bottlenecking of the parasite  
46 population and impose selection pressures not encountered in field conditions. Firstly,  
47 exposure of individual snails to single miracidia results in only 5 to 50% of successful  
48 infections, depending on the parasite strain used (Theron et al. 1997), thus between 50%  
49 and 95% of the parasite genetic diversity is lost. Secondly, as far as the vertebrate host  
50 is concerned, it has been shown that passaging through experimental models decreases  
51 the parasite genetic diversity in comparison to field isolates (Loverde et al. 1985). To  
52 circumvent ethical, technical and epidemiological disadvantages of the use of  
53 experimental hosts, methods for genotyping larvae have been recently proposed  
54 (Shrivastava et al. 2005; Sorensen et al. 2006). Due to their small size (450  $\mu\text{m}$  for

55 cercariae and 150 µm for miracidia), the main technical limitations of these studies were  
56 the available quantity of DNA to perform PCR amplifications. In 2005, Shrivastava et  
57 al. (2005) proposed a DNA extraction protocol allowing sufficient DNA for only one  
58 PCR reaction by larvae, thus for only one locus analyses. In 2006, Sorensen et al.  
59 (2006) proposed a more complex protocol, only tested on eggs and that required liquid  
60 nitrogen to disrupt the eggshell by heat shock and Instagen Matrix (Bio-Rad) to capture  
61 DNA. This last protocol permits multi-locus analyses but it requires a particular  
62 material and finally, the resulting analysis have been performed only on eggs. In this  
63 paper, we propose a novel assay for a very rapid, very low costly and efficient DNA  
64 extraction method of adult and free larval stages from individual *Schistosoma mansoni*.  
65 To investigate the efficiency of the method, we have performed DNA extraction of  
66 individual schistosomes from all life cycle stages (except intramolluskan stage) and  
67 used five microsatellite markers of various sizes (i) on 10 individual eggs derived from  
68 faeces of infected mice, (ii) on 10 individual miracidia obtained from eggs purified from  
69 the livers of infected mice, (iii) on 10 individual cercariae derived from  
70 monomiracidially infected mollusks, (iv) and finally, on 10 adults obtained from  
71 infected mice.

72         The *S. mansoni* strain was isolated from naturally infected mollusks collected in  
73 Guadeloupe (French West Indies) in December 2002. The intermediate host used was a  
74 Guadeloupean strain of *Biomphalaria glabrata* and the definitive host was the Swiss  
75 OF1 mouse strain. Detailed methods for the mollusk and mouse infections were  
76 previously described (Boissier and Moné 2000). The biological material (eggs,  
77 miracidia, cercariae and adults) was obtained around the parasite life cycle. (i) Eggs  
78 were recovered from faeces of two Swiss OF1 infected mice. Ten eggs were

79 individually isolated in 5  $\mu$ l of NaCl 8% and transferred in a PCR reaction tube using a  
80 20  $\mu$ l micropipette. The presence of only one egg in each tube was checked under a  
81 binocular microscope. (ii) Miracidia were hatched from eggs purified from the liver of  
82 one OF1 infected mouse. Ten miracidia were individually isolated in 5  $\mu$ l of spring  
83 water and transferred in a PCR reaction tube using a 20  $\mu$ l micropipette. The presence  
84 of only one miracidium in each tube was checked under a binocular microscope. (iii)  
85 Mollusks were individually exposed to individual miracidium which all originated from  
86 the same mouse. Five weeks later, mollusks were individually placed in spring water  
87 and exposed to artificial light to stimulate cercarial release. Ten cercariae derived from  
88 mollusks were individually isolated in 5  $\mu$ l of purified spring water and transferred in a  
89 PCR reaction tube using a 20  $\mu$ l micropipette. The presence of only one cercariae in  
90 each tube was checked under a binocular microscope. (iv) One mouse was infected  
91 using 120 cercariae. Seven weeks later, the mouse was sacrificed and 10 worms were  
92 recovered and individually isolated.

93         The same DNA extraction procedure was used, for either adult or larval stages.  
94 Before DNA extraction, individual eggs, miracidium, cercariae or adult worms were  
95 individually vacuum-dried for 15 minutes in a Speedvac evaporator. Then, 20  $\mu$ l of  
96 NaOH (250 mM) was added to each tube. After a 15 minutes incubation period at 25°C,  
97 the tubes were heated at 99°C for 2 minutes. 10  $\mu$ l HCl (250 mM), 5  $\mu$ l of Tris-HCl  
98 (500 mM) and 5  $\mu$ l Triton X-100 (2%) were added and a second heat shock at 99°C for  
99 2 minutes was performed. The products were stored at -20°C. The PCR amplifications  
100 were performed in duplicate using 5 microsatellite markers (Table 1). The PCR  
101 reactions were carried out in a total volume of 20  $\mu$ l containing 4  $\mu$ l of 5x buffer (10  
102 mM Tris-HCl, pH 9.0 at 25°C, 50 mM KCl, 0.1% Triton X-100), 0.2  $\mu$ M of each

103 oligonucleotide primer, 200  $\mu$ M of each dNTP (Promega), 1 unit of GoTaq polymerase  
104 (Promega, Madison, Wisconsin), 1  $\mu$ l of extracted DNA and DNase-free water qsp 20  
105  $\mu$ l. The PCR programme consisted in an initial denaturation phase at 95°C for 5 min,  
106 followed by 40 cycles at 95°C for 30 s, annealing temperature for 20 s (Table 1), 72°C  
107 for 30 s, and a final extension at 72°C for 10 min in a thermocycler (Bio-Rad, Hercules,  
108 USA). For each marker, the forward PCR primer was 5' fluorescein labelled (Proligo,  
109 Cambridge, UK) allowing a precise analysis in an automated DNA sequencer. The  
110 microsatellite PCR products were diluted in Sample Loading Solution (Beckman  
111 Coulter, Villepinte, France) with a red labelled size standard (CEQ<sup>TM</sup> DNA size  
112 standard kit, 400, Beckman Coulter) and electrophoresed using an automatic sequencer  
113 (CEQ<sup>TM</sup> 8000, Beckman Coulter) with CEQ<sup>TM</sup> 8000 sequence analysis software. The  
114 sizes of the alleles were calculated with the fragment analyzer package.

115         From the 10 eggs, we obtained, during the first amplification, 52% of success. A  
116 second amplification on the same extracted DNA gave 92% of success. From the 10  
117 miracidia, we obtained, during the first amplification, 90% of success. A second  
118 amplification on the same extracted DNA gave 100% of success. From the 10 cercariae,  
119 during the first PCR amplifications performed, we obtained 98% of success. A second  
120 amplification of the same extracted DNA gave 100% of success. From the 10 adults,  
121 during the first PCR amplifications performed, we obtained 98% of success. A second  
122 amplification of the same extracted DNA gave 100% of success. The amplification  
123 failures were independent from the locus tested. Furthermore, it is likely that 100% of  
124 DNA had been extracted because after one or two PCR reactions, all expected PCR  
125 products gave at least one result in one microsatellite marker. DNA extraction methods  
126 are generally complex and time consuming, or quick and usually more expensive due to



127 the use of commercial kits. Table 2 shows a comparison between our method and the  
128 two previous ones (Shrivastava et al. 2005; Sorensen et al. 2006). Our DNA extraction  
129 protocol is efficient on all parasite stages and makes it possible to obtain an extracted  
130 DNA for PCR amplification at top speed (15 minutes of incubation), with few handling  
131 steps (5) and at a very low cost (1 Euro is sufficient to perform more than 60 000 DNA  
132 extraction reactions). This extraction procedure yields 40 µl of DNA from individual  
133 egg, miracidium, cercaria or adult that allows for 40 PCR amplifications, according to  
134 our protocol. This method could be perform in 96-well microplates allowing several  
135 hundreds DNA extractions in one hour.

136

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193 Table 1: Microsatellite markers used to test the extraction procedure.

<b>Locus</b>	<b>Genbank accession number</b>	<b>Annealing temperature</b>	<b>Amplicon size (bp)</b>	<b>Number of alleles detected</b>	<b>Authors</b>
SMD011	AF325698	60°C	351-383	7	Curtis et al. 2001
SMC1	AF325694	56°C	287-303	5	Curtis et al. 2001
SMD57	AF202967	52,5°C	278-300	11	Durand et al. 2000
R95529	R95529	52,5°C	229-274	3	Durand et al. 2000
SMBR16	LO4480	59,5°C	337-341	4	Rodrigues et al 2007

194

195 Table 2: Comparison of DNA extraction procedures.

<b>Protocol from</b>	<b>Sorensen et al. (2006)</b>	<b>Shrivastava et al. (2005)</b>	<b>Present study</b>
Tested parasite stages	egg	miracidium, cercaria	egg, miracidium, cercaria, adult
Incubation time	20 min	2 h 25	15 min
Number of steps	8	6	5
Number of reactions with 1 Euro	7	300	60 000
Number of allowed PCR reactions	25	1	40

196