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PRIMER NOTE

# Isolation and characterization of microsatellite DNA markers in the malaria vector *Anopheles funestus*

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## Abstract

**Screening of the *Anopheles funestus* genomic DNA library detected 18 new sequences with dinucleotide tandem repeats. Primers were designed to amplify the loci and 14 out of 18 gave a repeatable and scorable amplification. Deviations from Hardy–Weinberg expectations were tested for each locus in a sample of 30 wild *Anopheles funestus* females. No heterozygote deficiency was detected for 11 loci of 14, thus revealing the absence of null alleles. The number of alleles per locus ranged from 5 to 15, and observed heterozygosity from 0.13 to 0.85.**

*Keywords*: *Anopheles funestus*, malaria, microsatellites, polymorphism

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*Anopheles funestus* is an important vector of human malaria in sub-Saharan Africa, being the main vector in some areas. Chromosomal inversion polymorphism suggests a genetic substructure within wild *A. funestus* populations, sometimes at a microgeographic scale (Costantini *et al*. 1999; Dia *et al*. 2000). Neutral molecular markers are needed to decipher further the genetic population structure. Twenty-two microsatellite DNA loci were recently characterized by Sinkins *et al*. (2000) but additional loci would be required for high throughput investigation of the distribution of neutral variability, within and between natural *A. funestus* populations. Development of new markers would eventually benefit gene mapping and 'quantitative trait loci' analysis in this important malaria vector. This study reports the characterization of a new set of polymorphic microsatellite markers.

Microsatellite loci were isolated as described by Estoup *et al*. (1993) using the detailed protocols of A. Estoup and O. Martin that are available at HTTP://www.inapg.inra.fr/dsa/microsat/microsat.htm. Genomic DNA was extracted from a pool of 20 *A. funestus* specimens and was totally digested by *Sau*3A. Size-selected fragments (400–900 base pairs) were ligated into a pUC18 vector (Pharmacia) digested by *Bam*HI, and plasmids were used to transform XL1-blue competent cells (Stratagene). Approximately 3000 recombinant clones were transfered onto Hybond-N + nylon membranes (Amersham) and screened with an equal mixture of $(TC)_{10}$ and $(TG)_{10}$ digoxigenine end-labelled oligonucleotide probes (Boehringer Mannheim). Plasmid DNA from 21 positive clones was purified using QIAprep Spin Miniprep Kit™ (Qiagen) and sequences of inserts were obtained with an ABI 310 sequencer (Perkin-Elmer).

Among 21 sequences, three were identical to formerly described loci (Sinkins *et al*. 2000). Polymerase chain reaction (PCR) primers were designed flanking each of 18 remaining microsatellite sequences using the computer program OLIGO™ (version 4.0, National Biosciences). Primer pairs were chosen to amplify short (80–240 base pairs) PCR products.

Microsatellite variability was analysed using 30 females from Cameroon. DNA was isolated from single specimens following Collins *et al*. (1987). PCR amplifications were carried out in a 25-μL reaction volume, from approximately 5–10 ng of template DNA. The reaction mixture contained 1× Qiagen PCR buffer (1.5 mM $MgCl_2$), 200 μM each dNTP, 10 pmol of each primer, and 1 U Qiagen *Taq* Polymerase. The forward primer was 5′ modified with either TET, HEX, or FAM fluorescent labels (Eurogentec) to allow multiplex

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**Table 1** Characteristics of 14 microsatellite loci of *Anopheles funestus*

| Locus | Repeat motif | Primer sequences (5′→3′) | No. of alleles | Allele size range (bp) | $H_O$ | $H_E$ | GenBank Accession no. |
|---|---|---|---|---|---|---|---|
| FunD | $(CT)_{18}GTCT(GT)_6$ | F:GCTAACTACTCCGAAGCGCT<br>R:GATCGCAAAACTTCCGGTT | 15 | 145–177 | 0.85 | 0.89 | AY6008 |
| FunE | $(CA)_7TA(CA)_3$ | F:GACCGGTTCTGGTATCGTC<br>R:ATCGAGTCACCCAATTCTCC | 9 | 136–154 | 0.85 | 0.88 | AY6009 |
| FunF | $(TG)_9$ | F:GCCTTCAGTTTCGATTGGCG<br>R:AATAAGATGCGACCGTGGC | 7 | 104–118 | 0.79 | 0.78 | AY6010 |
| FunG | $(TG)_9$ | F:GAGCAAGCAGCTTACTGCAC<br>R:ACGTTCAGTGCACATCAATG | 11 | 146–168 | 0.82 | 0.84 | AY6011 |
| FunH | $(GT)_{11}$ | F:ACCACCCGAAGGCATCTA<br>R:ATTCCTTCGCGTCTACAGTG | 10 | 134–164 | **0.57** | 0.85 | AY6012 |
| FunI | $(CA)_{11}$ | F:GTCAGGGTGGTACACGAATA<br>R:GCATCTAACCCTGCTGCTT | 9 | 181–197 | **0.36** | 0.80 | AY6013 |
| FunJ | $(GT)_2GC(GT)_{23}$ | F:GGGCTCCATTCTAAATGCC<br>R:GTGACGTTTCGCGATAAGG | 10 | 190–212 | 0.86 | 0.84 | AY6014 |
| FunK | $(GT)_9$ | F:GCGCTTCCGCAAACATAC<br>R:ACTCACACCCCATTCTTGTG | 10 | 184–202 | 0.81 | 0.92 | AY6015 |
| FunL | $(GT)_8$ | F:AACAGTGGAAGGCAAATTGC<br>R:GCACGGTTACCACTGCTCA | 12 | 140–166 | 0.83 | 0.87 | AY6016 |
| FunN | $(TG)_8$ | F:ATCCGAAAACAGAACGGG<br>R:GGTACCAAACAACGCAAATA | 5 | 234–244 | **0.13** | 0.48 | AY6018 |
| FunO | $(CA)_6TA(AC)_4$ | F:GCACACATTTCAGGCAGC<br>R:GCCCACATTCTGCACCTT | 10 | 110–132 | 0.70 | 0.76 | AY6019 |
| FunP | $(AC)_9$ | F:GACCGGCTTCAAAACGAG<br>R:GTTCGGCATGTTCTTTCCTC | 9 | 84–104 | 0.62 | 0.80 | AY6020 |
| FunQ | $(TG)_9$ | F:GCAAACTGCTAGTAAATGTTTCC<br>R:ACATTTCCACAATTTGCGC | 7 | 84–98 | 0.65 | 0.75 | AY6021 |
| FunR | $(TG)_8T(TG)_3$ | F:GTAGTCGATGGTGCCGTGTG<br>R:ACCGTCCCTTCCATCTGTGA | 8 | 132–148 | 0.76 | 0.79 | AY6022 |

The foward primer is labelled for each locus and the annealing temperature is 54 °C for all primer pairs. $H_O$, observed heterozygosity; $H_E$, expected heterozygosity under Hardy–Weinberg equilibrium, tested on 30 *Anopheles funestus* females; bold characters denote a significant ($P < 0.05$) heterozygote deficiency, taking multiple tests (Bonferroni method) into account, estimation of exact *P*-values by the Markov chain method.

electrophoresis. Amplifications were performed using a Mastercycler gradient Eppendorf thermocycler under the following conditions: an initial denaturation at 94 °C for 2 min followed by 36 cycles of 30 s at 94 °C, 30 s at 54 °C, 30 s at 72 °C and a final elongation step of 10 min at 72 °C. Fragment analyses were conducted with an ABI PRISM 377. Alleles were sized relative to an internal standard using GENESCAN version 3.1 (Applied Biosystems).

Of the 18 primer pairs, four failed to amplify or gave a banding pattern that was difficult to interpret (available sequences in GenBank: AY116005, AY116006, AY116007, AY116017). The 14 other loci yielded repeatable and scorable results (Table 1). All markers were polymorphic, showing five to 15 alleles. Expected and observed counts of homozygotes/heterozygotes were determined using GENEPOP version 3.2 (Raymond & Rousset 1995). These tests for homozygote excess were significant at three loci, which may suggest one or more null alleles operating at these loci. Linkage disequilibrium between all pairs of loci was not detected ($P > 0.05$ Fisher's exact test) when using GENEPOP. The loci have not yet been localized relative to the known chromosomal inversions.

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