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Molecular Epidemiology of *Schistosoma mansoni*:

A Robust, High-throughput Method to Assess Multiple Microsatellite Markers from Individual Miracidia

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Abstract

Schistosomiasis is one of the major unconquered infectious diseases afflicting people of developing countries, particularly in Africa. A deeper understanding of the epidemiology of schistosomes is complicated by the intravascular location of adult worms which makes them routinely unavailable for study. Their progeny, miracidia, which are hatched from eggs that are passed in feces, are available and can provide valuable insights about human infections, but they are small in size, hindering robust molecular analyses. Here we present a new high-throughput technique to assess the genotypes at 21 previously published microsatellite loci for individual miracidia of *S. mansoni*. The 21 loci can be amplified in four multiplexed PCR reactions; however, enough template is produced for approximately six PCR reactions, which allows for additional PCR reactions for resampling or obtaining additional data. We validated this technique using a pedigree study employing laboratory crosses of *S. mansoni* from Kenya to obtain sets of parents and offspring. Of 23 loci examined, 21 loci were found to be reliable: false alleles were rare and missing alleles due to allelic dropout occurred at only two loci in approximately 5% of the offspring. The latter type of error can be further reduced by reamplification which is possible with our method. This technique is amenable to a 96-well format thus facilitating analysis of larger samples of miracidia, allowing more robust molecular epidemiological studies of *S. mansoni* to infer population size, population structure, gene flow, mating systems, speciation, and host race formation.

Keywords

Schistosoma mansoni; microsatellites; genotyping errors; molecular epidemiology; miracidia; sampling

INTRODUCTION

Schistosomiasis is a neglected parasitic disease caused by trematodes that inhabit the circulatory system of their vertebrate hosts. Eight species of schistosomes infect humans, and it is estimated that 200 million people are infected worldwide with many suffering from severe

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morbidity due to these parasites (Crompton, 1999; Chitsulo et al., 2000). *Schistosoma mansoni* is one of the most common etiological agents of schistosomiasis and is estimated to infect more than 83 million humans in 54 countries (Crompton, 1999). Control of this disease is mainly achieved through repeated population based chemotherapy to reduce intensity of infection and thus morbidity (Gryseels et al., 2006). This method does not eliminate transmission and therefore requires regular drug treatment to be sustainable. Although possible drug resistance in schistosomes has been reported (Gryseels et al., 2001; Alonso et al., 2006), it has not become a widespread phenomenon. However, with repeated selection pressure, there is a distinct probability that drug resistance will evolve in these organisms. The development of new tools and approaches to study the epidemiology and evolution of these parasites is needed to monitor the impact of control programs and other environmental changes likely to affect schistosome distribution, transmission, and evolution.

Molecular or evolutionary epidemiology is a developing field that uses genetic data and population genetic analyses to answer epidemiological questions. These techniques are powerful because they can assess population size, population structure, gene flow, mating systems, speciation, and host race formation of disease-causing organisms, and with these data, inferences about parasite transmission, recruitment, and drug resistance can be made (Nadler, 1995; Tibayrenc, 1998; Curtis and Minchella, 2000; Criscione et al., 2005; de Meeus et al., 2007). However, these techniques can only be applied properly if the appropriate data can be obtained, which has been problematic for schistosome parasites. Adult schistosomes reside in the circulatory system of their hosts; therefore, sampling adults from humans is problematic if not impossible. Traditionally, for genetic analysis schistosome adults can be obtained from humans only by hatching eggs from stool samples, using the miracidia to infect snails, the cercariae to infect mice, and collecting the adults from mice via perfusion (Curtis et al., 2001; Curtis et al., 2002; Rodrigues et al., 2002b; Stohler et al., 2004; Agola et al., 2006). This technique has many disadvantages, particularly the introduction of sampling bias through the loss of genotypes by sampling error or selection (Rodrigues et al., 2002a; Stohler et al., 2004; Gower et al., 2007). In fact, false geographic structuring has been induced by laboratory passaging of material (Gower et al., 2007). Besides possible biased results, additional disadvantages include the need to use and care for laboratory-reared animals, and unavoidable redundant sampling since clonal genotypes are often present in the same host due to asexual reproduction within the snail.

The need for a technique to genotype individual eggs or miracidia has been recognized by the scientific community and two research groups have published new methodology (Sorensen et al., 2006; Gower et al., 2007). These pioneering new methods determine genotypes of individual eggs or miracidia at 6-7 microsatellite loci, which is a moderate number for population genetic analyses. Both techniques are limited in the number of loci that can be analyzed due to the small amount of template DNA and the inability of one method (Sorensen et al., 2006) to multiplex loci in PCR reactions, which adds additional cost and time to this technique. Additionally, these techniques can not be directly validated because the samples yield low quantities of template DNA, and low quantity DNA samples are particularly vulnerable to genotyping errors such as lack of amplification, allelic dropout, and amplification of false alleles (Gerloff et al., 1995; Taberlet et al., 1996; Pompanon et al., 2005; Gunn et al., 2007). These are errors that can bias the results of downstream analyses (Pompanon et al., 2005).

The aim of this study was to improve on these methodologies and develop a reliable and economical technique to assess the genotypes of individual miracidia at multiple microsatellite loci. Using previously published loci, we developed 4 multiplex PCR reactions that amplify a total of 23 loci from individual miracidia. Reliability of the procedure was assessed using a pedigree technique in which adults and their offspring from laboratory crosses of natural

populations of *S. mansoni* were examined. With this system, parents of an offspring can be inferred and errors can be detected based on Mendelian inheritance of alleles. Additionally, enough template DNA is obtained to perform additional PCR reactions so that error checking can be performed, and other markers of interest may be analyzed. Although the markers are specific for *Schistosoma mansoni*, the technique described here can be modified to obtain similar data from other small organisms.

MATERIALS AND METHODS

Mouse Infections

Schistosoma mansoni was originally obtained from 3 human fecal samples from the Lake Victoria region of Kenya and each of the 3 isolates used had been kept in the laboratory for 0-3 generations. Laboratory raised snails (*Biomphalaria sudanica*) were infected by exposing each to 3-5 miracidia (pooled among fecal samples) in an individual well of a 24 well plate. Cercariae were obtained from the snails 30 days post exposure by isolating the snails in 24 well plates and exposing them to bright light. Cercariae from 14 snails were used to infect two mice, which was done by placing them into individual 10 cm fingerbowls, each with 12 cercariae from each snail, for 30 minutes. Adult worms were obtained 11 weeks post-exposure via portal perfusion (Smithers and Terry, 1965). The organs and body cavity of each mouse were extensively searched to find as many adults as possible. Paired worms were maintained together and their relationships recorded.

The liver, recently passed fecal pellets and fecal material from the intestine were collected for hatching of miracidia. The liver was blended with water in a Waring blender for 6 seconds, the homogenate was poured into a 500 mL conical flask, and allowed to settle for 10 minutes. The supernatant was poured off and replaced with water. The fecal material was homogenized with water and poured into a 250 mL conical flask and filled with water. Both flasks were exposed to bright light for 30 minutes to encourage egg hatching, then covered with a black cloth and aluminum foil so that only the tops of the flasks were exposed to light. The miracidia are attracted to light and swim to the top of the flask where they can be collected. Miracidia from the liver and feces were collected for DNA analysis.

DNA Preparation and Microsatellite Loci

DNA from individual miracidia and adults was prepared for PCR in a 96 well format using a variation of the HotSHOT method (Truett et al., 2000). For miracidia, each well of a 96 well PCR plate was filled with 5 μ L of HotSHOT lysis reagent, sealed with pierceable aluminum tape, and chilled with a PCR cooler during specimen collection. A single miracidium was collected in 3 μ L of water with a micropipette which was then used to pierce the foil to enable addition of the miracidium to a well. Pipetting through the foil eliminates the possibility of adding miracidia into the same well twice. Each plate was then spun down in a centrifuge, sealed with a silicone sealing mat, and heated in a PCR machine with a heated lid at 95°C for 30 minutes. Plates were periodically removed from the PCR machine and spun down to ensure that the liquid remained in the bottom of the wells. After heating, the plates were cooled, spun down, and 5 μ L of HotSHOT neutralizing reagent was added to each well. The plates were sealed and stored below 0°C. Adults were processed in a similar manner; however, the volume of lysis and neutralizing reagents was increased to 20-30 μ L and the samples were heated until no remaining tissue could be detected in the wells.

A total of 23 previously published microsatellite loci were investigated and were PCR amplified in 4 panels or 4 separate multiplex reactions named P17, P22, P23, and P24 (Table 1). These multiplexes were created by grouping the loci based on their annealing temperature, strength of amplification, size of markers so they do not overlap if the primers contain the same

fluorescent tag, if they were polymorphic, and if they successfully coamplified together in several trial reactions. Fluorescently labeled primers (Applied Biosystems) were labeled with 6-FAM, HEX, or NED (Table 1) and mixed together in a 2 μ M stock concentration. The QIAGEN Multiplex PCR Kit (Qiagen) was used for PCR amplifications of 2 μ l of template in 5.5 μ l reactions according to the manufacturer's directions. Thermocycling was carried out in an Eppendorf® Mastercycler ep Systems thermocycler with the following annealing temperatures: 48°C for P22, 52°C for P17 and P24, and 54°C for P23. For panel P23, the final extension step was increased to 60 minutes to reduce +A/-A peaks, which are an artifact of the PCR process as the polymerase adds an extra "A" on the end of the products, but if the nucleotide is not added to all of the products, two peaks one base pair apart will be visualized. Increasing the extension time allows a greater percentage of products to receive the "A", reducing the -A peak and creating a cleaner signal. Multiplexed PCR products were genotyped using an ABI 3100 automated sequencer (Applied Biosystems) and scored with GeneMapper® v. 4.0 (Applied Biosystems) software. All genotype calls were verified manually.

Initial validation of the multiplex PCR reactions was carried out by amplifying the loci individually in PCR reactions and together in multiplex PCRs for at least 8 adult *S. mansoni* specimens, which resulted in identical results for all loci whether amplified individually or in multiplex. Further validation was carried out to ensure the reliability of the loci for miracidia. For a subset of the offspring, the parental individuals and the alleles contributed by each was determined manually and checked for errors. Three errors were considered: no amplification, missing alleles, and false alleles. Loci that do not amplify when others in the multiplex are successful indicate which loci are "weak amplifiers". This type of error will not bias downstream analyses, but instead represents missing data that may be obtained by repeating the PCR. Missing alleles at a locus are alleles that are expected to be inherited from a parent, but are not detected in the offspring due to the presence of null alleles, allelic dropout, or mutation between generations. Allelic dropout is common when template quantity is low and occurs when one allele (usually the shortest) is favored over the other allele so that the individual appears to be a homozygote when it is actually a heterozygote (Gerloff et al., 1995). False alleles are those that are called for an offspring, but do not exist in either parent and can be the result of a mutation between generations or a false peak due to "noise" in the data (Broquet and Petit, 2004). Sample sizes for offspring varied among loci and are given in Table 2. Because this technique relies on a limited amount of genomic DNA, we sampled 144 individuals in an attempt to genotype all miracidia at all 4 panels and calculated our success rate to collect data from all four microsatellite panels without running out of DNA. We collected additional data for panel P17, which contains the most loci, to investigate its usefulness in determining parent offspring relationships in the absence of other data.

Diversity statistics including observed heterozygosity and number of alleles for each locus for adults and all offspring sampled was calculated using GenAlEx (Peakall and Smouse, 2006). For the adults, populations from the mice were combined and individuals with identical alleles at all 21 loci were analyzed using GENCLONE 1.1 (Arnaud-Haond and Belkhir, 2007) to determine the probability that these multilocus genotypes were derived from separate sexual reproductive events rather than polyembryony within the snail host that creates identical cercariae. Individuals that were determined to be clones were removed from the dataset so that diversity calculations would not be biased by clones and that the numbers would reflect unique individuals.

RESULTS

After perfusion and genotyping of adults, Mouse 1 was found to harbor 15 female worms comprising 5 multilocus genotypes and 14 male worms comprising 9 multilocus genotypes while Mouse 2 harbored 9 female worms comprising 5 multilocus genotypes and 11 male

worms comprising 6 multilocus genotypes. The mice shared some multilocus genotypes and together harbored 18 unique multilocus genotypes. Mouse 1 harbored 13 unique male and female pairs and Mouse 2 harbored 8 unique pairs, all of which produced offspring and can be thought of as different families that were used in this analysis. Results of the GENCLONE analysis indicated that the probability that individuals that were identical at all 21 loci were derived from separate sexual reproductive events, P_{sex} , was very low, with values ranging from 2.38×10^{-20} to 2.82×10^{-124} . Therefore, these individuals were considered to be derived from polyembryony within the snail host, which produces identical cercariae. All loci were polymorphic, the number of alleles per locus ranged from 2-17 from 18 individuals with a mean of 8.24 alleles per locus, and adult heterozygosity at each locus ranged from 0.11 to 1.0 with a mean of 0.751 (Table 2). Heterozygosity and the number of alleles per locus for the offspring sampled were similar to those of the adults, and only 3 alleles observed in the adult population were not observed in the offspring population.

For each microsatellite panel, 142-303 individual miracidia were included in the validation analyses. Complete linkage between loci L46951 and AF009659 suggested that they amplify the same repeat with different priming sites, and further evidence was obtained by comparing their GenBank reference sequences, which overlap by 330 bp and share almost 98% sequence identity. Therefore, locus AF009659 was discarded from further analyses.

Diversity among microsatellite markers was high and assignment of offspring to parents was possible for all offspring if at least two microsatellite panels were considered, and 99.4% of offspring could be assigned to parents with only one panel, the P17 panel, which was the most informative because it comprises the most loci. Error rates were low for most loci except X77211, which was difficult to score due to stutter and long allele drop out and is therefore considered unreliable (Table 1). Loci AF325698 and BF936409 were subject to missing alleles in 5.40 and 3.47% of the offspring, respectively. In these cases, the data showed an allele for only one parent and was missing an allele for the other parent; however, the offspring could be easily traced to the other parent using the additional markers. Allelic drop out is most likely the cause since the missing alleles were relatively long compared to the observed allele. Amplification of loci in multiplex does not appear to be a significant problem and may only affect one locus, AF202965, which did not amplify in 4.20% of the specimens, and the signal for this marker is generally lower than the other markers in the multiplex. False alleles were rare and will have little effect on analyses with these multiplexed microsatellite markers. Success rate for obtaining data from all four microsatellite panels was 68% (98/144) and for three panels 86% (124/144).

DISCUSSION

The techniques presented represent a method to produce reliable multilocus microsatellite data from individual miracidia of *S. mansoni*. Although separately, some of the aspects of this method have been described previously for other uses, this report is novel in that these methods have been modified and combined with four new panels or multiplexes of microsatellite markers to obtain reliable data for 21 microsatellite loci, and to have additional template to perform other PCR reactions for error checking or obtaining further data. The technique reported here improves on other published techniques (Sorensen et al., 2006; Gower et al., 2007) that only amplify up to 7 loci, because it yields results from three times as many loci, which generally increases statistical power of downstream analyses and allows more robust conclusions to be drawn (Ryman et al., 2006). Also, the technique presented here has been validated for errors using a direct pedigree approach. With previously published methods, validation was achieved only indirectly by comparing the miracidial data to data obtained from adults derived from the same population of eggs or miracidia that were passaged through snails and mice. Observed levels of heterozygosity were lower in the egg/miracidia data compared

to the adult data which was considered a result of selection or heterozygote advantage for individuals passaged through a mouse. However, an alternative explanation, and perhaps a more plausible one given that the loci are presumably neutral markers, is that the heterozygote deficiency is due to allelic dropout, a common genotyping error.

One of the greatest challenges in genotyping individual miracidia is the limited amount of template, which can lead to genotyping errors including lack of amplification, allelic dropout, and false alleles. Reducing genotyping error is critical for obtaining reliable results from downstream analyses. The best error detection technique is to test independent samples from the same individual (Pompanon et al., 2005); however, this is not possible with small organisms. The pedigree analysis used in this paper is an alternative approach, and allows a close examination of potential errors and error rates for each marker so that these errors can be anticipated even prior to data collection. A downside to this approach is that it cannot detect errors that are consistent with Mendelian inheritance (i.e. heterozygous offspring that appear homozygous due to error, but both parents have the detected allele). Knowledge of error rates can add power and accuracy to any study for two reasons. First, software packages that allow the incorporation of error into their algorithms are becoming increasingly available and the use of these along with real estimates of error rates can improve analysis power and overall accuracy of results (Wang 2004). Second, knowledge of loci with higher error rates can be avoided (as we have with one locus in this study) or selectively subjected to validation. Since allelic dropout was the most common error in this analysis, a more targeted approach can be used in which individuals that appear to be homozygotes at loci that are more prone to allelic dropout can be singled out and resampled. Since heterozygosity is relatively high in this system, only a few samples would have to be reanalyzed. Also, in future studies for which pedigree information is unavailable, regenotyping a subset of individuals will allow further confirmation of the reliability of the data. Genotyping errors cannot be eliminated with certainty; however, with our procedure, we have identified and quantified potential errors so that we can work to minimize them and even incorporate them into downstream statistical analyses.

Another challenge with limited templates is that there is little room for laboratory error which could cause entire samples to be used up before all data are obtained. Our success rate for obtaining data from all four panels (21 loci) was 68%, and for three panels (up to 18 loci), 86%. We feel these rates are acceptable and sample collection can be adjusted to accommodate for sample loss. Also, depending on the research problem, 21 loci may not be necessary and instead 18 loci in 3 PCR reactions, or 13 loci in 2 PCR reactions may be sufficient to address the problem. This is an important consideration because reducing marker number not only reduces cost through reducing the sample failure rate but also through reduction of PCRs and samples for genotyping not to mention work effort and labor costs.

One potential problem with laboratory developed techniques is that they are not useful in the field where data will be collected. The method of microsatellite genotyping of miracidia described by Gower et al. (2007), is intended for field sampling with minimal equipment because it preserves miracidial DNA on Whatman FTA® cards, which can be stored at room temperature and can be transported easily. The benefits of this assay are obvious and it is very practical for a field situation in which an equipped laboratory is not available; however, the downside is that limited amounts of data can be obtained from individual miracidia and the reliability of the assay is unknown. We have successfully used our technique in western Kenya gathering data not presented here. Collection of miracidia and DNA lysis was performed in a laboratory in Kenya and samples were then transported to the University of New Mexico on ice where PCR amplifications were performed. The data indicate that the samples are robust enough to be collected in an endemic area and transported across continents as long as they are stored around 0°C during transport and stored below -20°C for the longer term (up to eight months so far). One limitation of the technique is having the necessary equipment in the

location where the miracidia will be collected including a microscope, centrifuge that spins 96 well PCR plates, thermocycler, and freezer. In our experience, this tissue lysis method will also work in individual tubes rather than the 96 well plate format; however, handling time greatly increases when this change is made.

In summary, we present a technique to reliably assess genotypes of *S. mansoni* at 21 microsatellite loci. These data will allow more robust analyses to be performed to infer population size, population structure, gene flow, mating systems, speciation, and host race formation of *S. mansoni*, in the human host. These data will also permit new inferences about transmission, recruitment, and gene flow to be made. With the development of techniques like the one presented here, we hope in the near future that the field of molecular or evolutionary epidemiology can potentially flourish, enabling us to finally begin to open the “black box” with respect to infection of humans with *S. mansoni* and other schistosomes.

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Descriptive statistics and validation of 23 previously reported microsatellite loci (given by GenBank accession number) that were PCR amplified in 4 multiplex panels (Pan). Descriptive statistics for each locus including the reference (Ref) from which it originated, repeat length, size range, and fluorescent tag used (H=HEX, F=6-FAM, N=NED) are given. The sample size (n) of individual miracidia included in the validation and the percentage of specimens in which the locus did not amplify (No Amp), percentage with missing alleles, and percentage with detected false alleles is given.

Table 1

Pan	Locus Accession	Ref	Repeat Length	Dye	Size Range	n	No Amp	Missing Alleles	False Alleles
P17	AF325695	2	4	H	81-125	333	0.30	2.10	0.30
P17	AF325697	2	4	F	120-198	333	0.60	0.90	0
P17	AF325698	2	4	F	240-406	333	1.50	5.40	0
P17	AF202965	3	2	N	282-302	333	4.20	1.80	0
P17	AF202966	3	3	F	226-240	333	0.60	0	0.30
P17	AF202968	3	2	N	147-157	333	0.90	0	0
P17	L46951	3	3	H	158-223	333	0	0.30	0
P22	AI067617	6	4	H	205-238	144	0	0	0
P22	AI395184	6	3	H	166-204	144	0	0	0.69
P22	BF936409	6	3	F	189-229	144	0	3.47	0.69
P22	AF325694	2	3	H	281-317	144	0	0	0
P22	M85305	3	3	F	252-303	144	2.78	1.39	0
P22	R95529	3	3	N	221-271	144	0	0	0
P23	L81235	4	2	H	150-160	165	0	0	0
P23	AF009659	4	3	H	247-302	165	ND	ND	ND
P23	BH795456	5	3	F	150-196	165	0	0	0.61
P23	AI068335	1	2+2	H	217-233	165	1.21	1.82	0
P23	AI067567	6	3	N	174-223	165	1.21	0.61	0.61
P24	X77211	4	2	N	264-307	142	0	17.61	0.70
P24	L25065	4	3	N	323-341	142	0.70	0.70	0.70
P24	AF325694	6	3	N	211-236	142	0	0	0
P24	M85304	6	3	H	259-302	142	1.41	0.70	0
P24	AI110905	6	3	F	173-239	142	0	0.70	0.70

References:

¹ Blair et al. 2001
² Curtis et al. 2001
³ Durand et al. 2001
⁴ Rodrigues et al. 2002a
⁵ Rodrigues et al. 2002b
⁶ Silva et al. 2006.

Table 2
Descriptive statistics for adults and offspring of *Schistosoma mansoni* genotyped at 21 microsatellite loci. The sample size (N), Number of alleles (Na) and observed heterozygosity (Ho) are given.

Locus	Adults			Offspring		
	N	Na	Ho	N	Na	Ho
AF325695	18	2	0.111	329	2	0.228
AF325697	18	7	0.500	330	7	0.458
AF325698	18	17	0.778	299	16	0.870
AF202965	18	6	0.722	301	6	0.721
AF202966	18	9	1.000	331	9	0.852
AF202968	18	5	0.611	331	5	0.574
L46951	18	15	0.889	332	15	0.958
AI067617	18	6	0.667	144	6	0.569
AI395184	18	4	0.556	143	4	0.804
BF936409	18	8	0.778	138	7	0.812
AF325694	18	7	1.000	144	7	0.785
M85305	18	11	0.778	138	11	0.928
R95529	18	13	0.944	144	13	0.924
L81235	18	5	0.778	100	5	0.580
AF009659	18	13	0.889	100	12	0.930
BH795456	18	5	0.722	97	5	0.680
AI067567	18	10	0.778	99	10	0.747
X77211	17	7	0.824	102	7	0.814
AF325694	18	7	1.000	105	7	0.733
M85304	18	8	0.667	100	8	0.680
AI10905	18	8	0.778	101	8	0.881