PRIMER NOTE

Isolation and characterization of polymorphic microsatellite markers from the mosquito *Anopheles moucheti*, malaria vector in Africa

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Abstract

Anopheles moucheti **is a major human malaria vector in Equatorial Africa. The screening of an** ***Anopheles moucheti*** **genomic microsatellite library allowed us to select 36 sequences with AC/GT dinucleotide tandem repeats. Primer pairs were designed to amplify the loci and 25 out of 36 gave a repeatable and scorable amplification. In total, 17 loci were selected for their high degree of polymorphism (the number of alleles per locus ranged from four to 16, and observed heterozygosity from 0.43 to 0.87) and suspicion of absence of null alleles, using 30 wild females from South-Cameroon. No linkage disequilibrium was found between the loci.**

Keywords: *Anopheles moucheti*, malaria vector, microsatellites, population structure

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Anopheles moucheti is a major human malaria vector in villages and towns situated in forest areas along large rivers or slow moving streams of Equatorial Africa. This species was shown to be responsible for *Plasmodium falciparum* entomological inoculation rates reaching 300 infective bites per person per year, with sporozoite rates ranging up to 4% (Fontenille & Lochouarn 1999). Despite this important role on malaria transmission, few studies were carried out on this species since its identification by Evans in 1925 (Evans 1931). As part of a large scale ongoing investigation of the African malaria vectorial system, a study based in South-Cameroon was undertaken on this vector. Biological observations and allozyme analysis on specimens from several villages of this region revealed this species to be morphologically polymorphic and genetically homogenous (Antonio-Nkondjio *et al*. 2002). Nevertheless, because allozyme loci might not be sufficiently variable to reveal *Anopheles moucheti*'s population level structuring, we undertook the development of microsatellite loci, known to be highly polymorphic markers.

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We report here the isolation and characterization of the first microsatellite loci from the genome of *Anopheles moucheti*, following the protocol described by Estoup *et al*. (1993) (detailed protocols of A. Estoup and O. Martin available at: *http://www.inapg.inra.fr/dsa/microsat/microsat.htm*).

Genomic DNA was extracted from a pool of 20 *Anopheles moucheti* specimens and totally digested by *Sau*3A. Size-selected fragments (400–900 pb) 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 using an equal mixture of $(TC)_{10}$ and $(TG)_{10}$ digoxygenin-end labelled oligonucleotide probes (Boehringer Mannheim).

Of the 78 positive clones detected, inserts from 50 of those harbouring a strong hybridization signal were polymerase chain reaction (PCR) amplified with the M13/pUC18 universal primers (Stratagene), and sequenced

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Table 1 Primer sequences and characteristics of 24 *Anopheles moucheti* microsatellite loci. Sequence interruptions of less than 5 bp between repeats are indicated as '+'. For locus AM8, the interruption between the two motifs is superior to 20 bp (juxtaposed motif). The annealing temperature is 55 °C for all primer pairs. Number of alleles are based on 3 samples of 10 specimens collected in 3 different villages of South-Cameroon. F and R: forward and reverse primers. H_O: observed heterozygosity, H_E: expected heterozygosity under Hardy–Weinberg equilibrium tested on the 30 specimens above. Bold locus are those for which a significant ($P < 0.05$) heterozygote deficiency was observed (estimation of exact P-values by the Markov chain method)

Locus	Repeat motif	Primer sequences (5′–3′)	Number of alleles	Allele size range (bp)	Heterozygosity		GenBank accession number
					H_O	H_E	
AM1	$(AC)_{8+2}$	F: ATA CCC TGC ATC CTC TGC R: GTA CGC AAA TGG TTG TTG C	7	94–122	0.47	0.64	AJ496752
AM2	$(GT)_9$	F: GCT CGC ATG GAT AAC CAC R: GTT CTA GGG CTT GGT TGG	7	104–118	0.53	0.63	AJ496753
AM3	$(GT)_9$	F: GAA TGG GAA AGA GAG ACG G R: AAG AAG CAG CAG ACA GAC G	6	188–199	0.67	0.73	AJ496754
AM4	$(AC)_9$	F: ATC CTT CAC CTT TTC GTG C R: ATC ATG TGC CTA TGA TGA GC	9	224–240	0.87	0.87	AJ496755
AM5	$(AC)_{18}$	F: GTT CCC TAG GCA AGA AAT TG R: AAG GTA ATC CCA CAG TAC GC	11	121–145	0.87	0.87	AJ496756
AM6	$(GT)_{4+6}$	F: ACG GTA GGT TAT CGA CAT GC R: ACG GAG CAC CGA ACA TG	9	150–168	0.80	0.76	AJ496757
AM7	$(GT)_{8+2}$	F: GTG CAA CAC GAA TTG CT R: AAA CGG ACG ACT GCT CG	8	218–246	0.73	0.78	AJ496741
AM8	$(GT)_{6+4}(AT)_8$	F: ATC ACG TGT GAC GCA CC R: ATC AGC GAA AGT GAG AGC TG	8	202–228	0.67	0.82	AJ496742
AM9	$(AC)_{4+4}$	F: AAT TTA CGC CAC ACG TAC C R: GAC GCA GTG TAA GAC GCT G	12	146–174	0.70	0.76	AJ496743
AM10	$(AC)_9$	F: GAT TTC ACT TCC TCA TTC GC R: GAT ACG TTT AAG CTG GCA GC	5	132–142	0.67	0.73	AJ496744
AM11	$(AC)_{8+4}$	F: AGA TTT AGC AAA GTT TGC CC R: AAT TGT TTG TTT GAC CGA GC	5	162–174	0.53	0.57	AJ496745
AM12	$(GT)_{16+6}$	F: ACA GAA CAG GTC ATT GTG GG R: AGC ACG TTT AAT AAT TTG GC	9	183–204	0.76	0.82	AJ496746
AM13	$(GT)_7$	F: GGG TGT TTC GTT TTG TTG TC R: ATT TCA CTT TCT CCT TTG GC	8	126–140	0.83	0.84	AJ496747
AM14	$(GT)_8$	F: GGG TGT TTC GTT TTG TTG TC R: ATT TCA CTT TCT CCT TTG GC	7	150–162	0.83	0.83	AJ496748
AM15	$(AC)_{13}$	F: TCA ATT TAT GAA AAC GGT CC R: GCA AAT GTT AAA TCC TAC GC	14	102–133	0.73	0.85	AJ496749
AM16	$(GT)_{3+6}$	F: GAG CCA AAG GTA AAT GAA GC R: AAG GAT GTC GAG CAC ACG	8	130–143	0.73	0.79	AJ496750
AM17	$(GT)_7$	F: GAT CGA CAC CGG TCG GTG A R: GCT TTT ATG ATG CGC AAC CC	4	218–224	0.86	0.63	AJ496751
AM18	$(GT)_{10}$	F: GCG CCA ATG TCG TAC ATC R: ATA ACA GAC ACC CTC ACA CG	16	204–240	0.70	0.91	AJ504655
AM19	$(GT)_{2+8}$	F: GGT AAC GAA ATA GTT TCC CG R: GCT ACA CCG GCT CCT GT	7	94–106	0.43	0.59	AJ504656
AM20	$(GT)_{8+2}$	F: GTC GTT GAA TTT GTC AAT GC R: GGT GGG AAT TGG ATG AGC	9	147–177	0.60	0.81	AJ504657
AM21	$(GT)_9$	F: GCT AGG AAT CGA AGC CAT C R: GAA TGC ATT TGC AAG TTG G	11	150–180	0.43	0.69	AJ504658
AM22	$(GT)_{11}$	F: AAT CAA TTG CAC TTG TAG GG R: GGT TTG TCG ACA ACG CTG	10	181–203	0.53	0.76	AJ504659
AM23	$(AC)_8$	F: ATA GTT GCA GCA TAG TCG CA R: ACT CCA TTG TGT GGA GCG	10	122–166	0.43	0.69	AJ504660
AM24	$(GT)_{16}$	F: GTG AAA CAT CCC AAC GAA GT R: ACA CCA TCA AAG GAC ACT GG	9	90–110	0.50	0.72	AJ504661

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using an ABI 310 sequencer (Perkin-Elmer). Primer sets were designed using OLIGO software version 4.04 (National Biosciences) to amplify short (90–230 bp) PCR products for 36 microsatellite sequences (the remainder were unsuitable because of incomplete sequences obtained). Microsatellite polymorphism was assessed using 30 females from three forest villages of South-Cameroon (10 specimens per village), distant from each other by about 200 km. DNA for genotyping was extracted from single specimens following Collins *et al*. (1987). PCR amplifications were carried out in 25 µL reaction volume, from approximately 5–10 ng of template DNA. Reaction mixture contained 1X Qiagen PCR buffer [Tris Cl, KCl $(NH4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7 (20 °C)], 2 mM $MgCl_2$ (Qiagen), 5 µL of Qiagen Q-Solution 5X, 200 µM each dNTP, 10 pmol of each primer, and 1 U Qiagen *Taq* Polymerase. Amplifications were performed using a Mastercycler Gradient thermocycler (Eppendorf) under the following conditions, common for all the loci amplified: an initial denaturation step at 94 °C for 5 min is followed by 34 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and a final elongation step of 10 min at 72 °C. Of the 36 primer pairs designed, 25 reliably produced a successful and consistent amplification under these conditions, yielding repeatable and scorable results evaluated on a 2% agarose gel as a single strong band. The forward primer of each of these 25 pairs was labelled with either TET, HEX or 6-FAM fluorescent dyes (Eurogentec). Resulting PCR products were resolved using an ABI Prism 377 Genetic Analyser (Perkin-Elmer). Alleles were sized relatively to an internal standard using GENESCAN software version 3.1 (Applied Biosystems).

In total, 24 loci revealed extensive allelic polymorphism with a number of alleles per locus ranging from four to 16 and an observed heterozygosity from 0.43 to 0.87 (Table 1), one locus being monomorphic (sequence available in EMBL database: AJ504662). The tests for heterozygote deficiency were performed for each locus on the 30 specimens using GENEPOP version 3.2 (Raymond & Rousset 1995). They were significant for 7 loci out of the 24 tested, which may suggest the presence of one or more null alleles operating at these loci (Table 1). Linkage disequilibrium between all pairs of loci was not detected ($P > 0.05$ Fisher's exact test) when using GENEPOP. In total, 17 polymorphic loci without any detected heterozygote deficiency are thus available for population genetics studies of *Anopheles moucheti* in Africa.

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