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New microsatellite markers for the neotropical malaria vector *Anopheles nuneztovari sensu lato*

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ABSTRACT. *Anopheles nuneztovari sensu lato* consists of cryptic species and genetic lineages, one of which is an important human malaria vector in the northern part of South America. Population structure and evolutionary genetics studies may help in the definition and delimitation of the species and lineages within this species complex, which is relevant information for organizations involved in malaria control efforts. In this study, 10 new microsatellite markers were isolated from 2 repeat-enriched genomic libraries of *A. nuneztovari s.l.* and were characterized in 37-48 mosquitoes of this species. All loci were highly polymorphic and encompassed 5-25 alleles per locus. The observed (H_O) and expected (H_E) heterozygosities ranged from 0.354 to 0.866 and from 0.613 to 0.932, respectively. Six of the 10 new loci showed significant deviations from the Hardy-Weinberg equilibrium, and no linkage disequilibrium was detected. The loci described in this study were more polymorphic than the 18 previously characterized loci and appear to be promising markers for use in investigating the fine-scale population genetic structure

and the boundaries of the cryptic species and lineages within the *A. nuneztovari* complex.

Key words: Malaria vector; Microsatellite markers; Genetic diversity; Population genetics; Speciation

INTRODUCTION

Anopheles nuneztovari sensu lato (order Diptera, family Culicidae) has a large geographic distribution in northern South America. Currently, *A. nuneztovari s.l.* comprises a species complex with 3 cryptic species: *Anopheles nuneztovari sensu stricto*, *Anopheles goeldii*, and *Anopheles dunhami* (Scarpassa et al., 1996, 1999; Foster et al., 2013). *A. nuneztovari sensu stricto*, which is geographically distributed in Colombia and Venezuela, is predominantly anthropophagic and has proven to be an efficient human malaria vector (Gabaldón, 1981). *A. goeldii*, which is geographically distributed in the Amazon Basin comprising the Brazilian Amazon and Suriname, has been found to be infected with 3 *Plasmodium* species in 5 states of the Brazilian Amazon region (de Arruda et al., 1986; Tadei et al., 1998; Póvoa et al., 2001; Galardo et al., 2007). Although the status of *A. goeldii* as a malaria vector has not yet been clarified in the Brazilian Amazon region because it is predominantly zoophagic, it is likely to be a local malaria vector or a secondary vector (which can contribute to malaria transmission, but cannot sustain it without the presence of a primary vector). *A. dunhami* is morphologically and genetically closely related to *A. nuneztovari s.s.* and *A. goeldii* (Lounibos et al., 1998; Trindade and Scarpassa, 2002) and has a large area of overlap with *A. goeldii* (Scarpassa and Conn, 2011). However, *A. dunhami* has not been found to be infected with the malaria parasite. Along with the presence of three cryptic species in this complex, previous studies have also shown the existence of genetic lineages within *A. nuneztovari s.l.* in the Brazilian Amazon region (Mirabello and Conn, 2008; Scarpassa and Conn, 2011), suggesting an intriguing evolutionary history for this species complex. Thus, studies based on highly polymorphic markers with a faster evolutionary rate, such as microsatellites, may provide a better understanding of the genetic structure of *A. nuneztovari s.l.* Furthermore, the definition and delimitation of these cryptic species and lineages could illuminate the manner in which *A. nuneztovari s.l.* contributes to the distinct patterns of malaria transmission that are found in South America. A previous study (Cardoza et al., 2011) isolated and characterized 18 microsatellite loci in *A. nuneztovari s.l.*, which have served as powerful markers that have allowed the investigation of the fine-scale population structure and evolutionary genetics of this species complex.

In the present study, we isolated 10 new microsatellite markers from 2 repeat-enriched genomic libraries of *A. nuneztovari s.l.* from Manaus (State of Amazonas, Brazil) and Nova Mazagão (State of Amapá, Brazil) and characterized these loci in 37-48 specimens from Manaus, Brazil.

MATERIAL AND METHODS

Two microsatellite-enriched libraries were constructed from 2 pools of *A. nuneztovari s.l.* (each containing 8 specimens) that were collected in either Manaus (03°06'S, 60°01'W; State of Amazonas, Brazil) and Nova Mazagão (00°07'S, 51°17'W; State of Amapá, Brazil). Genomic DNA was extracted from fourth instar larvae of *A. nuneztovari s.l.* using the phenol-

chloroform method (Sambrook and Russell, 2001). The genomic DNA samples (5 µg) extracted from each pool were digested separately with *Rsa*I, and the resulting DNA fragments were linked to *Rsa*21 and *Rsa*25 adapters. Pre-amplified ligated DNA was obtained by PCR using *Rsa*21 primers and purified with the QIAquick PCR Purification Kit (Qiagen, Germantown, MD, USA). DNA fragments containing putative markers were selected by hybridization with $(CT)_8$ and $(GT)_8$ repeats and biotin-linked probes and recovered with streptavidin-linked particles (Streptavidin MagneSphere Paramagnetic Particles; Promega, Madison, WI, USA). Selected fragments were linked to the pGEM-T easy vector (Promega), which was used to transform *Escherichia coli* XL1-blue competent cells, which were plated on X-Gal/IPTG Luria-Bertani (LB) agar and ampicillin (100 mg/mL) and allowed to grow overnight at 37°C. Single white colonies were transferred onto microplates with HM/FM medium containing ampicillin (100 mg/mL) and allowed to grow overnight. After the overnight growth period, 192 clones were selected and bi-directionally sequenced on an automated ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using T7 and SP6 primers, and the resulting sequences were assembled and edited using the SeqMan software (DNAStar Inc., Madison, WI, USA). Restriction sites were removed using the Microsat software (Risterucci et al., 2005) and microsatellite motifs were identified using the Simple Sequence Repeat Identification Tool (SSRIT), which is available online (Temnykh et al., 2001). Fifty-eight primer pairs were designed using the Primer Select software (DNAStar Inc.). For this purpose, we considered dinucleotides and trinucleotides with 5 or more repeats, as well as tetranucleotides and 4 or more repeats, and pentanucleotides with 3 or more repeats.

Of the 58 primer pairs designed, 28 successfully amplified their respective products. Of these successful primers, 18 loci were previously validated and characterized by Cardoza et al. (2011). The other 10 primer pairs were validated and characterized in the present study; 9 of these loci (*Anu19* to *Anu27*) were from the Manaus library and 1 (*Anu28*) was from the Nova Mazagão library (Table 1). The level of polymorphism was estimated using 37-48 specimens of *A. nuneztovari s.l.* sampled from Manaus, Brazil. The microsatellite fragments were amplified by polymerase chain reaction (PCR) in a final volume of 10 µL reaction solution containing 10-20 ng (1.0 µL) DNA template, 1.0 µL 10X buffer, 2.1 µL 1 mM dNTPs, 0.3 µL 50 mM $MgCl_2$, 0.4 µL 4 mM M13-tailed forward primer (Schuelke, 2000), 0.4 µL 4 mM M13-labeled primer (FAM, HEX, and TAMRA), 0.8 µL 4 mM reverse primer, 0.2 µL 5 U/µL Platinum Taq DNA Polymerase (Invitrogen Inc., Carlsbad, CA, USA), and 3.8 µL sterile water to complete the total volume. PCR was carried out in 2 steps: the first step consisted of denaturation (68°C for 2 min, 95°C for 30 s) followed by 30 cycles of 35 s at 92°C, 35 s at the primer-specific annealing temperature (Table 1), and 35 s at 72°C. The second step consisted of 15 cycles of 30 s at 92°C, 35 s at 53°C, 35 s at 72°C, which were followed by a final extension at 72°C for 30 min. PCR products were visualized on an automated ABI 3130 xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), and allele sizes were scored using GeneScan 500 ROX dye (Applied Biosystems) and analyzed with the GeneMapper software (version 4.0; Applied Biosystems).

After genotyping, the dataset was checked with the Micro-Checker program (version 2.2.3) (Van Oosterhout et al., 2004) to detect potential errors that might have occurred at each locus during the genotyping, such as null alleles, stuttering, and large allele dropouts. Next, the genetic diversity measures were calculated. The heterozygosities (observed and expected) and the Hardy-Weinberg equilibrium (HWE) were estimated using the MSTOOLS (version 3) (Park, 2001) and GENEPOP (Rousset, 2008) programs, respectively. The descriptive sta-

Table 1. Characterization of ten new microsatellites loci in 37-48 specimens of *Anopheles nuneztovari s.l.*

SSR locus	GenBank. accession No.	Repeat motif	Primer sequences (5'-3')	Ta (°C)	Size range (bp)	PIC	N	A	H_O	H_E	P-HWE	F_{IS} (f)
Anu19[1]	KJ676993	$(CA)_6$	F^{TAMRA}: TCAAGGGCACCACACCACAACT R: AATGAAGGCGCCAGAAGGAAGC	60	259-269	0.728	37	6	0.567	0.778	0.0232	0.274
Anu20[1]	KJ676994	$(GAC)_2GCA(GAC)_4$	F^{FAM}: CCGGACTGCAACATGATACGAG R: GAACGGAGCTGTGGACTGTGGT	60	225-249	0.656	45	5	0.400	0.713	**0.0000**	0.442
Anu21[1]	KJ676995	$(TG)_5$	F^{HEX}: CCCGGCGGCATCGTATTC R: CCTGCTGGTATCGGGGTATTGG	60	243-271	0.660	48	7	0.354	0.699	**0.0000**	0.496
Anu22[1]	KJ676996	$(CA)_7$	F^{TAMRA}: ATATACGGGTCAACAAATGGTG R: CAAACGATGCAAAAACAGTCA	52	251-275	0.833	48	12	0.625	0.859	0.0054	0.275
Anu23[1]	KJ676997	$(AC)_{14}$	F^{FAM}: AGAATAAAAGGGAGAGCGAGA R: CTACTACACCCAACGAAGGA	65	167-221	0.885	47	21	0.596	0.904	**0.0011**	0.343
Anu24[1]	KJ676998	$(CA)_8$	F^{TAMRA}: TCCCCCATCACCGACCATC R: ATTTCCCGCCCGCCAGTG	60	256-312	0.906	45	21	0.866	0.923	**0.0000**	0.062
Anu25[1]	KJ676999	$(GCA)_6$	F^{HEX}: GAAAAACTCATCCCATTGTC R: TTTTTCATTGCTTTCCTGTA	52	215-241	0.542	48	6	0.520	0.613	0.4467	0.152
Anu26[1]	KJ677000	$(CA)_{20}$	F^{TAMRA}: GGGGGATAGGGAGAAGTGAGGT R: GAACATTCCAGCAGCGGGTAAG	58	274-314	0.871	47	12	0.489	0.893	**0.0000**	0.454
Anu27[1]	KJ677001	$(TC)_{23}TT(TC)_{11}$	F^{FAM}: ATTTAACCGTATCCCTTTTT R: AACCGAACCATACCTGAGA	60	192-252	0.916	45	25	0.511	0.932	**0.0000**	0.454
Anu28[2]	KJ677002	$(GT)_{16}$	F^{HEX}: AGCCAGAATATGAGTAAAGT R: ATGTTGCTGGAATAAATC	48	123-141	0.797	48	9	0.750	0.829	0.0512	0.096

[1]Loci isolated from the Manaus library. [2]Locus isolated from the Nova Mazagão library. Ta = annealing temperature (°C); PIC = polymorphism information content; N = number of individuals screened; A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; P-HWE = values of P for the HWE test; P < 0.005 = significance threshold after Bonferroni correction. The significant values are in bold. F_{IS} = inbreeding coefficient.

tistics, number of alleles per locus, linkage disequilibrium (LD), inbreeding coefficient (F_{IS}), and allele size range were inferred using the FSTAT program (version 2.9.3) (Goudet, 2002).

RESULTS AND DISCUSSION

Of the 10 microsatellite loci characterized in this study, 2 were trinucleotides and 8 were dinucleotides (Table 1), and all were highly polymorphic. The number of alleles per locus ranged from 5 (locus *Anu20*) to 25 (locus *Anu27*) (Table 1), with a mean of 12.4. The observed heterozygosity (H_O) ranged from 0.354 (locus *Anu21*) to 0.866 (locus *Anu24*), whereas the expected heterozygosity (H_E) ranged from 0.613 (locus *Anu25*) to 0.932 (locus *Anu27*).

After Bonferroni correction, 6 of the 10 loci showed significant deviation from the HWE, suggesting a heterozygote deficit; consequently, the F_{IS} values were positive, ranging from 0.062 (locus *Anu24*) to 0.496 (locus *Anu21*), which indicated inbreeding. No significant LD was detected among the loci after Bonferroni correction. The significant deviation from the HWE suggests the presence of null alleles in most of the loci and may reflect accumulation of different mutations in primer-flanking regions, which would prevent PCR amplification of one or more alleles and result in significant heterozygote deficits. In the case of locus *Anu24* with its 21 alleles, deviation from HWE ($P < 0.0001$) may have occurred due to the very low expected values for genotypic classes, which would have increased the chi-square values, thus causing disequilibrium. This hypothesis is supported by the low F_{IS} values (0.062) for locus *Anu24*. However, for the other loci in disequilibrium we cannot completely rule out the possibility that such deviations may be a result of population subdivision (the Wahlund effect) in the sample from Manaus. This hypothesis arose from analyses of the *COI* gene (Scarpassa and Conn, 2011), which revealed 2 sympatric well-supported clades in samples from Manaus, suggesting 2 sympatric genetic lineages or species.

The novel *A. nuneztovari s.l.* microsatellite loci set described in this study is a marker set that will be highly useful in investigations of the population structure and evolutionary genetics of this neotropical malaria vector, and thus, will support the production of information that is of vital importance for malaria control efforts in areas where *A. nuneztovari s.l.* occur.

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REFERENCES

Cardoza TB, Sousa ACB, Lima MP, Souza AP, et al. (2011). Microsatellite markers for the neotropical malaria vector *Anopheles nuneztovari sensu lato*: isolation, development and characterization of eighteen loci. *Mol. Ecol. Resour.* 11: 1-3. Available at [http://tomato.bio.trinity.edu/manuscripts/11-4/mer-11-0032.pdf].

de Arruda M, Carvalho MB, Nussenzweig RS, Maracic M, et al. (1986). Potential vectors of malaria and their different susceptibility to *Plasmodium falciparum* and *Plasmodium vivax* in northern Brazil identified by immunoassay. *Am. J. Trop Med. Hyg.* 35: 873-881.

Foster PG, Bergo ES, Bourke BP, Oliveira TM, et al. (2013). Phylogenetic analysis and DNA-based species confirmation

in *Anopheles* (*Nyssorhynchus*). *PLoS One* 8: e54063.
Gabaldón A (1981). *Anopheles nuneztovari*: important vector y agente de malaria refractaria en Venezuela. *Bol. Dir. Malariol. Saneam. Amb.* XXI: 28-38.
Galardo AK, Arruda M, D'Almeida Couto AA, Wirtz R, et al. (2007). Malaria vector incrimination in three rural riverine villages in the Brazilian Amazon. *Am. J. Trop. Med. Hyg.* 76: 461-469.
Goudet J (2002). FSTAT Version 2.9.3: A Program to Estimate and Test Gene Diversities and Fixation Indices. Available at [http://www2.unil.ch/popgen/softwares/fstat.htm]. Accessed November 1, 2013.
Lounibos LP, Wilkerson RC, Conn JE, Hribar LJ, et al. (1998). Morphological, molecular, and chromosomal discrimination of cryptic *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae) from South America. *J. Med. Entomol.* 35: 830-838.
Mirabello L and Conn JE (2008). Population analysis using the nuclear white gene detects Pliocene/Pleistocene lineage divergence within *Anopheles nuneztovari* in South America. *Med. Vet. Entomol.* 22: 109-119.
Park SDE (2001). Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection. Ph.D. thesis, University of Dublin, Dublin.
Póvoa MM, Wirtz RA, Lacerda RNL, Miles MA, et al. (2001). Malaria vectors in the municipality of Serra do Navio, State of Amapá, Amazon Region, Brazil. *Mem. Inst. Oswaldo Cruz* 96: 179-184.
Risterucci AM, Duval MF, Rohde W and Billote N (2005). Isolation and characterization of microsatellite loci from *Psidium guajava* L. *Mol. Ecol. Notes* 5: 745-748.
Rousset F (2008). Genepop'007: a complete re-implementation of the Genepop software for Windows and Linux. *Mol. Ecol. Resour.* 8: 103-106.
Sambrook J and Russell DW (2001). A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
Scarpassa VM and Conn JE (2011). Mitochondrial DNA detects a complex evolutionary history with Pleistocene Epoch divergence for the neotropical malaria vector *Anopheles nuneztovari sensu lato*. *Am. J. Trop. Med. Hyg.* 85: 857-867.
Scarpassa VM, Tadei WP and Suarez MF (1996). Allozyme differentiation among allopatric populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Braz. J. Genet.* 19: 265-269.
Scarpassa VM, Tadei WP and Suarez MF (1999). Population structure and genetic divergence in *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. *Am. J. Trop. Med. Hyg.* 60: 1010-1018.
Schuelke M (2000). An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* 18: 233-234.
Tadei WP, Thatcher BD, Santos JM, Scarpassa VM, et al. (1998). Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. *Am. J. Trop. Med. Hyg.* 59: 325-335.
Temnykh S, DeClerck G, Lukashova A, Lipovich L, et al. (2001). Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. *Genome Res.* 11: 1441-1452.
Trindade DB and Scarpassa VM (2002). Genetic differentiation and diagnostic loci among *Anopheles* (*Nyssorhynchus*) *rangeli*, *An*. (*Nys*.) *nuneztovari*, and *An*. (*Nys*.) *dunhami* (Diptera: Culicidae) in the Brazilian Amazon. *J. Med. Entomol.* 39: 613-620.
Van Oosterhout C, Hutchinson WF, Wills DPM and Shipley P (2004). MICRO-CHECKER: software for identifying and correction genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4: 535-538.