

## PRIMER NOTE

# Isolation and characterization of microsatellite markers from malaria vector, *Anopheles culicifacies*

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## Abstract

*Anopheles culicifacies*, an important vector in the Indian subcontinent is a complex of five sibling species of which four are vectors. We describe the isolation of 31 microsatellite markers from the recently recognized isomorphic species A of which 13 were characterized in sympatric populations of *Anopheles culicifacies* isomorphic species A and B. The allele frequencies ranges from two to 12 in species A and two to seven in species B. Species A being a vector, and that these markers can be used in closely related species, makes the isolation of these markers important to study population structure of all sibling species in this complex.

**Keywords:** *Anopheles culicifacies*, malaria vectors, microsatellites, sibling species

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*Anopheles culicifacies* is the major vector in the Indian subcontinent and extends its role up to Yemen in the West and Sri Lanka in the South. This taxon has five isomorphic species provisionally designated as A, B, C, D and E (Subbarao 1998). Extensive studies carried out in India have shown distinct distribution and differences in vectorial potential, seasonal prevalence and host feeding preferences and responses to insecticides (Subbarao & Sharma 1997). Of these five species, *Anopheles culicifacies* species A is widely distributed and is an important vector of *Plasmodium falciparum* and *Plasmodium vivax* (Subbarao *et al.* 1988). The importance of this species has necessitated the need to examine the gene flow and understand its population structure for planning effective vector control strategies. Keeping this in view, microsatellite markers were isolated in species A and their use in species B was studied.

Total genomic DNA from 50 *An. culicifacies* species A mosquitoes from a laboratory colony was extracted and was digested with a mixture of restriction enzymes including *Sau3AI*, *RsaI*, *HaeIII*, *EcoRV*, and *SspI* (New England Biolabs, UK). The size selected fragments (300–1000 bp) were repaired with Klenow (New England Biolabs, UK), ligated into the

*SmaI* site of pBluescript KS(+) (Stratagene) and subsequently transformed into competent DH10B cells. Colonies from each plate were lifted onto nylon membranes (Sigma) and were screened with  $\gamma^{32}\text{P}$ -labelled oligonucleotide probes ( $\text{GA}_{(15)}$  and  $\text{GT}_{(15)}$ ). Plasmid DNAs were purified from the putative positive clones, digested with *PvuII*, subjected to Dot blot/Southern blotting, followed by hybridization with the radioactively labelled probes. Clones with strong positive signals were sequenced with T7 and M13 primers on automated ABI 310 Sequencer (Applied Biosystems) using Big Dye Terminator kits. For all the sequences with the target sequences, using software PRIMERSELECT (DNASTAR, Inc., Madison, WI), primers were designed from the flanking unique regions (Table 1) to give short amplified products (104–184 bp). PCR amplifications were carried out in 25  $\mu\text{L}$  reaction volume from 10 to 20 ng of genomic template DNA extracted from whole single mosquitoes. Reaction mixture (25  $\mu\text{L}$ ) contained 1X buffer, 1.5 mM of  $\text{MgCl}_2$ , 200  $\mu\text{M}$  of each dNTP, 0.65 unit of *Taq* polymerase (Ampli-Taq Gold, Perkin Elmer) and four picomoles of each primer. The PCR conditions were: an initial denaturation step at 94 °C for 10 min followed by 30 cycles of 30 s at 94 °C, 30 s at 48–58 °C for the different primers, 30 s at 72 °C and a final elongation step at 72 °C for seven minutes. In total, 31

**Table 1** Primer and core sequences of the 31 microsatellite loci developed from *Anopheles culicifacies* species A

S.No	Markers	Repeats	Primer sequences 5'-3'	Allele size (bps)	Accession numbers
1	AcAIIIB5*	CA <sub>(3+1+2)</sub>	CGGAAAACGTTGCAACAAAATC ATCCAACCGTAGCCATAACAAAAC	110	AJ417869
2	AcAIIIB7*†	GT <sub>(5)</sub>	GCAGGCAGACCACCTCACAATCTG GACTCTGCTGCTGCCACACTTG	149	AJ417870
3	AcAIVB129*†	GT <sub>(7)</sub>	TCTCCTTTTGCATATCTTTCGTG TAGATTTCGGTTGTAGTTTTCCTGC	107	AJ417871
4	AcAVB93*	GT <sub>(3)</sub> + TG <sub>(4)</sub>	GTCCCTTGCAAATCACATCGG T'TAATGACTTCAATCCACAAACCC	140	AJ420078
5	AcAVB93A*	CA <sub>(2+3)</sub>	GTGGCCGTTGTTTCGTCCCTTTTG TGCTCGTGCCGTTTCGTGAGTC	117	AJ420079
6	AcAVB194*	GT <sub>(6)</sub>	TGTCGTGAAGGCATGTTTGAG AAT'TAT'TGCAT'TCTAGCGGGTG	184	AJ420081
7	AcAVB221*	CT <sub>(3+7)</sub>	ACTCACGGGAAGCCAAAATACC AAGGAGAAGGATACATCGCTGGAG	115	AJ420073
8	AcAVIB134*	CA <sub>(3+1+1+6)</sub>	TGGCGATGATGATGATGGCG AGCAGT'TTTCGCCGAAGGAGAG	166	AJ420074
9	AcAVIB213*	GA <sub>(7)</sub>	ATAAAAACGCCCCGCATCATAATG ACGGCACAT'TCCCTCCCATAG	116	AJ420076
10	AcAVIIB46*	CA <sub>(1+4)</sub>	AACCGGAAGCAGTATCGCACAC GAGGCTCCTTCGT'TCCATCCG	140	AJ420075
11	AcAVIIB40*	CA <sub>(5)</sub>	TCAAGCTGGACAATGTAACTCAAC TTCAATCAAACCCAGCCAAAC	118	AJ420077
12	AcAVIIB182a*†	GT <sub>(1+6)</sub>	TTTAGCTTCGGCCCTTTCATAC AGATACAACCCGGTGCCTCAGC	166	AJ420080
13	AcA36*	GT <sub>(8+2)</sub>	CACTGATGACGTTTCGTTCG GGGCAAACCTGAAAAGGTTG	206	AJ616757
14	AcA59*	GT <sub>(6+2+2)</sub>	GCGTAGGTCAACCGTAATGC TCCCACATACCGATACACCA	210	AJ616758
15	AcA61*	GT <sub>(3+4+3+4+2)</sub>	CGAATGCAT'TTCGCTTGATA TTCCTACTCACCAGCTGAAC	204	AJ616759
16	AcA43c30*†	GT <sub>(38+2)</sub>	ATCACACACCGCGTACAGA CCT'TGGAGAGGGCTGTAGAA	200	AJ616760
17	AcA75*	GT <sub>(10)</sub>	AACGCAGTCACAAGGCAGTA TCTGGAGATCGAGCACGAGT	91	AJ616761
18	AcAI12a	GT <sub>(9)</sub>	AAT'TAT'TAT'TTGACTGAT'TATGGAT AGTGGTGATGCTGCTGTGTTG	104	AJ616743
19	AcAI12b	CA <sub>(6)</sub>	GCCTCTATGCTTTCGTGCTGA TAGGGTCGGGAAAT'TAGGAAAAA	125	AJ616744
20	AcAII83	GT <sub>(9)</sub>	CCTAGGATAGTGAGAAATACC CTCTACTCTCCCGCTTCT	104	AJ616745
21	AcAIV140a	CA <sub>(1+4+2)</sub>	ATAACTGAAAAGACAT'TCTTC CGACAAGTATCGTGTGTAT	178	AJ616746
22	AcAIV141a	GT <sub>(6)</sub> + TG <sub>(3)</sub> + CG <sub>(3)</sub>	CCACGACTAT'TTCATAT'TTAAAC CCGTTT'TAT'TGTTGGTGCTG	117	AJ616747
23	AcAIV141b	AC <sub>(10)</sub>	GCTT'TCCTACAT'TACACTCCG TGGCTGCTACGGACTGGAG	158	AJ616748
24	AcAIV198a	TG <sub>(5)</sub>	TGAAAAT'TTAAATCGCAACCATA GTAAGAAGGGACAACCATCA	167	AJ616749
25	AcAIV198b	CT <sub>(4+2)</sub>	AGT'TTACTGCAGCTTCTCATCA GACTCTCAGACGTCAGCAAC	127	AJ616750
26	AcAIV220	GAAT <sub>(4)</sub>	AACAACAT'TTCCTTAGCCT GCGAAGCGGTTGTAGCG	107	AJ616751
27	AcAIV62	TG <sub>(11)</sub>	AAACCGAATGCGCTCACCC CACGTCGGACACAAAACCAC	126	AJ616752
28	AcAIV93a	GT <sub>(3)</sub> + TG <sub>(8)</sub>	AACGCATGCGGTCGGTG TAT'TCCGGACGGTTGCTTAC	128	AJ616753
29	AcAIV93b	CA <sub>(5+1)</sub>	AGTGAAC'TAGAATGGAACGAG CCAAAGCAAAATAATATCAAACG	118	AJ616754
30	AcAV78a	CT <sub>(7)</sub>	CGCGCAGACCAATCCAC CATACCCTCTCTTACCCTTCA	112	AJ616755
31	AcAV78b	GT <sub>(14)</sub>	CATGGAGAGTGGACGGTAA CTCAAGATCCTAGCCTTTC	121	AJ616756

\*Markers used for genotyping.

†Markers not amplified.

**Table 2** Characteristics of microsatellite loci in *Anopheles culicifacies* species A and species B

Locus	<i>An. culicifacies</i> A (n = 28)			<i>An. culicifacies</i> B (n = 14)		
	No of alleles	$H_O$	$H_E$	No of alleles	$H_O$	$H_E$
AcA59	5	0.32143	0.65130	7	0.53846	0.79385
AcAVIIB46	3	0.14286	0.56039	2	0.15385	0.44308
AcA36	9 <sup>(2)</sup>	0.53571	0.84156	3	0.53846	0.61231
AcAVB221	9 <sup>(1)</sup>	0.37037	0.80922	4 <sup>(7)</sup>	0.00000	0.90909
AcA61	8	0.60714	0.83571	6 <sup>(2)</sup>	0.09091	0.81818
AcAVB93	5	0.46429	0.67857	5 <sup>(1)</sup>	0.41667	0.75725
AcAVIIB40	3	0.21429	0.52987	3	0.00000	0.58333
AcA75	1	0.00000	0.51039	Not done		
AcAVIB213	12 <sup>(5)</sup>	0.34783	0.93430	4	0.07692	0.63077
AcAVB194	6 <sup>(10)</sup>	0.11765	0.46346	4 <sup>(7)</sup>	0.16667	0.86364
AcAVIB134	3 <sup>(6)</sup>	0.04545	0.66385	Not done		
AcAIB5	6	0.07143	0.61753	3	0.23077	0.28615
AcAVB93A	2	0.00000	0.17013	2	0.00000	0.51692

Superscript numbers denote individuals in a population showing no amplification.  $H_E$  determinations were performed in ARLEQUIN ver 2.000 (Schneider *et al.* 2000).

markers were isolated (Table 1). Of the 31 primer sets, 27 produced successful and consistent amplification under the conditions, yielding repeatable and scorable results evaluated on a 2% agarose gel as a single strong band.

Of the 31 markers, 17 were used to study polymorphism in a population. One of each primer pairs was labelled at 5' with TET, HEX or 6-FAM fluorescent dyes (Microsynth Corporation, Switzerland). Multiplex PCRs were set up by grouping two to three primers together, depending on their annealing temperatures, dyes and sizes. The resulting amplified products were resolved using an ABI Prism 310 Genetic Analyser (Applied Biosystems). Alleles were sized relative to an internal standard using GENESCAN software version 3.1 (Applied Biosystems).

The variability of each locus was assessed in two sibling species of *Anopheles culicifacies*, species A and B found sympatric in district Sonapat of Haryana state (India). These mosquitoes were collected in July / August 2001. Except for marker AcA 75, all the markers were polymorphic in both species studied with the allele frequency ranging from two to 12 in species A and two to seven in species B. The observed heterozygosity varied from 0.00 to 0.6 (Table 2). Deficiency of heterozygotes was observed in most of the loci. The presence of null alleles could be a possible reason

for the deficiency observed. This consistent deviation from Hardy–Weinberg equilibrium could be due to inbreeding or population subdivision.

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