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# POPULATION STRUCTURE AND GENETIC DIVERGENCE IN *ANOPHELES NUNEZTOVARI* (DIPTERA: CULICIDAE) FROM BRAZIL AND COLOMBIA

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*Abstract.* *Anopheles nuneztovari* is considered an important vector of human malaria in several localities in Venezuela and Colombia. Its status as a vector of human malaria is still unresolved in areas of the Brazilian Amazon, in spite of have been found infected with *Plasmodium* sp.. For a better understanding of the genetic differentiation of populations of *A. nuneztovari,* electrophoretic analysis using 11 enzymes was performed on four populations from Brazil and two from Colombia. The results showed a strong differentiation for two loci: α-glycerophosphate dehydrogenase (*α-Gpd*) and malate dehydrogenase (*Mdh*) from 16 loci analyzed. Diagnostic loci were not detected. The populations of *A. nuneztovari* from the Brazilian Amazon showed little genetic structure and low geographic differentiation, based on the $F_{IS}$ (0.029), $F_{ST}$ (0.070), and genetic distance (0.001–0.032) values. The results of the isozyme analysis do not coincide with the indication of two lineages in the Amazon Basin by analysis of mitochondrial DNA, suggesting that this evolutionary event is recent. The mean $F_{ST}$ value (0.324) suggests that there is considerable genetic divergence among populations from the Brazilian Amazon and Colombia. The genetic distance among populations from the Brazilian Amazon and Colombia is ranges from 0.047 to 0.148, with the highest values between the Brazilian Amazon and Sitronela (SIT) (0.125–0.148). These results are consistent with those observed among members of anopheline species complexes. It is suggested that geographic isolation has reduced the gene flow, resulting in the genetic divergence of the SIT population. Dendrogram analysis showed three large groups: one Amazonian and two Colombia, indicating some genetic structuring. The present study is important because it attempted to clarify the taxonomic status of *A. nuneztovari* and provide a better understanding of the role of this mosquito in transmission of human malaria in northern South America.

The existence of sibling species complexes, frequently found in the genus *Anopheles,* makes epidemiologic studies difficult since the precise identification of a vector is essential to comprehend the dynamics of the disease transmission cycle, and to help make appropriate decisions for malaria vectors control. *Anopheles (Nyssorhynchus) nuneztovari* Gabaldón, 1940, is a neotropical anopheline found in northern South America and eastern Panama.[1] In Brazil, it has been found in the Amazon region states.[2,3] As a consequence of its wide distribution, populations of this species occupy large geographic areas separated by geographic barriers, such as the Andes Mountains and the main rivers, the Amazon and the Negro, in the Amazon. In addition, the degree of involvement of *A. nuneztovari* in human malaria transmission seems to differ among localities. *Anopheles nuneztovari* is considered an important vector of human malaria in areas of Venezuela and Colombia.[4] In eastern Peru, *A. nuneztovari* was found to be infected with *Plasmodium vivax.*[5] In the Brazilian Amazonian states, its importance as a human malaria vector is still unresolved,[6] in spite of being infected with *P. vivax* and *P. falciparum.*[7,8] According to Deane,[6] no correlation was ever found between the presence of *A. nuneztovari* and malaria endemicity. Therefore, the genetic analysis of *A. nuneztovari* populations is relevant since the vectorial competence can differ among members of species complexes.[9] Studies on behavior, polytene chromosomes, isozymes, male genitalia, eggs, sequences of nuclear genes, and mitochondrial (mt) DNA of geographic populations of *A. nuneztovari* have shown different degrees of conspecific divergence or even evidence for a species complex.[10–20] Elliott suggested two allopatric forms or ecotypes for this species based on behavioral differences.[10] Populations from western Venezuela and northern Colombia described as human malaria vectors were mainly anthropophilic and endophagic, with a biting peak around midnight. Populations from other localities, including Brazil, not regarded as malaria vectors showed high zoophily and exophagy, with biting activity often during the first hours of the night.[10] Kitzmiller and others, studying polytene chromosomes of samples of *A. nuneztovari,* found a fixed inversion in the XR chromosome arm in populations of Tibú (Colombia) and Barinas (Venezuela), and the absence of this inversion in the populations of Manaus (Brazil).[11] Conn later named these two allopatric races as A (Amazon Basin) and B (western Venezuela-east Andes) cytotypes.[12] A third one, a C cytotype, has a chromocenter and an large complex inversion (2Lb) in the chromosome II; it is found in populations from Colombia and western Venezuela west of the Andes.[13] Isozyme studies, carried out in populations from Brokopondo (Suriname) and Barinas (Venezuela), as well as on samples from both sides of the Andes Mountain range in Venezuela (B and C cytotypes), showed low differentiation.[14,15] Preliminary studies of isozyme performed on six populations of *A. nuneztovari* from Brazil and Colombia indicated greater differences in the alleles frequencies to populations from western Colombia.[16] Studies of male genitalia of nine populations of this species indicate that males from the B cytotype are more differentiated than the other two cytotypes (A and C).[17] Morphometric analysis of eggs has shown differences between populations from Venezuela and the Brazilian Amazon.[18] However, populations from the western Amazon did not cluster with other collections from Brazil.

Recent molecular studies do not support the hypotheses that *A. nuneztovari* consists of a species complex. Sequence analysis of the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA indicated little variation among geographically distant populations. However, three geographic groups were obtained: 1) Bolivia, Colombia and Venezuela,

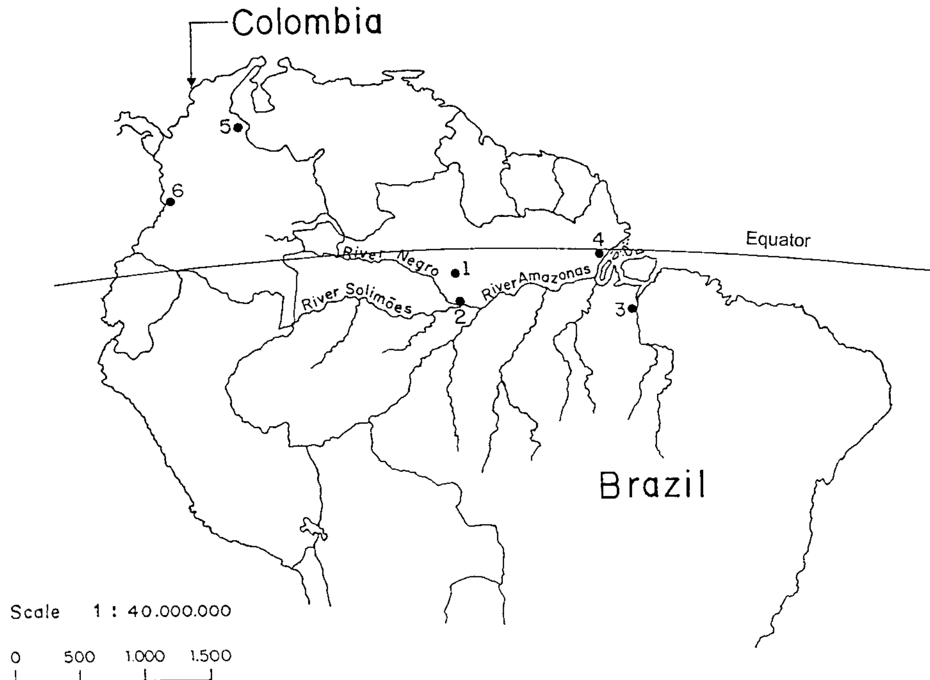


FIGURE 1. Collection sites of *Anopheles nuneztovari.* 1 = Km 206 of the BR-174 Highway (1°16′S, 60°23′W); 2 = Puraquequara (3°6′7″S, 60°1′30″W); 3 = Tucuruí (3°42′S, 49°27′W); 4 = Nova Mazagão (0°7′S, 51°17′W); 5 = Tibú = (8°39′N, 72°42′W); 6 = Sitronela (3°49′N, 77°4′W).

2) Northern Brazil and Suriname, and 3) central and eastern Brazil.[19] Studies of mtDNA from 12 populations in South America also showed the existence of three distinctive lineages: one in Venezuelan/Colombian and two within the Amazon Basin.[20]

The present study examined the variation of protein coding genes, using electrophoresis, as an attempt to better understand the geographical differentiation of populations of *A. nuneztovari.* These markers were used to estimate the amount of genetic divergence among geographic populations of *A. nuneztovari* from Brazil and Colombia, as well as to analyze the genetic structure of these populations. The results presented will provide additional information on the genetic divergence of *A. nuneztovari,* and will help explain the differences in malaria transmission patterns throughout its geographic range.

## MATERIALS AND METHODS

Adult female *A. nuneztovari* were collected at four sites in the Brazilian Amazon (Brazil) and two sites in Colombia (Figure 1). Samples from Km 206 of Highway BR-174, Amazonas (BR-1, BR-2, and BR-3), Puraquequara, Amazonas (PUR), and Nova Mazagão, Amapá (NOMA) were collected when feeding on pigs and cattle or resting on stable walls. The samples from Tucuruí, Pará (TUC-1 and TUC-2) were collected in the forest by human biting catches. For temporal genetic variation analysis, both populations from Highway BR-174 and Tucuruí were sampled several times. The samples from Highway BR-174 were obtained in July (BR-1) and October (BR-2) 1991 and in March (BR-3) 1992. The samples from Tucuruí were collected in August and October of 1992, which were called TUC-1 and TUC-2, respectively. In Tibú, Santander do Norte (TIBÚ) and Sitronela, Valle (SIT), mosquitoes were collected from the interior walls of houses or by human biting catches. The SIT sample included collections from Palo Grande Calle Larga, Sitronela, and Sabaleta. These three localities are close to each other and, for the analysis, were grouped under SIT. In all six populations, the collections were made between 6:00 PM and 9:00 PM.

Electrophoretic analysis was carried out using $F_1$ progeny from female mosquitoes captured in the wild. After capture, the females were individually isolated in plastic cups for egg laying. Following oviposition and hatching, the fourth instar larvae and adults were kept at −70°C until analysis. Fourth instar larvae were used for most enzymes, with the exception of α-glycerophosphate dehydrogenase (*α-GPD*), for which adults were used. An average of 2–4 individuals from each progeny were used. Morphologic identifications were done on egg and adults.[21,22] Voucher specimens have been deposited in the Malaria Vectors Laboratory of the Instituto Nacional de Pesquisas da Amazônia (Manaus, Amazonas, Brazil).

This study was reviewed and approved by Board of Research of the National Institute of Research of Amazonia, the Brazilian Ministry of Science and Technology, and the Colombian Ministry of Health.

**Electrophoretic analysis.** Isozymes were separated in two types of horizontal electrophoretic support: starch gel, at a concentration of 12% and starch-agarose gel, at concentrations of 2% and 0.8%, respectively. Samples were homogenized in 0.5% β-mercaptoethanol solution (v:v), absorbed onto Whatman (Hillsboro, OR) no. 3 filter papers, and applied to the gels. Each individual homogenate was assayed for up to four enzymes. After electrophoresis, the

TABLE 1
Allelomorph frequency of the 11 enzyme loci for all *Anopheles nuneztovari* populations studied

| | | Population* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Locus† | Allele | BR-1 | BR-2 | BR-3 | PUR | TUC-1 | TUC-2 | NOMA | TIBÚ | SIT |
| *Pgm* | n | 141 | 111 | 72 | 158 | 52 | 90 | 136 | 85 | 85 |
| | 113 | 0.039 | 0.063 | 0.063 | 0.013 | 0.000 | 0.000 | 0.026 | 0.000 | 0.000 |
| | 108 | 0.429 | 0.369 | 0.340 | 0.516 | 0.019 | 0.000 | 0.371 | 0.265 | 0.412 |
| | 100 | 0.362 | 0.437 | 0.396 | 0.263 | 0.875 | 0.883 | 0.445 | 0.735 | 0.588 |
| | 94 | 0.149 | 0.122 | 0.181 | 0.196 | 0.096 | 0.067 | 0.011 | 0.000 | 0.000 |
| | 91 | 0.021 | 0.009 | 0.021 | 0.013 | 0.010 | 0.050 | 0.121 | 0.000 | 0.000 |
| | 89 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.026 | 0.000 | 0.000 |
| $\chi^2_{het}$ | | | 5.83 (df = 8) | | | 3.72 (df = 2) | | | | |
| $\chi^2_{H-W}$ | | 10.648 | 12.736 | 49.983‡ | 6.555 | 1.011 | 1.570 | 9.705 | 1.298 | 0.034 |
| *6Pgd* | n | 130 | 130 | 124 | 124 | 89 | 164 | 67 | 64 | 62 |
| | 108 | 0.000 | 0.000 | 0.008 | 0.012 | 0.000 | 0.030 | 0.007 | 0.000 | 0.000 |
| | 100 | 0.988 | 1.000 | 0.992 | 0.988 | 0.983 | 0.966 | 0.985 | 1.000 | 0.952 |
| | 92 | 0.012 | 0.000 | 0.000 | 0.000 | 0.017 | 0.003 | 0.007 | 0.000 | 0.048 |
| $\chi^2_{het}$ | | | 6.64 (df = 4) | | | 3.89§ (df = 1) | | | | |
| $\chi^2_{H-W}$ | | 0.018 | | 0.008 | 0.019 | 0.026 | 0.197 | 0.015 | | 0.160 |
| *Acon* | n | 112 | 60 | 124 | 124 | 76 | 164 | 51 | 64 | 60 |
| | 106 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.102 | 0.275 |
| | 103 | 0.000 | 0.008 | 0.024 | 0.004 | 0.000 | 0.018 | 0.108 | 0.156 | 0.042 |
| | 100 | 0.991 | 0.983 | 0.952 | 0.960 | 1.000 | 0.979 | 0.892 | 0.742 | 0.667 |
| | 98 | 0.009 | 0.008 | 0.024 | 0.036 | 0.000 | 0.003 | 0.000 | 0.000 | 0.017 |
| $\chi^2_{het}$ | | | 7.74 (df = 3) | | | 4.31 (df = 2) | | | | |
| $\chi^2_{H-W}$ | | 0.009 | 0.017 | 0.321 | 0.219 | | 0.078 | 0.745 | 8.606§ | 4.035 |
| *Mdh* | n | 104 | 127 | 126 | 152 | 99 | 96 | 71 | 72 | 73 |
| | 113 | 0.000 | 0.012 | 0.000 | 0.026 | 0.005 | 0.000 | 0.014 | 0.000 | 0.000 |
| | 100 | 1.000 | 0.988 | 0.996 | 0.947 | 0.949 | 0.964 | 0.986 | 0.319 | 0.322 |
| | 94 | 0.000 | 0.000 | 0.004 | 0.026 | 0.035 | 0.036 | 0.000 | 0.681 | 0.678 |
| | 78 | 0.000 | 0.000 | 0.000 | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 |
| $\chi^2_{het}$ | | | 4.33 (df = 2) | | | 33.50‡ (df = 2) | | | | |
| $\chi^2_{H-W}$ | | | 0.018 | 0.002 | 0.469 | 40.498‡ | 0.137 | 0.014 | 5.554§ | 8.904¶ |
| *Idh-1* | n | 138 | 128 | 126 | 169 | 99 | 112 | 83 | 66 | 54 |
| | 106 | 0.029 | 0.035 | 0.020 | 0.012 | 0.030 | 0.000 | 0.102 | 0.000 | 0.000 |
| | 100 | 0.964 | 0.965 | 0.976 | 0.988 | 0.934 | 1.000 | 0.898 | 1.000 | 1.000 |
| | 93 | 0.007 | 0.000 | 0.004 | 0.000 | 0.035 | 0.000 | 0.000 | 0.000 | 0.000 |
| $\chi^2_{het}$ | | | 2.51 (df = 3) | | | 14.09‡ (df = 1) | | | | |
| $\chi^2_{H-W}$ | | 0.195 | 0.170 | 0.075 | 0.024 | 16.135¶ | | 1.080 | | |
| *α-Gpd* | n | 55 | 111 | 137 | 66 | 56 | 135 | 75 | 44 | 97 |
| | 107 | 0.000 | 0.000 | 0.000 | 0.000 | 0.036 | 0.033 | 0.000 | 0.000 | 0.000 |
| | 100 | 1.000 | 1.000 | 1.000 | 1.000 | 0.964 | 0.967 | 1.000 | 0.989 | 0.052 |
| | 90 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.011 | 0.948 |
| $\chi^2_{het}$ | | | | | | 0.02 (df = 1) | | | | |
| $\chi^2_{H-W}$ | | | | | | 0.077 | 0.161 | | 0.006 | 2.376 |
| *Lap-1* | n | 156 | 122 | 128 | 145 | 130 | 55 | 80 | 31 | 69 |
| | 100 | 1.000 | 1.000 | 1.000 | 0.976 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| | 98 | 0.000 | 0.000 | 0.000 | 0.024 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| $\chi^2_{H-W}$ | | | | | 45.607‡ | | | | | |
| *Lap-5* | n | 160 | 146 | 183 | 147 | 21 | 83 | 120 | 87 | 72 |
| | 100 | 1.000 | 0.979 | 0.973 | 1.000 | 0.857 | 0.898 | 0.967 | 0.937 | 0.938 |
| | 98 | 0.000 | 0.021 | 0.027 | 0.000 | 0.143 | 0.102 | 0.033 | 0.063 | 0.063 |
| $\chi^2_{het}$ | | | 10.23‡ (df = 2) | | | 0.02 (df = 1) | | | | |
| $\chi^2_{H-W}$ | | | 0.064 | 5.767§ | | 7.843¶ | 1.080 | 0.143 | 23.055‡ | 0.320 |
| *Est-5* | n | 142 | 128 | 155 | 128 | 100 | 41 | 68 | 78 | 80 |
| | 111 | 0.007 | 0.004 | 0.019 | 0.008 | 0.025 | 0.037 | 0.074 | 0.000 | 0.000 |
| | 109 | 0.190 | 0.207 | 0.106 | 0.180 | 0.330 | 0.061 | 0.368 | 0.058 | 0.019 |
| | 106 | 0.239 | 0.258 | 0.248 | 0.234 | 0.485 | 0.341 | 0.110 | 0.942 | 0.981 |
| | 100 | 0.532 | 0.488 | 0.610 | 0.516 | 0.145 | 0.463 | 0.367 | 0.000 | 0.000 |
| | 97 | 0.032 | 0.043 | 0.016 | 0.063 | 0.015 | 0.098 | 0.081 | 0.000 | 0.000 |
| $\chi^2_{het}$ | | | 10.22§ (df = 4) | | | 30.78‡ (df = 4) | | | | |
| $\chi^2_{H-W}$ | | 5.456 | 16.848 | 4.459 | 24.077¶ | 13.699 | 11.915 | 11.455 | 0.292 | 0.029 |
| *Pgi-1* | n | 73 | 84 | 84 | 123 | 28 | 56 | 75 | 69 | 69 |
| | 100 | 1.000 | 1.000 | 1.000 | 1.000 | 0.911 | 0.929 | 1.000 | 1.000 | 1.000 |
| | 96 | 0.000 | 0.000 | 0.000 | 0.000 | 0.089 | 0.071 | 0.000 | 0.000 | 0.000 |

TABLE 1
Continued

| Locus† | Allele | BR-1 | BR-2 | BR-3 | PUR | TUC-1 | TUC-2 | NOMA | TIBÚ | SIT |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Population* | | | | | | | | |
| $\chi^2_{het}$ | | | | | | 0.12 (df = 1) | | | | |
| $\chi^2_{H\text{-}W}$ | | | | | | 17.052‡ | 0.331 | | | |
| *Pgi-2* | n | 65 | 82 | 68 | 78 | 28 | 56 | 75 | 69 | 69 |
| | 110 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.036 | 0.000 | 0.000 | 0.000 |
| | 100 | 1.000 | 1.000 | 1.000 | 1.000 | 0.929 | 0.964 | 1.000 | 1.000 | 1.000 |
| | 93 | 0.000 | 0.000 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.000 |
| $\chi^2_{het}$ | | | | | | 8.97§ (df = 2) | | | | |
| $\chi^2_{H\text{-}W}$ | | | | | | 0.166 | 0.077 | | | |

* BR = Highway BR-174; PUR = Puraquequara; TUC = Tucuruí; NOMA = Nova Mazagão; TIBÚ = Tibú; SIT = Sitronela; df = degrees of freedom.
† *Pgm* = phosphoglucomutase; $\chi^2_{het}$ = Chi-square heterogeneity used in the comparations of the samples obtained of the populations from BR-174 Highway (BR-1, BR-2 and BR-2) and Tucuruí (TUC-1 and TUC-2); $\chi^2_{H\text{-}W}$ = Hardy-Weinberg equilibrium; *6Pgd* = 6-phosphogluconate dehydrogenase; *Acon* = aconitase; *Mdh* = malate dehydrogenase; *Idh-1* = isocitrate dehydrogenase-1; α-*Gpd* = α-glycerophosphate dehydrogenase; *Lap-1* = leucine aminopeptidase-1; *Est-5* = esterase-5; *Pgi-1* = phosphoglucose isomerase-1.
‡ $P < 0.001$
§ $P < 0.05$
¶ $P < 0.01$.

starch gels were cut in two equal parts, and staining solutions were applied on the exposed internal surfaces. For starch-agarose gels, the staining solutions were applied directly on the gel. Eleven enzymes were studied and 21 loci were developed, although only 16 of them were analyzed due to their greater accuracy for genotype counts. The enzyme systems, including the symbol, Enzyme Commission number, and number of analyzed loci were esterase (EST, 3. 1. 1. 1, 1 locus), leucine aminopeptidase (LAP, 3.4.11.1, 4 loci), α-glycerophosphate dehydrogenase (α-GPD, 1.1.1.8, 1 locus), 6-phosphogluconate dehydrogenase (6PGD, 1.1.1.44, 1 locus), aconitase (ACON, 4.2.1.3, 1 locus), isocitrate dehydrogenase (IDH, 1.1.1.42, 2 loci), malate dehydrogenase (MDH, 1.1.1.37, 1 locus), malic enzyme (ME, 1.1.1.40, 1 locus), phosphoglucomutase (PGM, 5.4.2.2, 1 locus), phosphoglucose isomerase (PGI, 5.3.1.9, 2 loci), and xanthine dehydrogenase (XDH, 1.2.1.37, 1 locus). The methods for the EST and LAP analysis were described by Scarpassa and others,[23] and the methods for α-GPD were described by Scarpassa and Tadei.[24] The Tris-citrate buffer system was used for 6PGD and ACON according to Steiner and Joslyn,[25] and the staining methods used were those of Steiner and Joslyn[25] and Harris and Hopkinson,[26] respectively. For the analysis of IDH, MDH and ME, the phosphate-citrate buffer system (0.245 M monobasic sodium phosphate and 0.15 M citric acid), pH 5.9, was used in the electrode, and a 1:40 dilution was used in the gel.[27] The staining methods used were those of Lima and Contel.[27] During the analysis of PGM and PGI, the buffer system used in the electrode was TEMM (0.1 M Tris, 0.01 M EDTA, 0. 1 M maleic anhydride, 0.001 M $MgClO_4$, pH 7.4, and a 1:15 dilution was used in the gel.[27] The staining methods were those of Lima and Contel[27] and Steiner and Joslyn,[25] respectively. For XDH, the lithium hydroxide buffer system and staining methods used were those of Steiner and Joslyn.[25] After the electrophoretic pattern development, the gels were typed and photographed using Pan AHU film (Agfa Copex, Ridgefield Park, NJ). In this study, the enzyme LAP showed four loci: *Lap-1, Lap-2, Lap-4,* and *Lap-5.* The *Lap-3* locus was not analyzed because it is exclusive to pupal and adult stages.[23]

**Statistical analysis.** The genetic variability for each population was estimated using the number of alleles per locus, proportion of polymorphic loci, mean heterozygosity observed and expected, and tests for Hardy-Weinberg equilibrium. A locus was considered polymorphic if any variation was observed, independent of the frequency of alleles detected.[28] The population genetic structure was estimated by Wright's F statistic, using six geographical populations (BR-1, PUR, TUC-1, NOMA, TIBÚ, and SIT) for analysis between Brazil and Colombia, and four geographic populations (BR-1, PUR, TUC-1, and NOMA) for analysis within Brazil. Genetic differentiation among all populations was measured by the Wright's $F_{ST}$ statistic, Nei's genetic distances values and by the resulting clusters.[29] The Biosys-1 Program[28] was used for this analysis. For temporal genetic variation analysis among samples from BR-174 Highway and Tucuruí, the chi-square heterogeneity test was used.

## RESULTS

Five of 16 loci analyzed (*Lap-2, Lap-4, Idh-2, Me,* and *Xdh*) were monomorphic in all samples. In the populations from Brazil, relatively homogenous allelic frequencies were found for 10 of the 11 polymorphic loci (Table 1). However, the *Pgm* locus showed differences in the frequencies for the $Pgm_{100}$ allele between TUC (TUC-1 = 0.875 and TUC-2 = 0.883) and PUR (0.263) populations. In the populations from Colombia, six of the seven polymorphic loci showed homogenous allelic frequencies, except for the α-*Gpd* locus, which showed high differentiation. The α-$Gpd_{100}$ allele was very frequent in TIBÚ (0.989), and the α-$Gpd_{90}$ allele had a high frequency in SIT (0.948). When the populations from Brazil and Colombia were compared, differences in allelic frequencies were found for the α-*Gpd, Mdh, and Est-5* loci, and smaller differences were found for the *Acon* locus. The α-*Gpd* locus had the α-$Gpd_{100}$ allele fixed (1.000) in the populations from the BR- 174 Highway, PUR, and NOMA. This allele was very frequent in the TUC-1 (0.964), TUC-2 (0.967), and TIBÚ (0.989) populations, while the α-$Gpd_{90}$ allele showed a high frequency (0.948) in the SIT population. For the *Mdh* locus, the $Mdh_{100}$ allele had frequencies > 94% for all populations from Brazil, while the $Mdh_{94}$ allele was the most frequent in the populations of TIBÚ (0.681) and SIT (0.678). The $Est\text{-}5_{106}$ allele of the *Est-5* locus had frequencies > 94% in the populations from TIBÚ and SIT.

TABLE 2

Estimate of measures of genetic variability in populations of *Anopheles nuneztovari**

| Population† | Mean number of alleles per locus | Percentage of polymorphic loci‡ | Mean heterozygosity | |
|---|---|---|---|---|
| | | | $\bar{H}_{obs}$ | $\bar{H}_{esp}$ |
| BR-1 | 1.80 (0.30) | 31.3 | 0.090 (0.056) | 0.087 (0.055) |
| BR-2 | 1.80 (0.30) | 37.5 | 0.087 (0.051) | 0.092 (0.055) |
| BR-3 | 1.90 (0.30) | 43.8 | 0.078 (0.043) | 0.092 (0.053) |
| PUR | 1.90 (0.30) | 43.8 | 0.088 (0.048) | 0.097 (0.053) |
| TUC-1 | 2.10 (0.30) | 56.3 | 0.088 (0.039) | 0.109 (0.041) |
| TUC-2 | 1.90 (0.30) | 56.3 | 0.090 (0.035) | 0.094 (0.042) |
| NOMA | 1.90 (0.40) | 43.8 | 0.117 (0.056) | 0.116 (0.057) |
| TIBÚ | 1.40 (0.20) | 37.5 | 0.093 (0.044) | 0.094 (0.041) |
| SIT | 1.60 (0.20) | 43.8 | 0.122 (0.053) | 0.110 (0.046) |

* Values in parentheses are standard errors.
† For definitions of populations, see Table 1.
‡ A locus was considered polymorphic independent of the frequency of the detected alleles.[28]

TABLE 3

Genetic structure analysis of *Anopheles nuneztovari* populations in Brazil and Colombia, using Wright's F statistics*

| Locus† | $F_{IS}$ | $F_{ST}$ | $F_{IT}$ |
|---|---|---|---|
| *Pgm* | 0.000 | 0.102 | 0.102 |
| *6Pgd* | −0.026 | 0.013 | −0.013 |
| *Acon* | −0.044 | 0.143 | 0.105 |
| *Mdh* | −0.209 | 0.508 | 0.405 |
| *Idh-1* | −0.018 | 0.033 | 0.016 |
| α-*Gpd* | 0.093 | 0.901 | 0.910 |
| *Lap-1* | 0.561 | 0.018 | 0.569 |
| *Lap-5* | 0.241 | 0.043 | 0.274 |
| *Est-5* | 0.046 | 0.256 | 0.290 |
| *Pgi-1* | 0.780 | 0.084 | 0.799 |
| *Pgi-2* | −0.077 | 0.067 | −0.005 |
| Mean | 0.007 | 0.324 | 0.329 |

* $F_{IS}$ = coefficient of inbreeding among individuals in the subpopulations; $F_{ST}$ = degree of genetic differentiation among the subpopulations; $F_{IT}$ = degree of genetic differentiation in the total population.
† For definitions of loci, see Table 1.

This allele was found at a frequency of 11% in the NOMA population.

The temporal genetic variation analysis performed on samples from BR-1, BR-2, and BR-3 indicated differences in the frequencies of the alleles, with significant chi-square values for heterogeneity ($\chi^2_{het}$) only for the loci *Lap-5* and *Est-5* of the 16 loci studied (Table 1). In the samples from TUC-1 and TUC-2, the $\chi^2_{het}$ values were significant for the *6Pgd, Mdh, Idh-1, Pgi-2,* and *Est-5* loci. It is possible that the presence of rare alleles has influenced the increase in the $\chi^2_{het}$ values for the *6Pgd, Mdh, Idh-I,* and *Pgi-2* loci (Table 1). However, the significant value for the *Est-5* ($\chi^2_{het}$ = 30.78) locus could be due either to higher differences in the frequencies of the *Est-5*$_{109}$ and *Est-*$_{100}$ alleles, between the TUC-1 and TUC-2 samples (Table 1), or to the small number of individuals analyzed in the second sample (n = 41).

The Hardy-Weinberg test indicated that most of the loci are at equilibrium in all populations (Table 1). However, 12 loci among 63 comparisons (19.05%) showed significant deviations for the expected Hardy-Weinberg equilibrium. Most of the deviation was due to observations of homozygous individuals for one rare allele, or to heterozygous individuals having two rare alleles (frequency $< 0.05$). The significant deviation of the *Mdh* locus was due to the excess of observed heterozygous individuals (n = 40, n = 43) in comparison with the expected numbers (n = 31.306, n = 31.870) for the populations from TIBÚ and SIT, respectively.

The genetic variability measurements (Table 2) showed that the mean ± SE number of alleles by locus ranged from 1.40 ± 0.20 to 2.10 ± 0.30, and the percentage of polymorphic loci ranged between 31.3% and 56.3%, with the highest percentages for the TUC-1 and TUC-2 samples. The observed mean ± SE heterozygosity ranged from 0.078 ± 0.043 to 0. 122 ± 0.053, with higher values for the populations from NOMA (0.117 ± 0.056) and SIT (0.122 ± 0.053).

Table 3 shows that the mean value of $F_{IS}$ was low (0.007), indicating that matings are occurring randomly within subpopulations, and there is no evidence of intra-subpopulation differentiation. The $F_{ST}$ statistic had a mean value of 0.324, mainly due to the α-*Gpd* and *Mdh* loci, which showed high differentiation, with the values 0.901 and 0.508, respectively. The high value of the α-*Gpd* locus was due to differences in the allelic frequencies between SIT and all other studied populations, while the *Mdh* locus showed differences between populations from Brazil and Colombia. The *Est-5* locus showed differentiation, but at a lower level (0.256), with higher differences in the frequency of the *Est-5*$_{106}$ allele between populations of Colombia and NOMA (Table 1). The genetic structure analysis performed among populations from the Brazilian Amazon showed low mean values of the $F_{IS}$ (0.029), $F_{ST}$ (0.070), and $F_{IT}$ (0.097). This suggests that the amount of intrapopulational and interpopulational genetic differentiation is small.

The genetic distances analysis (Table 4) showed a high level of differentiation between the populations from the Brazilian Amazon and SIT (0.125–0.148). The highest within country value was detected between TIBÚ and SIT (0.066), which was higher than the value observed between TIBÚ and TUC (TUC-1 = 0.047, TUC-2 = 0.057), which are separated by a greater geographic distance. The populations from Brazil had low distance values (0.001–0.032).

The genetic distance dendrogram (Figure 2) indicates three large groups. One consists of the populations from Brazil, where the populations from TUC-1 and TUC-2 were the most distant. The second group is represented only by the population from TIBÚ. The population from SIT is found in a third group, the most distant population among all analyzed.

## DISCUSSION

One of the most important implications of Hardy-Weinberg equilibrium is that when an allele is rare, most of the individuals should be heterozygous.[30] The significant excess of heterozygotes for the *Mdh* locus for the populations from

TABLE 4
Matrix of genetic distance of *Anopheles nuneztovari**

| Population† | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| 1. SIT | 0.000 | | | | | | | | |
| 2. TIBÚ | 0.066 | 0.000 | | | | | | | |
| 3. TUC-1 | 0.125 | 0.047 | 0.000 | | | | | | |
| 4. TUC-2 | 0.135 | 0.057 | 0.007 | 0.000 | | | | | |
| 5. NOMA | 0.148 | 0.072 | 0.021 | 0.019 | 0.000 | | | | |
| 6. PUR | 0.141 | 0.072 | 0.032 | 0.025 | 0.007 | 0.000 | | | |
| 7. BR-2 | 0.138 | 0.066 | 0.019 | 0.014 | 0.004 | 0.002 | 0.000 | | |
| 8. BR-1 | 0.143 | 0.071 | 0.025 | 0.018 | 0.005 | 0.001 | 0.000 | 0.000 | |
| 9. BR-3 | 0.144 | 0.071 | 0.025 | 0.015 | 0.007 | 0.002 | 0.001 | 0.001 | 0.000 |

* Values are Nei[29] unbiased genetic distance.
† For definitions of populations, see Table 1.

TIBÚ and SIT can be explained by one of two hypotheses. In the first, the excess of heterozygotes could be an indication of overdominance, conferring a higher fitness to this genotype. However, it is very difficult to demonstrate that specific loci confer higher fitness when they are heterozygous because closely linked loci, which are not directly observed, can interfere, producing a linkage disequilibrium.[31] Alternatively, Nevo suggested that an excess of heterozygotes could be due either to epistatic interactions or to frequency-dependent selection.[32] The second hypothesis would be the association of the *Mdh* locus with the 2La and/or 2Lb inversions of the polytene chromosomes observed in populations of *A. nuneztovari* from western Venezuela by Conn[12] and Conn and others.[13] These investigators found similar results with a significant excess of heterozygotes for both inversions. We propose that the $Mdh_{94}$ and $Mdh_{100}$ alleles of the *Mdh* locus may be situated inside one of these inversions,[33] producing a hitchhiking effect.

The temporal genetic variation analysis carried out for populations from the BR-174 Highway and TUC did not show significant changes for the great majority of loci studied. Despite the limited data, some observations are pertinent. The small temporal allelic variation observed for these populations can be correlated with the high population density of *A. nuneztovari,* mainly for population from BR-174 Highway, which remained high over the last few years due to the abundance of breeding sites arising after the construction of the Balbina Hydroelectric Dam. In *Culicoides variipennis,* similar results were observed, in which the genetic stability was consistent with a high number of specimens, characterized by a permanent population.[34]

The mean heterozygosity observed for populations of *A. nuneztovari* (0.078–0.122) is similar to those obtained for other species of anophelines of the subgenus *Nyssorhynchus,*[35] and are very close to those for populations of *A. nuneztovari* from Suriname (0.117), Venezuela (0.143),[14] and from both sides of the Andes Mountains in Venezuela (0.086 and 0.118).[15] Of the 16 loci, monomorphism was observed in loci *Idh-2, Me, Xdh, Lap-2,* and *Lap-4.* The *Idh-2* and *Me* loci were also monomorphic in localities from Suriname.[14] However, in populations from Táchira, Venezuela, the *Idh-2* locus had two alleles, both with frequencies near 0.50.[15]

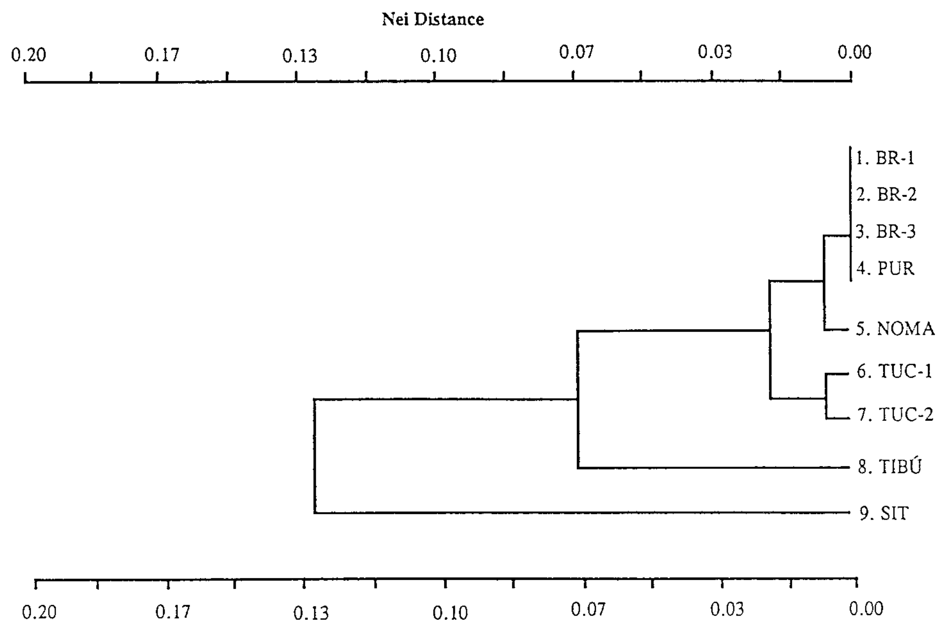


FIGURE 2. Unweighted pair group method using the arithmetic average phenogram from the Nei[29] unbiased genetic distance matrix for all *Anopheles nuneztovari* populations (cophenetic correlation = 0.967). For definitions of populations, see Table 1.

This locus may indicate differences between Venezuelan populations and the six populations in the present study.

In populations from the Brazilian Amazon, the low $F_{ST}$ and $F_{IS}$ values are indicative of small genetic differentiation among subpopulations, as well as random mating within them, resulting in little genetic structuring. Among the 11 polymorphic loci, the *Pgm* locus showed some evidence for differentiation between the populations from PUR and TUC. Corroborating the $F_{ST}$ values, the genetic distances were also low (0.001–0.032), even though the population from TUC was the most diverged within Brazil (Table 4 and Figure 2). In the TUC population, the percentage of the polymorphic loci was higher due to the presence of rare alleles. The presence of rare alleles can indicate mixing of populations, although it contributes little to increasing the level of heterozygosity in natural populations (Table 2).[36] In the early 1980s, the Tucuruí site under went great environmental changes caused by the construction of Tucuruí Hydroelectric Dam, which inundated an area of approximately 2,430 km.[2,37] It is believed that several subpopulations of *A. nuneztovari* were forced to inhabit the same location after they were displaced by the reservoir. The genetic distances values found among the four populations from the Brazilian Amazon are within the limits proposed for intraspecific variation in anopheline mosquitoes,[38] indicating genetic homogeneity on a macrogeographic spatial scale, in spite of the differentiation in the locus *Pgm* between PUR (central Brazilian Amazon) and TUC (eastern Brazilian Amazon). Our results are consistent with the analysis of the ultrastructure of eggs and the ITS2 sequence, which showed genetic similarity between the central and eastern Brazilian Amazon,[18,19] as did populations from Amazon Basin analyzed by polytene chromosomes.[11,12] On the other hand, allozyme similarity is not compatible with the results of mtDNA, which clearly show the existence of two lineages in the Amazon Basin: 1) Belém and Capanema (eastern Brazilian Amazon) and 2) Boa Vista, Puraquequara, and Victoria (northern and central Brazilian Amazon and Suriname).[20] These results underscore the differences between the nuclear and mitochondrial genomes, implying distinctive rates of evolution.[39] Alternatively, nuclear DNA (allozyme) is more susceptible to genetic interpopulation homogenization via gene flow, becoming less sensitive to population subdivision, because of Mendelian inheritance and recombination.[40]

Little genetic structure and low geographic differentiation, supported by the values of $F_{IS}$, $F_{ST}$, and genetic distance, suggest that populations of *A. nuneztovari* from the Brazilian Amazon are recent, with insufficient evolutionary divergence for allozyme differentiation having taken place.

Although the results indicate larger differences in the alleles frequencies for two (α-*Gpd* and *Mdh)* of the 16 loci analyzed between populations from Brazil and Colombia, diagnostic loci were not observed among the populations of *A. nuneztovari* in this study.[41] The degree of genetic divergence obtained by the $F_{ST}$ statistic (0.324) between populations from the Brazilian Amazon and Colombia was higher than that observed between species $C_1$ and $C_2$ of the *A. quadrimaculatus* complex ($F_{ST}$ = 0.219)[42] and lower than the divergence found among the four species of the *A. dirus* complex ($F_{ST}$ = 0.334).[43] The low mean value of $F_{IS}$ (0.007) indicates that random mating among the populations of *A. nuneztovari* is occurring.[44] The degree of divergence among populations from the Brazilian Amazon and SIT measured by distance (0.125–0.148) is within the limits proposed for *Anopheles* species complexes.[38] The geographic distance and presence of the Andes Mountains may have restricted the gene flow, resulting in the higher divergence for the population from SIT. In addition, the presence of an additional band was observed at the α-*Gpd* locus only in the population from SIT, indicating post-translation changes (post-synthesis).[16]

The slightly low genetic divergence among populations from the Brazilian Amazon and Tibú (0.047–0.072) agrees with the high genetic similarity (identity = 0.933) observed among populations from Barinas and Brokopondo.[14] However, our results do not reflect the level of chromosomic differentiation found between the Brazilian Amazon and Tibú, which were called two chromosomal races.[11] The low allozymatic divergence among chromosomally distinct populations can be interpreted as recent evolutionary history.[14]

The phenogram of Figure 2 shows clearly that the populations of *A. nuneztovari* were genetically clustered into three groups: Brazilian Amazon, Tibú, and Sitronela. This result may be indicating the decrease of gene exchange, based in the differentiation of α-*Gpd, Mdh, Est-5,* and *Acon* loci, resulting in structuring groups. These clusters are in partial agreement with the ITS2 sequence and mtDNA analysis, whereas Colombian and Venezuelan populations were gathered in one single and distinctive group from the one in the Amazonian Basin.[19,20]

Analysis of polytene chromosomes in *A. nuneztovari* indicates the occurrence of three cytotypes. Cytotypes B and C differ from the cytotype A by a fixed inversion in the X chromosome, inversion frequencies in the autosomic chromosomes, and one chromocenter.[11–13] Fixed inversions in the X chromosome are frequent among anopheline mosquitoes, and can be involved in the speciation process.[45] A method to assess the existence of reproductive isolation (post-zygotic) between populations from Brazilian Amazon (cytotype A) and Colombia (cytotype C) is experimental crossings.[46,47] Unfortunately, laboratory colonies of *A. nuneztovari* have not been successfully maintained, hampering a better knowledge on the existence of reproductive barriers between these populations. Analysis of mtDNA showed three distinct lineages among the 12 populations, one of which consisted of populations from SIT and Venezuela. However, the population from SIT does not share haplotypes with the other populations studied, which according to Conn and others is in part due to the geographic isolation.[20] Even though diagnostic differences was not demonstrate, hierarchical analysis in combination with multiple lineages strongly suggests that speciation events are in progress.[20] Consensus analysis of the ITS2 sequence showed low or no difference among nine populations from South America, including SIT, which could indicate a slow rate of evolution for this region in anophelines species.[19] Fritz and others have discussed the possibility that natural selection can be influencing the establishment and maintenance of the similarity of the ITS2 region among geographically distant populations and among closely related species of the genus *Anopheles.*[19] Morphologic similarity of male genitalia does not coincide with allozyme differentiation between Brazilian Amazon and SIT.[17] These results re-

inforce the hypothesis that the speciation process in anopheline mosquitoes is frequently accompanied by few morphologic changes.[9]

In summary, the absence of diagnostic loci and the degree of the genetic divergence observed may indicate a separation that has occurred recently among populations of *A. nuneztovari,* suggesting recent speciation. The degree of genetic divergence detected for the population from Sitronela suggests that this population can eventually be considered an independent evolutionary unit.[48] Additional molecular studies and detailed morphologic analysis of all stages of development need to be conducted with *A. nuneztovari* from Sitronela to provide a better understanding of the high allozyme differentiation that has reached the interspecific level estimated by genetic distances. Allozyme data may clarify the taxonomic status of *A. nuneztovari* and aid in understanding its role in human malaria transmission in northern South America.

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