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PRIMER NOTE

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

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Abstract

The mosquito *Anopheles sacharovi*, a member of the *A. maculipennis* complex, is an important malaria vector in the Middle East. Here we describe the isolation of 15 microsatellite polymorphic loci from the *A. sacharovi* genome, displaying a high among-individual diversity (0.30–0.92) in a sample from Turkey. Seven loci displayed a significant departure from Hardy–Weinberg proportions, suggesting a substantial frequency of null alleles. The remaining eight loci are good candidates for further genetic studies in this species.

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

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The most important malaria vectors in the geographical area represented by the World Health Organization European Region, including the Middle East and the countries of the former Russian Federation, the Newly Independent States (NIS), belong to a group of anopheline mosquitoes, i.e. the *Anopheles maculipennis* complex (Romi *et al*. 2000). Species from this group, *A. maculipennis* s.s. and *A. sacharovi*, were responsible for the recent *Plasmodium vivax* malaria outbreaks in Turkey, Armenia, Azerbaijan and other NIS. The quite high frequency of these outbreaks, resulting from the difficulties of the local health system in implementing efficacious control measures together with massive population movements, could represent a situation of high risk for malaria reintroduction in European temperate areas, where potential vectors are still present (Majori *et al*. 1999). *Anopheles sacharovi* is the main vector in malarious areas of Turkey, which is relatively close to other mediterranean countries of southern Europe. The main reasons for malaria recrudescence in this country are (i) the great expansion of the irrigation network, that resulted in a dramatic increase in *A. sacharovi* density and (ii) the resistance in the vector populations to organochlorine and several other organophosphorous compounds used in residual spraying activities (Kasap *et al*. 2000; Sabatinelli *et al*. 2000).

In order to study the population structure of this malaria vector, we developed and characterized microsatellite loci for this species. Microsatellite loci were isolated as described by Estoup *et al*. (1993) using the detailed protocols of Estoup and Martin available at http://www.inapg.inra.fr/dsa/microsat/microsat.htm. Genomic DNA was extracted from a pool of 20 *A. sacharovi* specimens and 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 transfered 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 45 positive clones was purified using the 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 18 microsatellite sequences using the computer program OLIGO™ (version 4.0; National Biosciences). Primer pairs were chosen to amplify short (90–260 bp) PCR products.

Microsatellite variability was analysed using 41 individuals from the locality of Adana in Turkey. DNA was isolated from single specimens following Collins *et al*. (1987). Either the shorter of the two primers or, if primer lengths were equal, the forward primer had a 19-base extension at its 5′ end with the sequence 5′-CACGACGTTGTAAAACGAC-3′ (Roy *et al*. 1996). This sequence is identical to an Infra-Red (IR)-labelled (LI-COR dye, IRD-700 or IRD-800) universal M13 forward primer (Steffens *et al*. 1993). The PCR amplifications

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were carried out in a 20-μL reaction volume from approximately 5–10 ng of template DNA. The reaction mixture contained 1× Qiagen PCR buffer, 200 μM of each dNTP, 20 pmol of the primer with M13 tail, 10 pmol of the non-tailed primer, 20 pmol of IR-labelled M13 forward primer, 1 U Eurogentec Taq Polymerase and 2.5 mM of $MgCl_2$ for all loci except for SachA3 and SachA16 where 2.8 mM of $MgCl_2$ was used. Amplifications were performed using a Mastercycler gradient Eppendorf thermocycler using a touchdown procedure: an initial denaturation at 94 °C for 2 min followed by 10 cycles of 30 s at annealing temperature decreasing by 1 °C per cycle from 57 to 47 °C, followed by 20 cycles of 30 s at 94 °C, 30 s at 50 °C 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 PCR product size was determined by comparison 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 18 pairs of primers, three failed to amplify. The other 15 loci yielded repeatable and scorable results. All markers were polymorphic showing three to 17 alleles. In order to assess the usefulness of these 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), eight loci displayed a significant ($P < 0.05$) departure from Hardy–Weinberg equilibrium, due to excess of homozygotes (Table 1A), and eight loci displayed no significant

Table 1 Characteristics of 15 microsatellite loci of *Anopheles sacharovi*

Locus	Repeated motif in cloned allele (bp)	Primer sequences (5′–3′)	Accession no.	Size of the cloned allele (bp)	ASR	Adana (Turkey)			
						n	No. alleles	1-Qinter	Fis
A									
SachA16	$(GT)_{11}G_2T_4A(TG)_3$	F: GGGATGGGTTGAATG R: GCATAAGTTTCGCCTC	AJ539386	97	189–211	41	11	0.78	**0.34**
SachCU	$(CA)_{18}$	F: TACCTAAATCGCATCGT R: TCCTGAAATCCTGTTGA	AJ539387	185	175–227	40	17	0.92	**0.46**
SachQ7	$(CA)_9$	F: GCGCACCGAGTAAAG R: CATCCCCAGCAAAAG	AJ539388	101	101–111	41	6	0.64	**0.62**
SachK161	$(AC)_{12}$	F: CACAAATCTCCGAACC R: TATTGCTTTCTTGTCTATCC	AJ539389	253	241–285	40	16	0.90	**0.31**
SachC55	$(CA)_8$	F: CGCCATTAGACACCC R: CTGACGAAGACAACTCCT	AJ539390	170	168–182	38	13	0.88	**0.19**
SachOB	$(CT)_3(GT)_{14}N_7(TG)_3N_{11}(TG)_7$	F: GATACGCACAACTCCCT R: CAACGCTATTTGCCC	AJ539391	223	205–235	38	15	0.92	**0.23**
SachK89	$(GT)_2AT(GT)_7$	F: GCACTAACACACCGACA R: CACATCGCCTCCAAC	AJ539392	112	110–120	41	5	0.70	**0.23**
B									
SachO27	$(AC)_3GA(AC)_{10}$	F: CAAAGTGAGGCAGGG R: AGTGTGCGTTGGCTT	AJ539393	262	254–266	38	7	0.68	0.15
SachQ82	$(AT)_2(AC)_2AT(AC)_3$ $AT(AC)_6AT(AC)_4T_5(AT)_3$	F: GTAGTTACGAGGGCGA R: CAGTTTCAGGATTCAGTTT	AJ539394	248	224–264	37	9	0.70	0.19
SachA3	$(GT)_8TT(GT)_2$	F: GCCGTAGAAAATCGTG R: GTCCTTCCCAGTCCTT	AJ539395	127	121–129	41	3	0.30	–0.15
SachG49	$(AC)_2(CA)_3TA(CA)_8TA(CA)_3$	F: GTGCGAAAACTCAACC R: ATGATGCTGCCTGAAC	AJ539396	263	249–277	29	8	0.63	0.13
SachG97	$(CA)_{12}$	F: GATGCTGATGAAGATGG R: GACGGATGGAAAAGTG	AJ539397	119	111–129	40	9	0.77	0.03
SachG103	$(CA)_3CGCAAA(CA)_6CG(CA)_5$	F: CACGGGATATGTTAAGAA R: CTTCCTAGTGAGTGGAGC	AJ539398	169	159–175	40	5	0.52	0.19
SachMB	$(GA)_{20}$	F: GTGTTACTTCAACCTGTCC R: TGCGTTAGTTTACCTCCT	AJ539399	161	137–165	40	14	0.86	0.15
SachM1	$(CT)_5CG(CT)_4TT(CT)_3$	F: GCTATGTCCCATCGTAA R: CCAAATACAGCCATCC	AJ539400	124	123–128	41	5	0.58	–0.006

ASR, Allele size range (bp); *n*, sample size; 1-Qinter, diversity among individuals within samples (Raymond & Rousset 1995); Fis, estimates following Weir & Cockerham (1984); bold characters denote a significant ($P < 0.05$) heterozygote deficiency, taking multiple tests (sequential Bonferroni method) into account. Loci are presented according to whether they display (A) or not (B) a significant heterozygote deficiency.

($P > 0.05$) heterozygote deficiency (Table 1B). No case of significant linkage disequilibrium was detected among all pairs of loci in Table 1B (Fisher's exact test, $P > 0.05$, taking into account multiple testing), suggesting that these loci are statistically independent. Fis estimates following Weir & Cockerham (1984) and diversity among individuals within samples (1-Qinter) were determined using GENEPOP (Raymond & Rousset 1995). In total, eight polymorphic loci without significant heterozygote deficiency are thus available for population genetics studies of *A. sacharovi* in the Middle East and NIS.

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