Identification and validation of the first EST-SSR markers based on transcriptome of Anopheles darlingi, the primary transmitter of malaria in Brazil

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Abstract

Background *Anopheles darlingi* is a monotypic species in terms of its morphological, genetic, and behavioral aspects and is the primary transmitter of human malaria (99%) in Brazil, especially in the Brazilian Amazon. In this pioneering study, 15 expressed sequence tag (EST)-simple sequence repeat (SSR) markers were obtained and characterized in samples from the municipality of São Gabriel da Cachoeira, Amazonas state, Brazil, with polymorphisms that can be used for further genetic research.

Methods and Results The specimens (from egg to larval stage) collected were bred in the insectary at INPA (National Institute for Amazonian Research). The SSR repeats within the contigs of the *A. darlingi* EST banks were confirmed on the Vector Base site. DNA was extracted and amplified using polymerase chain reaction and then genotyped. Fifteen polymorphic SSR loci were identified and characterized. The number of alleles totaled 76, and ranged from 2 to 9. The observed heterozygosity varied between 0.026 and 0.769, the expected heterozygosity between 0.025 and 0.776, and the mean polymorphism information content was 0.468. Eight loci showed Hardy-Weinberg equilibrium (HWE) after Bonferroni correction (P: (5%) $\leq$ 0.0005). No linkage disequilibrium was found among the loci.

Conclusions The polymorphic SSRs of the loci have been shown to be efficient for investigation of the variability and genetic population structure of *A. darlingi.*

Introduction

Anopheles (*Nyssorhynchus*) *darlingi* Root, 1926 is the most endophagic and anthropophilic malaria vector in the Neotropical region [1, 2], and its contact with humans is due to its anthropogenic actions, which increases the risk of disease transmission [2]. There were 140,974 cases of malaria registered in Brazil in 2020 and, worldwide, in the same year, 241 million cases of this disease, with 140,974 deaths [3].

Given these indices, research on genetic structures has been carried out in several vector species [4, 5] using previously isolated and characterized SSR microsatellite markers. A total of 11 loci have been isolated and characterized in 323 *Anopheles marajoara* from northern Brazil [6]; Twenty five loci in 24 to 36 *Anopheles albitarsis sensu lato* from Puraquequara, Manaus, Amazonas state [7]; Twelve loci in 20 to 25 *Anopheles triannulatus s. l.* from Manaus [8]; Ten loci in 37 to 48 *Anopheles nuneztovari s. l* from Manaus and Nova Mazagão, Amapá state [9]; Eight loci in 253 *Anopheles darlingi* from the states of Amapá, Pará and Mato Grosso [10]; Seven loci in 377 *A. darlingi* from the states of Acre, Rondônia and Amazonas [11]; Twenty four loci in 21 to 32 *A. darlingi* from Coari, Amazonas state [12]; ten loci in 440 *A. darlingi* from Rondônia (found along the Madeira River) [1].

Isolations and characterizations from *A. darlingi* Express Sequence Tags (EST) banks are also being developed (Rafael, personal communication; [13]), and are single-pass sequences produced from cDNA present in expressed and more conserved regions of the genome.

In order to provide the first DNA microsatellite markers of EST regions for the study of variability, and the evolution and population structure of *A. darlingi*, the present study identified and characterized markers for this species using the contigs of this mosquito, which were grouped in clusters.

Material And Methods

The ESTs of *A. darlingi*, containing 568 UniGenes, were obtained from a library of adults and larvae of *A. darlingi* grouped in clusters (Rafael, personal communication; [13]). By means of the BLASTn tool, this EST bank was used to identify, quantify and locate the repeats motifs of the SSRs that flank exon regions from dinucleotide to hexanucleotide repeat motifs within the contigs. Among the repeat motifs, scores were established for the homology or protein similarity of the EST contigs that contain SSRs.

Pairs of primers, which flanked the repeat motifs of each contig sequence, were designed using the WEBSAT program [14]. Stringent criteria were adopted, which were the conditions that interfere with the association of nucleic acid strands, primer size (18 to 24 bp); annealing temperature (50 ºC to 65 ºC) with 3 ºC difference (forward and reverse primers).

Adult *A. darlingi* females were captured on the BR 307 highway, at highway marker 20, in São Gabriel da Cachoeira (SGC), Amazonas state, Brazil (00º 07' 48" S, 67º 05' 20" W), which is an area without insecticide pressure. The collection of *A. darlingi* was carried out under approval from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (license number 32941) and the Sistema de Autorização e Informação em Biodiversidade (SISBIO), Brazil (permanent license number 32941). Samples were packed in plastic

cups with thin mesh and transported to the insectarium at INPA for taxonomic identification [15]. After oviposition, the eggs were kept in plastic cups until hatching. The larvae were fed until the 4th larval stage, then individually packed in microtubes in a freezer at -80°C.

The genomic DNA of *A. darlingi* from SGC was extracted [16] from each larva of the 4th stage larvae (N = 40), digested in lysis buffer {Tris-HCl [50 mM]; EDTA [25 mM]; SDS [0.05%]; NaCl [25 mM] and sterilized Milli-Q water} at pH 8.0, then incubated (65 ºC/1 h with RNAse 10 mg/mL) and centrifuged (14,000 rpm/30 s with potassium acetate 3M, pH 7.2/10 min). The DNA pellet was eluted in TE 10X (Tris 10 mM, EDTA 1 mM) and preserved at -20 ºC. Electrophoresis (0.8% agarose) was performed and visualization was via a photo-documentation system (Alpha Imager MINI).

The microsatellite DNA reaction was prepared for amplification in a mixture of 1 µL of 10X buffer (200 mM Tris-HCl, pH 8.4 and 500 mM KCl, without $MgCl_2$); 2 µL dNTP at 1 mM; 0.2 µL Taq DNA polymerase [5 u/µL] (Promega); 0.3 µL of magnesium chloride ($MgCl_2$) at 50 mM; 0.4 µL of primer M13F (FAM, HEX or NED); 0.4 µL forward primer at 4 µM; 0.8 µL reverse primer at 4 µM; 1 µL genomic DNA at 10 ng/µL and ultrapure water (Milli-Q).

The PCR was performed in the following 4 steps: denaturation (68 ºC/2 min; 92 ºC/30 s); 30 cycles (92 ºC/30 s, each annealing temperature of each SSR loci was 68 ºC/35 s); addition of fluorescence M13F tail (FAM, HEX or NED) with 20 cycles (92 ºC/20 s, 53 ºC/30 s and 72 ºC/30 s); final extension (72 ºC/15 min and 68 ºC/15 min). DNA amplification was verified in agarose gel 1.5%, electrophoresis at 100 v, 170 mA, 140 W, 90 min, stained with GelRed (Biotium) and was visualized using an UV transilluminator.

The genotyping of the PCR products [1 µL PCR product + M13f Label primer (FAM, HEX, NED) + 7 µL (Tween 20 to 0.1%) + 1 µL ET-550 ROX (GE Healthcare) (1:10)] was performed in five multiplex systems, developed and used to optimize this step (Table 2). These were transferred to an automatic DNA Analyzer (ABI PRISM 3130xL, ThermoFisher) for sequencing and were visualized using GENEMARKER software (v2.6.4) (Soft Genetics, LLC).

The polymorphism information content (PIC) was estimated using MSTOOLS v.3 [17] and the Hardy-Weinberg equilibrium (HWE), and expected (H_E) and observed (H_O) heterozygosity were analyzed in GenAlEx v.6.5 [18]. The MICROCHECKER 2.2.3 [19] analysis showed the null alleles, dropouts, and stutters. The FSTAT v2. 9.3.2 [20] program provided descriptive statistics, such as the inbreeding index (F_{IS}) and linkage disequilibrium (D_L).

Results And Discussion

A total of 59 contigs with *A. darlingi* transcriptome SSRs were identified (34 dinucleotides, 23 trinucleotides, 1 tetranucleotide and 1 hexanucleotide). Thirty-seven pairs of *primers* were designed. The type of repetition of most of the SSRs was perfect simple and the most frequent repeat motif was dinucleotide (AC/AG).

The characterization of 40 *A. darlingi* in the 37 pairs of primers generated the amplification of 28 SSR loci of which 15 were polymorphic and amplified at 56 ºC to 65 ºC with size ranged from 115 to 368 bp (Table 1).

In all the 15 polymorphic SSR loci the dinucleotide repeat motif (AC/AG) was the most frequent. This motif was also found in *A. darlingi* from Coari, Amazonas state (Brazil) [12], in *A. gambiae* from Africa [21], in *A. culicifacies* from India [22], and in *A. triannulatus* from Manaus, Amazonas state, Brazil) [8]. However, in *A. darlingi* from Capanema, Pará state (Brazil) [10], the repeat motif that stood out was the (TC/GA). This indicates that dinucleotide is the most frequent type of SSR, and this is irrespective of the species.

The 15 SSR loci had a total of 76 alleles, ranging from 2 to 9, with a mean of 5.0 alleles per locus; variation of observed heterozygosity was 0.026–0.769 with a mean H_O of 0.402 and an expected H_E of 0.025–0.776, with a mean H_E of 0.509 (Table 2).

Considering the genetic variability, the allelic frequency found in *A. marajoara* (11 to 52) [11], in *A. albitarsis* (2 to 10) [7], in *A. triannulatus* (3 to 10) [8], in *A. stephensi* (3 to 16) [4], in *A. darlingi* (7 to 44) [10], (4 to 11) [12] and from 2 to 9 (Table 2) in this study, indicated that SSRs are efficient for the investigation of the genetic structure of populations due to their high polymorphism.

The variations of observed (H_O = 0.026–0.769) and expected heterozygosity (H_E = 0.025–0.776) found in this study were similar (Table 2) when compared to *A. culicifacies* (H_O = 0.045–0.607 and H_E = 0.170–0.841) [22], *A. gambiae* (H_O = 0.216–0.894) [21], and *A. nuneztovari* (H_O = 0.354–0.866 and H_E = 0.613–0.932) [9]. Comparing the heterozygosity of *A. darlingi* in this study with those of other

populations of the same species, such as those from Capanema, in the Pará state (H_O = 0.368–0.769 and H_E = 0.785–0.987) [10] and Coari, Amazonas state (H_O = 0.037–0.833 and H_E = 0.177–0.871) [12], higher values are noted.

Based on the values of the PIC scale [23], eight highly informative loci (Adar4. Adar11, Adar13, Adar16, Adar24, Adar30, Adar34, Adar36) and five moderately informative loci (Adar1, Adar3, Adar6, Adar19, Adar32) were observed. Thus, new loci with this performance are made available for future studies of the species.

Eight loci presented significant HWE after Bonferroni correction (P: (5%) ≤ 0.0005) [24]. The HWE deviation present in the other loci may have been caused by the presence of null alleles, the bottleneck effect, or due to mating between related individuals. No linkage disequilibrium was found among the loci (Table 2).

HWE was found in all SSR loci of *A. moucheti* with no null alleles in 3 loci of *A. maculipennis*, which presented some null alleles due to the deficiency in heterozygotes [13], and in 2 loci of *A. triannulatus*, with the presence of null alleles due to excess homozygotes [8]. On the other hand, HWE was found in 11 loci of *A. albitarsis* [7], in 6 loci of *A. nuneztovari* [9], in 10 loci of *A. darlingi* [12] and in 7 short tandem repeat (STR) loci of *A. darlingi* [5], which is similar to what was found in this study. This is due to the presence of null alleles and/or a bottleneck effect.

In addition, ESTs such as cuticular protein 23 RR-1 family, troponin C, ribosomal genes, and myosin light chain 2 from a cDNA library of *A. darlingi* (Rafael, personal communication; [13]), were submitted to the basic local alignment search tool (BLAST) against *A. gambiae*, the primary malaria vector in Africa, via an in silico hybridization method. These gene sequences showed regions of similarity in the 2L, 2R, 3L and X chromosome arms between both species, as well as with ESTs from other insects such as *Aedes aegypti*, *Drosophila melanogaster* and *Culex* spp (L. Bridi, personal communication). Furthermore, troponin (L. Bridi, personal communication) and actin [25] were hybridized as probes via a fluorescent in situ hybridization method in the polytene chromosomes of *A. darlingi*, which showed markers located in the arm 2R, section 14B, and in the arm 2L, section 23, respectively. These results provide a suitable basis for establishing chromosomal and evolutionary genomics homologies among major malaria vectors.

The present work provides 15 new microsatellite markers for *A. darlingi*, totaling 64 SSRs, when considering the studies that developed and characterized SSR loci, which are the first EST-SSR markers.

Considering the great capacity of *A. darlingi* for the transmission of malaria and its resistance to synthetic insecticide, the EST-SSR markers characterized are potential tools in future populational studies of this mosquito and actions for its control, especially in the Amazon.

Conclusion

The 15 microsatellite loci of the EST regions characterized in *A. darlingi* from São Gabriel da Cachoeira, identified in contigs with SSRs of the transcriptome of this mosquito, were polymorphic and can have potential for the population study of this species.

Declarations

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Conflicts of interest: The authors declare that they have no conflicts of interest. This manuscript contains original data and has not been published and is not being considered for publishing in any other journal.

Author contributions: MSR and JSB conceived the study; MSR collected the specimens; ATS and GMGM conducted the experiments and analyzed the data; ATS, JSB, GMGM and MSR wrote the manuscript. All authors read and approved the final version.

Ethics approval: The collection and transport of A. darlingi was carried out under approval from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (license number 32941) and the genetic access from the Sistema de Autorização e Informação em Biodiversidade (SISBIO), Brazil (permanent license number 32941).

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Tables

Table 1. Putative functions and characterization of 15 polymorphic EST-SSR loci from *Anopheles darlingi* from São Gabriel da Cachoeira, Amazonas state, Brazil.

VectorBase Accession No.	GenBank Accession No.	Locus	Primer sequence (5'-3')	Repeat motif	Dye	Ta (°C)	Size range (bp)	Putative function, accession code
ADAC008921	XP_049546846.1	Adar1	F: [M13] CTGAAGTCCATCGTTACCAGC	$(TC)_8$	FAM	60	175 - 181	ATP synthase subunit alpha [*Anopheles darlingi*] ADAC008921-RA
			R: CAATTCTCAGCCTCTCTCACG					
ADAC003454	XP_049548517.1	Adar3	F: [M13] CGCTTCGTCGTCTTCTTCTAC	$(CTA)_6$	FAM	58	222 - 228	unspecified product
			R: CACCTGTGTTCAGTGCTCCTT					
ADAC006317	XP_049547296.1	Adar4	F: [M13] CGACGAATGATGTGGATGTC	$(AC)_6$	FAM	60	152 - 176	Troponin C [*Anopheles darlingi*] ADAC006317-RA
			R: TGGCTCTAACAAAGATCGGC					
ADAC008921	XP_049547296.1	Adar6	F: [M13] CTGAAGTCCATCGTTACCAGC	$(TC)_8$	FAM	60	173 - 181	ATP synthase subunit alpha [*Anopheles darlingi*] ADAC006317-RA
			R: CAATTCTCAGCCTCTCTCACG					
ADAC001844	XM_049691057.1	Adar11	F: [M13] TAAGGAAGACTCAGGGGCAAC	$(CAG)_7$	FAM	58	115 - 150	Stoned-A [*Anopheles darlingi*] ADAC001844-RA
			R: CTTCTCATCCACGTCAGTGCT					
ADAC007453	XP_049536131.1	Adar13	F: [M13] ATCGGTCGAGTCCGGTTC	$(ACG)_6$	HEX	56	115 - 181	unspecified product
			R: CGGTAGCCAGAAGCAGATGTA					
ADAC007453	XP_049536131.1	Adar16	F: [M13] AGTGGTAGCCAAGGAGAAGGA	$(TCA)_4$	HEX	60	258 - 267	unspecified product
			R: TATCGTAAATGGGCTGAGGG					
ADAC001738	XP_049548491.1	Adar19	F: [M13] TCGGAGACGATCTGGATACTG	$(CAG)_6$	HEX	61	359 - 368	Cuticular protein 23 RR-1 family [*Anopheles darlingi*] ADAC001738
			R: CGCTACGTGGACATTAGAGGA					
ADAC007403	XP_049537137.1	Adar21	F: [M13] GACAGTACCTGATGCCGAACA	$(GAG)_5$	HEX	64	337 - 340	Ribosomal protein L32 [*Anopheles darlingi*] ADAC007403-RA
			R: CTTGACACCAATCGCTGATG					
ADAC000938	XP_049546089.1	Adar24	F: [M13] GAGCACCGACTGAAACTGTGA	$(CTA)_5$	HEX	65	320 - 354	Ubiquitin-related modifier 1 [*Anopheles darlingi*] ADAC000938-RA
			R: CGAGCGTCTGATAGATGGAAG					
ADAC008019	XP_049533270.1	Adar30	F: [M13] CCCTACCATTGCTGGACG	$(CAC)_5$	NED	60	186 - 210	unspecified product
			R: AGTCGATACGGAAGGAAGCAC					

ADAC003721	XP_049546781.1	Adar31	F: [M13] ACTGCTCGGTGGTAAAGTGC R: GACGGTTGTACTGGATGCGT	$(AAG)_5$	NED	60	143 - 148	40S ribosomal protein S30 [*Anopheles darlingi*] ADAC003721-RA
ADAC008233	XP_049532280.1	Adar32	F: [M13] TTTGCTCCTTCTCCTACCTCC R: GTTCGTGATGATACCGTGCTC	$(AC)_6$	NED	60	334 - 338	Actin [*Anopheles darlingi*] ADAC008233-RA
ADAC008233	XP_049532280.1	Adar34	F: [M13] GTGGATCTCGAAGCACGAGTA R: GACAGACACCACCACAAGAGC	$(AAC)_4$	NED	60	271 - 288	Actin [*Anopheles darlingi*] ADAC008233-RA
ADAC000311	XP_049531897.1	Adar36	F: [M13] ACACCTCTTGTGGAAGTGGG R: CATCTCCGCACCTCTTTAGG	$(AAC)_3$ tga $(AAC)_7$ ac $(AAC)_2$ g $(AAC)_5$	NED	60	185 - 200	Myosin light chain 2 [*Anopheles darlingi*] ADAC000311-RA

Ta: annealing temperature (°C).

Table 2. Parameters of genetic variability of each of the 15 expressed sequence tag-derived simple sequence repeats (EST-SSR) from *Anopheles darlingi*.

Multiplex Systems	Locus	H_O	H_E	N	Na	PIC	P-HWE	FIS (*f*)
1	Adar1	0.410	0.368	39	4	0.343	0.793	-0.102
2	Adar3	0.410	0.443	39	3	0.356	0.303	0.088
3	Adar4	0.667	0.776	39	9	0.750	0.040	0.154
4	Adar6	0.410	0.422	39	5	0.395	0.099	0.042
5	Adar11	0.769	0.688	39	7	0.654	0.929	-0.105
2	Adar13	0.564	0.700	39	7[a]	0.667	0.069	0.206
1	Adar16	0.550	0.618	40	4	0.542	0.230	0.123
3	Adar19	0.325	0.417	40	4	0.357	0.008	0.233
4	Adar21	0.026	0.025	39	2	0.025	0.000*	0.000
5	Adar24	0.385	0.713	39	8[a]	0.668	0.000*	0.471
2	Adar30	0.658	0.641	38	7	0.593	0.000*	-0.014
4	Adar31	0.026	0.026	38	2	0.026	0.000*	0.000
1	Adar32	0.162	0.444	37	3[a]	0.400	0.000*	0.643
3	Adar34	0.289	0.594	38	5[a]	0.517	0.000*	0.522
5	Adar36	0.385	0.763	39	6[a]	0.726	0.000*	0.505

Observed (H_O) and expected (H_E) heterozygosity, sample size (N), number of alleles (Na), Polymorphism Information Content (PIC); P-HWE* loci that did not show Hardy-Weinberg equilibrium after Bonferroni correction; FIS(f) = inbreeding index; *P: (5%) ≤ 0.0005 after Bonferroni correction.