Published in final edited form as:

Mol Ecol Resour. 2008 May ; 8(3): 647–649. doi:10.1111/j.1471-8286.2007.02031.x.

Isolation and characterization of the first polymorphic microsatellite markers for *Schistosoma haematobium* and their application in multiplex reactions of larval stages

R. GOLAN^{1,*}, C.M. GOWER^{1,2}, A.M. EMERY², D. ROLLINSON², and J. P. WEBSTER¹

¹Department of Infectious Disease Epidemiology, Faculty of Medicine, Imperial College, London, Norfolk Place, London W2 1PG, UK

²Wolfson Wellcome Biomedical Laboratories, Department of Zoology, Natural History Museum, Cromwell Road, London SW7 5BD, UK

Abstract

The ability of microsatellite loci to reveal genetic diversity within the trematode *Schistosoma haematobium* is demonstrated for the first time. Nine novel polymorphic microsatellite markers were isolated and their viability assessed on 36 *S. haematobium* adult worm individuals from three geographic populations. Allelic diversity and gene diversity ranged from two to seven and 0.29–0.76 respectively, suggesting high variability between individuals and between unrelated populations. Three primers also amplified *Schistosoma mansoni* and two *Schistosoma japonicum*. The results suggest these primers are useful for population genetic analyses of *S. haematobium*.

Keywords

Schistosoma haematobium; microsatellites; multiplex; polymorphism; miracidia

Schistosomiasis is a chronic and debilitating disease, second only to malaria in terms of parasite-induced human morbidity and mortality. Among the species of schistosomes infecting humans, *Schistosoma haematobium* is responsible for the largest number of infections. It has been estimated that in sub-Saharan Africa 112 million people are infected with this species, compared with 54 million infected with *Schistosoma mansoni* (van der Werf, 2003). However, the genetic diversity of *S. haematobium* remains largely unstudied in comparison to *S. mansoni*, primarily due to the more demanding conditions for laboratory maintenance and a lack of available molecular markers. Here we present the first isolation and characterisation of *S. haematobium* DNA microsatellite markers and their use in multiplex reactions. The utility of these novel microsatellites for the two other major schistosome species infecting humans was also examined.

Four genomic libraries enriched for simple sequence repeats (CA, AAT, ATG, TAGA) were constructed using *E. coli* DH5alpha and pUC19 vector by Genetic Identification Services (GIS, Chatsworth, CA, USA). For these libraries, a mixture of *S. haematobium* DNA was used derived from adult worms originating from Mali, Tanzania, Zanzibar, Zambia, Uganda and Zimbabwe. Eighty-seven microsatellite-containing clones were sequenced and PCR primers designed for 29 suitable unique sequences. Nine primers were selected based on their polymorphic content, absence of null alleles and reproducibility (Table 1). In addition,

* Author for correspondence r.shahar-golan@imperial.ac.uk, Tel: 0207 594 3819, Fax: 020 7402 3927 .

three of the novel *S. haematobium* primers amplified *S. mansoni* (originally from Egypt) DNA and two amplified *Schistosoma japonicum* (originally from the Philippines).

All optimizations were conducted under conditions suitable for multiplexing of loci, since only a single PCR reaction is possible from individual schistosome larvae (Shrivastava et al., 2005; Gower et al., 2007). Optimization and characterization of the reported primers was, however, performed on adult *S. haematobium* worms, because of their individually greater DNA content relative to miracidia and hence their suitability for comparing multiplex and single locus reactions in order to check for multiplex artefacts. Polymorphic loci were characterized using groups of 12 individual *S. haematobium* adult worms from each of three geographic regions: Kano, Nigeria; Loum, Cameroon and Vallee Pitot, Mauritius. Genomic DNA was extracted from ethanol-preserved individual worms by digesting for 2 hours with proteinase K (200µg/ml) at 55°C in a 200µl of a solution containing 1% SDS, 0.05M EDTA and 0.1M Tris (pH. 8). DNA was then transferred onto Indicating FTA Classic cards (Whatman, Middlesex, UK) in drops of 10µl and left to dry for one hour. A 2.0mm sample disk was removed from the FTA cards using a Harris micro punch, and washed twice with FTA reagent (Whatman, Middlesex, UK) and twice with TE solution (pH=8.0).

Forward primers were fluorescently labelled using 6-FAM, VIC, NED and PET dyes (dye set – G5) (Applied Biosystems, Cheshire, UK), using different colours for alleles with overlapping size ranges. PCR reactions were performed using a PCR-200 Thermal Cycler (MJ Research, UK). Amplifications were performed in 25µl reactions containing template 1µg DNA approximately and a ratio of 1:9:10 between primer mix (0.05µM of each primer), distilled water and a multiplex PCR master mix (Qiagen® Multiplex PCR kit, West Sussex, UK) containing 3mM MgCl₂, ultra pure quality dNTP mix and HotStarTaq DNA Polymerase. Thermal cycling was performed with a step-down PCR beginning with an initial hot-start activation of 15 minutes at 95°C, followed by 20 cycles with the following conditions: 30 seconds at 94°C, 90 seconds at annealing temperature (2 cycles at each temperature stepping down from 63 to 54°C every 1°C), 60 seconds at 72°C, followed by 20 cycles with the following conditions: 30 seconds at 94°C, 90 seconds at 53°C, 60 seconds 72°C, followed by a final extension at 60°C for 30 minutes. Products were diluted five-fold in autoclaved deionised water, run with 0.18µl Genescan®-LIZ500 size standard and analysed using an ABI 3730 automated sequencer. All samples were run with positive and negative controls. Allele sizes were determined using Genemapper v.4.0 (Applied Biosystems, California, USA).

There was 97% identity between single-locus and multiplex comparison of four adult worms due to the presence of two null alleles (for primer A6) in the multiplex. Allelic diversity and heterozygosity, linkage disequilibrium and deviation from Hardy-Weinberg Equilibrium were calculated using GDA version 1.1 (Lewis & Zaykin, 2001). Allelic diversity was high with 2-7 alleles and gene diversity ranging from 0.29-0.76. There was no evidence of linkage disequilibrium between pairs of loci. The comparison of observed and expected heterozygosity is less useful in schistosomes since asexual multiplication in an intermediate host means that adult worms may be clonally related. Results are consistent with a small number of sibling/half sibling families, indicating a very small number of clonal parental types, as individuals were likely to be highly related. The current *H_o* results are thus likely to reflect only massive bottlenecking at the first laboratory passage, as we have demonstrated previously for both *S. mansoni* and *S. japonicum* (Shrivastava et al. 2005; Gower et al. 2007), and/or that there were very few genetic “individuals” at the original sampling locations. Three loci showed evidence of deviation from H-W equilibrium due to a deficiency of homozygote genotypes. A reduction in homozygosity/excess of heterozygotes have also previously been reported in adult *S. mansoni* as a result of laboratory passage (Gower et al., 2005; Sorensen et al., 2006).

Estimates of Wright's F_{st} statistics (Weir & Cockerham, 1984) were calculated in Arlequin 3.1 (Schneider *et al.*, 2000). P-values were calculated by 100,000 random permutations. There was evidence of significant differentiation between all three populations (Mauritius: Nigeria: $F_{st} = 0.17$; Mauritius: Cameroon: $F_{st} = 0.11$; Nigeria: Cameroon: 0.08; $p < 0.001$ for all pair wise comparisons) with evidence of a greater distance between Nigeria and Cameroon from that of the more geographically separated Mauritian population. The number of private alleles in the Mauritius population sample (seven) in comparison to those in Nigeria (two) and Cameroon (one) are in concordance with the F_{st} estimates.

We thus conclude that these first polymorphic microsatellite markers described here should provide a valuable initial tool for fine scale studies of the population genetics of *S. haematobium*.

Acknowledgments

We thank Dr. Jaya Shrivastava for supplying *S. japonicum* samples, Michael Anderson and Jayne King for schistosome culture. This work was supported by grants from the Royal Society (JPW), the Bill and Melinda Gates foundation (JPW, RG, AFG) and the Wellcome Trust (CMG, DR, JPW; grant number GR063774).

References

- Goudet, J. Fstat: A Computer Program to Calculate F-Statistics. version 2.9.3.2.2002. Free program distributed by the authors over the internet from <http://www2.unil.ch/popgen/softwares/fstat.htm>
- Gower CM, Webster JP. Intraspecific competition and the evolution of virulence in a parasitic trematode. *Evolution*. 2005; 59:544–553. [PubMed: 15856697]
- Gower CM, Shrivastava J, Lamberton PHL, et al. Development and application of an ethical and epidemiological appropriate assay for the multi-locus microsatellite analysis of *Schistosoma mansoni*. *Parasitology*. 2007; 134(4):523–536. [PubMed: 17096873]
- Lewis, PO.; Zaykin, D. Genetic Data Analysis: Computer Program for the Analysis of Allelic Data. 2001. Free program distributed by the authors over the internet from <http://lewis.eeb.uconn.edu/lewishome/software.html>
- Schneider, S., et al. ARLEQUIN, A software for Population Genetics Data Analysis. Genetics and Biometry Laboratory, University of Geneva; Geneva, Switzerland: 2000.
- Sorensen RE. Genetic filtering and optimal sampling of *Schistosoma mansoni* populations. *Parasitology*. 2006; 133:443–451. [PubMed: 16817994]
- Shrivastava J, Gower CM, Balolong EJ, et al. Population genetics of multihost parasites – the case for natural sampling of *Schistosoma japonicum* larval stages. *Parasitology*. 2005; 131:617–626. [PubMed: 16255820]
- van der Werf MJ, de Vlas SJ, Brooker S, et al. Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. *Acta Tropica*. 2003; 86:125–139. [PubMed: 12745133]
- Weir BS, Cockerham CC. Estimating F statistics for the analysis of population structure. *Evolution*. 1984; 38:1270–1359.

Table 1

Primer sequences and characteristics of *Schistosoma haematobium* microsatellite loci, including GeneBank accession no., repeat structure, clone size and range of alleles in base pairs (bp). Also shown expected and observed heterozygosity from multiplex reactions of *Schistosoma haematobium* adult worms from each of three geographical isolates (each n=12 worms), and cross-species amplification results.

Locus (GenBank Acc. No.)	Primer sequences (5'-3')	Repeat motif	Vallee Pitot, Mauritius		Kano, Nigeria		Lom, Cameroon		Clone size (Allele size range)	Amplification in other species	
			He	Ho	He	Ho	He	Ho		S.m.	S.j.
A1* (EF608039)	F: 6FAM-TTG-CAT-TCT-CCT-ACC-AAC-ATG R: TCC-ATC-AAA-CAA-CCA-GTT-GAC	(TCAA) ₈ n ₄ (TCAG) ₅	0.64	1	0.65	1	0.59	1	233 (222-230)	+	+
A4 (EF608040)	F: NED-CGA-ACT-CCA-ACG-AGC-ATC R: GGG-TGT-GGG-AAT-GAC-TTG	(CA) ₂ (TACA) ₂ (CA) ₂ (CACACG) ₃ (CA) ₇	0.65	1	0.65	1	0.69	1	292 (281-298)	+	+
A6 (EF608041)	F: 6FAM-AAG-GAG-GAT-GGC-TCT-TGT-G R: TGG-AAA-ACT-TGT-GGA-GAA-GG	(CA) ₆ n ₄ (CA) ₉ n ₂ (CA) ₁₀	0	0**	0	0**	0	0**	189 (185-193)	-	-
B2 (EF608042)	F: NED-ATA-GCC-CTC-ACC-TTC-TTG-TTC R: GTT-TTC-CTG-GTA-GAC-TTC-TTC-A	(CAA) ₇	0.69	1	0.59	1	0.67	1	266 (256-267)	-	-
B4* (EF608043)	F: VIC-AAG-CCG-ACC-ATT-TGA-CTC R: GTT-GCT-GTT-GAT-GAC-GAT-G	(CAACAT) ₃ (CAA) ₇	0.52	1	0.72	1	0.78	1	217 (193-218)	-	-
C2 (EF608044)	F: NED-AAG-AAT-GCC-CCT-TGT-CTT-C R: ACG-TCT-AAC-TGG-CGA-TCA-C	(TTA) ₁₀	0.56	0.33	0.69	0.44	0.64	0.20	212 (199-222)	-	-
C131 (EF608045)	F: PET-AGC-CTC-AAC-ACT-TGT-CAT-TTG R: TCC-GTC-TAC-ACA-GTC-TGA-AGG	(TTA) ₁₆	0.64	1	0.70	1	0.75	1	233 (204-256)	-	-
C140 (EF608046)	F: PET-TCC-TTG-AAG-CAA-TGA-ATT-TCA-C R: CCA-GGC-AGT-ACC-ACA-GTC-C	(AAT) ₁₂	0.63	0.71	0	0**	0.10	0.10	286 (270-278)	-	-
D3 (EF608047)	F: 6FAM-ATA-GGA-TTC-GAT-CTG-CAC-TAT-G R: GAC-CAC-TTG-TTG-AGA-TTG-AIT-T	(TAGA) ₁₀ (TTGA) ₉	0.71	0**	0.24	0.17	0.44	0**	298 (287-306)	+	-

S.m. = *Schistosoma mansoni*, S.j. = *Schistosoma japonicum*; + = amplification using PCR, - = no amplification.

* Caution is advised for this primer due to presence of large stutter bands. Genotype of individuals was confirmed by concordancy in three independent amplifications.

** Ho of 0 shows monomorphism for the primer within the population.