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Short technical report

Efficient genotyping of *Schistosoma mansoni* miracidia following whole genome amplification

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ABSTRACT

Small parasites and larval stages pose a problem for molecular analyses because limited amounts of DNA template are available. Isothermal methods for faithfully copying DNA have the potential to revolutionize studies of such organisms. We evaluated the fidelity of multiple displacement amplification (MDA) for amplifying DNA extracted from a single miracidium of *Schistosoma mansoni*. To do this we genotyped DNA extracted from 28 F1 miracidia following MDA using 56 microsatellite markers. Because these miracidia were obtained from a cross between a male and female worm of known genotypes, we were able to predict the alleles present in the progeny and quantify the genotyping error rate. We found just 8/1568 genotypes deviated from Mendelian expectations. Furthermore, because 1 of these resulted from a genuine mutation, the error rate due to MDA is 7/1568 (0.45%). We conclude that many hundreds of microsatellites or other genetic markers can be accurately genotyped from a single miracidium using this method, greatly expanding the scope of population genetic, epidemiological and evolutionary studies on this parasite.

The genome sequence and genetic map of *Schistosoma mansoni* [1,2] open the door to genome-wide analyses involving hundreds or thousands of genetic markers. However, adult worms live in the mesenteric veins of human hosts. Thus, only larval stages, from which limited amounts of DNA can be extracted, are available for genetic analyses when working with samples from humans. Researchers have used two approaches to get around this problem. First, eggs obtained from human hosts can be hatched in the laboratory and the miracidia used to infect the appropriate freshwater snail. Clonally produced cercariae shed from snails or adult worms generated from passage through rodents can then be used for genetic analysis. This approach is cumbersome and time consuming, and has the additional problems of creating genetic bottlenecks and possibly causing host (snail or rodent)-induced selection at pertinent loci [3]. Hence, the parasites genotyped may be poorly representative of those initially sampled. Second, PCR-based methods have been used to amplify and genotype DNA extracted from a single egg or miracidium [4–7]. Using this approach up to 21 loci have been genotyped from a single miracidium [6]. However, PCR genotyping of miracidia using this approach requires careful optimization and pooled PCR reactions. Observations of strong deviations from Hardy–Weinberg proportions in the genotypes generated may indicate that template is limiting or of poor quality and suggest caution is needed when using these methods [4]. Furthermore, these methods [4,5] do not leave enough template DNA for repeat genotyping, which precludes the assessment of genotyping error rates in field studies.

Isothermal amplification of template DNA using Phi-29 DNA polymerase provides a simple approach for generating microgram amounts of DNA template from a few nanograms of starting material [8]. This method is now widely used for copying DNA for use in human genetic studies and has been carefully validated for use in a variety of organisms [9]. This approach has also been used to generate large amounts of DNA template from single sperm [10] and from a variety of parasite species, such as *Plasmodium* sp. [11] and *Trypanosoma* sp. [12]. We have evaluated the use of Phi-29 to faithfully replicate DNA extracted from a single miracidium. To measure the reliability of this approach we genotyped F1 miracidia generated from a genetic cross between parental worms in which genotypes were known. Therefore, we were able to predict the expected genotypes at each locus genotyped and quantify genotyping error rates. We observed just 7 discrepant genotypes due to whole genome amplification among 28 miracidia genotyped at 56 microsatellites markers (1568 genotypes) giving an error rate of 0.45%. While this validation experiment is specific to *S. mansoni*, we expect that similar success will be found for other schistosome species and for helminth parasites for which only eggs or larval stages are easily accessible.

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We set up a genetic cross between single genotypes of a female schistosome from the NMRI (Puerto Rico) strain with a male schistosome from the LE (Brazil) strain to generate the F1 progeny. To do this we exposed snails (*Biomphalaria glabrata*) to a single miracidium. After 28–29 days, the cercariae were collected by exposure to light. We extracted DNA from 20 to 50 cercariae from each shedding snail in 50 μL of 6% chelex containing 0.2 mg/mL of proteinase K. This was incubated at 56 °C for 2 h and at 100 °C for 8 min. We sexed cercariae by amplifying cercarial DNA using the W1 primers, which are specific for a repetitive region in the W chromosome of female worms [13]. As male worms are not expected to show any amplification, we ran a control PCR with the autosomal microsatellite locus sc18 (forward CAGTTTGTCAACAAGTAACGAG and reverse AAAAGACAAATTCGTTGTGG) in order to confirm the DNA was extracted. PCR was performed with 15 μL reactions containing 2.4 μL of extraction supernatant, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.4 μM each primer, and 0.75 units (0.15 μL) Taq DNA polymerase (Takara Shuzo Co., Otsu, Shiga, Japan). PCR cycling was 95 °C for 3 min, once; 94 °C for 45 s, 54 °C for 30 s, 72 °C for 45 s, 35 times; 72 °C for 7 min, once. We then infected a hamster with LE male cercariae of a single clonal genotype and NMRI female cercariae of a single clonal genotype to set up the cross. After 45 days, the hamster was perfused and the parental worms collected. We removed the liver, hatched the eggs in freshwater, and collected single miracidia in 10 μL H_2O in 0.5 mL microcentrifuge tubes for DNA extraction.

We extracted DNA from individual adult worms and 29 F1 miracidia in 200 μL and 20 μL of 5% chelex, respectively, as described above. We then used Phi-29 polymerase to expand the DNA template available using the GenomiPhi v2 DNA Amplification Kit (GE Healthcare) according to the manufacturer's manual, using 2.5 μL of the chelex-extracted DNA in each reaction. The total reaction volume of 50 μL was incubated at 30 °C for 4 h and terminated at 65 °C for 10 min. After amplification, the whole genome amplified DNA was purified through Sigma Spin Post Reaction Purification columns (Sigma, St. Louis, Missouri). The final volume of the DNA product was adjusted to 150 μL with water. DNA concentration was determined using a Hoefer DyNS Quant 200 Fluorometer and diluted to a final concentration of 15 ng/μL. Two microliters (30 ng) of product were used as template for genotyping of each microsatellite marker.

We used an economical method for fluorescent labeling of microsatellites [14], in which oligos are unlabeled, but attached to the 5′-end of the forward primer is the M13 oligo (5′-TGTAAAACGACGGCCAGT-3′). The PCR cocktail contains a fluorescent-labeled M13 primer, the reverse primer, and the forward primer with an M13 tail. The concept underlying this approach is simple: the first rounds create the specific product and then the last rounds label the product. This method allows one to order a single labeled primer (i.e., M13) that can be used to label multiple loci. We also "pig-tailed" the reverse primers by adding GTTTCTT to the 5′-ends [13] to minimize stutter bands. Amplifications were performed in 5 μL reactions containing 1× buffer, 1.5 mM of $MgCl_2$, 0.2 mM each of dNTP, 0.16 μM of fluorescent label M13(-21), 0.08 μM of the forward primer, 0.16 μM of the reverse primer, 0.15 units (0.03 μL) of Takara Taq (Takara Shuzo Co., Otsu, Shiga, Japan) and 30 ng of DNA template. For each reaction, the labeled M13(-21) primer was fluorescently marked with 6-FAM, VIC, NED, or PET. PCR cycling was as follows: 94 °C (5 min), 30 cycles at 94 °C (30 s)/56 °C (45 s)/65 °C (45 s), followed by 8 cycles 94 °C (30 s)/53 °C (45 s)/65 °C (45 s), and 65 °C for 10 min. The PCR products were pooled in groups of four and analyzed on an ABI 3130*xl* genetic automated analyzer with GENESCAN software (Applied Biosystems). Allele sizes were calculated using GENOTYPER software (Applied Biosystems) and visually confirmed.

For this study, we used 56 microsatellite markers distributed across the genome of the parasite (Supplementary Table 1). These markers have known chromosomal locations and are a subset of 243 microsatellite markers used to construct a genetic linkage map for *S. mansoni* [2]. Forty-eight of the loci have autosomal inheritance (including eight pseudoautosomal markers on the Z and W chromosomes) while eight loci were located in the Z-specific region, which does not recombine with the W chromosome [2]. When females are genotyped for these Z-specific loci, only the allele inherited from the male parent is present. Fifty-one loci were fully informative because the parental parasites did not share alleles. Thus, we could directly assess the genotyping error rate at these loci because at an autosomal locus, F1 offspring should have two alleles. Five loci were partially informative because the parents shared one of the alleles.

Table 1
Summary of results.

Category	No. of genotypes	%
Expected	1561	99.55
Discrepant	8	0.51
Additional allele	4	0.26
Missing allele	3	0.19
Mutation	1	0.06

The 1568 genotypes were characterized as being either expected or discrepant with respect to Mendelian segregation. See Figs. 1 and 2 for illustrative examples in each category. The italicized categories show different types of discrepant genotypes.

The final data set included parental genotypes for the 56 loci as well as for the 28 F1 miracidia, giving a total of 1568 F1 genotypes. We were able to chelex extract DNA from all 29 miracidia attempted. However, one sample contained two miracidia or was contaminated, because more than two alleles were seen at multiple loci. This sample was not included in the study. The results are summarized in Table 1. We observed a single allele from each of the two parents (or for Z-specific loci, a single allele from the male parent in F1 female offspring) as expected for Mendelian segregation for 1560/1568 genotypes, giving an overall success rate of 99.49% (see Fig. 1 for example). This error rate is similar to that observed in studies in which the same individuals are re-genotyped to measure reproducibility [15], giving us considerable confidence in this method. Just 8/1568 (0.51%) of genotypes showed patterns that differed from those expected under Mendelian segregation. Of these 8, discordant genotypes resulting from generation of additional non-parental alleles were observed in 4/1568 genotypes (Fig. 2(a)). In these cases the expected parental alleles were present, but a third non-parental allele was also observed. Discordant genotypes resulting from failure to genotype one of the parental alleles were observed in 4/1568 cases. In this situation one of the parental alleles was present but the other allele was shifted by 2 bp (two cases) (Fig. 2(b)) or absent (two cases). These eight discordant genotypes could have resulted from natural mutation events (e.g., occurring during the clonal expansion of sporocysts within the snail host). Alternatively they may represent mutations occurring during MDA or slippage during the PCR reaction. To differentiate between these explanations, we repeated PCR reactions using miracidial DNA pre-MDA (i.e., a PCR using the chelex-extracted DNA). In seven cases, the discordant patterns were not observed when using extracted miracidial DNA as template for PCR. Hence, in a small number of genotypes (0.45% [95% confidence intervals: 0.22–0.92%]) MDA-induced artifactual genotypes. We note that in about 6% of the genotypes the shorter allele had a lower peak height than the larger allele. These genotypes were not restricted to a subset of miracidia but were randomly distributed among individuals. This pattern appeared to result from the MDA because a repeat of the MDA from a few of these samples reversed this pattern (data not shown). A lower shorter allele could be problematic for scoring loci with a large degree of stutter. However, we feel this is not an insurmountable problem as there are methods that can reduce or eliminate microsatellite stuttering (e.g., using a polymerase that is highly

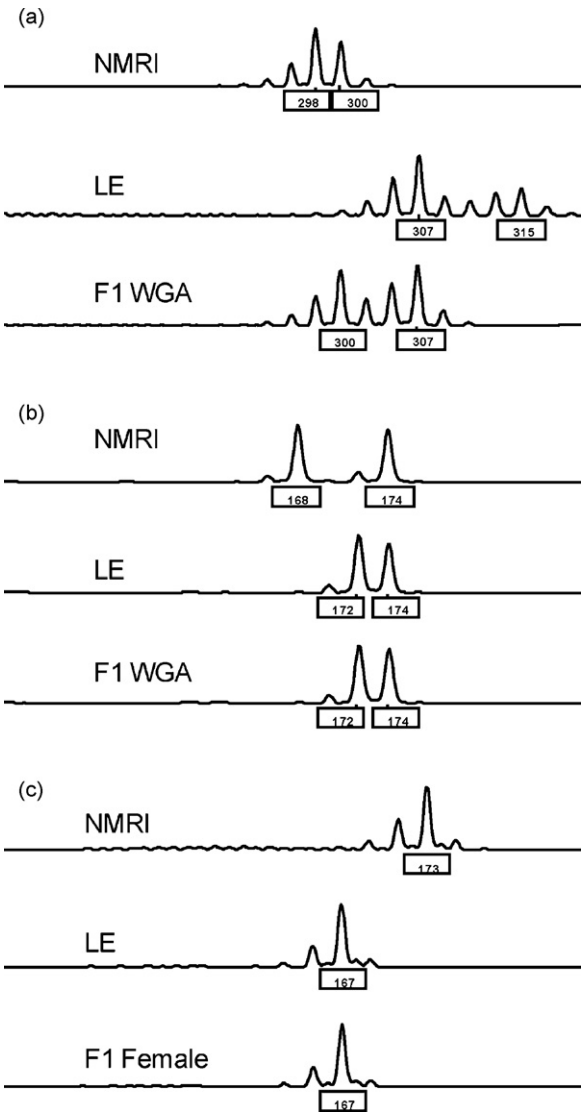

Fig. 1. Examples of genotypes consistent with Mendelian segregation. The traces generated on GENOTYPER show the alleles present in the two parents followed by the genotype observed in a miracidium. Such patterns were observed in the vast majority of genotypes. The boxes show the estimated allele size relative to an internal size standard. (a) Fully informative autosomal locus (Locus sc239). Here the parental parasites do not share any alleles and the miracidium inherits one allele from each parent. (b) Partially informative autosomal locus (Locus sc349). Here the parental parasites have one allele in common and the miracidia genotypes are consistent with one allele from each parent. (c) Z-linked locus (Locus sc16b). In this case female miracidium (ZW) inherits a single allele from the male parent, while males (ZZ) (not shown) inherit an allele from each parent.

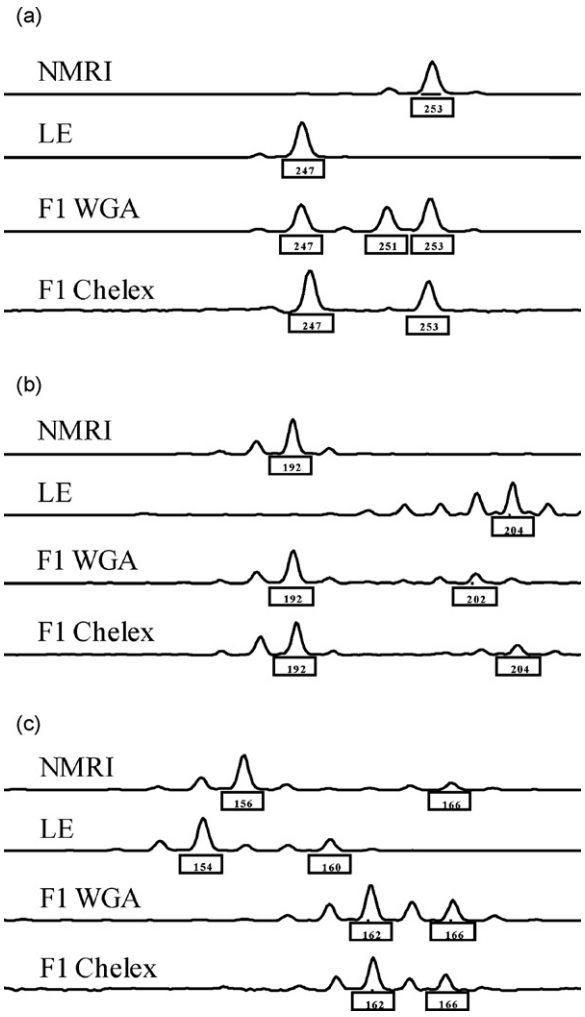

Fig. 2. Examples of genotypes inconsistent with Mendelian segregation. A small number of genotypes (0.45%) showed discrepant patterns of segregation as illustrated below. (a) Additional non-parental alleles (Locus sc40, Miracidium 21). The two parents are homozygous for alternative alleles. However an additional non-parental allele occurs following WGA. This additional allele was not present in genotypes generated from chelex-extracted material prior to WGA. (b) Missing allele (Locus sc168, Miracidium 14). The two parents are homozygous for alternative alleles. However, while the NMRI allele is present in the miracidium, the LE allele has been shifted by 2 bp. The expected LE allele is observed in chelex-extracted miracidium prior to WGA. (c) Genuine mutation (Locus sc453, miracidium 24). Both parents are heterozygous. While the NMRI allele is present in the F1 miracidium the LE allele has been shifted by 2 bp. The shift occurs in miracidial DNA both before and after WGA demonstrating that this is a genuine mutation.

processive). One could also repeat MDA on a subset of samples in order to determine if lower shorter alleles result in genotyping errors. Interestingly, one of the eight Mendelian discrepancies resulted from a genuine mutation in the miracidia genotyped. In this case both chelex-extracted and WGA DNA-generated genotypes with a 2-bp shift relative to the allele from the LE parent (Fig. 2(c)). This gives an estimated mutation rate of 3.7×10^{-4} [95% confidence intervals: 1.0×10^{-4} to 2.2×10^{-3}] per generation for autosomal loci [1/(48 autosomal loci × 28 miracidia × 2 chromosomes)], similar to that observed in humans and maize, but higher than in *Drosophila* [16]. This mutation was not included in the overall error rate, because the Mendelian discrepancy observed was not due to WGA.

Only two markers (sc290, and sc19; $p < 0.01$) showed a deviation from expected Mendelian ratios. This was tested by comparing observed and expected numbers of genotypes using chi-squared tests. Both markers were fully informative and all offspring had two alleles. Thus, these deviations are not the result of MDA. Interestingly, sc290 and sc19 are only 2.4 cM apart in the linkage map [2]. It is not uncommon to find genomic regions with segregation distortion when crossing diverged populations due to the evolution of coevolved gene complexes or of incompatible regions [16].

These results demonstrate that MDA can generate large amounts of DNA from single miracidia with high fidelity. In this work we used the high yield GenomePhi kit (GenomePhi HY DNA Amplification Kit) giving an average (±1 s.d.) yield of 53.5 ± 3.6 μg. Thus, 2.5 μL of chelex-extracted miracidium can provide enough DNA to run up to 1500 microsatellite genotyping reactions (assuming 30 ng of template/reaction). Hence, up to 12,000 reactions could be generated from the 20 μL chelex extraction. The total amount of DNA available from a single MDA is also sufficient for SNP typing assays (e.g., Illumina Golden Gate) and next-generation sequencing using

platforms such as the Illumina Genome analyzer (Solexa). An advantage of generating large amounts of DNA is that genotypes that fail to amplify at first attempt can be retried. In addition, samples can be regenotyped to provide reproducibility estimates in field-based studies. MDA currently costs between $4 and $16 depending on whether low or high yield is required. For most applications low yield kits generate ample DNA (approximately 5 μg). Hence the method is relatively inexpensive.

The *Schistosoma mansoni* genome sequence (v3.0 of the sequence assembly) contains approximately 16,757 dinucleotide repeats (≥8 perfect repeats) and 4976 trinucleotide (≥7 perfect repeats) and 1422 tetranucleotide repeats (≥6 perfect repeats). Some of these have been previously used for population studies of *S. mansoni*, while 243 markers have recently been used to generate a genetic map for this organism [2]. Furthermore, multiple SNP polymorphisms are present in the genome sequence data and from the examination of EST sequences [17]. MDA gives researchers the ability to genotype many hundreds of microsatellite or SNPs from single miracidia. Hence, genome-wide studies of genetic variation are now feasible. We anticipate that genotyping of miracidia after WGA will allow detailed studies of parasite population genetics at a variety of spatial scales, improving our understanding of mating systems and transmission dynamics of this parasite. Genome-wide genotyping of miracidia could also be used to systematically search for genome regions that have been exposed to strong selection, for example by drug treatment, and for genome-wide association studies of schistosome traits of biomedical importance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2009.02.010.

References

[1] LoVerde PT, Hirai H, Merrick JM, Lee NH, El Sayed N. *Schistosoma mansoni* genome project: an update. Parasitol Int 2004;53:183–92.
[2] Criscione CC, Valentim CLL, Hirai H, LoVerde PT, Anderson TJ. A genomic linkage map of the human blood fluke *Schistosoma mansoni*. Submitted for publication.
[3] LoVerde PT, DeWald J, Minchella DJ, Bosshardt SC, Damian RT. Evidence for host-induced selection in *Schistosoma mansoni*. J Parasitol 1985;71:297–301.
[4] Sorensen RE, Rodrigues NB, Oliveira G, Romanha AJ, Minchella DJ. Genetic filtering and optimal sampling of *Schistosoma mansoni* populations. Parasitology 2006;133:443–51.
[5] Gower CM, Shrivastava J, Lamberton PH, et al. Development and application of an ethically and epidemiologically advantageous assay for the multi-locus microsatellite analysis of *Schistosoma mansoni*. Parasitology 2007;134:523–36.
[6] Steinauer ML, Agola LE, Mwangi IN, Mkoji GM, Loker ES. Molecular epidemiology of *Schistosoma mansoni*: a robust, high-throughput method to assess multiple microsatellite markers from individual miracidia. Infect Genet Evol 2008;8:68–73.
[7] Beltran S, Galinier R, Allienne JF, Boissier J. Cheap, rapid and efficient DNA extraction method to perform multilocus microsatellite genotyping on all *Schistosoma mansoni* stages. Mem Inst Oswaldo Cruz 2008;103:501–3.
[8] Dean FB, Hosono S, Fang L, et al. Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA 2002;99:5261–6.
[9] Lovmar L, Syvanen AC. Multiple displacement amplification to create a long-lasting source of DNA for genetic studies. Hum Mutat 2006;27:603–14.
[10] Spits C, Le Caignec C, De Rycke M, et al. Optimization and evaluation of single-cell whole-genome multiple displacement amplification. Hum Mutat 2006;27:496–503.
[11] Wang Y, Nair S, Nosten F, Anderson T. Multiple displacement amplification for malaria parasite DNA. J Parasitol 2009;95:253–5.
[12] Morrison LJ, McCormack G, Sweeney L, et al. Use of multiple displacement amplification to increase the detection and genotyping of trypanosoma species samples immobilized on FTA filters. Am J Trop Med Hyg 2007;76:1132–7.
[13] Gasser RB, Morahan G, Mitchell GF. Sexing single larval stages of *Schistosoma mansoni* by polymerase chain reaction. Mol Biochem Parasitol 1991;47:255–8.
[14] Schuelke M. An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 2000:233–4.
[15] Hoffman JI, Amos W. Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Mol Ecol 2005;14:599–612.
[16] Vigouroux Y, Jaqueth JS, Matsuoka Y, et al. Rate and pattern of mutation at microsatellite loci in maize. Mol Biol Evol 2002;19:1251–60.
[17] Simoes M, Bahia D, Zerlotini A, et al. Single nucleotide polymorphisms identification in expressed genes of *Schistosoma mansoni*. Mol Biochem Parasitol 2007;154:134–40.