Gene Differentiation in Chromosome Races of *Anopheles nuneztovari* (Gabaldon)[1]

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ABSTRACT. In the subgenus *Nyssorhynchus* of the Neotropical anophelines, *An. nuneztovari* is a well-known malaria vector in portions of Colombia and Venezuela, but evidently not east of the Orinoco or in the Amazon basin. Populations previously found to differ in behavioral ecology and by a fixed chromosome inversion were examined for up to 23 electrophoretically detectable allozyme loci. We found genetic heterozygosity to be on a par with other insects studied by this method. However, we found that genetic identities estimated from the allozyme data suggest a lack of genic differentiation, indicating that divergence may be a relatively recent event in the evolutionary history of the chromosomally distinct populations.

INTRODUCTION

It is a well-known fact that sibling species are of common occurrence in the Culicidae, as witnessed by the occurrence of at least six such species in the *Anopheles gambiae* complex. White (1974) has reviewed and discussed this complex from a historical perspective, discussing the identification of the species, their relative distributions and biology, and their role in malaria and filariasis transmission. Although morphologically indistinguishable, a variety of methods have shown that the species can be recognized on the basis of differences in egg features, physiological tolerances of sea water, behavior, the statistical means or ratios for certain characteristics, cytogenetics, and disease transmission capabilities. Kitzmiller et al. (1973) have pointed out that the problem is one involving evolution in the physiological sense without a corresponding degree of morphological differentiation in these organisms.

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The evidence collected to date suggests that *Anopheles nuneztovari*, of the "tarsimaculatus" group of the Neotropical subgenus *Nyssorhynchus*, may also consist of a sibling species group. Although Rozeboom and Gabaldon (1941) included six species in the "tarsimaculatus" group of *Nyssorhynchus*, of which *An. nuneztovari* was one, *An. nuneztovari* was actually described on the basis of differences in male terminalia by Gabaldon (1940) a year earlier. Kitzmiller et al. (1973) point out that the distribution of *An. nuneztovari* includes all of the Amazon basin, the South American countries of the Caribbean Coast, and Colombia, Ecuador, Peru and Bolivia.

Populations of *An. nuneztovari* differ in two characteristics that have been useful in differentiating species of the *An. gambiae* complex. First, Elliott (1968) summarizes the data to suggest that there are at least two sibling species or ecotypes; one which is a vector for malaria and one which is not. Evidently, the malaria vector predominates in Colombia and Venezuela west of the Orinoco River and is a late night biter. The non-vector type can be found east of the Orinoco River and especially in Brazil. It prefers to take a blood meal at sundown. Panday's (1977) suggestion that this type may be involved in malaria transmission in two recent outbreaks was never confirmed and the presence of *An. oswaldoi* and *An. aquasalis* offers alternative vector species.

The second feature useful in differentiating vector and non-vector groups involves the chromosomes. Kitzmiller et al. (1973) have described and compared the chromosomes of both types. These workers show that a fixed inversion difference in the X-chromosome differentiates vector populations from the state of Barinas, Venezuela, and the department of Norte de Santander, Colombia, from non-vector populations sampled in Manaus, Amazonas, Brazil. No heterozygotes were found in the 416 chromosome slides Kitzmiller et al. examined.

Mahon et al. (1976) and Miles (1978) have clearly demonstrated that allozymes, heritable mutant forms of enzyme proteins, can be used to differentiate the various species of the *Anopheles gambiae* complex. Accordingly, we have examined putative vector and non-vector populations for their electrophoretically determined enzyme variation in hopes of uncovering suitable, diagnostic loci. The results, not totally expected by us, are reported here.

MATERIALS AND METHODS

Biting collections of *An. nuneztovari* were made near Brownsweg, District Brokopondo, Suriname, on June 1-3, 1979, at sundown by Dr. Roy S. Panday. These were returned on dry ice to the Pan American Health Organization (PAHO) Laboratory at Maracay, Venezuela, for electrophoretic analysis. In Venezuela, late night biting collections of *nuneztovari* were made near Puerta Vivas (Barinas) on June 15, 1979, and returned alive to the PAHO laboratory. At the laboratory, mosquitoes were kept in liquid nitrogen until electrophoretic analysis was done, mosquitoes from each population being run on the same gels to determine allele homologies.

Statistical analysis of the data included contingency χ^2 tests to evaluate the hypothesis that population x was similar in genotype frequencies to population y. This test was performed using the TI-59 program ST-14 corrected for inflated χ^2 values by substituting a 1 for each empty cell in the R x C matrix. Rare genotypes were pooled into one class to keep within the limits of the R x C program. In addition, after calculating gene frequencies for each locus, the data were analyzed for Nei's (1972) index of genetic similarity.

The electrophoretic techniques and equipment we used are described in Steiner and Joslyn (1979). The electrophoretic loci we analyzed include: aldehyde oxidase (Aldox; E.C.1.2.3.1), esterase (5 loci: Est-1, Est-2, Est-3, Est-4, Est-5; E.C.3.1.1.1), glutamate oxaloacetate transaminase (2 loci: Got-F, Got-S; E.C.2.6.1.1), α-glycerophosphate dehydrogenase (α-gpdh-F; E.C.1.1.1.8), hydroxybutyrate dehydrogenase (H-β-bdh; E.C.1.1.1.30), hexokinase (3 loci: Hk-F, Hk-M, Hk-S; E.C.2.7.1.1), isocitrate dehydrogenase (2 loci: Idh-F, Idh-S; E.C.1.1.1.42), leucine aminopeptidase (Lap-F; E.C.3.4.11), malate dehydrogenase (Mdh-F; E.C.1.1.1.37), malic enzyme (Me; E.C.1.1.1.40), 6-phosphogluconate dehydrogenase (6-pgdh-F; E.C.1.1.1.44), phosphoglucose isomerase (Pgi; E.C.5.3.1.9.), phosphoglucomutase (Pgm; E.C.2.7.5.1), and xanthine dehydrogenase (2 loci: Xdh-F, Xdh-S; E.C.1.2.1.37). In this paper, each band is interpreted as a single allele, although in reality they may consist of several alleles which are not separable by charge differences. In the latter case, the name "electromorph" has been used to define the theoretical case of bands which may consist of more than one allele (King and Ohta, 1975). We use the term allele throughout this report because to date only one study (for Pgm; Scozzari et al., 1977) has investigated the possibility that electromorphs exist in mosquitoes, and because Cochrane (1976) has shown that electromorphs within a *Drosophila* species are relatively rare and have little effect on gene frequencies of common alleles. To date, no studies have confirmed that electromorphs are structural gene mutations.

In this paper, the electromorphs or alleles are numbered such that the most commonly occurring allele in the Barinas, Venezuela, sample is assigned the number 1.00 and is taken as the standard for that locus. Slower migrating alleles on the starch gels have a lower numerical designation and faster migrating alleles have a higher numerical designation. The numerical differences between the alleles within loci reflect the millimeters of distance from the standard allele (i.e., $Pgm^{\cdot 98}$ migrates 2 mm slower than $Pgm^{1\cdot 00}$ and 4 mm slower than $Pgm^{1\cdot 02}$). Although no attempt to determine the genetic basis for our observations is made here, the segregation and linkage genetics for most of these loci have been reported for other mosquito species and are reviewed by Steiner and Joslyn (1979), Kitzmiller (1976) and Munsterman and Craig (1979). Banding patterns on the starch gels in the present study were always consistent with respect to genetic interpretations. Where allele interpretations were in doubt, the individual for that locus was dropped from the data file, resulting in approximately a 6% reduction in total sample numbers.

RESULTS

Although we exposed a total of 23 enzyme loci, only 22 were examined in each population. In this study, the same loci were examined in each population with the exception of Aldox which was not analyzed in the Suriname sample, and H-β-bdh which was not analyzed in the Venezuela sample. Monomorphic loci are listed as follows with the number analyzed for Suriname and Venezuela in the first and second parentheses, respectively: Pgi (215) (196), Xdh-S (7) (83), Aldox (0) (15), Est-1 (51) (92), Est-2 (20) (39), Hk-S (62) (21), Got-S (93) (27), Got-F (77) (57) and H-β-bdh (69) (0).

The loci which had two or more alleles present are listed in Table 1. Not every locus was polymorphic when only a single population was considered; for example, of the 14 loci segregating when both populations were considered, only 8 were segregating in Suriname and 11 in Venezuela. Only 7 loci (Pgm, Xdh-F, Mdh-F, Idh-F, Est-3, Est-4 and Lap-F) were segregating in both populations. Even then, unique low frequency (1%-5%) alleles showed up in 4 loci in Venezuela and for one locus in Suriname.

The data of Table 1 indicate a fairly large number of alleles to be segregating in each population. Better ways of representing this genetic variability are shown in Table 2, where two indices of variation are presented. The first index estimates the percentage of loci segregating for alleles that occur in greater than 1% frequency. The loci sampled are assumed to be representative of all loci in the genome; and we can conclude that 36.4% of the loci are segregating in the Suriname sample and 50% are segregating in the Venezuela sample. The second index is based on the number of heterozygotes observed in the population, summed for each locus analyzed per individual and divided by the product of the sample size and the total number of loci studied. This index of the heterozygote frequency (heterozygosity) is 11.7% in Suriname and 14.3% in Venezuela. In either case, the levels of genetic variation are similar to those seen for other insects (Selander, 1976; Steiner et al., 1976).

A locus-by-locus χ^2 analysis was also done to determine if genotype frequencies in each population were similar. The results in Table 3 show that nine of the 21 loci examined in both species were significantly different at the 1% level or lower. Examination of Table 1 shows that for these 9 loci, the majority of cases are due to strong differences in the allele frequencies of the most common allele.

The genetic identity values for each locus and the average genetic identity are shown in Table 4. The high genetic identity occurs even though one locus appears to be diagnostic. This is Est-5 which has alleles Est-$5^{.98}$ and Est-$5^{1.02}$ segregating in population Suriname and Est-$5^{1.00}$ unique to and fixed in Venezuela. Although Lap-F has a relatively low genetic identity index, it has allele Lap-$F^{1.00}$ as the most common albeit at greatly different frequencies. We cannot conclude that allele Lap-$F^{1.02}$, found only in the Venezuela sample, is unique in nature to that population. Further sampling may reveal it to occur in low frequency in Suriname as well. This allele may eventually prove to be diagnostic for Venezuela and certainly contributes to the relatively low genetic identity observed for this locus.

DISCUSSION

The evidence presented in Tables 1 and 2 indicate that relatively high levels of genetic variation are present, which should facilitate genetic differentiation. The levels observed in the *An. nuneztovari* populations are similar to those observed in *Drosophila* (Steiner et al., 1976; Saura et al., 1973; Ayala et al., 1971), *Tephriditae* (Berlocher, 1976), *Heliothis* (Sluss et al., 1978), and other insects, including mosquitoes (Miles, 1978).

It is interesting, however, that very little genetic differentiation has actually taken place between the chromosome races of *Anopheles nuneztovari*. The differences in allele frequencies and presence or absence of low-frequency alleles can be easily explained if the populations differ in environmental, ecological, or behavioral patterns. Even features such as the breeding structure of the population, or the nature of the founding of the population, etc., can affect the amount of differentiation (Roughgarden, 1979).

The studies on insects indicate that genetic identities at various taxonomic levels are usually distinct (Table 5). However, in the *Drosophila bipectinata* group, a high genetic identity of .92 seen at the sibling species level matches the high estimate seen for *An. nuneztovari*. This occurs between the sibling species pairs *D. pallens* (The Philippines) and *D. malerkotliana* (S. E. Asia), and *D. pallens* and *D. bipectinata* (Fiji and Samoa). In these cases, although overlap occurs in some areas, geographical isolation involving oceanic barriers is complete with respect to the center of the distribution of each *Drosophila* species, a feature which remains to be investigated in *nuneztovari*.

There is evidence that recently evolved species have high genetic identities. For example, two Hawaiian *Drosophila* species have been studied morphologically (Val, 1977) and chromosomally (see Sene and Carson, 1977). Ahearn and Val (1975) have found these species, *D. silvestris* and *D. heteroneura*, to produce fertile offspring regardless of the direction of the cross. Because the island of Hawaii is geologically young, the *Drosophila* that occur there are less than half a million years old and so allozyme differentiation has probably not had time to occur (Sene and Carson, 1977). This implies that speciation is relatively recent for the *D. bipectinata* complex as well, and that the *nuneztovari* chromosomal races may represent speciation in progress.

Interestingly, even though the populations we analyzed have a high genetic identity, it appears that the Est-5 locus may be diagnostic for chromosomal type (Ayala and Powell, 1972). The Est-$5^{1.00}$ allele is unique to the Venezuela sample, while Est-$5^{\cdot 98}$ is unique to the Suriname sample. Relatively small sample sizes and limited population samples preclude use of the locus, however, to differentiate the chromosome races, at least until more populations can be sampled. This particular locus may provide the type of species-specific characterization which Faran (1979) points out will be useful for mosquito systematists.

The high genic similarity between the chromosomal races is unexpected in light of the genetic differences found to exist between the sibling species of the *Anopheles gambiae* complex (Miles, 1978; Mahon et al., 1976) and between ecotypic races of *Aedes aegypti* (Scott and McClelland, 1975). These parallel a recent case we have described for the Neotropical species *Anopheles aquasalis* and its relative, *An. emilianus* (Steiner et al., submitted). Table 5 shows that a genetic identity of more than 91% exists for this species pair. These can be related to an estimate greater than 0.65 for the Nearctic anopheline species, *An. quadrimaculatus, An. walkeri* and *An. atropos* (Kitzmiller et al., 1978), an estimate that appears to be in line with species estimates for other *Insecta*. It leaves us with the interesting question of why these "sibling" species of Neotropical anophelines should show such apparently recent genetic divergence? Future studies in preparation and in planning will establish what the genetic identity estimates will be for Neotropical "non-sibling" species, and if other vector-nonvector species pairs have similar levels of genetic identity.

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Table 1. Allele frequencies at 23 enzyme loci in two populations of *Anopheles nuneztovari*.

Locus	Allele	Suriname	Venezuela
Pgm	.98	.075	.079
	1.00	.661	.777
	1.02	.259	.144
	1.04	.005	.000
	N	199	184
Xdh-F	.99	.016	.076
	1.00	.943	.867
	1.01	.041	.058
	N	157	165
Mdh-F	.97	.019	.302
	1.00	.974	.613
	1.03	.006	.085
	N	78	53
Est-5	.98	.982	.000
	1.00	.000	1.00
	1.02	.018	.000
	N	27	44
Hk-F	.98	.000	.018
	1.00	1.00	.982
	N	65	28
Hk-M	.98	.000	.018
	1.00	1.00	.982
	N	65	28
Idh-F	1.00	.963	.931
	1.01	.020	.028
	1.03	.020	.042
	N	27	108
6-pgdh	.98	.008	.000
	1.00	.992	1.00
	N	63	55
α-gpdh	.97	.000	.038
	1.00	1.00	.962
	N	90	172
Me	.99	.000	.004
	1.00	1.00	.996
	N	21	120
Idh-S	.98	.000	.022
	1.00	1.00	.903
	1.02	.000	.074
	N	89	135
Est-3	.99	.409	.205
	1.00	.579	.785
	1.01	.012	.011
	N	126	137
Est-4	.98	.009	.000
	1.00	.547	.585
	1.02	.444	.415
	N	106	130
Lap-F	.98	.150	.115
	1.00	.850	.490
	1.02	.000	.395
	N	20	52
Got-F	1.00	1.00	1.00
	N	77	57

Table 1, cont'd.

Locus	Allele	Suriname	Venezuela	Locus	Allele	Suriname	Venezuela
Got-S	1.00	1.00	1.00	Est-1	1.00	1.00	1.00
	N	93	27		N	51	92
Pgi	1.00	1.00	1.00	Est-2	1.00	1.00	1.00
	N	215	196		N	20	39
Xdh-S	1.00	1.00	1.00	Hk-S	1.00	1.00	1.00
	N	7	83		N	62	21
Aldox	1.00	UN	1.00	H-β-bdh	1.00	1.00	UN
	N		15		N	69	

N = Number of individuals analyzed

UN = Unanalyzed

Table 2. Heterozygosity estimates for two populations of *An. nuneztovari*. A locus is considered polymorphic if the allele frequency is 1% or greater, which reduces the possibility of the polymorphism being maintained by mutation pressure.

	Suriname	Venezuela
No. of loci polymorphic	8	11
Total no. of loci analyzed	22	22
% loci heterozygous	36.4	50.0
% heterozygotes in population sample[1]	11.7	14.3

[1]Summed over all loci.

Table 3. Significant χ^2 values for between sample comparisons of two *Anopheles nuneztovari* populations. The degrees of freedom are listed in parentheses. All values are significant at the 0.1% level unless otherwise indicated. All other enzyme loci were not significantly different in genotype numbers.

Locus	χ^2/(df)	Locus	χ^2/(df)
Pgm	19.34 (6)*	Idh-S	19.39 (2)
Xdh-F	14.41 (4)*	Est-3	23.83 (4)
Mdh-F	36.04 (5)	Lap-F	20.67 (4)
Est-5	71.00 (2)*	α-gpdh	7.15 (1)*
Idh-F	14.22 (4)*		

*.01 > P > .001

Table 4. Locus and average genetic identity values for 14 polymorphic and 7 monomorphic loci in two populations of chromosomally differentiated *Anopheles nuneztovari*.[1]

Locus	Genetic Identity	Locus	Genetic Identity
Pgm	.982	Idh-S	.996
Xdh-F	.997	Est-3	.936
Mdh-F	.899	Lap-F	.785
Est-5	0	Hk-F	.999
Idh-F	.999	Hk-M	.999
α-gpdh	.999	Est-4	.998
6-pgdh	.999	Me	.999

Average = .933

[1]Monomorphic loci include Pgi, Xdh-S, Est-1, Est-2, Got-S, Got-F and Hk-S, each fixed for the same allele in both populations.

Table 5. Comparative genetic identities at three recognized evolutionary levels in different genera of insects.

Species group	No. of Species	Genetic Identity: Within Species	Genetic Identity: Sibling Species	Genetic Identity: Non-sibling Species	Author(s)
Drosophila willistoni	4	.97	.489-.623	.287-.489	Ayala and Tracey, 1974
Drosophila mesophragmatica	6	--	.626-.767	.259-.767	Yang et al., 1972[a]
Drosophila bipectinata	6	--	.821-.922	.649-.911	Yang et al., 1972[a]
Drosophila mulleri	4	.99	.756-.884	.722-.749	Zouros, E., 1974
Heliothis zea & H. virescens	2	--	--	.874	Sluss et al., 1978
Dendroctonus ponderosae	1	.92-.99	--	--	Stock and Guenther, 1979
Anopheles aquasalis	2	--	.91-.94	--	Steiner et al., submitted
Anopheles nuneztovari	2	.95	.93	--	This study[b]
Anopheles quadrimaculatus	3	--	--	.65	Kitzmiller et al., 1978

[a]Calculated using Rogers' (1972) coefficient of similarity.

[b]Sibling species status is dependent upon the outcome of hybridization studies which remain to be done.