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PRIMER NOTE

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

A. BERTHOMIEU,* P. KENGNE,†‡ P. AWONO-AMBENE,‡ M. RAYMOND,* D. FONTENILLE† and M. WEILL*

**Institut des Sciences de l'Evolution, Laboratoire de Génétique et Environnement, UMR CNRS 5554, Université de Montpellier II, France, †Laboratoire de Lutte contre les Insectes Nuisibles, IRD, BP 64501, 34394 Montpellier Cedex 05, France, ‡Organization de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), BP 288, Yaoundé, Cameroon*

Abstract

The mosquito *Anopheles nili* is widespread across tropical Africa and appears to be the major vector of malaria in some rural forested areas of central Africa. Here we describe the isolation of 11 microsatellite polymorphic loci from the *A. nili* genome, displaying a high among-individual diversity (0.58–0.96) in samples from west Africa. Two loci displayed a significant departure from Hardy–Weinberg proportions across all samples, suggesting a substantial frequency of null alleles. The remaining nine loci are good candidates for further genetic studies in this species.

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

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Anopheles nili (Theobald) (Hamon & Mouchet 1961) appears to be the major vector of malaria in some rural forested areas of central Africa, with entomological inoculation rates reaching 100 infective bites per person and per year (Carnevale *et al*. 1992). Larvae of *A. nili* are typically found in vegetation or in dense shade along the edges of streams and large rivers. The extensive morphological, ecological and ethological variations among *A. nili* populations have been reported by many authors (Gillies & De Meillon 1968; Carnevale *et al*. 1992; Brunhes *et al*. 1999) suggesting that *A. nili* is a species complex. This species complex includes *A. nili sensu stricto*, which is anthropophilic and displays several morphological variants (Gillies & De Meillon 1968), the recently described *A. carnevalei* (Brunhes *et al*. 1999) as well as the rare zoophilic and highly exophilic *A. somalicus* characterized by slight differences observable only at the larval and pupal stages (Gillies & De Meillon 1968; Gillies & Coetzee 1987). The development of methods allowing precise characterization of genetic variation and population structure will improve our understanding of *A. nili* transmission patterns. In this work we describe the first microsatellite loci from *A. nili s.s.*, the most widespread species of the complex.

Correspondence: Mylène Weill. Fax: (00 33) 467143622 Email: weill@isem.univ-montp2.fr

Microsatellite loci were isolated as described by Estoup *et al*. (1993) using the 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 *A. nili s.s.* specimens and totally digested by *Sau*3A. Size-selected fragments (400–900 bp) were ligated into the pUC18 vector (Pharmacia) digested by *Bam*HI and plasmids were used to transform XL1-blue competent cells (Stratagene). Approximately 3000 recombinant clones were transferred onto Hybond-N+ nylon membranes (Amersham) and screened with an equal mixture of $(TC)_{10}$ and $(TG)_{10}$ digoxigenin-end-labelled oligonucleotide probes (Boehringer Mannheim). Plasmid DNA from 69 positive clones was purified using a QIAprep Spin Miniprep Kit™ (Qiagen) and the inserts were sequenced on an ABI 310 sequencer (Applied Biosystems) with the universal pUC18 primers PU 5′-GTTTTCCCAGTCACGACGTTGTA-3′ and PR 5′-TTGTGAGCGGATAACAATTTC-3′ and the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems). The polymerase chain reaction (PCR) primers were designed flanking 22 microsatellite sequences using the software PRIMER 3 (Rozen & Skaletsky 2000). Primer pairs were chosen to amplify short (100–260 bp) PCR products.

Microsatellite variability was analysed using 30 individuals from Cameroon, 10 from Burkina Faso and seven from

Table 1 Characteristics of 11 microsatellite loci of *Anopheles nili*

Locus	Repeat motif	Primer sequences	Accessio no.	T_a (°C)	No. of alleles	Allele size range (bp)	Cameroon			Burkina Faso			Côte d'Ivoire			All
							N	F_{IS}	1-Qinter	*N*	F_{IS}	1-Qinter	*N*	F_{IS}	1-Qinter	F_{IS}
1G13	$(CA)_{11}$	5′-AGCGCATCGCGTAGGTAGC-3′ 5′-CCCGTCTATCGTCACTCTGGG-3′	AJ536271	56	9	174–198	29	0.064	0.736	6	0.429	0.583	7	−0.18	0.726	0.065
A14	$(GT)_{10}GA(GT)_2GC(GT)_3$	5′-TTTCGGAGTGTGCTTT-3′ 5′-CGACTAAACTGACCGAG-3′	AJ536274	52	12	92–200	30	0.037	0.761	8	**0.417**	0.857	7	−0.075	0.798	0.093
1D80	$(CA)_{18}$	5′-CGCTTCCAGATGAGTG-3′ 5′-GATTTGTGTCGCTAAAGG-3′	AJ536269	52	19	115–177	30	0.087	0.913	9	0.145	0.910	7	0.089	0.94	0.099
F41	$(CT)_{11}TT(CT)_8$	5′-TACCGGAAAACGAATG-3′ 5′-CGAGGGCTAGTAGACTGA-3′	AJ536277	52	28	179–233	27	0.036	0.96	8	0.215	0.955	7	−0.05	0.952	0.056
1A27	$(TC)_{15}GC(TC)_2AC(TC)_2$	5′-GGTTTGCAAACTCCCACC-3′ 5′-TGGTTGTCGTTCGATTCG-3′	AJ536268	55	22	114–166	30	**0.093**	0.918	4	−0.043	0.958	7	**0.259**	0.964	**0.108**
2Ateta	$(AC)_9$	5′-TTTTGCATCGCATCACCG-3′ 5′-TTCCATGCGGAATGTGGC-3′	AJ536272	56	13	195–229	30	0.145	0.858	8	−0.037	0.723	7	−0.12	0.893	0.074
B115	$(CA)_{10}$	5′-TATACTCATGCCCAGCGCG-3′ 5′-CCATGCTTTTTCACATTTGGC-3′	AJ536276	56	13	175–201	30	0.050	0.878	7	−0.043	0.821	5	−0.032	0.775	0.027
F56	$(TC)_{11}$	5′-TGTCCTTGCTCTCATGAGG-3′ 5′-AACAGGACTCATTGACTGGG-3′	AJ536278	55	18	125–167	28	**0.051**	0.903	7	**0.446**	0.774	7	0.178	0.869	**0.130**
2C157	$(GT)_{12}$	5′-AATTCCGGACCGTACCG-3′ 5′-AGACTTCTCGCATTGGCG-3′	AJ536273	52	8	123–137	28	0.157	0.763	7	0.104	0.798	7	0.077	0.774	0.014
1F43	$(AC)_3GC(AC)_7AT(AC)_2$	5′-AGACAAGAGCCGGCCCAG-3′ 5′-CCTTTGATTCGGGACGGC-3′	AJ536270	56	9	166–188	29	0.095	0.8	5	0.273	0.825	7	0.048	0.75	0.11
A154	$(AC)_{13}$	5′-CTGACAATAAACCACCCT-3′ 5′-GAAATGTGACAGCGAAC-3′	AJ536275	52	14	200–232	30	0.124	0.799	6	0.231	0.867	6	0.143	0.583	0.143

F_{IS} are estimated following Weir & Cockerham (1984). Bold characters denote a significant ($P < 0.05$) heterozygote deficiency. *N*, Population size; T_a, polymerase chain reaction annealing temperature; 1-Qinter, diversity among individuals within populations.

Côte d'Ivoire. DNA was isolated from single specimens following Collins *et al*. (1987). The shortest primer (or the forward primer if both were of the same size) had a 19-base extension at its 5′ end with the sequence 5′-CACGACGTT-GTAAAACGAC-3′ (Roy *et al*. 1996). This sequence is identical to an IR-labelled (LI-COR dye; IRD-700 or IRD-800) universal M13 forward primer (Steffens *et al*. 1993). The PCR amplifications were carried out in a 20-μL reaction volume from approximately 5–10 ng of template DNA. The reaction mixture contained 1× PCR buffer (Eurogentec), 200 μM each dNTP, 1.5 mM $MgCl_2$, 50 pmol of the primer with M13 tail, 100 pmol of the nontailed primer, 10 pmol of IR-labelled M13 forward primer and 1 U Goldstar Taq Polymerase (Eurogentec). Amplifications were performed using a Mastercycler gradient thermocycler (Eppendorf) under the following conditions: an initial denaturation at 94 °C for 4 min followed by 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature (T_a; Table 1) and 30 s at 72 °C and a final elongation step of 2 min at 72 °C. Amplified fragments were separated on 6.5% polyacrylamide denaturing gels using a LI-COR (IR2 LI4200S2G). The size of the alleles was determined relative to the amplified clone size, loaded on the gel every six lanes. If necessary, two or more runs were performed to verify the allele typing by re-ordering the samples.

Fifteen of 22 pairs of primers flanking microsatellite sequences showed repeatable and scorable results. The number of alleles detected varied from eight to 28. The presence of null alleles was tested for each locus in each sample using GENEPOP version 3.3 (Raymond & Rousset 1995). Four loci displayed a high and systematic homozygote excess, indicating that null alleles were present at a high frequency. These four loci were discarded. For the remaining 11 loci, heterozygote deficiency was not apparent for the overall estimate of F_{IS} across populations, except for loci A14 and 1A27 (Table 1). Linkage disequilibrium between all pairs of loci was not detected ($P > 0.05$, Fisher's exact test) when using GENEPOP. At least nine highly polymorphic loci without apparent heterozygote deficiency are thus available for population genetics studies of *A. nili* in Africa.

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