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PRIMER NOTE

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

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Abstract

The *Anopheles maculipennis* complex includes the most important malaria vectors of the western Palearctic. *Anopheles maculipennis* s.s., one member of this complex, is a reported vector in the Middle East. Here we describe the isolation of 15 microsatellite polymorphic loci from the *An. maculipennis* s.s. genome, displaying a high among individual diversity (0.37–0.77) in a sample from France. Three loci displayed a significant departure from Hardy–Weinberg proportions, suggesting a substantial frequency of null alleles. The remaining 12 loci are good candidates for further genetic studies in this species.

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

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The *Anopheles maculipennis* complex includes the most important malaria vectors of the western Palearctic (White 1978; Stegniy 1982). One member of this complex, *An. maculipennis* s.s. was the common malaria vector in Bosnia Herzegovina and a reported vector in Eastern Europe (Jetten & Takken 1994). Its role as a malaria vector in northern and western Europe is considered minor, because of its reduced anthropophily (Jaenson *et al*. 1986), although there is the intriguing possibility that the present zoophily is a recently evolved trait, triggered by agricultural and social changes during the nineteenth century (Wesenberg-Lund 1920–21). In Armenia in 1997, results of entomological investigations carried out after the reappearance of malaria indicated *An. maculipennis* s.s. as a possible vector responsible for the transmission (Romi *et al*. 2002) because of its wide distribution, high densities and its propensity to enter houses and buildings (i.e. endophily).

In order to study the population structure of this potential malaria vector, we developed and characterized microsatellite loci for this species. Microsatellite loci were isolated as described by Estoup *et al*. (1993) using 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 female *An. maculipennis* s.s. collected on 14 January 1999, in a natural cave near Montpellier ('Grotte du Zoo') where they were overwintering. DNA was totally digested by *Sau*3A, size-selected fragments (400–900 bp) 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 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 59 positive clones was purified using QIAprep Spin Miniprep Kit™ (Qiagen) and sequences of inserts were obtained with an ABI 310 sequencer (Perkin-Elmer).

Polymerase chain reaction (PCR) primers were designed flanking 26 microsatellite sequences using the computer program OLIGO (version 4.0, National Biosciences). Primer pairs were chosen to amplify short (80–260 bp) PCR products.

Microsatellite variability was analysed using 53 individuals from Montpellier, France. DNA was isolated from single specimens following Collins *et al*. (1987). The shortest primer, or if they were of the same length the forward primer, has a 19-base extension at its 5′ end with the sequence 5′-CACGACGTTGTAAAACGAC-3′ (Roy *et al*. 1996). This sequence is identical to an infrared-labelled

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Table 1 Characteristics of 15 microsatellite loci of *Anopheles maculipennis* s.s.

Locus	Repeated motif in cloned allele (bp)	Primer sequences (5′–3′)	Accession no.	ASR (SCA)	PCR conditions (T_a °C/mM $MgCl_2$)	n	No. alleles	Gene diversity	F_{IS}
MacuKY	$(CA)_5N_6(CA)_2/(CA)_{11}$	F: CTTCAGGGCATTTATTTG R: ATGGATGGAGTGGATTG	AJ550005	176–190 (186)	50/1.2	52	7	0.76	0.32
MacuW161	$(CA)_9$	F: CAAGATGGCGGAGAA R: TTTCTTTGGAACGGC	AJ550006	113–119 (117)	56/1.2	53	4	0.61	0.05
MacuGQ	$(AC)_9$	F: GCCTTTCGTCCACCT R: GCTAACACGCTCCAAAC	AJ550007	179–193 (187)	56/1.2	49	5	0.54	−0.21
MacuW149	$(AG)_2AA(AG)_{12}$	F: TGCTGTTTTCGTGCT R: TCCAGTAGCTTTCCGT	AJ550008	242–258 (254)	56/1.2	46	6	0.49	0.65
MacuU182	$(AC)_3AG(AC)_8$	F: ATCTTGATGGCAAGGG R: GAAGGGCAGGGAATG	AJ550009	115–147 (123)	56/1.2	53	8	0.71	0.10
MacuUF	$(CA)_8TA(CA)_4$	F: CCGTCAAAACGACTTC R: GATCTGTCCCATCCTGT	AJ550010	158–182 (174)	55/1.2	53	8	0.77	0.16
MacuU125	$(CA)_4CG(CA)_2CG(CA)_4TA(CA)_3G(CA)_4$	F: CCCACGATAATTGGTTC R: TCGCGCTCGTAAATC	AJ550011	253–263 (259)	57→47/2.5	43	5	0.55	0.03
MacuQ72	$(CA)_9$	F: AGTGGAGAAGTGGCAAA R: CGTTCGTGTCCATAAATC	AJ550012	157–169 (161)	57→47/2.5	50	4	0.65	0.23
MacuO177	$(CA)_7$	F: TGGCAAGGCAATGT R: GATCCCGTGGCTG	AJ550013	163–185 (169)	57→47/2.8	51	4	0.57	0.03
MacuM34	$(GT)_2ATC(TG)_5TA(TG)_2CG(TG)_2TA(TG)_2$	F: CATTGCCTTCGTGACC R: CTTGGCCGCTACCTC	AJ550014	140–152 (150)	57→47/2.5	52	2	0.38	0.09
MacuO150	$(CA)_6TA(CA)_4$	F: CGTAACGAGCCAAAC R: GATCGTCCCTATGTAC	AJ550015	66–76 (72)	57→47/2.8	52	3	0.53	0.53
MacuI3	$GCCC(GC)_2(GT)_8$	F: ATCTCGTTTCGCACTC R: ATCGCTTCCGTTCAC	AJ550016	105–129 (115)	47/2	53	7	0.62	−0.12
MacuO185	$(CA)_3GA(CA)_7$	F: GAGTCGAGCCGAAAAG R: CCGTTGGGAAATGTATG	AJ550017	93–105 (101)	57→47/2.5	52	5	0.37	0.37
MacuA159	$(AC)_3GC(AC)_2AA(AC)_2CC(AC)_6$	F: CGTGGGTCGTAAGCA R: ACAGGGTAAACTATCGCC	AJ550018	140–152 (140)	57→47/2.5	52	5	0.65	0.24
MacuG66	$(CA)_3(CG)_2(CA)_7$	F: CGTTCCGATAGTTGCT R: ATGATTGTTGCGTGTG	AJ550019	85–89 (85)	57→47/2.5	52	2	0.50	0.01

ASR, allele range size (bp); SCA, size of cloned allele (bp); n, sample size; gene diversity (or 'expected heterozygosity', measured as one minus frequency of identical pairs of genes among different individuals); F_{IS} estimates following Weir & Cockerham (1984); bold characters denote a significant ($P < 0.05$) heterozygote deficiency, taking multiple tests (Bonferroni method) into account. P-values were estimated by the Markov chain method when there were more than four alleles.

(LI-COR dye: IRD-700 or IRD-800) universal M13 forward primer (Steffens *et al*. 1993). PCR amplifications were carried out in 20-μL reaction volume, from approximately 5–10 ng of template DNA. Reaction mixture contained 1 × Eurogentec PCR buffer [750 mM Tris-HCl pH 8.8, 200 mM $(NH4)_2SO_4$, 0.1% (v/v) Tween 20], 200 μM each dNTP, 20 pmol of the primer with M13 tail, 10 pmol of the non-tailed primer, 20 pmol of infrared-labelled M13 forward primer and 1 U Eurogentec *Taq* polymerase. Amplifications were performed using a Mastercycler gradient Eppendorf thermocycler under the following conditions: an initial denaturation at 94 °C for 2 min followed by 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature T_a (Table 1), 30 s at 72 °C and a final elongation step of 2 min at 72 °C. To optimize the PCR reaction for some primers, alternate touchdown annealing temperature were used (Table 1) based on the above profile with annealing temperature decreasing 1 °C per cycle during 10 cycles, followed by 20 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C. Amplified fragments were separated on 6.5% polyacrylamide denaturing gels, using a LI-COR (ScienceTec IR2 LI4200S2G). Size of the PCR products was determined by comparing with the clone size (amplification of the clone loaded every six lanes on each gel). If necessary, two or more runs were performed to verify the allele typing, by re-ordering the samples.

Of the 26 pairs of primers, 10 failed to amplify. The 16 other loci yielded repeatable and scorable results. All except one were polymorphic, showing two to eight alleles (Table 1). In order to assess the usefulness of the loci for population genetic analysis, heterozygote deficiency (an indication of the presence of null alleles at high frequency) was studied using Genepop version 3.3 (Raymond & Rousset 1995). Taking into account multiple testing (Hochberg 1988), three loci displayed a significant ($P < 0.05$) departure from Hardy–Weinberg equilibrium, due to excess of homozygotes. Contingency table tests for linkage disequilibria were computed for all pairs among the remaining 12 loci. No test remained significant after correcting for multiple tests. Diversity among individuals within samples and F_{IS} estimates following Weir & Cockerham (1984) were determined using GENEPOP version 3.3 (Raymond & Rousset 1995). In total, 12 polymorphic loci without significant heterozygote deficiency are thus available for population genetics studies of *Anopheles maculipennis*.

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