GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Isolation and characterization of polymorphic DNA microsatellite loci for *Anopheles triannulatus sensu lato* (Diptera: Culicidae) and cross-amplification in congeneric species

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Genet. Mol. Res. 12 (3): 3088-3092 (2013)
Received May 21, 2012
Accepted November 2, 2012
Published March 13, 2013
DOI http://dx.doi.org/10.4238/2013.March.13.11

ABSTRACT. *Anopheles* (*Nyssorhynchus*) *triannulatus* is a complex of 3 species. Thirteen polymorphic microsatellite loci were isolated and characterized in 20 to 25 individuals from Manaus (AM, Brazil). The number of alleles per locus varied from 3 to 10 (mean = 6.0). The observed heterozygosity ranged from 0.250 to 0.875 (mean = 0.680) and expected heterozygosity ranged from 0.376 to 0.844 (mean = 0.698).

Two loci exhibited null alleles and all loci were in Hardy-Weinberg equilibrium. No linkage disequilibrium between loci was observed. These loci were used in 4 congeneric species and provide a useful tool for studying population genetics and other aspects of the biology of this and other *Anopheles* species.

Key words: *Anopheles triannulatus*; Microsatellite; Species complex; Malaria

INTRODUCTION

Anopheles (*Nyssorhynchus*) *triannulatus* belongs to a complex (Galvão and Lane, 1941) of 3 species: *A. triannulatus sensu strictu* (Neiva and Pinto, 1922), *Anopheles halophylus* (Silva do Nascimento and Lourenço-de-Oliveira, 2002), and another as yet morphologically unidentified *A. triannulatus* C. They are closely related species differentiated by the 4th-instar larvae and the ventral lobe of the clasper in the male genitalia (Silva do Nascimento and Lourenço-de-Oliveira, 2002). This species has a wide geographical distribution, occurring on the east of the Andes in Argentina, Brazil, Paraguay, Bolivia, and the Guyanas, Colombia, Venezuela, Ecuador, and Peru. It is also found in Central America, extending to Nicaragua, west of Peru, on the western side of the Andes (Faran, 1980). It is mainly a zoophilic and exophilic species commonly found around forests (Arruda et al., 1986). *A. triannulatus* is endophagic and anthrophophilic, making it a possible vector of human malaria at high densities (Lourenço-de-Oliveira, 1989; Tadei et al., 1993).

MATERIAL AND METHODS

A genomic library enriched with DNA microsatellites was constructed (Billote et al., 1999) from a pool of genomic DNA from 15 mosquitoes collected in Manaus, AM, Brazil. The DNA was digested with *Rsa*I (Invitrogen, USA) and ligated to adapters RSA21 and RSA25 (Integrated DNA Technologies, USA). DNA fragments were selected by hybridization with $(TC)_8$ and $(TG)_8$ biotin-linked probes and recovered with streptavidin-linked particles (Streptavidin MagneSphere® Paramagnetic Particles, Promega, USA). The selected fragments were inserted into the cloning vector pGEM-T (Promega), transformed into *Escherichia coli* electrocompetent cells from the TOPO TA cloning kit (Invitrogen), inoculated onto X-Gal/IPTG/LB agar plates, and incubated at 37°C. White colonies were transferred to a microplate for overnight growth in LB medium. The extracted plasmid DNA (Sambrook and Russell, 2001) from 96 clones with inserts was sequenced on an ABI 3130 (Applied Biosystems, USA), using the primers SP6 and T7 and Big Dye Terminator 3.1 (Applied Biosystems). The sequences were edited in CHROMAS 2.33 and BioEdit 7.0.9.0 (Hall, 1999). The primers were designed in WebSat (Martins et al., 2009) and an M13 tail was added to the 5'-end of each forward primer (Schuelke, 2000). Thirty-seven primer pairs for microsatellite fragments were amplified (Lima et al., 2010) in a 10-µL reaction volume containing 10-50 ng genomic DNA, forward M13 primer labeled with FAM or HEX (0.4 µM), 0.8 µM reverse primer, 200 µM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, and 0.5 U Taq DNA Polymerase (Fermentas, Canada). PCR was performed in 2 steps: denaturation (68°C, 1 min; 94°C, 30 s) followed by 30 cycles of

30 s at 93°C, 35 s at 60°C, 40 s at 68°C. The second step included 15 cycles of 25 s at 93°C, 35 s at 53°C, 30 s at 72°C, and a final extension at 72°C for 30 min. PCR products were visualized on MegaBACE 1000 (GE Healthcare, UK); allele sizes were scored against the size standard ROX ET-550 (GE Healthcare) and analyzed in FRAGMENT PROFILER v1.2 (GE Healthcare). The polymorphism information content (Botstein et al., 1980) and descriptive statistics were estimated by MSTOOLS v3 (Park, 2001). Hardy-Weinberg equilibrium (HWE) was evaluated with GENEPOP v4 (Raymond and Rousset, 1995). The presence of null alleles was suggested by MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

We obtained 72 non-redundant clones containing 83 microsatellites. Thirteen polymorphic microsatellite loci (Table 1) were characterized in 20-25 *A. triannulatus s.l.* individuals collected in Manaus, AM, Brazil. The number of alleles varied from 3 (Atr48) to 10 (Atr13) per locus, with an average of 6 alleles. Observed heterozygosity ranged from 0.250 (Atr48) to 0.875 (Atr34), with an average of 0.680, while expected heterozygosity ranged from 0.376 (Atr48) to 0.844 (Atr44), with an average of 0.698. The polymorphism information content value ranged from 0.326 (Atr48) to 0.789 (Atr44), with an average of 0.639. All microsatellite loci were in HWE after Bonferroni's correction (Rice, 1989). These data did not show significant linkage disequilibrium between loci in the study population. The value of F_{IS} ranged from -0.213 (Atr24) to 0.362 (Atr13), with an average of 0.125.

Table 1. Characterization of 13 polymorphic microsatellite loci for *Anopheles triannulatus sensu lato* from Manaus (AM, Brazil).

SSR locus	GenBank accession No.	Repeat motif	Primer sequences (5'- 3')	N	N_A	Ta (°C)	Size range (bp)	PIC	H_O	H_E	P-HWE	F_{IS}
Atr04	JQ920418	$(AC)_6$	FFAM: GGGAAGACAGTAATAGCCGGA R: GACCGACTTAACGACCCAAA	21	8	60	156-174	0.721	0.762	0.762	0.704	0.000
Atr13	JQ920407	$(CT)_{13}$	FHEX: TGGGGAACACACTAGCATCTC R: GTGGCGTTTGTGACCATACA	23	10a	63	207-229	0.768	0.522	0.811	0.015	0.362
Atr18	JQ920408	$(GT)_8$	FFAM: CTAGCCAGCAAACGAGCAC R: TCACTCGACGGACATACACAG	22	5	60	219-231	0.588	0.545	0.654	0.167	0.170
Atr19	JQ920409	$(GT)_6$	FHEX: ACTGCGTGGGTATGAGTGAGT R: TCCGTAAATCCAGCGGTATC	24	5	60	141-151	0.513	0.708	0.592	0.603	-0.201
Atr21	JQ920410	$(CTG)_6$	FHEX: CACTCAGAAGAAGGAGCAGCA R: GCAGCACGGAGAAGGAATTA	21	6	60	303-330	0.571	0.619	0.635	0.227	0.026
Atr23	JQ920411	$(TG)_7$	FHEX: TAATCCTCCCTTCCACAGTCC R: AACTACCACCATAGAACCAGGG	22	6	63	290-306	0.673	0.682	0.735	0.755	0.074
Atr24	JQ920412	$(TA)_6$	FFAM: AGACACTAAGCACCAGCAACC R: GCCCGTTTCTCTAATACGCA	20	5	65	247-257	0.654	0.867	0.720	0.303	-0.213
Atr30	JQ920413	$(CACG)_4$	FHEX: ACTCTGCGTCTTCTGCTTCTG R: ACTAACCACCCTTCTGCCATC	25	5	59	223-243	0.644	0.722	0.713	0.353	-0.014
Atr32	JQ920414	$(CA)_8$	FHEX: GGAGGAAGGAGTGGAAGAATG R: GCTCGTGGATGTGTGTATGTG	24	6	59	155-165	0.558	0.583	0.621	0.556	0.061
Atr34	JQ920415	$(CA)_9$	FFAM: ACGTTCCTTTCACAACTGGC R: GCGAACAAGACCAGATCACTC	22	5	60	171-179	0.744	0.875	0.804	0.708	-0.091
Atr39	JQ920419	$(GA)_{19}$ $GC(GA)_8$	FFAM: CTGTCGAGAAGAATACCGAGG R: ACCACCTCCATCCACC	20	8	64	316-334	0.759	0.842	0.807	0.522	-0.045
Atr44	JQ920416	$(CA)_{10}$	FFAM: CATCCTGCCACACGAAGGTAT R: CGGTAATAAAGGACACCGAACG	25	6	65	130-140	0.789	0.867	0.844	0.899	-0.028
Atr48	JQ920417	$(AG)_{18}$	FFAM: CGATTTGTGTCGCGTAGAGA R: GACTAGCGGTTGGCGTAAGTA	25	3a	65	167-171	0.326	0.250	0.376	0.023	0.340

N = number of individuals; N_A = number of alleles (a = presence of null alleles); Ta = annealing temperature; PIC = polymorphism information content; H_O = observed heterozygosity; H_E = expected heterozygosity; P-HWE = Hardy-Weinberg equilibrium; F_{IS} = coefficient of inbreeding.

Data concerning the isolation and characterization of microsatellite markers in *Anopheles* species of the *Nyssorhynchus* subgenus are rare. Only 3 species have been cited: *A. marajoara* has 11 characterized microsatellite loci (Li et al., 2005), *A. darlingi* has 8 (Conn et al., 2001) and 24 loci characterized (Lima et al., 2010), and 18 loci have been characterized for *A. nuneztovari* (Austin et al., 2011). The 13 loci developed for *A. triannulatus* brought the total to 74 SSR loci for this subgenus.

Cross-amplification in 4 species of *Anopheles*

We performed interspecific amplification of 13 SSR loci (Table 2) from 4 species of the same subgenus (*A. oswaldoi*, *A. rangeli*, *A. darlingi*, and *A. benarrochi*) sharing 6 loci. Of these, 4 showed polymorphisms (2-3 alleles): Atr04 in *A. rangeli*; Atr13 in *A. darlingi*, *A. rangeli*, and *A. benarrochi*; Atr24 in *A. darlingi* and *A. rangeli*; Atr39 in *A. benarrochi*. These microsatellite loci will be useful as genetic markers for population genetic studies in *Anopheles*.

Table 2. Cross-species amplification of 13 *Anopheles triannulatus sensu lato* microsatellite loci.

Loci	*A. darlingi**		*A. rangeli**		*A. oswaldoi**		*A. benarrochi**	
	Size range (bp)	N_A	Size range (bp)	N_A	Size range (bp)	N_A	Size range (bp)	N_A
Atr04	x		169-177	2	177	1	177	1
Atr13	210-230	3	205-230	2	230	1	205-230	3
Atr18	x		x		x		x	
Atr19	x		x		x		x	
Atr21	225	1	x		x		x	
Atr23	x		x		x		x	
Atr24	170-200	3	190-200	2	200	1	200	1
Atr29	x		x		x		x	
Atr30	x		x		x		x	
Atr32	x		x		x		x	
Atr34	x		145	1	x		x	
Atr37	x		x		x		x	
Atr39	300	1	x		x		280-300	2
Atr44	x		x		x		x	
Atr48	x		x		x		x	

*PCR annealing temperature in Table 1. N_A = estimated number of alleles; x = no amplification.

Cross-amplification of SSR loci in the genus *Anopheles* was performed from only 24 microsatellites of *A. darlingi* tested for *A. triannulatus*, *A. benarrochi*, and *A. rangeli* (Lima et al., 2010). At least 17 loci were amplified in 1 of the 3 species and 8 were detected in all species ranging from 2-5 alleles per locus. That study cross-amplified 13 polymorphic SSR loci for *A. triannulatus*; with the 13 loci developed in this study, 26 loci are available for this species.

ACKNOWLEDGMENTS

The authors are grateful for the support of the FAPEAM Agency, CTPetro-Network Malária. Research supported by FAPEAM, CTPetro-Network Malária/CNPq, and the PROCAD-Amazônia-INPA/UNICAMP/UFRGS/CAPES (#023/2006) Program. The authors thank the LTBM/INPA, where most of the experiments were performed, and the helpful technical staff of the Malaria and Dengue/INPA Laboratory, who kindly helped capture and identify the collection of mosquitoes for this study.

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