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PRIMER NOTE

Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes

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Abstract

Reptile and bird hosts of malaria parasites (*Plasmodium*) have nucleated erythrocytes. Infected blood thus contains a mix of abundant host and scant parasite DNA which has prevented identification of *Plasmodium* microsatellites. We developed a protocol for isolation of microsatellite markers for *Plasmodium mexicanum*, a parasite of lizards. The ATT repeat was common in the genome of *P. mexicanum*, but most (87%) of these repeats were exceptionally long (50–206 + repeats). Seven microsatellite markers with polymerase chain reaction primers are described. The protocol should allow discovery of microsatellites of malaria parasites (with AT-rich genomes) infecting bird and reptile hosts.

Keywords: malaria parasite, microsatellites, *Plasmodium mexicanum*

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Within the vertebrate host, infections of a malaria parasite (*Plasmodium*) may consist of a single genotype of haploid cells (single clone) or several genotypes. Clonal diversity appears important for the parasite's life history and transmission biology (deRoode *et al*. 2005), and therefore assaying the number of genotypes within infections is important for studies of the ecology of *Plasmodium*. Full-genome sequencing has revealed numerous microsatellites in *Plasmodium falciparum*, the major human malaria parasite, that are useful for studies in ecological genetics (Awadalla *et al*. 2001). However, most *Plasmodium* species are parasites of birds and reptiles. These parasites are phylogenetically distant to the *Plasmodium* of mammals (Perkins & Schall 2002), and we found that 20 primer pairs for *P. falciparum* microsatellites failed to produce product for *Plasmodium mexicanum*, a parasite of fence lizards (*Sceloporus occidentalis*). Use of standard techniques for discovery of microsatellites is unproductive for the saurian/avian malaria parasites because the vertebrate hosts have nucleated erythrocytes. In a blood sample, concentration of host DNA is much greater than that of the haploid parasite. Here we report the first identification of microsatellite markers for a malaria parasite infecting a vertebrate host with nucleated erythrocytes, *P. mexicanum*. The method (details at www.uvm.edu/~jschall/malaria.html) is based on that of Hamilton *et al*. (1999).

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The genome of most malaria parasites (*Plasmodium*) appears to be highly AT-rich (*P. falciparum*, *P. yoelii*, *P. berghei*, *P. gallinaceum*, but not *P. vivax*). Such AT-rich DNA will denature near standard polymerase chain reaction (PCR) extension temperature (72 °C) (Su *et al*. 1996). Therefore, to favour PCR amplification of *P. mexicanum* DNA, a low denaturation temperature (84 °C) and low extension temperature (60 °C) were used. Genomic DNA was enriched for an ATT repeat, the most common motif for *P. falciparum* microsatellites (Su & Wellems 1996), but one that is relatively rare in the vertebrate genome (BLAST search).

DNA was extracted from blood of fence lizards heavily infected with *P. mexicanum*, and was digested with *Sau*3AI. Fragments of 400–1200 bp were isolated from an agarose gel. Damage to the low concentration of parasite DNA was prevented by cutting off the two edge ladder lanes from the gel and staining only these in ethidium bromide. The ladder lanes were replaced on the gel with a piece of aluminium foil under the target DNA lanes. The gel was examined under UV light illumination and the proper section removed. DNA was purified from the gel, and then ligated onto a double-stranded linker that matched the

Sau overhang. The linker was designed for a low PCR annealing temperature and was produced from: LinkA = 5′-GATATACTAATGAATGTTGG-3′ LinkB = 5′-GATC-CCAACATTCATTAGTATATC-3′. For PCR amplification of the DNA, only LinkA was needed as the primer using the program: 84 °C 2 min, 32 cycles of 84 °C 1 min, 52 °C 10 s, 48 °C 10 s, and 60 °C 4 min, followed by a final extension of 60 °C 4 min.

Three enrichments for the target microsatellite used a $(TAA)_{10}$ biotinylated oligonucleotide. The PCR product was hybridized with the labelled repeat, and avidin beads (Vector) captured the DNA fragments attached to the biotin-labelled repeat. After each round of enrichment, the DNA was cleaned and amplified again using the above PCR program. The final PCR product was used as template for TOPO (Invitrogen) cloning. Cloned fragments were sequenced (ABI BigDye). Selection of sequences for further study was based on a high AT-rich flanking regions and a BLAST search that found at least 15 bases overlap with *P. falciparum* (the latter method was a poor indicator because of the divergence of the two *Plasmodium* species). Primers were designed and the locus was scored as 'parasite' if the PCR product produced a band for lizards infected with *P. mexicanum*, but not for uninfected lizards.

A total of 156 cloned fragments were sequenced, with 86% revealing the ATT repeat. Of sequences containing the repeat, 15% were identified as from the lizard genome. Fully 99 sequences containing the microsatellite revealed a very long repeat. Of 65 with sufficient flanking region for study, 53 were unique sequences, indicating a true abundance of these long repeats. Long repeats entirely contained in sequences ranged from 50 to 176 × ATT (median = 83× $N = 39$), and those that ran off at the end of the sequence ranged from 48 to 206 × ATT (median = 93). We suspect these are from the parasite's genome. A BLAST search of the *Mus* and *Gallus* genomes, found no ATT repeats as long as 50×, whereas such repeats are present for *P. falciparum* (although the ATT 100× repeat is rare). We obtained sufficient flanking sequence to perform a successful BLAST search for 16 of the long repeats and all matched one or more species of *Plasmodium*. We were able to design primers for only one sequence with a long repeat, and confirmed this locus (Pmx308) was parasite. All of the sequences identified as lizard contained a far shorter repeat (12–35× median = 15). Subsequent to the described search, we altered the protocol in a way to avoid the expense of sequencing long repeats. A third primer, the $(TAA)_{10}$ without the biotin molecule, was added to the PCR mixture when amplifying the cloned DNA fragments. On an agarose gel, clones that did not contain a repeat produced a single, sharp band. Clones with a long repeat produced a long smear because the third primer annealed at many places on the repeat region. Clones with a short repeat produced a short smear.

Of 14 microsatellite loci from the *P. mexicanum* genome, seven were duplicates. Primers were designed for amplification of the remaining seven loci (Table 1). Six loci displayed useful variation, providing markers for study of the clonal diversity of the parasite (Table 1). The locus with

Table 1 Primer sequences for microsatellite markers of *Plasmodium mexicanum*. The expected allele size is presented for a given repeat length. PCR amplification is achieved with the program 94 °C for 2 min, followed by 32 cycles of 94 °C for 1 min, annealing (given in the table), 60 °C for 1 min, followed by a final extension of 60 °C for 4 min. For fragment analysis, the forward primer was dye-labelled. Number of alleles given is based on an initial survey of 100 infected fence lizards from a site in Mendocino County, California, near the town of Hopland. Many infections contained > 1 allele (clone of haploid parasite). All loci except Pmx308 revealed substantial diversity among infections. For these loci, half or more of alleles were at least 5% frequency among all alleles scored. An allele association analysis revealed no evidence for linkage of the loci

Locus	Forward/Reverse primer 5′–3′	Annealing conditions	No. of alleles	Size	GenBank Accession no.
Pmx306	GATCACATTTTGCTATTTTAGTATT AACTTTTGATTCTTCTATAACAG	60 °C/10 s + 50 °C 20 s	16	203 @ 27x	DQ503416
Pmx308	CTTTTGTTATAATTATTCATATTTT CAATAATAATAAATATAATAACAACA	47.5 °C/30 s + 60 °C/20 s	2	312 @ 74x	DQ503422
Pmx328	TATTATTTAAGTTTTGAATGG CTTCTTTACTTTACAAAAAAT	42.5 °C/30 s + 43.6/20 s	5	261 @ 18x	DQ503417
Pmx710	GCCGTCTTATGAATTAAGTGAACAAG CATTTTGCTATTTTAGTATTTTCTA	55 °C/50 s	16	318 @ 16x	DQ503418
Pmx732	CAGGTAGATATTTTTTGATG GATGTAAATGAGATAAAATCC	56 °C/50 s	17	255 @ 22x	DQ503421
Pmx747	CACAAATTCAAGATAATTCAAAAG TCTTTTTCGAGACATATTATTGC	51 °C/30 s + 49 °C/20 s	13	182 @ 24x	DQ503420
Pmx839	CATTGAGAATAATCCGTTAAG GGGACCATAATGAATTTGATTC	47 °C/30 s + 44 °C/20 s	14	255 @ 57x	DQ503419

only two alleles, Pmx308, was also the longest (primary allele = 74× repeat).

Despite the low concentration of parasite DNA in blood extract, the protocol was highly successful in enriching for *P. mexicanum* DNA containing the ATT microsatellite. However, the genome of *P. mexicanum* appears unusual in containing primarily very long ATT repeats. The protocol should be useful in locating microsatellites in other malaria parasites of birds and reptiles, provided those species have a highly AT-rich genome.

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