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Isolation and characterization of 25 microsatellite DNA loci for *Anopheles albitarsis sensu lato* and inter-specific amplification in 5 congeneric species

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ABSTRACT. The *Anopheles albitasis* complex includes 6 species, and 3 are considered as malaria vectors in Brazil. Twenty-five polymorphic microsatellite DNA loci were isolated and characterized in 24-36 individuals from the neighborhood of Puraquequara, Manaus, Amazonas State, Brazil. The number of estimated alleles ranged from 2 to 10, the observed heterozygosity ranged from 0.182 to 0.897, and the expected heterozygosity ranged from 0.260 to 0.854. Eleven loci showed significant deviation from Hardy-Weinberg equilibrium. Eleven loci were cross-

amplified successfully in 5 *Anopheles* species. These microsatellite loci will be useful in studies investigating population structure and evolutionary genetics in *A. albitarsis sensu lato* and other *A. albitarsis* complex species.

Key words: *Anopheles albitarsis*; Species complex; SSR; Malaria vectors; Microsatellite-enriched library; Cross-amplification

INTRODUCTION

The complex *Anopheles* (*Nyssorhynchus*) *albitarsis* consists of 6 sibling species: *A. albitarsis sensu stricto* Lynch-Arribálzaga, *A. deaneorum* Rosa-Freitas, *A. albitarsis* F Brochero, *A. marajoara* Galvão and Damasceno, *A. oryzalimnetes* Wilkerson and Motoki, and *A. janconnae* Wilkerson and Sallum.

These species are not distinguishable morphologically, with the exception of *A. deaneorum* (Rosa-Freitas et al., 1998). Of these species, the following are considered as malaria vectors in Brazil: *A. deaneorum* in Rondônia (Klein et al., 1991), *A. marajoara* in São Paulo, and Pará and Amapá (Conn et al., 2002; McKeon et al., 2010) and *A. janconnae* in Roraima (Povoa, 2006). Biochemical and/or molecular differences, eating habits, and anthropophily reflect the heterogeneity and capacity of the *A. albitarsis s.l.* vector (Consoli and Lourenço-de-Oliveira, 1994).

In this study, 25 microsatellite markers for *A. albitarsis s.l.* were isolated, characterized, and cross-amplified in 5 species of the *Nyssorhynchus* subgenus: *A. darlingi*, *A. oswaldoi*, *A. triannulatus*, *A. nuneztovari*, and *A. braziliensis*.

MATERIAL AND METHODS

Microsatellite-enriched library construction and sequencing

A genomic library enriched for microsatellite regions of *A. albitarsis s.l.* was constructed following the method described by Billotte et al. (1999). Genomic DNA extraction was performed on a pool of 12 larvae in the 4th instar, collected in the Coari locality, Amazonas State, Brazil, according to the method described by Wilkerson et al. (1995).

The DNA samples were digested with the restriction enzyme *Rsa*I (Invitrogen, USA), and the digested fragments were ligated to adapters RSA21 and RSA25 (Integrated DNA Technologies, USA). DNA fragments were selected by the hybridization method with $(CT)_8$ and $(GT)_8$ repeat biotin-linked probes and recovered with streptavidin-linked particles (Streptavidin MagneSphere® Paramagnetic Particles, Promega, USA). Select fragments were linked into a pGEM-T easy vector (Promega) and transformed using *Escherichia coli* XL1-blue competent cells, which were subsequently inoculated onto plates with X-Gal/IPTG Luria-Bertani (LB) agar and ampicillin (100 mg/mL) for overnight growth at 37°C. Single white colonies were transferred to microplates with an HM/FM medium with ampicillin (100 μg/mL) for overnight growth. The plasmid DNA was extracted using the protocol reported by Sambrook et al. (1989). The 96 clones were sequenced in an ABI PRISM 3130 (Applied Biosystems), using the primers T7 and SP6, with the Big Dye terminator v3.1 kit (Applied Biosystems).

From the 96 colonies obtained, 88 clones were extracted and sequenced with an average enrichment of DNA microsatellites of 95.8%, including nucleotide sequences of 70 clones

(72.9%), which were edited with CHROMAS 2.23, CHROMAS PRO, and BIOEDIT 7.0.9.0 programs (Hall, 1999).

Primer design, PCR, and genotyping

Contigs of 35 non-redundant simple sequence repeats (SSRs) with an average size of 506 bp were used to design 34 primer pairs. These primers were designed according to WEBSAT (Martins et al., 2009) and PRIMER3 programs (Rozen and Skaletsky, 2000). The mean GC content was 52%, and the average size of the amplified fragment was 201 bp. An M13 tail was added at the 5'-end of each forward primer to allow for fluorescence marking, in accordance with the protocol suggested by Schuelke (2000).

Genomic DNA used for characterization of the microsatellite fragments was obtained from 36 larvae in the 4th instar, collected in the neighborhood of Puraquequara, Manaus, Amazonas, Brazil. DNA extraction was performed according to the protocol described by Wilkerson et al. (1995). The microsatellite fragments were amplified by PCR (Batista et al., 2010) in a 10-µL final volume containing the following components: 10 ng genomic DNA, 0.4 mM each forward primer, 0.4 mM fluorescence-labeled M13 (FAM, HEX, and NED), 0.8 µM of the reverse primer, 200 µM dNTP, 1.5 mM $MgCl_2$, 1X buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.4), and 0.5 U *Taq* DNA polymerase (Promega).

PCR was performed in 2 stages: the 1st consisted of denaturation (68°C, 2 min; 92°C, 30 s) followed by 30 cycles of 30 s at 92°C, 35 s at the annealing temperature of the specific primer (Table 1), and 35 s at 68°C. The 2nd step consisted of 20 cycles with the following time and temperature profile: 20 s at 92°C, 30 s at 53°C, 30 s at 72°C, and a final extension at 72°C for 15 min and at 68°C for 15 min.

The amplified PCR product was run on a 1.5% agarose gel stained with GelRed (Biotium, CA, USA). The PCR product was visualized on an ABI PRISM 3130 automated Genetic Analyzer (Applied Biosystems). The size of the alleles was estimated using standard genotyping ROX ET-550 (GE Healthcare, UK) and analyzed with the GENEMARKER v.1.97 program (SoftGenetics).

Statistical analysis

The test for Hardy-Weinberg equilibrium (HWE) was estimated with the GENEPOP V.4.0 program (Raymond and Rousset, 1995). The MSTOOLS program (Park, 2001) was used to calculate observed (H_O) and expected (H_E) heterozygosities. All tests were corrected for multiple comparisons by Bonferroni's correction (Rice, 1989). Descriptive statistics (F_{IS}) were inferred by the FSTAT v2.9.3.2 program (Goudet, 2001).

RESULTS AND DISCUSSION

Twenty-five polymorphic microsatellite loci were obtained and analyzed in 24-36 samples of *A. albitarsis s.l.* collected in Puraquequara, Manaus, whose annealing temperature varied from 56° to 65°C (Table 1).

We obtained a total of 127 alleles (average, 5.0), and the number of alleles per locus ranged from 4 to 10. The observed H_O ranged from 0.182 to 0.897 (Aa01-Aa27, respectively)

Table 1. Characterization of 25 polymorphic microsatellite loci in *Anopheles albitarsis sensu lato* from Puraquequara, Manaus, Amazonas State, Brazil.

SSR locus	Genbank accession No.	Repeat motif	Primer sequences (5'-3')	Ta (°C)	Size range (bp)	N	N_A	H_O	H_E	P-HWE	F_{IS} (*f*)
Aa01	JQ697071	$(CT)_{16}$	F^{NED}: CTGATGTCTGCATTTGACGG R: TGAGTGAGTGAGTGATGACGG	57	132-152	33	4	0.182	0.410	0.000*	0.560
Aa05	JQ697072	$(GT)_7$	F^{FAM}: TGTTGTTGCTCCTCCTTCTGT R: CAGAGAGAACGCATTACGGAG	56	184-194	33	5	0.727	0.658	0.453	-0.107
Aa06	JQ697073	$(CA)_5$	F^{HEX}: GCTACGCCCTGTTTTAACTCC R: GGTGTGTTCGTGTATGTGTGTG	62	216-244	31	7	0.667	0.785	0.003	0.160
Aa07	JQ697074	$(CA)_9$	F^{HEX}: GAAGGGATAGTCAGCAATCACG R: TCCAGTGAGAGGGAGAGAGGT	65	297-301	31	9[a]	0.290	0.854	0.000*	0.644
Aa08	JQ697075	$(AC)_8$	F^{HEX}: TCTATCGAGCAACGTCATGC R: CCTCGCAGCTCACACATTA	56	124-134	36	5[a]	0.472	0.674	0.017	0.302
Aa09	JQ697076	$(TG)_8$	F^{HEX}: AGATGATGAATGGGAGTGGG R: AAATACGACGCCAGGAAGGT	60	238-256	36	7	0.722	0.644	0.557	-0.124
Aa10	JQ697077	$(TG)_9$	F^{FAM}: CGAGAATAGCCACCGTATGA R: TAAGCACCACATCACAATCC	60	126-136	35	5	0.514	0.532	0.641	0.033
Aa11	JQ697078	$(GT)_{12}$	F^{HEX}: GTTCTCGTCTCGTCTCGTTTG R: GGTCTGTCTGCTATCTGCTGG	60	269-283	33	7[a]	0.394	0.777	0.000*	0.497
Aa14	JQ697079	$(GT)_5AT(GT)_3$	F^{HEX}: GCAATTCCATGATTTACCCC R: AGGAGAAGGAGGAGAAGCAGA	60	315-321	34	4[a]	0.471	0.673	0.028	0.304
Aa16	JQ697080	$(GA)_9$	F^{HEX}: AGAGTAGAGTATCGGGTCGGC R: CAGTCGAAGCGCGTACTAAGA	60	107-113	36	4	0.806	0.640	0.013	-0.262
Aa18	JQ697081	$(TCAG)_4CA(TTCAG)_3$	F^{HEX}: TGCAACCCCTTACGTCCTAC R: GCTAAAAGCACAATCCACCG	60	165-177	35	4	0.229	0.260	0.141	0.121
Aa19	JQ697081	$(AC)_9$	F^{FAM}: CCTCACTTTCAATTCGGT R: TCGACTTTACATTAACAAGC	60	109-115	31	3[a]	0.194	0.574	0.000*	0.666
Aa20	JQ697082	$(GT)_{7v}$	F^{NED}: CACATGAACACCGACACGTAG R: CTGGGGATTGTGACTGGATAG	60	271-273	35	2	0.629	0.497	0.171	-0.270
Aa21	JQ697083	$(TG)_6$	F^{FAM}: GTCGTGCCTCTCTTTTCTAGG R: CCGCTCACTCACTGTTTCTCT	60	151-157	34	4	0.765	0.717	0.000*	-0.068
Aa22	JQ697083	$(AACA)_5$	F^{FAM}: CTACCACGCTTCGTTCGACT R: GCCGACTGAAAATAGCTTCC	65	184-192	35	3	0.514	0.612	0.186	0.162
Aa23	JQ697084	$(TG)_5A(TG)_{13}(GA)_{15}$	F^{FAM}: CTTCCTCTTGTCTGTCATCGC R: TGCTCGTGTCTGTATTTGCC	60	242-272	36	10[a]	0.278	0.802	0.000*	0.657
Aa25	JQ697085	$(AC)_5$	F^{NED}: AGGGATGATACTGGGGTATGG R: GAAGGGTGCGTAGAGGATTG	65	142-152	31	4[a]	0.387	0.685	0.003	0.439
Aa26	JQ697086	$(CA)_8CT(CA)_5$	F^{FAM}: ACATTTCCTGTGGTCCTGTGG R: GAGAGCTTCAGCGTAATCGTC	60	313-323	33	7[a]	0.394	0.753	0.000*	0.481
Aa27	JQ697087	$(TG)_5TTTA(TG)_8$	F^{HEX}: CGGCATTCAAACGCATCT R: GGAATACTGTTGACGCTGACC	65	101-127	29	5	0.897	0.630	0.000*	-0.434
Aa28	JQ697088	$(CAT)_7$	F^{HEX}: CTGAACGAGATGCTGGAGCTA R: TCACAGATGGAGTGGTTGGA	60	198-218	36	8[a]	0.222	0.533	0.000*	0.587
Aa30	JQ697089	$(GT)_5$	F^{NED}: TGCACCAGAATTGGCACG R: GAACCCTCTTCCCTCTGATTG	62	219-241	31	4	0.871	0.617	0.000*	-0.421
Aa31	JQ697090	$(ATC)_4A(GAG)_3$	F^{HEX}: GGCTAGGAGCGTAGAGAGAGG R: CTACCAGCATCCACCGTTCTA	65	216-231	35	4[a]	0.400	0.632	0.000*	0.371
Aa32	JQ697090	$(CA)_7$	F^{FAM}: AGAGTCCTTTCTGCCGCTAAC R: CATTAACCCCTTCCTTCGGT	59	203-209	28	4	0.500	0.659	0.479	0.245
Aa33	JQ697091	$(AGCC)_7$	F^{FAM}: CACATAGTTACGAGGACGAG R: GACTCTTAGGACTTCCAGGG	62	245-257	24	4	0.417	0.402	1.000	-0.038
Aa34	JQ697091	$(CT)_7$	F^{NED}: GAGAGAAACAGTGAGTGAGCGG R: ACGCCACGCCATCATCAG	60	95-105	30	4[a]	0.333	0.586	0.006	0.435

Ta = annealing temperature (°C); N = number of individuals genotyped; N_A = number of alleles ([a]presence of null allele); H_O = observed heterozygosity; H_E = expected heterozygosity; P-HWE* = loci that were not in Hardy-Weinberg equilibrium after Bonferroni's correction; F_{IS} = inbreeding coefficient.

with an average of 0.017, while the H_E varied from 0.260 to 0.854 (Aa18 and Aa07, respectively) with an average of 0.623 (Table 1). Eleven loci (Aa01, Aa07, Aa11, Aa19, Aa21, Aa23, Aa26, Aa27, Aa28, Aa30, and Aa31) showed significant deviation from HWE after Bonferroni's correction ($P \leq 0.002$) and was performed according to the method described by Rice

(1989). This deviation may be due to one or more factors such as the use of insecticides in the region where the samples were collected and insufficient sample size or bottleneck effects or even the presence of null alleles, as suggested by the MICROCHECKER v2.2.3 program (Van Oosterhout et al., 2004) in loci detected in Aa07, Aa08, Aa11, Aa14, Aa19, Aa23, Aa25, Aa26, Aa28, Aa31, and Aa34.

The polymorphic markers were tested for cross-species amplification in 5 other species of *Anopheles* (Table 2). One locus (Aa09) was amplified in all 5 species, and 11 loci (Aa01, Aa09, Aa14, Aa16, Aa20, Aa26, Aa27, Aa28, Aa30, Aa32, and Aa34) were amplified in at least one species. The number of loci amplified per species varied from 2 in *A. triannulatus* and 9 in *A. nunneztovari*. These characterized 25 polymorphic microsatellite markers can be used as an efficient tool for population studies in the *A. albitarsis* complex and other *Anopheles* sp.

Table 2. Cross-species amplification of 25 microsatellite markers in five species of the genus *Anopheles* (N = 4 for each species).

Locus	*A. darlingi*		*A. braziliensis*		*A. triannulatus*		*A. oswaldoi*		*A. nuneztovari*	
	Size (bp)	N_A	Size (bp)	N_A	Size (bp)	N_A	Size (bp)	N_A	Size (bp)	N_A
Aa01	x		138-152	4	x		138-168	4	x	
Aa05	x		x		x		x		x	
Aa06	x		x		x		x		x	
Aa07	x		x		x		x		x	
Aa08	x		x		x		x		x	
Aa09	244-268	3	238-252	2	238-268	3	252-268	6	240-286	5
Aa10	x		x		x		x		x	
Aa11	x		x		x		x		x	
Aa14	x		x		x		337-343	2	347	1
Aa16	x		125-129	2	109-125	4	103-125	5	115-137	4
Aa18	x		x		x		x		x	
Aa19	x		x		x		x		x	
Aa20	323	1	x		x		253-281	3	257-277	2
Aa21	x		x		x		x		x	
Aa22	x		x		x		x		x	
Aa23	x		x		x		x		x	
Aa25	x		x		x		x		x	
Aa26	230-282	3	x		x		330	1	x	
Aa27	136-148	2	x		x		x		132-142	2
Aa28	x		x		x		x		214	1
Aa30	x		251	1	x		251	1	251	1
Aa31	x		x		x		x		x	
Aa32	x		x		x		x		206-224	4
Aa33	x		x		x		x		x	
Aa34	110	1	x		x		105-113	2	119-125	2

Annealing temperature shown in Table 1; N_A = number of alleles; x = not amplified.

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