

Microsatellite Isolation and Linkage Group Identification in the Yellow Fever Mosquito *Aedes aegypti*

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Abstract

Microsatellites have proved to be very useful as genetic markers, as they seem to be ubiquitous and randomly distributed throughout most eukaryote genomes. However, our laboratories and others have determined that this paradigm does not necessarily apply to the yellow fever mosquito *Aedes aegypti*. We report the isolation and identification of microsatellite sequences from multiple genomic libraries for *A. aegypti*. We identified 6 single-copy simple microsatellites from 3 plasmid libraries enriched for (GA)_n, (AAT)_n, and (TAGA)_n motifs from *A. aegypti*. In addition, we identified 5 single-copy microsatellites from an *A. aegypti* cosmid library. Genetic map positions were determined for 8 microsatellite loci. These markers greatly increase the number of microsatellite markers available for *A. aegypti* and provide additional tools for studying genetic variability of mosquito populations. Additionally, most *A. aegypti* microsatellites are closely associated with repetitive elements that likely accounts for the limited success in developing an extensive panel of microsatellite marker loci.

Microsatellites or simple sequence repeats are short stretches of DNA sequence in which motifs of 1–6 bases are tandemly repeated (Weber and May 1989; Schlötterer 2000). They have become the genetic marker of choice for many eukaryotic species because of their high variability, codominant expression, and broad genome distribution (Tautz and Renz 1984; Tautz 1989; Webber and May 1989). In addition, they are easily developed into PCR-based molecular markers that are particularly useful for small organisms with limited DNA.

Microsatellites have been used as genetic markers for a number of arthropod vectors for human disease. One hundred and fifty polymorphic microsatellite loci have been characterized in the African malaria vector, *Anopheles gambiae* s.l. (Zheng et al. 1993, 1996). They have been employed to evaluate population genetics and structure, as well as gene flow within and among *A. gambiae* s.l. (Lehman et al. 1996, 1997; Kamau et al. 1998, 1999; Lanzaro et al. 1998), *A. gambiae* s.s. (Zheng et al. 1996; Tripet et al. 2001, 2005; Carnahan et al. 2002), and *Anopheles arabiensis* (Donnelly and Towson 2000; Nyanjom et al. 2003). Microsatellites have also been mapped to the *A. gambiae* s.s. genome (Zheng et al. 1993,

1996; Wang et al. 1999) and have proved useful in quantitative trait loci studies of susceptibility to *Plasmodium* (Gorman et al. 1997; Zheng et al. 1997) and of permethrin resistance (Ranson et al. 2004). Microsatellites have also been characterized in *Anopheles darlingi* (Conn et al. 2001) and *Anopheles funestus* (Sinkins et al. 2000; Sharakhov et al. 2001, 2004; Braginets et al. 2003), and have been used to investigate population genetics, structure, and gene flow within *Anopheles maculatus* (Rongnopard et al. 1996, 1999) and *Anopheles albimanus* (Molina-Cruz et al. 2004).

Microsatellites have also been characterized within the family Glossinidae (Solano et al. 1998, 1999; Luna et al. 2001) that serve as vectors for African trypanosomiasis. Microsatellites within the Glossinidae have been used for population genetic studies and evaluation of genetic structure and gene flow in the *Glossina morsitans* (Baker and Krafur 2001; Krafur et al. 2001; Krafur and Endsley 2002), *Glossina pallidipes* (Krafur 2002), and the *Glossina palpalis* complex (Solano et al. 1997, 1999).

Additionally, within other arthropods microsatellite marker loci have been identified and employed to study

population genetics and ecology, including investigations of gene flow, dispersal, migration, relatedness and parentage within ants (Gertsch et al. 1995; Tsutsui et al. 2003), honeybees (Estoup et al. 1995; Franck et al. 2001), parasitic wasps (Butcher et al. 2000), yellow-jacket wasps (Thoren et al. 1995), butterflies and moths (Bogdanowicz et al. 1997; Anthony et al. 2001; Keyghobadi et al. 2002), and aphids (Sloane et al. 2001).

In contrast to most other arthropods, microsatellites appear to be underrepresented within some members of the mosquito subfamily Culicinae. A limited number have been identified in *Culex pipiens* and *C. pipiens quinquefasciatus* (Fonseca et al. 1998; Keyghobadi et al. 2004; Smith et al. 2005). Within *Aedes aegypti*, the primary vector for the yellow fever and dengue viruses, 6 unique microsatellites have been identified using sequence databases (Barbazan et al. 1999; Huber et al. 2001; Ravel et al. 2001). Huber et al. (1999) isolated 4 microsatellites from an enriched partial library of 120 recombinant clones and successfully designed PCR primers for 2 of the microsatellites. They later screened an enriched (CAA)_n library and successfully designed primers for 2 additional microsatellites (Huber et al. 2001). Fagerberger et al. (2001) utilized an enrichment procedure to generate a number of microsatellite-containing libraries. They isolated 52 putative microsatellite-containing clones and successfully designed primers for 7 putative microsatellites from 3 different enriched libraries. Six of these putative microsatellites were monomorphic in the F₁ intercross families they tested. Only one locus, TAG66, was polymorphic in the F₁ intercross families tested (Fagerberger et al. 2001). Still, these limited numbers of microsatellites have been employed for several studies of *A. aegypti* population structure (Ravel et al. 2001, 2002; Huber et al. 2002a, 2002b, 2004).

The development of additional microsatellite markers for *A. aegypti* would greatly benefit both genetic and population studies, and thereby assist efforts to investigate the genetic basis for dengue vector competence and to assess population variation subsequent to developing genetic control strategies. Here, we report on our efforts to isolate and develop microsatellite markers from 3 libraries enriched for (GA)_n, (AAT)_n, and (TAGA)_n motifs from genomic DNA isolated from *A. aegypti*, as well as by the direct screening of selected clones from 3 different *A. aegypti*-derived libraries. We identified 11 new microsatellites that segregate as single locus, codominant markers, and determined the genetic map positions for 8 of these markers. We also describe the close association of microsatellites in *A. aegypti* with known repetitive elements that likely accounts for some of the limited success in developing an extensive panel of microsatellite marker loci from the present study, as well as previous attempts by other investigators (Fagerberger et al. 2001).

Materials and Methods

Microsatellite Enriched Library Construction, Sequence Analysis, and Primer Design

Genomic DNA was extracted from approximately 700 pupae from our *A. aegypti* Trinidad strain. Pupae were gently ho-

mogenized using a glass homogenizer in equal parts DNA extraction buffer (0.5% sodium dodecyl sulfate [SDS], 0.2 M NaCl, 25 mM ethylenediaminetetraacetic acid [EDTA], 10 mM Tris, pH 8.0) and phenol followed by phenol/chloroform extraction, including incubation with RNaseA (Severson 1997).

Genomic DNA was enriched for 3 microsatellite motifs (GA, AAT, and TAGA) by Genetic Identification Services (<http://www.genetic-id-services.com>, Chatsworth, CA). The enriched genomic fragments were ligated into the *Hind*III site of the pUC19 plasmid vector and transformed into *Escherichia coli* strain DH5 α cells via electroporation. White recombinant colonies were arrayed manually into 96-well microplates and stored at -80 °C. Sequencing was performed using an ABI 3700 DNA sequencer (PE Applied Biosystems, Crescent City, CA) at the Purdue University Genomics Center.

Sequence data were analyzed using RepeatMasker (Smit et al. 2004) and Sputnik (Abajian 1994). RepeatMasker screens DNA sequences against a library of known repetitive elements and returns a masked query sequence ready for database searches as well as a table annotating the masked regions. Here, we used a library of known *A. aegypti* repeats (Aaeg.lib.2, Brown SE, Knudson DL, unpublished data). Sputnik utilizes a recursive algorithm to search for 2–5 base repeated patterns of nucleotides. Identity searches were conducted on all clones by BLASTN analysis against the nonredundant GenBank database (Altschul et al. 1997).

Primers for putative unique sequences flanking microsatellites were designed using PRIMER3 (Rozen and Skaletsky 2000). Primers were designed to be 18–27 nt in length with a G + C content of 50%, and optimum anneal temperature of 60 °C, with a range of 57–63 °C. When possible, we included a GC clamp and selected primers that would yield a 150- to 300-bp product. A sequence similarity and homology search was conducted on all putative PCR primers using the FASTA computer program (Pearson 1990). This program was employed as it allows the user to set the KTUP (word size) to 1, allowing for a more sensitive search that facilitates avoidance of primer sequences that may have homology to known repetitive elements.

Existing Library Screening for Microsatellites

Three *A. aegypti*-derived libraries were screened with 3 dinucleotide motifs (GA, CA, and GC) and 5 trinucleotide repeats (ATA, AGT, ATC, CAA, and ATG) that included: 1) 85 cDNA and genomic DNA plasmid clones previously mapped as restriction fragment length polymorphism (RFLP) markers (Severson et al. 2002), 2) 207 cosmids from the *A. aegypti* ATC-10 cell line that had been previously mapped using Fluorescent In Situ Hybridization (S.E. Brown et al. 1995; Brown SE, Knudson DL, unpublished data), and 3) 36 cosmids prepared from the *A. aegypti* Liverpool strain known to contain mapped RFLP marker sequences.

Clones were manually arrayed in 96-well plates, transferred to nylon membranes (NEN™ Life Science Products, Boston, MA) using a 96-well plate replicator and grown overnight at 37 °C on LB plates selected with ampicillin. Clones

were fixed to the nylon membranes by placing them sequentially onto Whatman paper drenched with 10% SDS for 3 min, 0.5 M NaOH for 5 min, and 1.5 M NaCl/0.5 M Tris, pH 8.0 for 5 min. Membranes were allowed to dry for 1 h at room temperature, UV cross-linked at $1200 \times 100 \mu\text{J}/\text{cm}^2$, and then hybridized overnight at the probe-specific temperature in a hybridization oven (Hybaid, Milford, MA) in a prehybridization solution consisting of 0.1 M sodium phosphate buffer, pH 7.8, 20 mM sodium pyrophosphate, 5 mM EDTA, pH 8.0, 0.1% SDS, 10% (w/v) sodium dextran sulfate, 1.0 mM o-phenanthroline, 500 $\mu\text{g}/\text{ml}$ heparine sulfate, 50 $\mu\text{g}/\text{ml}$ yeast RNA.

Di- and trinucleotide oligonucleotides were end-labeled using [γ - ^{32}P] ATP and bacteriophage T4 polynucleotide kinase, and purified by ethanol precipitation as described by Sambrook et al. (1989). After addition of the labeled probe, membranes were hybridized overnight in a hybridization oven. Membranes were then washed for 15 min each in 0.1% SDS/2X SSC and 0.1% SDS/0.2X SSC, and exposed to X-ray film for 4–8 h.

Cosmid Subcloning, Sequencing, and Primer Design

Purified cosmid DNA from clones selected with oligonucleotide probes was isolated using the alkaline lysis miniprep protocol (Birnboim and Doly 1979). The purified DNA was then digested with *Sau3AI* according to supplier's recommendations (Promega, Madison, WI). One half of each digest was size fractionated in 0.9% agarose gels at 1.8–2.0 V/cm. Gels were UV nicked in a cross-linker at $800 \times 100 \mu\text{J}/\text{cm}^2$, the DNA transferred to nylon membranes, and the membranes hybridized with the appropriate end-labeled oligonucleotide as described above. The remaining clone digest was then size fractionated in 0.9% low melting temperature agarose and fragments positive for microsatellites were identified by comparison with positive fragments identified by autoradiography. Microsatellite-containing bands were excised and subcloned into the pGEM®-3Z (Promega) cloning vector. Subclones were plated onto LB ampicillin plates. DNA from the recombinant clones was transferred to nylon membranes and hybridized with the appropriate end-labeled oligonucleotide.

Sequencing of positive subclones was performed using an ABI 310 DNA sequencer (PE Applied Biosystems). Microsatellites were identified by visual inspection and subjected to BLASTX analysis (Altschul et al. 1997) against the GenBank database to identify sequences containing known *A. aegypti* repetitive elements. Primers for unique sequences flanking the microsatellites were designed using PRIMER3 (Rozen and Skaletsky 2000).

PCR Amplification of Microsatellites

PCR reactions were performed on genomic DNA isolated from individual mosquitoes in 25 μl volumes containing $1 \times$ Taq buffer (10 mM KCl, 2 mM Tris, pH 9.0, 0.02% TritonX), 1.5 or 3.0 mM MgCl_2 , 0.4 mM each dATP, dCTP, dGTP, dTTP, 5 pmol of each primer, 1 U Taq DNA polymerase and 5 ng genomic DNA. PCR reactions for micro-

satellite loci A10, H08, B07, F06, and G11 were performed under the following conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C anneal for 1 min, 72 °C extension for 2 min, and a final 72 °C extension for 10 min. PCR reactions for B19, M205, and M313 were performed under the following conditions: 94 °C for 5 min, followed by 39 cycles at 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, and a final 72 °C extension for 10 min. PCR products were size fractionated in 2% agarose gels and visualized with ethidium bromide under UV light.

Microsatellite Polymorphism Detection

For preliminary screenings and linkage analysis, microsatellite polymorphisms were resolved on $31.0 \text{ cm} \times 38.5 \text{ cm} \times 0.44 \text{ mm}$ denaturing acrylamide gels (4.0% acrylamide [19:1 polyacrylamide:bis] $1 \times$ TBE, 38% urea), preheated to 50 °C. Three microliters of PCR product was mixed with 5 μl of denaturing loading mix (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol). The denaturing acrylamide gels (SDS–polyacrylamide gel electrophoresis [PAGE]) were run at 2200 V for 45 min, fixed for 20 min in 10% acetic acid, and the DNA was then visualized by silver staining using the genePrint STR system (Promega).

For evaluating microsatellites among field-collected mosquitoes, PCR products were size fractionated and individual alleles identified using a Beckman-Coulter CEQ as described elsewhere (Michel et al. 2005). Briefly, microsatellites were amplified using dye-labeled primers (Prologo) and pooled into groups of 3 loci. Pools consisted of 0.4 μl of 400 bp standard (Beckman-Coulter), 30.0 μl of standard loading solution (Beckman-Coulter), and 1.0 μl of diluted amplified product. Alleles were resolved and analyzed using a Beckman-Coulter CEQ8000 and Beckman-Coulter CEQ8000 software.

Mosquito Strains, Crosses, and Genetic Mapping

Initial microsatellite polymorphism screens were evaluated among 4 *A. aegypti* laboratory colonies: Liverpool, RED, MOYO-R, and Trinidad. These strains have been maintained in the laboratory for an unknown number of generations, and the first 3 are long-standing colonies that likely carry reduced polymorphism.

To evaluate microsatellite polymorphisms among field populations, we collected eggs using standard ovitraps (Fay and Eliason 1966) from 2 sites in Trinidad, with 24 ovitraps placed at each site. Individuals were reared to adults in the laboratory and subjected to DNA extraction and microsatellite alleles identified as described above.

A segregating F_1 intercross mapping population, SDAM2, produced by pairwise matings between *A. aegypti* LIV^{SD} strain females and MOYO strain males was generated for a previous study and is described elsewhere (Severson et al. 1999). For this study, we examined 99 F_1 intercross progeny from the SDAM2 population that had been genotyped at 12 RFLP markers distributed across chromosomes 1 and 2. This included 5 RFLP loci on chromosome 1 and 7 on chromosome 2. For this study, we included 3 single nucleotide polymorphism markers, *ApolipoII*, *Cyp9*, and

Table 1. Analysis of enriched libraries with RepeatMasker^a and Sputnik^b

Analysis	No. of sequences
Total clones sequenced	253
RepeatMasker	
Clones positive for low-complexity and interspersed repeat sequences	110
Sputnik	
Clones positive for microsatellite sequences	127
Sputnik and RepeatMasker	
Clones positive for microsatellite sequences and negative for interspersed repeats	68
Clones positive for microsatellite sequences and interspersed repeats	59

^a Screens DNA sequences for interspersed repeats and low-complexity DNA sequences (Abajian 1994). Here, sequences were masked using an *A. aegypti* repeat library (Aeg.lib.2, Brown SE, Knudson DL, unpublished data).

^b Searches DNA sequences for microsatellites of between 2 and 5 nucleotides in length using a recursive algorithm (Smit et al. 2004).

LF108 located on chromosome 3 (Severson et al. 2002) as anchor loci for chromosome 3. Putative microsatellite loci identified as polymorphic by our preliminary screenings were amplified from the individual SDAM2 progeny and genotypes determined as described above. Multipoint linkage analysis was performed using the MapMaker/EXP 3.0 computer package (Lander et al. 1987) with the linkage logarithm of the odds score set at 3.0 with the Kosambi (1944) mapping function.

Results

Microsatellite-Enriched Libraries

We obtained sequence data for 253 putative microsatellite-containing GA-, AAT-, or TAGA-enriched clones from 3 microsatellite-enriched plasmid libraries prepared from the Trinidad strain of the yellow fever mosquito *A. aegypti*. These data were screened against a database of known *A. aegypti* interspersed repeats and low-complexity DNA sequences using RepeatMasker. This analysis indicated that 110 of the 253 clones (43.5%) contained known repetitive elements (Table 1), and because 182 repetitive elements were identified (Table 2), many clones contained multiple elements. The 182 repetitive elements consisted of 7 different repeat class family types represented by 15 different repeat types. The most common repeat type was the *Feilai* element, a member of the short interspersed repetitive element (SINE) class (Tu 1999). Sputnik analyses identified 127 clones (50.2%) that contained a microsatellite. However, for 59 of these clones (46.5%), the microsatellites were within or immediately adjacent to known repetitive elements or low-complexity DNA regions (Table 1). Of the remaining 68 clones, several were duplications of the same sequence resulting in a final total of 30 putative unique sequence clones containing a microsatellite.

We were able to design PCR primers for unique sequences flanking the microsatellite for 12 of the 30 putative unique sequences. The remaining sequences were unsuitable for

Table 2. Repeat classifications associated with *A. aegypti* microsatellites (after Severson et al. 2001)

Repeat class/family	Element	Total
LTR retrotransposons	<i>Zebedee</i>	7
Non-LTR retrotransposons	<i>Jam1</i>	10
LINES	<i>MosquiI</i>	22
	<i>JuanA</i>	14
	<i>Lion</i>	2
	<i>heF-A-like</i>	1
SINE	<i>Feilai</i>	86
DNA/LTR	<i>Schelli-like</i>	1
DNA/MITE	<i>Dufu</i>	1
	<i>Homey</i>	1
	<i>Pony</i>	7
	<i>Wukong</i>	13
	<i>Youzi</i>	7
DNA/MSITE	<i>Microuli</i>	8
Unknown	<i>P2405</i>	2
Total		182

primer design because the microsatellites were too close to the clone end or the base composition of the flanking regions was unsuitable for quality primer design. The 12 putative single-copy microsatellite loci were tested for amplification with genomic DNA from individual mosquitoes representing 4 laboratory strains of *A. aegypti*. Eight of the primer pairs amplified a single PCR product when visualized on agarose gels, whereas the remaining 4 primer pairs amplified multiple PCR products indicating that they likely recognized repetitive sequences. SDS-PAGE suggested that 7 of the 8 microsatellites were single locus markers. Five were polymorphic within and among the strains tested, whereas G11 was single locus and polymorphic between the RED and Liverpool strains, but was monomorphic between Liverpool and MOYO-R. F06 though single locus was monomorphic within the strains tested. These consisted of one perfect dinucleotide repeat (B07), 2 imperfect dinucleotide repeats (A10, B6), 3 perfect trinucleotide repeats (B19, G11, H08), and 1 perfect tetranucleotide repeat (F06). One of the trinucleotide repeats (B19) also contained an imperfect dinucleotide repeat.

Microsatellites from Cosmid Subclones

We isolated a total of 9 microsatellites by screening and subcloning a selected set of cosmid clones. SDS-PAGE indicated that M201, M205, and M313 were single locus and polymorphic between the 4 strains screened. However, the M313 subclone sequence was identical to the TAG66 locus reported by Fagerberger et al. (2001). M106 and M307 were single locus but monomorphic. We were unable to identify primer sequences suitable for PCR amplification from subclones M203 and M102, and the microsatellites in subclones M209 and M310 were located too close to one end of the subclone insert for primer design. All 5 of the single locus microsatellites were trinucleotide repeats. Three contained imperfect repeats (M205, M307, M313), whereas 2 contained perfect repeats (M106, M201).

Field Populations

The level of polymorphism for the 12 putative single-copy microsatellites was examined among *A. aegypti* that were collected from Curepe in northern Trinidad, near the capital city of Port of Spain, and from Oropouche, near the southern port city of San Fernando. A total of 159 mosquitoes were genotyped, 69 from Curepe, and 90 from Oropouche. Microsatellites M106, M205, and M307, although polymorphic among laboratory strain individuals, were monomorphic among individuals from Trinidad. Microsatellite B6 was determined to be multicopy. The number of alleles per locus for A10, B07, B19, F06, G11, H08, M201, and M313 ranged from 2 to 11 and the observed heterozygosity ranged from 0.04 to 0.80 (Table 3).

Linkage Analysis

We performed linkage analyses with 4 *A. aegypti* microsatellite markers isolated from the enriched plasmid libraries (A10, B07, B19, H08), as well as 2 microsatellite markers obtained by screening an *A. aegypti* cosmid library (M205, M313). These loci mapped to chromosome 2 or 3 on the *A. aegypti* linkage map (Figure 1). Two microsatellite loci, F06 and G11, were not polymorphic in our mapping population. Map positions for 2 microsatellite loci (M106, M201) were inferred by their physical location within cosmid clones also containing previously mapped RFLP marker loci. We have deposited sequence data for the 11 single locus microsatellites isolated in this study to GenBank (Table 3).

Discussion

Despite their demonstrated utility among a wide variety of arthropods, useful microsatellites are neither highly abundant nor easily isolated within all arthropod species. Within 4 different species of phlebotomine sandflies, *Lutzomyia whitmani*, *Lutzomyia longipalpis*, *Phlebotomus papatasi*, and *Phlebotomus langeroni*, microsatellites containing the (TG)_n motif varied greatly in abundance, with the average frequency of occurrence ranging from 1 per 100 kb in *P. langeroni* to 1 per 1000 kb in *P. papatasi* and *L. longipalpis* (Day and Ready 1999). In contrast, the (GT)_n motif repeats occur about once every 26 kb in *A. gambiae* (Knudson et al. 1996), once every 68 kb in *A. maculatus* (Rongneparut et al. 1996), once every 88–224 kb in *Drosophila melanogaster* (Schug et al. 1998) and once every 10 000 kb in *A. aegypti* (Knudson et al. 1996). The frequency of repeats exhibiting the (TC)_n motif was estimated to occur at a rate of 1 per 25 kb in *P. langeroni* and 1 per 250 kb in other sandfly species (Day and Ready 1999). Finally, (AAT)_n repeats were found to occur at a frequency of 1 per 12.5 kb in *P. papatasi* and at a frequency of 1 per 125 kb in other sandfly species (Day and Ready 1999). Two species of mites, *Amblyseius fallacies* and *Tetranychus urticae*, when tested for 10 repeat motifs, yielded considerably fewer microsatellites than the typical vertebrate species, and trinucleotide repeats were more abundant than dinucleotide repeats (Navajas et al. 1998). It is clear that the frequency and types of microsatellites can occur at widely varying rates within many arthropod species.

Microsatellites appear to be underrepresented and, therefore, of limited utility in *A. aegypti*. In this study, we screened genomic libraries enriched for 3 repeat motifs (GA)_n, (AAT)_n, (TAGA)_n and selected cosmid clones for useful, single locus microsatellite sequences. We have identified and characterized 11 single locus microsatellite loci within the *A. aegypti* genome and, therefore, they should prove useful for genetic mapping studies as well as tools for studying population genetics. With this study, we have increased the number of documented single-copy microsatellite markers in *A. aegypti* from 9 to 20. In addition, we were able to determine genetic map positions for 8 of these loci. These represent the first *A. aegypti* microsatellite loci placed to chromosomes that show polymorphism based on fragment length variation. Fagerberger et al. (2001) employed an enrichment protocol in attempts to identify microsatellites within *A. aegypti* and failed to identify length variation at any single microsatellite locus. Huber et al. (1999) using a similar enrichment protocol to identify (CAA)_n microsatellites isolated 4 imperfect repeats. Microsatellites appear to be underrepresented within some other culicine mosquitoes as both di- and trinucleotide repeats are underrepresented within *C. pipiens* (Keyghobadi et al. 2004; Smith et al. 2005) and *C. pipiens quinquefasciatus* (Fonseca et al. 1998).

Our results also indicate that microsatellites are not only underrepresented in the *A. aegypti* genome but also most of those that are present are associated with repetitive sequences. More than 46% (59/127) of the microsatellites identified by Sputnik were found directly within or closely linked with known *A. aegypti* repetitive elements. Further, only 30 of the remaining 68 putative single-copy microsatellite loci were unique. After PCR amplification and screening, only 6 microsatellites from our original 127 were demonstrated to be single copy and polymorphic, whereas an additional 6 amplified as multiple-copy loci, even though RepeatMasker did not detect repetitive elements in sequences flanking the microsatellites. This was not unexpected as our RepeatMasker database only contains known repetitive elements and many novel repetitive elements remain to be identified.

A number of retroelements and, to a lesser degree, DNA elements were commonly associated with microsatellites (Table 2). It was not surprising that the SINE element, *Feilai*, was frequently associated with microsatellites. These elements alone represented 47% of all repetitive element types associated with microsatellites in our study. *Feilai* elements are found throughout the *A. aegypti* genome, and account for up to 2% of the genome (Tu 1999). Other common repeat types associated with microsatellites included the *MosqI* (Tu and Hill 1999) and *Juan* (Mouches et al. 1992), members of the nonlong terminal repeat (non-LTR) or long interspersed repetitive element (LINE) family, and *Zebedee* (Warren et al. 1997), a member of the LTR family. Among the DNA elements most frequently associated with microsatellites were *Wukong* (Tu 1997) and *Pony* (Tu 2000), both miniature inverted-repeat transposable elements (MITEs), that together accounted for almost 11% of the repetitive elements associated with the microsatellites isolated in our study. Finally, several microsatellites were associated with *Microuli*,

Table 3. Single-copy microsatellite sequences identified from enriched plasmid libraries and selected cosmid subclones^a

Library source	Sequence ID	Repeat motif	Linkage group	Primers 5'-3'	No. of alleles	Allele size range (bp)	H _E	H _O	Accession no.
B ^b	A10	CT ₁₀ (TT)CT	III	F-AATCGTGACGCGTCTTTTG; R-TAACTGCATCGAGGGAAACC	4	233–239	0.45	0.49	DU169901
B ^b	B07	GA ₁₅	II	F-CAAACAACGAAGTCTCAG; R-TCGCAATTTCAACAGGTAGG	11	157–183	0.81	0.80	DU169902
B ^b	B19	CAT ₇	III	F-ATTGGCGTGAGAACATTTTG; R-GAGGAGTGAGCAGATAGGAGTG	5	156–186	0.65	0.40	DU169905
B ^b	H08	TCG ₇	II	F-AAAAACCACGATCACC GAAG; R-ACGCGATCACACACTGAAAAATG	3	199–205	0.60	0.68	DU169903
C ^c	G11	TTA ₁₆	Unknown	F-TGTCTCATGGATTGCCCTTATT; R-GTCAGA ACTTTTGGGGACCA	6	240–300	0.57	0.42	DU169906
D ^d	F06	TAGA ₈	Unknown	F-GCCAAAAACCAACAAACAGG; R-AATCGACCCGACCAATAACA	2	286–290	0.04	0.04	DU169907
113.9G7 ^e	M205	GAA(GTA) GAA ₄	II	F-CTATTAGTCCCAAGTCTCCGG; R-TGTGATAGGGATAAGAAGCTGC	1	266	—	—	DU169908
126.8E8 ^f	M313	ATG ₅ (ATA)ATG	III	F-CACCTCGTGACATACAAACACC; R-ACGTACCCAAGCCACGTACA	3	117–123	0.49	0.20	DU169909
102.1D5 ^g	M201	ATA ₃₆	II	F-GGAGCATTCATAGAGAATTGTCA; R-GAGATGAACCAGTCATAGGGC	3	110–116	0.51	0.77	DU635091
125.4D7 ^b	M106	ATG ₅	I	F-TGTA CT CAGTTCATCGGGGA; R-TTTTCATGCGAGAGATGACG	1	229	—	—	DU635090
6.3F1 ⁱ	M307	CAT ₃ (CGT)CAT (CGT)CAT(CGT)	III	F-CAGCTCCACCCATCAGATTT; R-GTTTTCCAGTCACGACGTT	1	260	—	—	DU635092

^a Allele data are for Trinidad field populations; H_E, expected heterozygosity, H_O, observed heterozygosity; all loci showed polymorphism among laboratory strain individuals tested.

^b Isolated from plasmid libraries enriched for (GA)_n motifs.

^c Isolated from plasmid libraries enriched for (AAT)_n motifs.

^d Isolated from plasmid libraries enriched for (TAGA)_n motifs.

^e Isolated from *A. aegypti* Liverpool cosmid clone containing previously mapped RAPD marker A13L975.

^f Isolated from *A. aegypti* Liverpool cosmid clone containing previously mapped *def* marker.

^g Isolated from *A. aegypti* Liverpool cosmid clone containing previously mapped *vcp* marker.

^b Isolated from *A. aegypti* Liverpool cosmid clone containing previously mapped LF198 marker.

ⁱ Isolated from *A. aegypti* ATC-10 cell line cosmid clone physically mapped to chromosome 3 as a Fluorescent In Situ Hybridization probe.

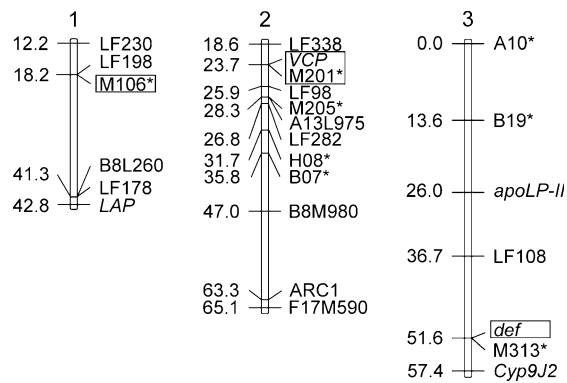


Figure 1. Genetic map positions for microsatellite loci (*) in *A. aegypti*. Map positions were determined relative to previously described RFLP loci, and map positions for the top-most loci on chromosomes 1 and 2 were anchored to the existing linkage map (Severson et al. 2002). Boxes indicate markers for which no linkage data were obtained and instead map positions were inferred from their association with cosmid clones that also contained known marker loci. Map distances are given in Kosambi centiMorgans.

a miniature subterminal inverted-repeat transposable element (MSITEs) (Tu and Orphanidis 2001).

It is likely not surprising that microsatellites within some Culicines, and within *A. aegypti* in particular, would be associated frequently with a variety of dispersed repetitive elements. Within humans, a large proportion of microsatellites are associated with *Alu* and other SINEs (Arcot et al. 1995; Nadir et al. 1996). Within plants, a large proportion of microsatellites from *Hordeum vulgare* have flanking sequences exhibiting homology to a number of retrotransposons and dispersed repetitive elements (Ramsay et al. 1999), whereas within *Oryza sativa*, the *micron* element that resembles a MITE is intimately associated with microsatellites (Akagi et al. 2001). Finally, within the Diptera a novel retrotransposon, *mini-me*, is associated with microsatellite genesis and dispersal. It provides a model for how microsatellites can be associated with both the 5' and internal regions of retrotransposons (Wilder and Hollacher 2001).

Despite the limited number, the 9 previously identified microsatellites have proved quite useful in evaluating the population genetics of *A. aegypti* in a number of populations including, Ho Chi Minh City, Vietnam (Huber et al. 2002a, 2002b), Phnom Penh, Cambodia (Huber et al. 2004), Cote d'Ivoire, Africa (Ravel et al. 2002), and Guaymas, Mexico (Ravel et al. 2001). Based on results from our initial studies with laboratory strains and in Trinidad field populations, the 11 new microsatellite markers identified in this study should provide additional tools for understanding population structure and gene flow within *A. aegypti*, and thereby facilitate efforts to understand disease transmission within these populations. Finally, the ongoing *A. aegypti* genome project (Severson et al. 2004) will undoubtedly reveal additional single-copy microsatellites for development as useful genetic

markers, although our results indicate the number of useful microsatellite loci is likely to remain low.

Acknowledgments

This work was supported by NIH/NIAID grants AI33127, AI34337, and AI59342.

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Received November 28, 2005

Accepted January 31, 2007

Corresponding Editor: Ross MacIntyre