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Genetic variability among *Anopheles* species belonging to the *Nyssorhynchus* and *Anopheles* subgenera in the Amazon region

Variabilidade genética entre espécies de *Anopheles* dos subgêneros *Nyssorhynchus* e *Anopheles* da região Amazônica

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ABSTRACT

Introduction: Isoenzymatic analyses were performed involving species of the *Nyssorhynchus* and *Anopheles* subgenera in order to estimate the intra and interspecies genetic variability. **Methods**: Mosquitoes were caught at different localities in the Amazon region. The collection and rearing of mosquitoes in the laboratory followed specific protocols. For the genetic variability analyses, the technique of horizontal electrophoresis on starch and starch-agarose gel with appropriate buffer systems was used. The alloenzyme variation was estimated using the Biosys-1 software. **Results:** Out of the 13 loci, eight were polymorphic. *Anopheles nuneztovari* presented the largest number of alleles per locus, while the smallest number was detected in *Anopheles marajoara* from Macapá. The largest number of polymorphic loci was found for *Anopheles marajoara* from Maruanum and the smallest for *Anopheles benarrochi* (Guayará Mirim). *Anopheles darlingi* (Macapá) presented the greatest heterozygosity (Ho = 0.167 ± 0.071), while the lowest heterozygosity (Ho = 0.045 ± 0.019) was observed in *Anopheles intermedius* (Pacoval) of the subgenus *Anopheles*. Wright's F coefficient revealed considerable genetic structuring between the populations of *Anopheles darlingi* (Fst = 0.110) and between the populations of *Anopheles marajoara* (Fst = 0.082). **Conclusions:** Considering all the species studied, the genetic distance ranged from 0.008 to 1.114. The greatest distance was between *Anopheles mattogrossensis* and *Anopheles oswaldoi*, while the smallest was between the *Anopheles benarrochi* populations.

Key-words: *Nyssorhynchus*. *Anopheles*. Genetic variability. Isoenzymes. Amazon.

RESUMO

Introdução: Análises isoenzimáticas foram realizadas envolvendo espécies dos subgêneros *Nyssorhynchus* e *Anopheles* para estimar a variabilidade genética intra e interespecífica. **Métodos:** Os mosquitos foram capturados em diferentes localidades da região Amazônica. A coleta e a criação dos mosquitos em laboratório foram conforme protocolos específicos. Na análise da variabilidade genética empregou-se a técnica de eletroforese horizontal em géis de amido e amido-agarose com sistemas tampões apropriados. A variação alozímica foi estimada pelo Programa Biosys-1. **Resultados:** Dos 13 *loci,* oito foram polimórficos. *Anopheles nuneztovari* apresentou o maior número de alelos por loco, sendo o menor detectado para *Anopheles albitarsis* (Macapá). O maior número de *loci* polimórficos foi detectado em *Anopheles marajoara* (Maruanum) e o menor, em *Anopheles benarrochi* (Guayará Mirim). *Anopheles darlingi* (Macapá) apresentou a maior heterozigosidade (H_o = 0,167 ± 0.071) e a menor (H_o = 0,045 ± 0.019) foi observada em *Anopheles intermedius* (Pacoval) do subgênero *Anopheles.* O coeficiente F de Wright evidenciou considerável estruturação genética entre populações de *Anopheles darlingi* (Fst= 0,110) e entre as populações de *Anopheles marajoara* (Fst= 0,082). **Conclusões:** Considerando todas as espécies estudadas, a distância genética variou de 0,008 a 1,144, onde a maior distância foi entre *Anopheles mattogrossensis* e *Anopheles oswaldoi* e a menor, entre as populações de *Anopheles benarrochi.*

Palavras-chaves: *Nyssorhynchus*. *Anopheles*. Variabilidade genética. Isoenzimas. Amazônia.

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INTRODUCTION

Improved understanding of malaria transmission and control among humans can be achieved by developing studies on morphological and molecular characteristics. Among the molecular markers used in population genetic research, isoenzymes are used to estimate genetic variability, as well as for helping to elucidate problems that can occur in relation to morphological identification of species, especially given the existence of species complexes such as in the genus *Anopheles*[1-5]. The *Anopheles albitarsis* complex consists of six cryptic species[6]. Li and Wilkerson[7] studied the species-specific variation of the ITS2 ribosomal DNA (rDNA) in order to identify the four species of the *Albitarsis* complex, and found complete correlation with the characteristics previously identified using random amplification of polymorphic DNA (RAPD). Merritt et al[8] examined phylogenetic relationship within the *Albitarsis* complex using a region of the white gene and found consistency with RAPD analysis: four species were distinguished in the *Albitarsis* complex and a fourth intron was detected only in *Anopheles marajoara*. Alternative topology placed *Anopheles marajoara* as a sister to *Anopheles albitarsis* B and revealed a sister relation between *Anopheles albitarsis sensu stricto* and *Anopheles deaneorum*. Based on the complete sequence of the cytochrome oxidase I (COI) gene, together with support from maximum parsimony, maximum likelihood and Bayesian analyses, Lehr et al[9] observed four members of the *Anopheles deaneorum* species that did not fit the sequences of the remaining *Albitarsis* complex species according to Bayesian topology, thus suggesting that these might represent a fifth species, *Anopheles albitarsis* E. Li and Wilkerson[7] cloned and sequenced the ITS2 region of rDNA extracted from the four species belonging to the *Albitarsis* complex and, despite obtaining significant divergence in one clone from two individuals of *Anopheles marajoara* from Venezuela, they were unable to distinguish *Anopheles albitarsis* E from *Anopheles marajoara*. However, while studying specimens from Colombia,

Brochero et al[6] used the same rDNA region and a partial sequence of the white gene and detected the presence of a new species, denominated *Anopheles albitarsis* F. Differing from *Anopheles marajoara*, but in common with other species of the complex, *Anopheles albitarsis* F does not possess the fourth intron of the white gene. Numerous immunoradiometric assays (IRMA) and enzyme-linked immunosorbent assays (ELISA) have shown *Anopheles albitarsis* specimens infected with *Plasmodium falciparum* and *Plasmodium vivax*, thus placing it as a potential malaria vector[10,11].

Studies on the genetic structure in *Anopheles* species have been conducted, notably on the *Nyssorhynchus* subgenus, since it includes the main vector species of human malaria in the neotropical region. Among these, *Anopheles darlingi* is the major malaria vector in Brazil and the most anthropophilic and endophagic in the Brazilian Amazon basin[12]. It is also a significant vector in other countries within its distribution range in South America, such as Peru, Colombia and Surinam[13]. The *Anopheles triannulatus* complex consists of three cryptic species: *Anopheles triannulatus s.s.*, *Anopheles halophylus* and *Anopheles triannulatus* "C"[14]. This complex occurs in Central and South America[15] and has not been incriminated as a malaria vector, although specimens infected by *Plasmodium vivax*[12,16] have been identified. *Anopheles oswaldoi* is largely distributed from the eastern region of South America up to Costa Rica in Central America. Together with *Anopheles konderi*, it forms a cryptic species complex. *Anopheles nuneztovari* is essentially a South American species, mainly in the Amazon region, and is an important vector in areas of Colombia, Venezuela and Peru; in Brazil it is considered to be a secondary vector. Cytogenetic, molecular and behavioral data indicate that this species forms a complex of two cryptic species[17]. *Anopheles rangeli* presents large distribution in South America, including Colombia, Ecuador, Guyana, Venezuela, Peru and Bolivia, and in Brazil it has been identified in the States of Amazonas, Acre, Rondônia, Pará and Roraima[12,18]. *Anopheles benarrochi* is considered to be predominantly zoophilic and not relevant to malaria transmission[15]. This species is found in Brazil, Venezuela and Colombia[19].

Species of the subgenus *Anopheles* found in Brazil have not been identified as malaria vectors[11], which is probably the reason for so few studies regarding the genetic structure of populations of these species[20]. *Anopheles intermedius* and *Anopheles mattogrossensis*, both belonging to the *Anopheles* subgenus, present wide geographic distribution. The former is found in Central and South America, and has been identified from Trinidad and Guyana up to Bolivia[21]. In Brazil, it is found throughout the country being abundant in the Amazon region, extending into the south, where it occupies coastal areas in the southern States[22]. *Anopheles mattogrossensis*, is found in Bolivia, Brazil, Colombia, Peru and Venezuela[23]. In Brazil, it has been identified in the States of Acre, Amazonas, Roraima, Rondônia, Pará and Amapá[24]. No evidence exists that this species is involved in malaria transmission.

Considering the epidemiological importance of the *Nyssorhynchus* subgenus, the present work aimed to determine the genetic variability and differentiation of seven species of this subgenus and two of the subgenus *Anopheles*.

METHODS

Species and collection locations

The collection locations of *Anopheles* species of the *Nyssorhynchus* and *Anopheles* subgenera are shown in **Figure 1**. Adult females of the *Anopheles* subgenus were collected from areas surrounding homes and specimens of *Nyssorhynchus* subgenus were collected both around and inside homes, between 18:00 and 22:00. They were placed in individual plastic cups for oviposition. The eggs were reared up to the adult stage in the laboratory, in accordance with Santos et al[25]. Female adults and 4th instar larvae were identified by means of the Consoli and Lourenço-de-Oliveira[26] keys.

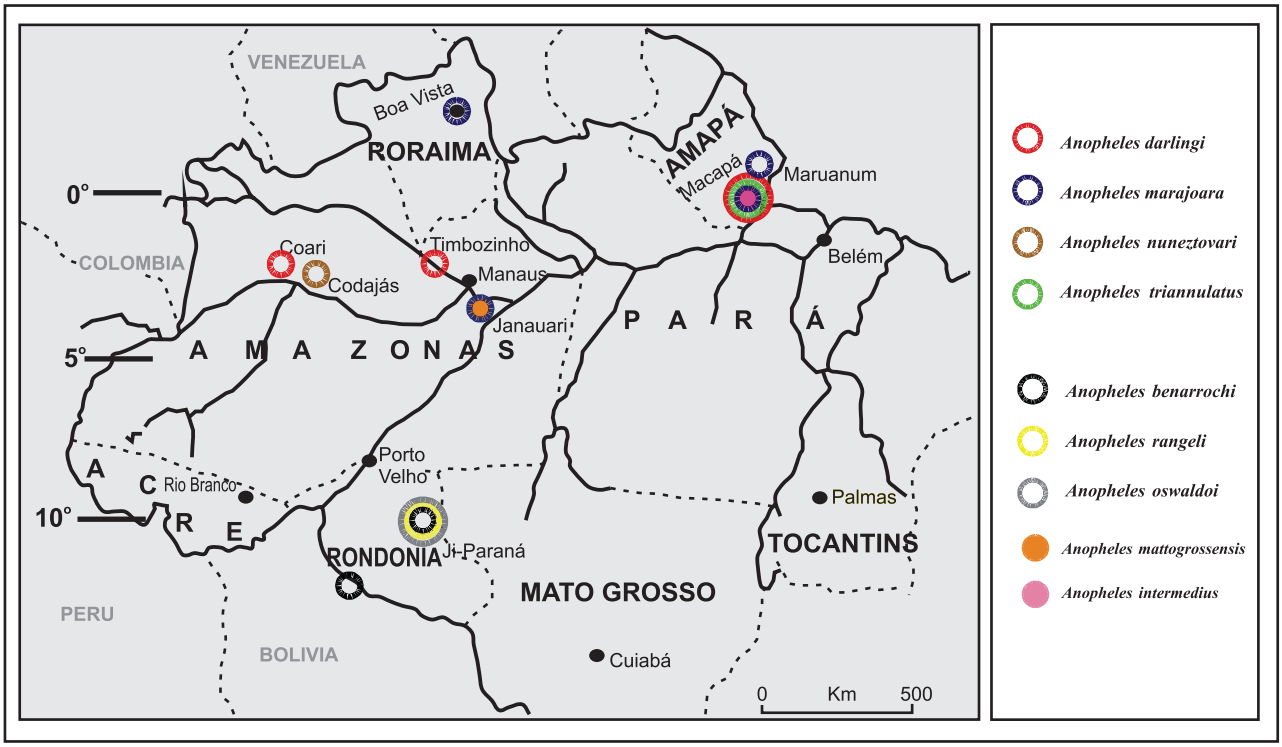

FIGURE 1 - Collection sites for *Anopheles* species belonging to the *Nyssorhynchus* and *Anopheles* subgenera, Amazon region.

Electrophoretic analysis

Four 4th instar larvae from each progeny for each enzyme system were used, with a mean of 75 larvae per population for each species. Of the eight enzyme systems studied, 13 loci were analyzed: esterase (*EST1, EST5* - E.C.3.1.1.1), leucine aminopeptidase (*LAP1, LAP2* - E.C.3.4.1.1), isocitrate dehydrogenase (*IDH 1* - E.C.1.1.1.42), phosphoglucomutase (*PGM1* - E.C.2.7.5.1), phosphoglucose isomerase (*PGI1* - E.C.5.3.1.9), hexokinase (*HK1, HK2, HK3, HK4* - E.C.2.7.1.1), malic enzyme (*ME1* - E.C.1.1.1.40) and malate dehydrogenase (*MDH1* - EC 1.1.1.37). The larvae were homogenized in 20µl of 0.5% b-mercaptoethanol, and a Whatman No. 3 (4.0cm^2) filter paper was used to absorb supernatant for horizontal electrophoresis. Isoenzymes were separated using two types of electrophoretic support: starch gel, at a concentration of 12%; and starch-agarose gel, at concentrations of 2% and 1%. CA-7[27], modified Poulik[28] and TEMM buffer systems[29] were used.

Statistical analyses

The alloenzyme variations were estimated using the Biosys-1 software program[30]. The genetic distances were calculated in accordance with Nei[31], and the UPGMA algorithm was used to generate a dendrogram.

RESULTS

Out of the 13 loci analyzed (**Table 1**), *HK1, HK2, HK4* and *PGI* were monomorphic and the EST5 and *PGM* loci were polymorphic for all the species studied. *EST1* was polymorphic, except in *Anopheles benarrochi* from Bolivia. *LAP1* was polymorphic only in *Anopheles marajoara* from Maruanum, *Anopheles intermedius* and *Anopheles mattogrossensis*, while *LAP2* was monomorphic only in *Anopheles darlingi* from Timbozinho and *Anopheles benarrochi* from Bolivia. *HK3* was polymorphic only in *Anopheles marajoara* from Macapá, *Anopheles rangeli* and *Anopheles oswaldoi*. *MDH1* was polymorphic for the majority of the species, except for *Anopheles triannulatus*, *Anopheles marajoara* from Macapá, *Anopheles darlingi* from Timbozinho and *Anopheles intermedius*. The *ME1* locus was polymorphic only in *Anopheles triannulatus*.

The chi-square test showed that 38% of the loci analyzed (*EST1, EST5, PGM, LAP1* and *LAP2*) presented deviations from Hardy-Weinberg genetic equilibrium.

The mean number of alleles per locus varied from 1.5 ± 0.2 for *Anopheles intermedius* and *Anopheles albitarsis* from Macapá, to 2.2 ± 0.1 for *Anopheles mattogrossensis*. Of the nine species analyzed, *Anopheles marajoara* from Maruanum was the most polymorphic (P = 53.8), while the least polymorphic species were *Anopheles darlingi* from Timbozinho and *Anopheles benarrochi* from Guayará Mirim (P = 30.8). *Anopheles darlingi* from Macapá revealed the highest value for heterozygosity (H_o = 0.165 ± 0.071, H_e = 0.172 ± 0.061), while the lowest value was found for *Anopheles intermedius* from Pacoval (H_o = 0.045 ± 0.019, H_e = 0.048 ± 0.018) (**Table 2**).

The genetic distance values (D) ranged from 0.003 to 1.114 (**Table 3**). The greatest distance (D = 1.114) was between *Anopheles* (*Nyssorhynchus*) *oswaldoi* and *Anopheles* (*Anopheles*) *mattogrossensis* and the smallest (D = 0.003) was between *Anopheles (Nyssorhynchus) benarrochi* from Bolivia and from Ji Paraná. For the *ME1* locus, alleles *105, 95, 90* and *85* were present only in species of the subgenus *Nyssorhynchus*, while the allele 100 was observed in species of both

TABLE 1- Frequencies of alleles at nine polymorphic loci in *Anopheles* species belonging to the *Nyssorhynchus* and *Anopheles* subgenera from the Amazon region.

		Anopheles													
			Subgenus *Nyssorhynchus*											Subgenus *Anopheles*	
Locus	Allele	*An tri*	*An mar*			*An dar*			*An ran*	*An osw*	*An nun*	*An ben*		*An int*	*An mat*
		Pac	Mac	BV	Mar	Coa	Tim	Mac	Ji-Par	Ji-Par	Cod	Ji-Par	Bol	Pac	Jan
EST1	N	76	39	42	55	42	46	67	74	72	59	63	56	131	136
	100	0.882	0.756	0.857	0.845	0.976	0.957	0.657	0.939	0.847	0.797	0.873	1.000	0.889	0.890
	97	0.118	0.244	0.143	0.155	0.024	0.043	0.343	0.061	0.153	0.203	0.127	0.000	0.111	0.110
EST5	N	43	36	40	75	32	38	71	42	40	28	36	56	131	123
	103	0.081	0.250	0.438	0.140	0.125	0.461	0.155	0.202	0.363	0.232	0.000	0.000	0.966	0.902
	100	0.628	0.375	0.450	0.413	0.531	0.145	0.606	0.357	0.338	0.179	0.000	0.027	0.034	0.098
	95	0.070	0.375	0.100	0.433	0.172	0.289	0.225	0.310	0.200	0.143	0.264	0.143	0.000	0.000
	90	0.140	0.000	0.013	0.013	0.172	0.105	0.014	0.095	0.100	0.357	0.528	0.661	0.000	0.000
	80	0.081	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.089	0.181	0.116	0.000	0.000
	78	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.054	0.000	0.000
LAP1	N	64	32	46	56	44	32	61	64	55	50	63	43	117	163
	100	1.000	1.000	1.000	0.920	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.987	0.975
	95	0.000	0.000	0.000	0.080	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.025
LAP2	N	63	32	46	65	44	32	56	62	55	50	63	43	120	161
	100	0.929	0.609	0.913	0.592	0.955	1.000	0.634	0.750	0.782	0.790	0.937	1.000	0.938	0.894
	98	0.056	0.391	0.087	0.408	0.045	0.000	0.366	0.194	0.164	0.190	0.048	0.000	0.063	0.106
	95	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.055	0.020	0.016	0.000	0.000	0.000
HK3	N	67	44	36	56	38	44	42	66	66	57	46	84	114	111
	100	1.000	0.955	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000
	95	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
	90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000

Continue.....

TABLE 1- Continuation.

		Anopheles													
		Subgenus *Nyssorhynchus*												Subgenus *Anopheles*	
Locus	Allele	*An tri*	*An mar*			*An dar*			*An ran*	*An osw*	*An nun*	*An ben*		*An int*	*An mat*
		Pac	Mac	BV	Mar	Coa	Tim	Mac	Ji-Par	Ji-Par	Cod	Ji-Par	Bol	Pac	Jan
IDH1	N	75	42	48	54	48	42	77	76	76	60	58	85	127	104
	105	0.013	0.000	0.000	0.018	0.021	0.012	0.013	0.000	0.000	0.008	0.000	0.000	0.016	0.000
	100	0.800	1.000	0.979	0.982	0.969	0.964	0.922	1.000	1.000	0.983	0.983	0.988	0.957	0.000
	95	0.187	0.000	0.021	0.000	0.010	0.024	0.065	0.000	0.000	0.008	0.017	0.012	0.028	1.000
MDH1	N	65	42	38	54	38	42	34	68	62	50	46	84	108	118
	112	0.000	0.000	0.066	0.000	0.145	0.000	0.279	0.000	0.000	0.010	0.891	0.964	0.000	0.000
	100	1.000	1.000	0.934	0.981	0.855	1.000	0.721	0.875	0.919	0.970	0.109	0.036	1.000	0.987
	95	0.000	0.000	0.000	0.019	0.000	0.000	0.000	0.125	0.081	0.020	0.000	0.000	0.000	0.013
ME1	N	74	44	44	56	44	44	42	73	74	60	54	70	40	40
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
	100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000
	95	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
	90	0.986	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	85	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGM	N	72	43	44	60	44	42	89	74	74	59	75	84	116	113
	110	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.068	0.213	0.155	0.000	0.000
	105	0.035	0.186	0.023	0.000	0.045	0.179	0.011	0.020	0.007	0.331	0.433	0.375	0.000	0.000
	100	0.132	0.709	0.466	0.925	0.739	0.679	0.882	0.932	0.966	0.458	0.260	0.315	0.069	0.000
	95	0819	0.105	0.455	0.075	0.216	0.143	0.107	0.047	0.027	0.136	0.093	0.155	0.931	0.000
	90	0.014	0.000	0.057	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.743
	88	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.257

***An tri*:** *Anopheles triannulatus*. ***An mar*:** *Anopheles marajoara*. ***An dar*:** *Anopheles darlingi*. ***An ran*:** *Anopheles rangeli*. ***An osw*:** *Anopheles oswaldoi*. ***An nun*:** *Anopheles nuneztovari*. ***An ben*:** *Anopheles benarrochi*. ***An int*:** *Anopheles intermedius*. ***An mat*:** *Anopheles mattogrossensis*. **Mac:** Macapá, **Mar:** Maruanum, **BV:** Boa Vista, **Coa:** Coari, **Tim:** Timbozinho, **Ji-Par:** Ji Paraná, **Bol:** Bolivia, **Pac:** Pacoval, **Cod:** Codajás, **Jan:** Janauari. **EST:** esterase, **LAP:** leucine aminopeptidase, **HK:** hexokinase, **IDH:** isocitrate dehydrogenase, **MDH:** malate dehydrogenase, **ME:** malic enzyme, **PGM:** phosphoglucomutase, **PG:** phosphoglucose isomerase. **N:** sample size.

TABLE 2 - Genetic variability at thirteen loci in nine *Anopheles* species belonging to the *Nyssorhynchus* and *Anopheles* subgenera from the Amazon region.

Population	Collection sites	Mean sample size/locus	Mean n. of alleles/locus	Polymorphic loci (%)*	**Mean heterozygosity** observed	expected**
An (*Nys*) *triannulatus*	Pacoval/AP	66.8± 2.6	2.0 ± 0.4	46.2	0.092 ± 0.037	0.122 ± 0.051
An (*Nys*) *marajoara*	Macapá/AP	39.6 ± 2.1	1.5 ± 0.2	38.5	0.140 ± 0.077	0.159 ± 0.067
An (*Nys*) *marajoara*	Boa Vista/RR	41.4 ± 1.4	1.8 ± 0.3	46.2	0.136 ± 0.063	0.135 ± 0.060
An (*Nys*) *marajoara*	Maruanum/AP	116.7 ± 3.3	1.7 ± 0.2	53.8	0.123 ± 0.052	0.133 ± 0.058
An (*Nys*) *darlingi*	Coari/AM	40.7 ± 1.4	1.8 ± 0.3	46.2	0.079 ± 0.034	0.116 ± 0.056
An(*Nys*) *darlingi*	Timbozinho/AM	41.4 ± 1.3	1.6 ± 0.3	30.8	0.086 ± 0.049	0.102 ± 0.061
An (*Nys*) *darlingi*	Macapá/AP	86.3 ± 14.9	1.8 ± 0.3	46.2	0.165 ± 0.071	0.172 ± 0.061
An (*Nys*) *rangeli*	Ji Paraná/RO	65.0 ± 3.8	1.8 ± 0.3	38.5	0.077 ± 0.039	0.123 ± 0.061
An (*Nys*) *oswaldoi*	Ji Paraná/RO	63.2 ± 4.0	1.7 ± 0.3	38.5	0.117 ± 0.055	0.119 ± 0.059
An (*Nys*) *nuneztovari*	Codajás/AP	51.7± 3.0	2.2 ± 0.4	46.2	0.128 ± 0.059	0.169 ± 0.076
An (*Nys*) *benarrochi*	Ji Paraná/RO	53.8± 3.4	1.8 ± 0.3	46.2	0.133 ± 0.062	0.146 ± 0.067
An (*Nys*) *benarrochi*	Guayará-Mirim/Bolivia	70.4 ± 4.6	1.7 ± 0.4	30.8	0.092 ± 0.057	0.103 ± 0.065
An (*Ano*) *intermedius*	Pacoval/AP	112.2 ± 6.3	1.5 ± 0.2	46.2	0.045 ± 0.019	0.048 ± 0.018
An (*Ano*) *mattogrossensis*	Janauari/AM	116.5 ± 8.3	1.5± 0.1	46.2	0.076 ± 0.037	0.078 ± 0.034

*the locus was considered polymorphic if more than one allele was detected in the same population.

**expected Hardy-Weinberg heterozygosity; Nei's unbiased estimate (Nei 1978). *An: Anopheles, Ano:* subgenus *Anopheles, Nys:* subgenus *Nyssorhynchus.*

TABLE 3 - Genetic identity (above diagonal) and distance coefficients (below diagonal) among fifteen populations of *Anopheles* (*Nyssorhynchus* and *Anopheles* subgenera) from the Amazon region.

Species/ Collection sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14
An (*Nys*) *triannulatus*/Pacoval-AP	_	0.761	0.804	0.751	0.789	0.774	0.715	0.671	0.668	0.685	0.603	0.602	0.446	0.421
An (*Nys*) *marajoara* /Macapá-AP	0.273	_	0.978	0.996	0.890	0.891	0.894	0.811	0.722	0.800	0.790	0.772	0.575	0.569
An (*Nys*) *marajoara*./Boa Vista-RR	0.218	0.023	_	0.967	0.900	0.899	0.877	0.799	0.715	0.799	0.801	0.794	0.627	0.603
An (*Nys*) *marajoara*/Maruanum-AP	0.287	0.004	0.034	_	0.891	0.855	0.896	0.818	0.727	0.788	0.777	0.763	0.547	0.549
An (*Nys*) *darlingi*/Coari-AM	0.236	0.117	0.105	0.115	_	0.986	0.979	0.729	0.814	0.891	0.734	0.734	0.497	0.488
An (*Nys*) *darlingi*/Timbozinho-AM	0.257	0.115	0.106	0.122	0.014	_	0.958	0.726	0.816	0.898	0.723	0.717	0.527	0.521
An (*Nys*) *darlingi*/Macapá-AP	0.287	0.112	0.131	0.110	0.021	0.043	_	0.712	0.803	0.870	0.712	0.702	0.447	0.456
An (*Nys*) *rangeli*/Ji Paraná-RO	0.398	0.209	0.224	0.201	0.316	0.320	0.340	_	0.824	0.798	0.801	0.793	0.479	0.393
An (*Nys*) *oswaldoi*/Ji Paraná-RO	0.403	0.326	0.336	0.320	0.206	0.204	0.219	0.194	_	0.889	0.705	0.698	0.406	0.319
An (*Nys*) *nuneztovari*/Codajás-AP	0.378	0.223	0.225	0.238	0.116	0.108	0.139	0.225	0.117	_	0.829	0.817	0.499	0.407
An (*Nys*) *benarrochi*/Ji Paraná-RO	0.506	0.236	0.221	0.252	0.309	0.324	0.340	0.222	0.350	0.187	_	0.997	0.496	0.410
An (*Nys*) *benarrochi*/Guayará-Mirim-Bolivia	0.507	0.259	0.230	0.270	0.309	0.333	0.354	0.232	0.359	0.202	0.003	_	0.496	0.407
An (*Ano*) *intermedius*/Pacoval-AP	0.807	0.554	0.468	0.603	0.699	0.641	0.804	0.736	0.902	0.695	0.702	0.701	_	0.534
An (*Ano*) *mattogrossensis*/Janauari-AM	0.864	0.564	0.505	0.600	0.718	0.651	0.784	0.934	1.144	0.898	0.891	0.899	0.628	_

An: *Anopheles*, ***Ano:*** subgenus *Anopheles*, ***Nys:*** subgenus *Nyssorhynchus.*

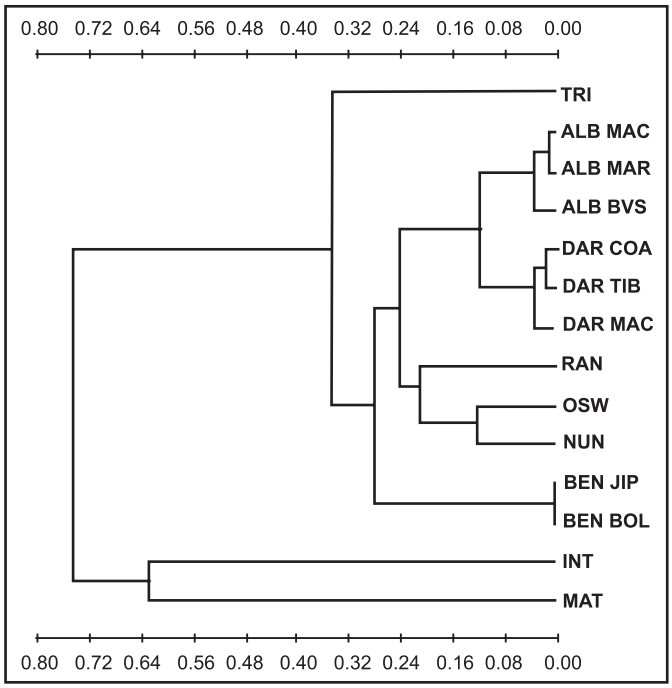

FIGURE 2 - UPGMA dendrogram for nine *Anopheles* species based on their genetic distances values (Nei, 1978). Unweighted method of population grouping using arithmetic mean (UPGMA). Species: TRI: *Anopheles triannulatus*. ALB: *Anopheles marajoara*. DAR: *Anopheles darlingi*. RAN: *Anopheles rangeli*. OSW: *Anopheles oswaldoi*. NUN: *Anopheles nuneztovari*. BEN: *Anopheles benarrochi*. INT: *Anopheles intermedius*. MAT: *Anopheles mattogrossensis*. Collection locations: MAC: Macapá. MAR: Maruanum. BVS: Boa Vista. COA: Coari. TIB: Timbozinho. JIP: Ji Paraná. BOL: Bolivia. Cophenetic correlation = 0.930.

subgenera. Alleles *110* and *105* of the *PGM1* locus were detected in the subgenus *Nyssorhynchus* and allele *88* was only observed in *Anopheles mattogrossensis* of the subgenus *Anopheles*. For the locus *PGI1,* alleles *95* and *90* were detected in species of the subgenus *Nyssorhynchus*. Allele *100* of the *HK1* and *HK2* loci was detected in the subgenus *Nyssorhynchus,* and in the subgenus *Anopheles*, allele *95* was observed in *Anopheles intermedius* and allele *90* in *Anopheles mattogrossensis.* For the locus *HK4*, only the allele *105* was observed in *Anopheles intermedius*. Allele *100* was detected in *Anopheles triannulatus*, *Anopheles marajoara, Anopheles darlingi* and *Anopheles mattogrossensis*, and allele *95* was observed in *Anopheles rangeli, Anopheles oswaldoi, Anopheles nuneztovari, Anopheles benarrochi* and *Anopheles mattogrossensis.* For the *PGI1* locus, allele 100 was observed in all species with the exception of *Anopheles marajoara, Anopheles rangeli* and *Anopheles oswaldoi*, in which allele 95 was detected. Allele *90* was observed only in *Anopheles triannulatus* (**Table 1**).

Based on the genetic distance values, the species were separated into four groups: three in the subgenus *Nyssorhynchus* and one in the subgenus *Anopheles*. The first group was composed of *Anopheles marajoara* and *Anopheles darlingi* (Argyritarsis section). *Anopheles darlingi* from Coari and *Anopheles marajoara* from Maruanum presented the highest identity value (I = 0.891), thus indicating low genetic differentiation. The second group was composed of *Anopheles benarrochi* (Albimanus section) and the third included *Anopheles rangeli, Anopheles oswaldoi* and *Anopheles nuneztovari* (Albimanus section). The fourth group was composed of *Anopheles mattogrossensis* and *Anopheles intermedius* (**Figure 2**).

DISCUSSION

Several studies involving the electrophoretic patterns of isoenzymes have been used for ontogeny analysis[32], as well as for analysis of genetic variability and differentiation in *Anopheles* populations[2,20,33]. Isoenzyme analysis associated with morphological and biochemical studies has contributed towards identifying species complexes, which are common among anopheline mosquitoes, and has been used to differentiate malaria vector and nonvector species[33], thus improving the current understanding of the mechanisms of malaria transmission and control[20]. Given that the subgenus *Nyssorhynchus* contains the principal malaria transmission species in Brazil, this subgenus has been investigated in general with the aim of achieving greater understanding of the genetic structure of its species and the dynamics of malaria transmission, as well as the possible mechanisms responsible for the vector capacity of the species involved[33]. In contrast, in the subgenus *Anopheles*, which does not contain species involved in malaria transmission in Brazil, such studies are at an incipient stage[20,34].

The variation observed in the percentages of polymorphic loci corroborate data obtained by Fritz et al[35], when studying *Anopheles rangeli*, *Anopheles nuneztovari* and *Anopheles trinkae* originating from Venezuela, Ecuador, Brazil and Bolivia (P = 20.3 - 58.3%). Scarpassa et al[17] analyzed 13 isoenzyme loci in populations from Colombia and Brazil and also observed *Anopheles nuneztovari* polymorphism levels (P = 31.3 - 56.6%) similar to those obtained in the present study. According to our data, Rodriguez[36] detected low polymorphism for *Anopheles intermedius* and *Anopheles mattogrossensis* (P = 35.3 and 47.1%, respectively). The low polymorphism and heterozygosity levels obtained for these species may be associated with low selection pressure because of their inability to cause *Plasmodium* infection. The most polymorphic species in the present study were *Anopheles triannulatus*, *Anopheles darlingi* and *Anopheles marajoara*, thus corroborating the results obtained by Santos et al[20].

We found genetic variability similar to other species of the same subgenera[33,35]. However, despite the high polymorphism presented, the heterozygosity levels were considered low for *Anopheles marajoara* from Maruanum (H_o = 0.123 ± 0.052, H_e = 0.133 ± 0.058). A high number of alleles per locus was observed for species of the subgenus *Nyssorhynchus*, with *Anopheles nuneztovari* presenting the highest value (2.2 ± 0.4). Low values were detected for *Anopheles intermedius* and *Anopheles mattogrossensis,* which are both species of the subgenus *Anopheles* (1.5 ± 0.2 and 1.5 ± 0.1). This shows the wide genetic variability in the *Anopheles* species and suggests that the variability is related to the innate population structure: mutation, preferential mating and selection or genetic drift.

The genetic distance values calculated for the *Anopheles marajoara* (D = 0.004 - 0.034) and *Anopheles darlingi* populations (D = 0.014 - 0.043) argues in favor of some differentiation. However, this was not observed in *Anopheles benarrochi* populations, in which the genetic distance values were very low (D = 0.003). Other studies have also found strong similarities among populations of different *Anopheles* species. Santos et al[33] obtained distance values from 0.010 to 0.024 for *Anopheles darlingi* populations from the Amazon region. Likewise, Manguin et al[37] reported a range of genetic distances for this species in South America, from 0.005 to 0.024.

The genetic distances observed among *Anopheles marajoara* populations belonging to the *Anopheles albitarsis* complex (D = 0.004 - 0.034) differed from those reported by Narang et al[38] in populations of this complex (D = 0.074 - 0.406). We found genetic distances among *Anopheles oswaldoi*, *Anopheles nuneztovari* and *Anopheles rangeli* ranging from 0.117 to 0.194, thus corroborating the results of Trindade[39], who obtained a genetic distance between *Anopheles rangeli* and *Anopheles nuneztovari* of 0.182. However, higher values between *Anopheles rangeli* and *Anopheles nuneztovari* were obtained by Fritz et al[35], in studying populations from Venezuela, Ecuador, Brazil and Bolivia (D = 0.319 - 0.419).

The genetic distance values obtained between *Anopheles triannulatus* and *Anopheles darlingi*, *Anopheles albitarsis*, *Anopheles intermedius* and *Anopheles mattogrossensis* were generally lower (D = 0.287; 0.287; 0.807 and 0.864, respectively) than those obtained previously[20] in analyses on genetic distances among populations of the same species (D = 0.524, 0.399, 0.989 and 0.789, respectively). Moreover, previous studies[4] on *Anopheles triannulatus* populations in the Brazilian Amazon region revealed much smaller genetic distances (D = 0.011 - 0.052). These data partially corroborate those reported by Sallum et al[40], which were based on molecular characteristics, and by Harbach[41] based on morphological and molecular studies.

Considering the subgenus *Nyssorhynchus*, our data revealed genetic similarity among the *Anopheles darlingi* and *Anopheles albitarsis* populations, while the *Anopheles benarrochi* and *Anopheles triannulatus* populations presented great divergence (**Figure 2**). In part, these data disagree with those reported by Sallum et al[42], who grouped *Anopheles albitarsis* and *Anopheles triannulatus* into the same cluster and separated these from *Anopheles darlingi*, based on morphological characteristics and on another study conducted by Sallum et al[40], using mitochondrial and ribosomal DNA sequences. However, Santos et al[20] studied five *Anopheles* species based on isoenzyme variation and reported results similar to those presented in this work.

In general, the species of the subgenus *Nyssorhynchus* showed greater variability than was shown by the species belonging to the subgenus *Anopheles*, thus corroborating the results obtained by Sallum et al[42] from studies on morphological characteristics. The low heterozygosity found in *Anopheles mattogrossensis* and *Anopheles intermedius* populations can be related to their specialized niches, in spite of showing wide geographic distribution. However, there is a need to amplify the enzyme systems used and define more conservative molecular markers for future studies, in order to the improve current understanding of the taxonomic position of species belonging to the subgenera *Nyssorhynchus* and *Anopheles*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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