DIFFERENTIATION AND GENETIC VARIABILITY IN NATURAL POPULATIONS OF *Anopheles (N.) triannulatus* (NEIVA & PINTO, 1922) OF BRAZILIAN AMAZONIA

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ABSTRACT

Populations of *Anopheles triannulatus* from Macapá (AP), Aripuanã (MT), Ji-Paraná (RO), and Manaus-Janauari Lake (AM) were studied using 16 enzymatic loci. The results of the isozyme analysis showed that the population of Macapá presented higher polymorphism (56.3%). The lowest variability was observed in the population of Manaus (p = 25.0; Ho = 0.077 ± 0.046). The results of Wright's F statistics showed unbalance due to excess of homozygotes ($F_{is} > F_{st}$), denoting a certain intrapopulational differentiation. Although the populations are genetically very close (D = 0.003 – 0.052), the dendrogram separates the populations in two groups: Macapá separated from that of Manaus, Ji-Paraná, and Aripuanã. This result may suggest a reduction in the genic flow, which possibly had some influence in the substructuration of the populations.

Key words: *Anopheles triannulatus*, isozymes, genetic of population, malaria.

RESUMO

Diferenciação e variabilidade genética em populações naturais de *Anopheles* (*N.*) *triannulatus* (Neiva & Pinto, 1922) da Amazônia brasileira

Populações de *Anopheles triannulatus* procedentes de Macapá (AP), Aripuanã (MT), Ji-Paraná (RO) e Manaus-Lago Janauari (AM) foram estudadas utilizando-se 16 locos enzimáticos. Os resultados mostraram maior polimorfismo (56,3%) na população de Macapá. A menor variabilidade foi verificada na população de Manaus (p = 25,0; Ho = 0,077 ± 0,046). Os resultados das estatísticas F de Wright mostraram desequilíbrio decorrente de excesso de homozigotos ($F_{is} > F_{st}$), denotando certa diferenciação intrapopulacional. Embora as populações sejam geneticamente muito próximas (D = 0,003 – 0,052), o dendrograma separa as populações em dois grupos: Macapá separado de Manaus, Ji-Paraná e Aripuanã. Isto pode ser indicativo de redução no fluxo gênico, que possivelmente influenciou a subestruturação das populações.

Palavras-chave: *Anopheles triannulatus*, isoenzimas, genética de população, malária.

INTRODUCTION

Anopheles triannulatus of the subgenus *Nyssorhynchus* presents wide distribution in South America and part of Central America. In South American it occurs mainly to the east of the Andes, in the Guyanas, Colombia, Venezuela, Ecuador, Peru, Bolivia, Paraguay, Argentina, and the whole Brazilian territory (Lane, 1953; Forattini, 1962; Faran, 1980). It shows zoophilic, exophilic, and crepuscular behavior for most of the Brazilian populations, placing this species among the

secondary or potential vectors of malaria (Forattini, 1962; Tadei *et al.*, 1983, 1988a, 1998; Deane, 1988).

This species is common in forests, where it shows hematophagic behavior in the upper strata of the vegetation (Deane *et al.*, 1971; Lourenço-de-Oliveira, 1993). However, it has also been found attacking man in the peridomicile as well as inside houses (Gabaldon, 1949; Charlwood & Wilkes, 1981; Tadei *et al.*, 1983, 1988a; Lourenço-de-Oliveira *et al.*, 1989; Rúbio-Palis, 1994). Nevertheless, its role as a vector of human malaria is still controversial. In Venezuela, *A. triannulatus* was identified as a possible vector during a malaria outbreak (Benarroch, 1931) and found to be naturally infected with plasmodian oocytes by Gabaldon & Cova Garcia (1946). Faran & Linthicum (1981) stated that *A. triannulatus* has no importance in malaria transmission in a large part of its occurrence area.

However, natural infections in this anopheline, detected by radioimmunoassay and immunoenzimatic tests with monoclonal antibodies for research of the protein CS in material from Peru, were reported by Hayes *et al.* (1987). The same is true for certain areas of Brazilian Amazonia, where *A. darlingi* is responsible for the transmission and continuation of malaria (Arruda *et al.*, 1986; Tadei *et al.*, 1988b; Oliveira-Ferreira *et al.*, 1990). Some authors believe that *A. triannulatus* presents epidemiological importance in relation to malaria when it occurs in areas of high population density (Hill, 1934; Charlwood & Wilkes, 1981). This species was separated from the subgroup Oswaldoi of the group Oswaldoi by Faran (1980) and, due to differences in taxonomic characters, placed in the monotypic subgroup Triannulatus.

Morphological, behavioral, and epidemiological differences have been observed, complicating the taxonomy states of this species, which is either considered to be a highly polymorphic single species or separated into two subspecies: *A. triannulatus triannulatus* and *A. triannulatus davisi* (Galvão & Lane, 1941; Forattini 1962; Nascimento, 1995). In spite of this variation and controversy regarding the taxonomic status of the species, it has been the object of few genetic studies, the first of which were carried out by Conn (1991). Whose polythenic chromossome analysis of samples from Venezuela showed some associations of the chromossomes arms which differentiate it in this particular from those of the other members of the subgenus *Nyssorhynchus*. Santos *et al.* (1992) studied a population of *A. triannulatus* from Balbina (AM) based on five esterase loci, all of them polymorphic. The values of χ^2 for the allelic frequencies were highly significant for all loci in that population.

Given these differences and the possibility of *A. triannulatus* being a complex of sibling species, populations from four places in Brazilian Amazonia were studied, with the aim of contributing towards elucidation of these taxonomic problems, and describing the genetic structure of the populations regarding their enzymatic variability.

MATERIAL AND METHODS

The mosquitoes were obtained from natural populations of Macapá (00º02'19.8"N, 51º03'40.7"W), Amapá State (AP); Aripuanã (10°10'00"S, 59°27'34"W), Mato Grosso State (MT); Ji-Paraná (10°53'07"S, 61°57'06"W), Rondônia State (RO); and the Janauari Lake Manaus (03º08'00.5"S, 60º00'34.7"W), Amazonas State (AM) (Fig. 1).

Adult mosquitoes were caught in animal enclosures next to human dwellings and in peridomestic sites between 18:00 and 22:00 h. After the collections, females were allowed to lay eggs separately and, soon after the egg hatching, larvae were maintained until becoming adults. Five fourth-instar progeny of each field-collected female were used for each enzymatic system, except for the Ji-Paraná population (three individuals on average). The adults were identified soon after their emergence and kept at –70ºC or deposited in the laboratory entomological collection. For identifying the mosquitoes, the keys of Gorham *et al*. (1967) and Consoli & Lourenço-de-Oliveira (1994) were used. The larvae were maintained in the laboratory according to methods described by Santos *et al.* (1981).

Sixteen enzymatic loci were analyzed: EST1, EST2, EST4, LAP2, LAP5, PGI, XDH, HK1, HK2, HK3, HK4, IDH, MDH, ME, 6-PGDH, and PGM in starch gel (12.5%) and starch-agarose (2% and 1%, respectively) electrophoresis following the method described by Steiner & Joslyn (1979), Lima & Contel (1990) and Santos *et al.* (1996).

Allelic frequencies were estimated directly from the data. Polymorphic loci ratio (P), heterozygosities found (Ho) and expected (He), and Wright's coefficients were estimated in each population by using the Biosys (Swofford & Selander, 1981) program. The dendrogram was constructed employing the UPGMA method (Nei, 1978).

RESULTS

Ten of the 16 loci analysed showed genetic variability in the four populations: EST1, EST2, EST4, LAP2, LAP5, IDH, MDH, ME, 6-PGDH, and PGM. The PGI, XDH, HEX1, HEX2, HEX3, and HEX4 loci were monomorphic in all populations. The LAP2 and ME loci were polymorphic only in the Macapá population, while EST1, LAP5, and IDH showed variation only in the Manaus population. The 6-PGDH locus showed variation only in the Aripuanã population (Table 1). Alleles B (EST1, LAP2, and LAP5), E (EST4), and C (IDH1, MDH, 6-PGDH, and PGM) presented the lowest frequencies in the four populations. Hardy-Weinberg's test indicated that most of the loci are in balance in all the populations.

Table 2 shows a genetic variability estimate of the studied populations. The Macapá population is the most polymorphic (p = 0.56) and its observed (Ho = 0.133 ± 0.054) and expected (He = 0.174 ± 0.055) average intralocus heterozygosity was higher. Lowest variability was observed in the Manaus population, with lower polymorphism (p = 0.25) and heterozygosity (Ho = 0.077 ± 0.046; He = 0.076 ± 0.046) and fewer alleles per locus (1.4 ± 0.2). In spite of the low variability detected in the Manaus population, the values of χ^2 were not significant at the 5% level in three of the four polymorphic loci. The populations of Macapá and Manaus showed excess heterozygotes in most of the loci, while excess of homozygotes was observed in the Ji-Paraná and Aripuanã populations.

Genetic structure analysis of the populations by Wright's F statistics showed an average F_{is} value higher than F_{st} (F_{is} = 189 > F_{st} = 0.110). Higher F_{is} values in relation to F_{st} were observed in all loci in the four populations (Table 3). This result may be largely due to the LAP2 and ME loci which were polymorphic only in the Macapá population and, thus have contributed to the high F_{is} values (0.525 and 0.968, respectively), indicating high genetic substructuration. The genetic distance values were low among the populations, showing little inter-populational differentiation (Table 4). The largest distance was found between Macapá and Manaus and the smallest between Aripuanã and Ji-Paraná. The genetic distance dendrogram separates the populations in three clusters. The first separates Manaus, Ji-Paraná, and Aripuanã from Macapá, the latter being isolated from the others in a single cluster. The second cluster separates Manaus from Ji-Paraná and Aripunã.

Fig. 1 — Collection sites of *Anopheles triannulatus*. Legend: ▲ Study sites.

TABLE 1
Allelic frequency of 16 enzymatic loci studied in populations of *Anopheles triannulatus*.

Locus	Allele	Population			
		Manaus	Macapá	Ji–Paraná	Aripuanã
EST1					
(N)		78	95	42	89
	A	1.000	0.600	0.821	0.848
	B	0.000	0.400	0.179	0.152
χ^2 H–W		–	0.155	15.995*	11.191**
EST2					
(N)		68	83	42	89
	A	0.059	0.494	0.429	0.399
	B	0.941	0.506	0.571	0.601
χ^2 H–W		0.231	1.886	0.790	32.861*
EST4					
(N)		69	104	42	90
	A	0.384	0.135	0.190	0.444
	B	0.246	0.572	0.321	0.300
	C	0.268	0.260	0.238	0.122
	D	0.101	0.034	0.190	0.072
	E	0.000	0.000	0.060	0.061
χ^2 H–W		16.101***	6.912	18.111	28.731**
LAP2					
(N)		58	95	42	114
	A	1.000	0.842	1.000	1.000
	B	0.000	0.158	0.000	0.000
χ^2 H–W		–	27.228*	–	–
LAP5					
(N)		58	84	42	118
	A	1.000	0.940	0.881	0.911
	B	0.000	0.060	0.119	0.089
χ^2 H–W		–	0.301	4.939***	12.906*
PGI1					
(N)		56	112	42	104
	A	1.000	1.000	1.000	1.000
χ^2 H–W		–	–	–	–
XDH1					
(N)		56	120	42	104
	A	1.000	1.000	1.000	1.000
χ^2 H–W		–	–	–	–
HEX1					
(N)		56	56	56	104
	A	1.000	1.000	1.000	1.000
χ2 H–W		–	–	–	–
HEX2					
(N)		56	56	56	104
	A	1.000	1.000	1.000	1.000
χ2 H–W		–	–	–	–

TABLE 1 (*Continued*).

Locus	Allele	Population			
		Manaus	Macapá	Ji–Paraná	Aripuanã
HEX3					
(N)		56	56	56	104
A	A	1.000	1.000	1.000	1.000
χ^2 H–W		–	–	–	–
HEX4					
(N)		56	56	56	104
A	A	1.000	1.000	1.000	1.000
χ^2 H-W		–	–	–	–
IDH1					
(N)		70	98	56	103
	A	0.000	0.005	0.000	0.000
	B	1.000	0.964	0.982	0.966
	C	0.000	0.031	0.018	0.034
χ^2 H-W		–	0.115	0.009	0.109
MDH1					
(N)		60	56	56	103
	A	0.083	0.018	0.054	0.053
	B	0.917	0.973	0.866	0.908
	C	0.000	0.009	0.080	0.039
χ^2 H-W		0.443	0.028	1.240	1.003
ME1					
(N)		56	63	56	104
	A	1.000	0.484	1.000	1.000
	B	0.000	0.516	0.000	0.000
χ^2 H-W		–	60.045*	–	–
6-PGDH					
(N)		56	56	56	103
	A	0.000	0.000	0.000	0.034
	B	1.000	1.000	1.000	0.898
	C	0.000	0.000	0.000	0.068
χ^2 H-W		–	–	–	6.670
PGM1					
(N)		62	178	50	108
	A	0.129	0.011	0.160	0.069
	B	0.863	0.885	0.810	0.894
	C	0.008	0.104	0.030	0.037
χ^2 H-W		0.151	97.121*	1.557	1.461

* = $p < 0.001$; ** = $p < 0.01$; *** = $p < 0.05$; – = not calculated.

DISCUSSION

The allelic frequencies were not uniform in the different loci, as indicated in the Hardy-Weinberg test of which are in balance in all populations. One of the most important implications in Hardy-Weinberg's balance is that when an allele is rare, many of the individuals can be hetero-

zygotes (Hartl, 1981). The heterozygote excess observed in most of the loci in the Macapá and Manaus populations may be an indication of greater adaptability of these genotypes in response to environmental alterations. In spite of these two populations occurring in different geographical areas, both were caught on cattle in enclosures close to homes and river margins greatly influenced by the anthropic and natural (flood/low water regime environment). Nevo (1978) suggests that the heterozygote excess may also be due to epistatic interactions or to frequency-dependent selection. Heterozygote excess was verified by Scarpassa *et al.* (1999), in an MDH locus of *A. nuneztovari* populations of Tibu and Sitronela, in Colombia. However, heterozygote excess was detected in the Ji-Paraná and Aripuanã populations, and Hardy-Weinberg's test showed balance for most loci in these populations. The same results have frequently been reported for other mosquito populations (Santos *et al.*, 1985; Van Driel *et al.*, 1987; Hii *et al.*, 1991; Scarpassa, 1988) and other insects (Sturgeon & Milton, 1986; Crouau-Roy, 1988). According to these authors, the deficiencies were attributable to differential genotypical selection, inbreeding, null alleles, a bottleneck effect, and the intrinsic population structure. In *A. nuneztovari* of Tucurui, Scarpassa (1988) attributed heterozygote deficiency in three esterase loci to ecological alterations in that area which possibly affected genotypical frequencies, resulting in different adaptive values for the homozygote classes.

TABLE 2
Estimate of genetic variability in 16 loci of *Anopheles triannulatus*.

Population	Mean sample size per locus	Mean n. of alleles per locus	Percentage of loci polymorphic*	Mean heterozygosity	
				Observed	Expected**
Manaus	60.7 ± 1.7	1.4 ± 0.2	25.0	0.077 ± 0.046	0.076 ± 0.046
Macapá	85.5 ± 8.4	1.9 ± 0.2	56.3	0.133 ± 0.054	0.174 ± 0.055
Ji-Paraná	49.5 ± 1.7	1.8 ± 0.3	43.8	0.117 ± 0.046	0.149 ± 0.057
Aripuanã	102.8 ± 2.0	1.9 ± 0.3	50.0	0.106 ± 0.039	0.139 ± 0.050

* A locus was considered polymorphic if more than one allele was detected; ** Expected heterozygosity of Hardy-Weinberg; Nei's unbiased estimate (Nei, 1978).

TABLE 3
Analysis of intra and interpopulational genetic structure by Wright's F statistics in populations of *Anopheles triannulatus*.

Locus	F_{is}	F_{it}	F_{st}
EST1	0.272	0.371	0.137
EST2	0.167	0.272	0.126
EST4	0.080	0.131	0.055
LAP2	0.525	0.584	0.123
LAP5	0.231	0.255	0.031
IDH1	–0.031	–0.021	0.009
MDH1	–0.089	–0.069	0.019
ME1	0.968	0.982	0.444
6–PGDH	0.121	0.174	0.061
PGM1	0.156	0.176	0.023
Mean	0.189	0.278	0.110

F_{is} = coeficient of inbreeding among individuals in the subpopulations; F_{it} = degree of genetic differentiation among the total populations; F_{st} = degree of genetic differentiation among the subpopulations.

Average heterozygosity observed in the populations of *A. triannulatus* (0.077-0.133) is similar to those obtained for other anopheline species of the subgenus *Nyssorhynchus* (Steiner *et al.*, 1982), and very close to the populations of *A. nuneztovari* of Brazil and Colombia (0.078-0.122) analyzed by Scarpassa *et al.* (1999), as well as that of Andean Venezuela (0.086-0.0118) described by Fritz *et al.* (1995). However, these results differ from those obtained by Santos *et al.* (1999) in populations of *A. darlingi* of Brazilian Amazonia (0.236-0.432).

Unbalance as a result of homozygote excess was observed in lineages of *A. stephensi* (Van Driel *et al.*, 1987), in which the value of F_{is} is higher than that of F_{st} for most loci analyzed. The average fixation index among the loci was low (F_{it} = 0.107). However, the GPDH locus of *A. stephensi*, and LAP2 and ME of *A. triannulatus* showed high fixation indexes (F_{it} = 0.831, 0.584, and 0.982, respectively). A similar result was observed by Santos *et al.* (1999) in the locus AO of populations of *A. darlingi* of Brazilian Amazonia (0.663) and by Scarpassa *et al.* (1999), in the loci α-GPDH, LAP1, and PGI (0.910, 0.569, and 0.799, respectively) of populations of *A. nuneztovari* of Brazil and Colombia. For *A. triannulatus*, the fixation index ($F_{is} > F_{st}$) may indicate some kind of isolation existing in some of the analyzed loci which interferes in random crossing. Genetic divergence among populations can be observed when a certain degree of reproductive isolation has already been established in the populations, as described by Narang *et al.* (1990).

The allelic frequency variability in *A. triannulatus* populations based on the fixation data is of intrapopulational origin and result in a certain differentiation among these populations. This differentiation may possibly be produced by homozygote excess in some loci and/or in the LAP2 and ME loci, which were polymorphic only in the Macapá population.

The distance values found among the four populations are in the range proposed for anophelines intra-specific variation (Bullini & Coluzzi, 1982), indicating genetic homogeneity on the macro-geographical scale, in spite of intrapopulational geographical differentiation. The small genetic divergence (D = 0.003-0.052) observed in *A. triannulatus* was similar to that shown in the data obtained by Santos *et al.* (1999) among *A. darlingi* populations of (D = 0.010-0.024) and by Scarpassa *et al.* (1999) among *A. nuneztovari* populations of Brazil (0.001-0.032).

Comparing these distance values and genetic similarity with those obtained for anopheline species, a similarity is detected, except for interspecific cases. For example, for the complex *A. marshalli*, the isoenzime analysis showed little divergence, with a genetic similarity of 0.803-0.972 (Lambert, 1983).

The phenogram (Fig. 2) shows that *A. triannulatus* populations are genetically separated in two groups: the Macapá population separated from a cluster of the Manaus, Ji-Paraná, and Aripuanã populations, and these separated in two clusters. This result may indicate a genic flow reduction, based on populations substructuration. Additional molecular studies will be necessary to better define *A. triannulatus* genetic structure and taxonomic status.

TABLE 4
Similarity matrix and genetic distance among four populations of *Anopheles triannulatus*.

Population	1	2	3	4
1. MANAUS	*****	0.949	0.987	0.989
2. MACAPÁ	0.052	*****	0.970	0.966
3. JI-PARANÁ	0.013	0.030	*****	0.997
4. ARIPUANÃ	0.011	0.035	0.003	*****

* Values above the diagonal are Nei's unbiased genetic identities, and below the diagonal are Nei's unbiased genetic distances (Nei, 1978).

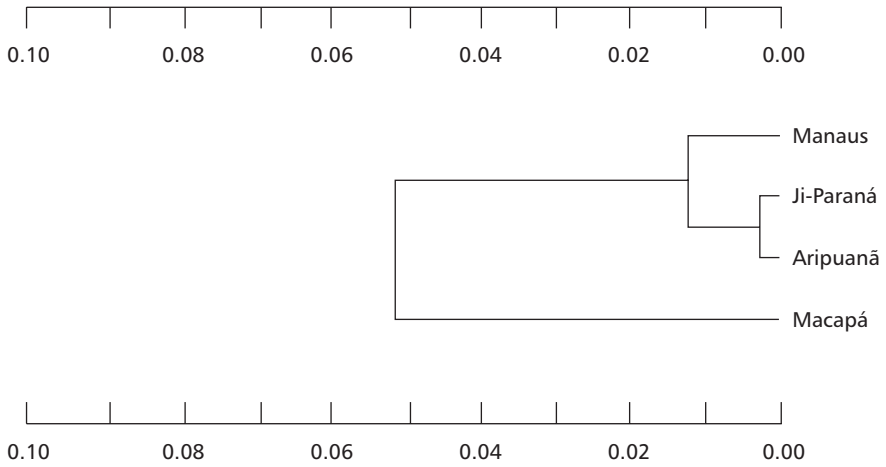

Fig. 2 — Resulting dendrogram of grouping of the populations based on genetic distance. Non-pondered method of grouping of populations with arithmetic mean (UPGMA).

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