Random Amplified Polymorphic DNA Analysis of *Anopheles nuneztovari* (Diptera: Culicidae) from Western and Northeastern Colombia

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Random amplified polymorphic DNA (RAPD) markers were used to analyze 119 DNA samples of three Colombian Anopheles nuneztovari *populations to study genetic variation and structure. Genetic diversity, estimated from heterozygosity, averaged 0.34. Genetic flow was greater between the two populations located in Western Colombia (F_{ST}: 0.035;* Nm*: 6.8) but lower between these two and the northeastern population (F_{ST}: 0.08;* Nm*: 2.8). According to molecular variance analysis, the genetic distance between populations was significant (Φ_{ST} 0.1131, P < 0.001). The variation among individuals within populations (Φ_{ST} 0.8869, P < 0.001) was also significant, suggesting a greater degree of population subdivision, not considered in this study. Both the parameters evaluated and the genetic flow suggest that Colombian* An. nuneztovari *populations are co-specific.*

Key Words: *Anopheles nuneztovari* - genetic diversity - malaria vectors - *Nyssorrhynchus* - ramdon amplified polymorphic DNA - polymerase chain reaction - Colombia

The application of molecular techniques such as single-strand conformation polymorphism (SSCP), random amplified polymorphic DNA (RAPD) and genomic mapping in the field of medical entomology has permitted new interpretations in disease vector systematics and has stimululated the discovery and characterization of cryptic species complexes, as well as providing new perspectives on relationships at higher taxonomic levels (Munstermann & Conn 1997). Nonspecific amplification methods such as RAPD and other multilocus techniques are very useful for analyzing genetic variation within species because they allow the rapid acquisition of a great deal of genetic information (Zhivotovsky 1999).

Molecular studies of *Anopheles nuneztovari* have included internal transcribed spacer 2 (ITS2) comparisons of ribosomal DNA for populations of the malaria vector collected in Colombia, Venezuela, Bolivia, Surinam and Brazil (Fritz et al. 1994). In this study mosquitoes from Colombia and Venezuela had identical ITS2 sequences and were distinguished from mosquitoes from Bolivia, Surinam and Brazil, suggesting that putative cryptic species of *An. nuneztovari* are distinguished by very minor differences in the DNA sequence of the ITS2 region.

Another study of variability in the mosquito populations from Bolivia, Brazil, Colombia, Surinam and Venezu-

ela, using restriction enzymes, was done by Conn et al. (1998), suggesting that *An. nuneztovari* is a monophyletic species. Genetic distance analysis distinguished five haplotype lineages, and the relationships among the 12 populations revealed distinctive lineages, one in Venezuela, Colombia and two within the Amazon Basin.

Onyabe and Conn (1999) evaluated the ITS2 intragenomic heterogeneity for five Neotropic populations of *An. nuneztovari*: three from Brazil, one from Colombia and one from Venezuela. Sequence divergence per genome was significantly higher in mosquitoes from Brazil than in those from Venezuela and Colombia.

Scarpassa et al. (2000) studied the genetic variability of mtDNA in four populations of *An. nuneztovari* (three from Brazil and one from Colombia), using restriction endonucleases. Haplotype diversity was slightly elevated in all populations. Nucleotide diversity was lowest in the Colombian population and highest in the Brazilian Amazon populations. Their study suggested that these geographic populations might eventually constitute separate species.

As can be seen from the foregoing, significant degrees of differentiation have been found among different populations of Venezuela, Colombia and Brazil; therefore the purpose of this study was to determine whether this divergence exists among the populations of Western and Northeastern Colombia. To study these populations, the RAPD-polymerase chain reaction (PCR) technique was used, and genetic parameters such as heterozygosity and genetic flow were evaluated using molecular variance and statistical F_{ST} analyses.

MATERIALS AND METHODS

Collection, manipulation and identification of An. nuneztovari - Samples of *An. nuneztovari* were collected in three geographic zones of Colombia where it is consid-

ered a primary malaria vector: Sitronela in the municipality of Buenaventura (3º54'N, 77º05'W), Valle Province; Tai in the municipality of Tierralta (8º10'N, 76º04'W), Cordoba Province; and Tibú (8º39'N, 72º 59'W) in the Province of Norte de Santander (Fig. 1). Mosquitos samples was realized using human-bait following standard WHO recommendations (WHO 1975) and identified on the basis of their morphological traits (Faran 1980, Delgado & Rubio-Palis 1993), and preserved individually in 100% ethanol in 1.5 ml microcentrifuge tubes at -20°C.

DNA extraction and RAPD-PCR conditions - DNA isolation was done according to Coen et al. (1982), modified as in Romans (in Black IV & DuTeau 1997). The conditions of the specific amplification reaction (1 ng/µl DNA, 1X buffer, 2.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.2 mM oligonucleotides, 1U *Taq*, adjusted to 25 µl with H$_2$O) for *An. nuneztovari* were standardized in order to obtain well-defined, consistent and reproducible banding patterns. Amplification products were visualized on 1.5% agarose gels according to the method of Sambrook et al. (1989). Gels were run at 300 V in a horizontal electrophoresis chamber (Life Technologies Inc.) for approximately 1 h. Lambda DNA, digested with PstI, was used as a molecular-size standard in each run. The bands were visualized with ethidium bromide (0.8 µg/ml). Gels and banding patterns were visualized and recorded with Eagleeye II (Stratagene) equipment.
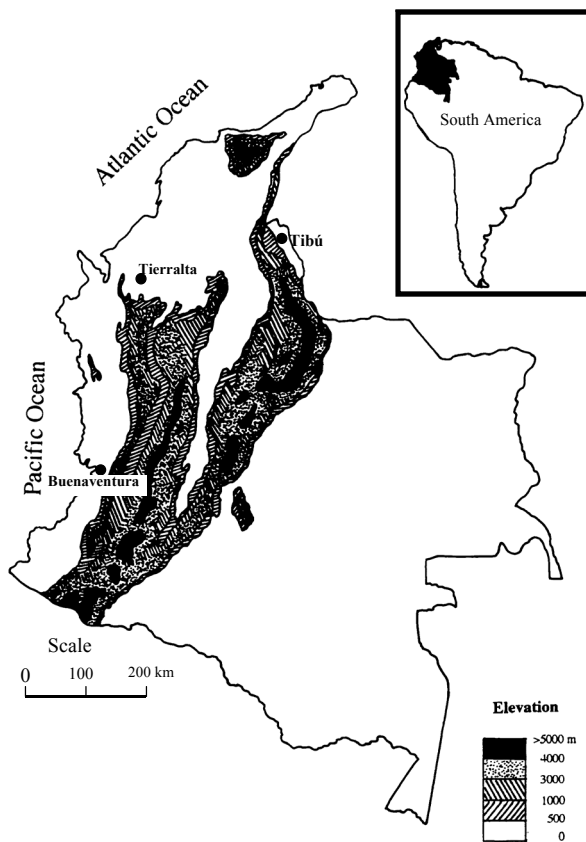

Fig. 1: geographic location of three Colombian *Anopheles nuneztovari* populations: Buenaventura (Valle), Tierralta (Córdoba) and Tibú (Norte de Santander) analyzed from 65 random amplified polymorphic DNA-polymerase chain reaction loci.

Selection of polymorphic oligonucleotides - In this study 554 oligos RAPD-PCR from Operon Technologies®, corresponding to the series A-Z and AA-AN, were analyzed for three individuals from each locality. Negative and positive controls (*An. darlingi* DNA) were used in all the reactions in order to detect artifacts of the technique and to verify the reliability of the amplification process. Twenty oligonucleotides selected from 70 polymorphic primers were used for PCR amplification of 42 individuals from Buenaventura, 39 from Tierralta and 38 from Tibú. For the final analysis, only 10 primers were selected.

Taxonomic analyses of the RAPD-PCR patterns - Different methods were used to analyze the RAPD-PCR fragments obtained from the three *An. nuneztovari* populations in Colombia. Genetic similarity analysis was performed using NTSYS software, vers. 2.0 (Rohlt 1994), in which dendrograms were generated by using the program's TREE option. The unweighted pair-group method with arithmetic means (UPGMA) described by Sneath and Sokal (1973), as well as the programs *RAPD-PLOT* and *RAPD-BOOT* written in FORTRAN language, developed for mosquito subspecies studies (Black IV 1995), were also used. Multiple correspondence analysis (MCA) using the SAS (1979) computer program was employed to study the relationships among the individuals of the three populations with a multidimensional vision of arrangement. Conglomerate analysis was applied, using the matrix for the MCA and UPGMA. The resulting groups were identified by symbols in a three-dimensional diagram.

The consensus tree was obtained using the program RAPBOOT.TREE (Black IV 1995), which creates a cluster of *n* dendrograms of genetic distance. A total of 100 replications were made, in which the length of the branches varied without changing the topology of the tree per se.

Genetic analysis of the An. nuneztovari populations - Genetic variation and levels of population structure of the three *An. nuneztovari* populations were assessed by estimating heterozygosity and F$_{ST}$ values, the migration rates (*Nm*) and AMOVA.

In order to calculate heterozygosity, three methods were used: the traditional square root of q (Apostol et al. 1996), the Lynch and Milligan (1994) method and a Bayesian method proposed by Zhivotovsky (1999). Heterozygosity was calculated according to Black IV and Munstermann (1996) and Apostol et al. (1996), which consider the absent bands (q) and estimates $q_j(i)$, the frequency of the null allele at locus *i* ($I = 1,...,L$) in population $j(j = 1,...,r)$ as: $q_j(I) = \sqrt{x_j(i)}$, where $x_j(i)$ is the frequency of null recessive homozygotes in population *j* at locus *i*, given the difficulties of obtaining the observed heterozygosity from dominant markers (Black IV 1993,1995, Black IV & Munstermann 1996, Tabachnick & Black IV 1996, Yan et al. 1999).

The statistical F$_{ST}$ is the ratio of the observed variance (S^2) in the frequency of an allele among populations in relation to its maximum variance in the total population. The F$_{ST}$ values and the *Nm* migration rates were calculated using the RAPDFST computer program (Black IV 1995), assuming locus RAPD dominance and a population at Hardy-Weimberg equilibrium. The RAPDFST program estimates the F$_{ST}$ from the formula proposed by

Wright (1951): $F_{ST} = s_q^2/(\bar{q}(1-\bar{q}))$ where s_q^2 is the variance in the frequency of a RAPD allele among subpopulations and $\bar{q}$ is the weighted average frequency among all sub-populations (Apostol et al. 1996). It also estimates the effective migration rate (Nm) by means of the equation $Nm = (1-F_{ST}) / (4F_{ST})$ and the θ_{ST}, which represents the population structure.

AMOVA was run using the WINAMOVA program, vers. 1.5 (Excoffier@sc2a.unige.ch), which calculates the components of the internal variances and the statistical F_{IS} values (Excoffier et al. 1992). The files were created with the AMOVA-PREP 1.01 program (Miller 1998) used for dominant markers like the RAPDs. The Φ_{ST} value, which is analogous to F_{ST}, represents the population structure. Significance levels and the Φ values were computed 1000 times by nonparametric procedures of exchange.

RESULTS

Descriptive analysis of RAPD-PCR loci - Of the 544 oligonucleotides screened, 104 did not produce amplification, 440 displayed variable amplification qualities, and 10 were highly polymorphic and reproducible, producing a total of 65 bands in the three populations analyzed (Table I). The frequency of the selected bands in the three populations ranged from 7-92%. The polymorphic bands ranged in size from 396-1782 bp.

Taxonomic analysis - RAPD-PCR polymorphic bands corresponding to phenotypic classes AA and Aa were considered as RAPD loci. The final analysis with 65 RAPD loci was done using a presence-absence matrix in 119 individuals. The results suggest the presence of a single *An. nuneztovari* species in the western and northeastern Colombia populations.

The NTSYS dendrogram of genetic similarity showed a greater tendency of clustering between Buenaventura and Tierralta individuals, discretely separated from Tibú individuals (Fig. 2). Analysis of polymorphic bands with the RAPDPLOT software generated a dendrogram of genetic-similarity in which the individuals from all three populations showed the same tendency to group, with no noticeable differences (results not shown).

Nei's consensus tree of genetic similarity, with a bootstrapping of 100 "pseudoreplicate" datasets, revealed a greater genetic similarity among individuals from Western Colombia than among those from the Northeast (Fig. 3). This may indicate that the geographic localization of the Tierralta and Buenaventura populations permits a greater genetic flow among individuals and that the separation gradient among the three populations is possibly not impeding this flow as can be seen in Fig. 3. The hight bootstrap scores (100%) is suggesting a strong support for the obtained cluster.

In a second approach to understanding the genetic relationship among the Colombian populations of *An. nuneztovari*, a multidimensional MCA identified in the one dimension, two subspaces, a negative one where all individuals of the Tibú population were concentrated and a positive one where individuals from the Tierralta and Buenaventura populations were concentrated. In the third dimension a clear separation was observed between these last two populations (Fig. 4). The similar positive values shared by individuals from the Buenventura and Tierralta populations in the first dimension were probably due to the common elements in the two; while the height similarities between each of these western populations with individuals from the northeastern population also express the common elements among the three populations.

To find clusters of individuals with similar banding patterns, a multiple-correspondence matrix was used. Fig. 4 shows the 16 clusters identified and located in a multidimensional diagram. Mixed groups indicated similarity in the band patterns of each. Mixtures between two populations were always formed between Buenaventura and Tierralta – never between these two populations and the one from Tibú.

Population genetics analysis - Average heterozygosity among the 65 RAPD bands was 0.34. Similar values of heterozygosity between and within populations were obtained when this parameter was calculated using different methods. The values ranged from 0.3217-0.3585 (Table II). In order to calculate heterozygosity with the Lynch and Milligan method (1994), the analysis was restricted to

TABLE I

Number of random amplified polymorphic DNA-polymerase chain reaction polymorphic bands produced by oligonucleotides (Operon Technologies®) applied to *Anopheles nuneztovari* populations

Oligonucleotides	Sequence	GC contents	Polymorphic bands
A07	5'- GAA ACG GGT G –3'	60%	5
A17	5'- GAC CGC TTG T –3'	60%	7
E03	5'- CCA GAT GCA C –3'	60%	6
L02	5'- TGG GCG TCA A –3'	60%	8
M04	5'- GGC GGT TGT C –3'	70%	4
M09	5'- GTC TTG CGG A –3'	60%	8
N08	5'- ACC TCA GCT C –3'	60%	6
P17	5'- TGA CCC GCC T –3'	70%	7
Y17	5'- GAC GTG GTG A –3'	60%	5
Z10	5'- CCG ACA AAC C –3'	60%	9
Total			65

bands with frequencies < 1-(3/N) to avoid selecting loci with a high frequency of null alleles. This analysis eliminated the bands with frequencies > 97.47. The resulting values demonstrate the existence of highly polymorphic populations.

The expected heterozygosity obtained with the Bayesian method (Zhivotovsky 1999) confirmed a high level of genetic polymorphism among *An. nuneztovari* in Colombia. The values of this parameter in each of the populations showed the same tendency toward diversity. There
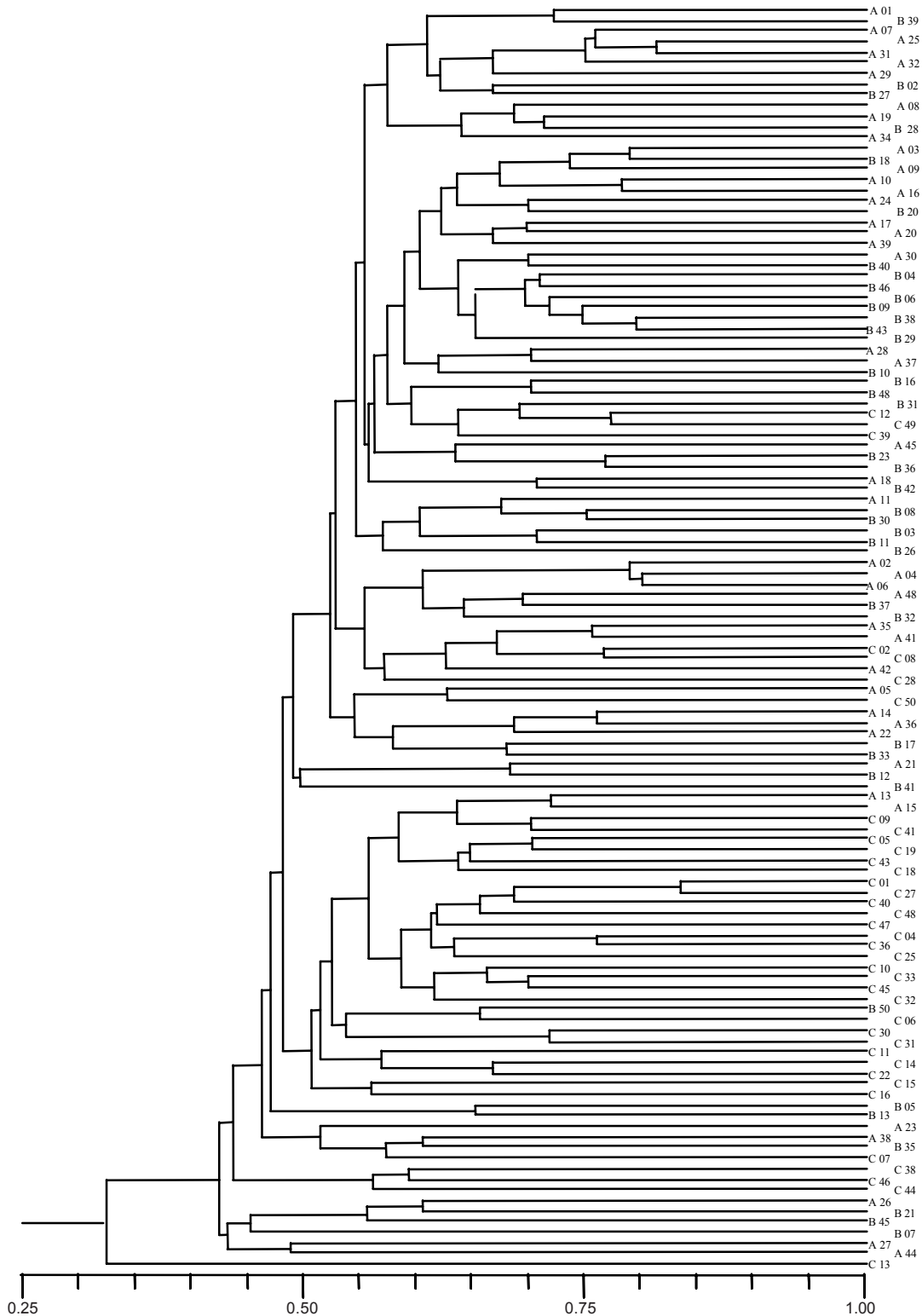

Fig. 2: NTSYS dendrogram of genetic similarity for Colombian *Anopheles nuneztovari* populations analyzed from 65 random amplified polymorphic DNA-polymerase chain reaction loci (A = Buenaventura; B = Tierralta and C = Tibú).
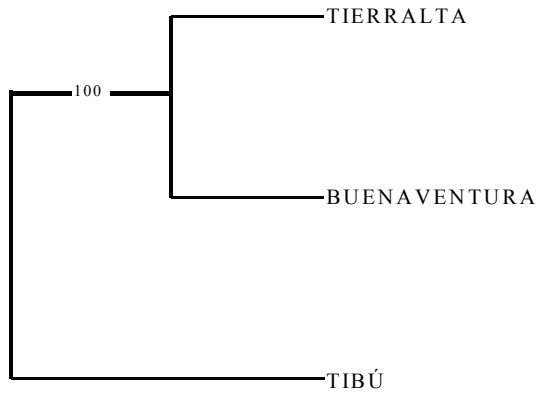
Fig. 3: majority consensus tree for three Colombian *Anopheles nuneztovari* populations based on analysis of 65 polymorphic random amplified polymorphic DNA-polymerase chain reaction loci. The number in the branch indicates the number of times that the topology was consistent.

was a uniform distribution of q (Table II).

The estimated F_{ST} averages among the three populations were slightly lower with the F coefficient of Wright (1951) than with the statistical θ and the Lynch and Milligan as well as the respective standard deviations (Table III). Nevertheless, the Pearson correlation coefficients ($r \geq 0.99$, $r \geq 0.95$ and $r \geq 0.96$; $P \leq 0.0001$) were high, both between values of F_{ST} estimated using Wright and Lynch and Milligan, and between θ and Lynch and Milligan through loci.

The migration rates obtained confirm a differential genetic flow between Colombian populations of *An. nuneztovari*. These values were greater between the populations from Buenaventura and Tierralta than between these and Tibú, where the number of migrants per generation was relatively smaller (Table III).

The AMOVA showed that Φ_{ST} among the three populations analyzed (0.1131) was highly significant (Table IV).
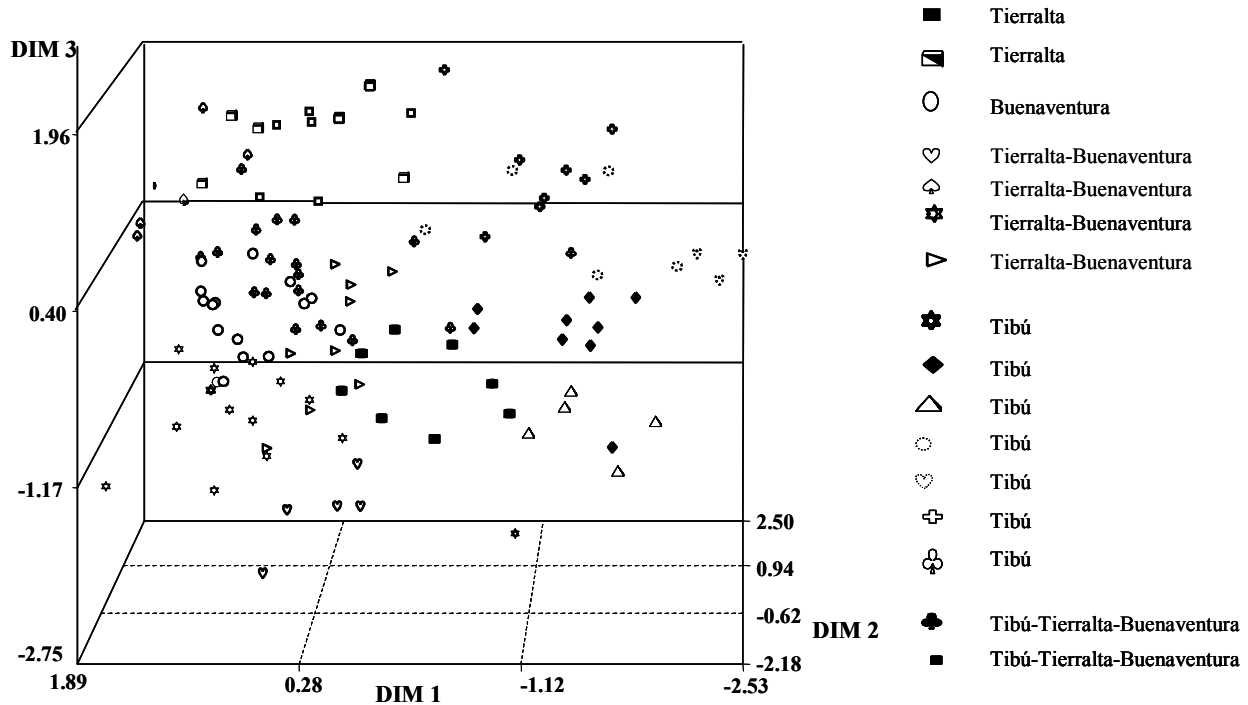

Fig. 4: UPGMA conglomerates in three Colombian *Anopheles nuneztovari* populations analyzed from 65 random amplified polymorphic DNA-polymerase chain reaction loci.

TABLE II

Heterozygosity values in three Colombian populations of *Anopheles nuneztovari* calculated using three methods

Population	Estimated square root of q	Lynch &Milligan	Bayesian method	
			Uniform q distribution	Nonuniform q distribution
Buenaventura	0.3217 ± 0.0127	0.3168	0.3306	0.3287
Tierralta	0.3274 ± .1280	0.3219	0.3364	0.3297
Tibú	0.3304 ± 0.0835	0.3266	0.3398	0.3388
Total	0.3434 ± 0.0092	0.3565	0.3464	0.3585

TABLE III

Estimation of F_{ST} and *Nm* among Colombian populations of *Anopheles nuneztovari*

Populations	F_{ST}			*Nm*		
	Wright	Theta (θ)	L&M	Wright	θ	L&M
Buenaventura/Tierralta	0.024	0.039	0.035	10.1	6.2	6.8
	(0.039)	(0.067)	(0.056)			
Buenaventura/Tibú	0.054	0.091	0.086	4.4	2.5	2.7
	(0.066)	(0.111)	(0.092)			
Tierralta/Tibú	0.051	0.085	0.081	4.7	2.7	2.8
	(0.059)	(0.099)	(0.082)			
All	0.058	0.072	0.079	4.1	3.2	2.9
	(0.060)	(0.081)	(0.083)			

L&M: Lynch & Milligan; SD in parentheses

TABLE IV

Results of AMOVA in three Colombian populations of *Anopheles nuneztovari*

Source of variation	DF	SS	SD	Componentsof variance (%)	P
Between populations	2	280.0218	140.011	2.9491 (11.31)	< 0.001
Within populations	116	2683.9267	23.137 (88.69)	23.1372	< 0.001
Total	118	2963.9486			

The Φ_{ST} values that represent the population structure statistically were calculated between pairs of populations: Buenaventura/Tierralta, Buenaventura/Tibú and Tierralta/Tibú (Table V). All the Φ_{ST} values between pairs of populations were highly significant and indicated a high degree of genetic differentiation among the three populations. The remaining total variation (0.8869) was found among individuals within populations and was similar in the three populations studied. The level of significance found may indicate other levels of subdivision not considered in this study.

DISCUSSION

The number of RAPD loci generated by the ten oligos in this study (Table I) were compared with those from other genetic-structure studies of *An. nuneztovari* populations and other mosquito species such as in Apostol et al. (1993; 46 RAPD loci), Apostol et al. (1996; 57 RAPD loci) and Manguin et al. (1999; 17 RAPD loci). It was concluded that in addition to the size of the sample, the number of oligos used to establish genetic parameters to compare populations of Colombian *An. nuneztovari* was adequate.

Individual differences in the frequencies of bands between each population (data not shown) suggest a degree of geographic differentiation among them. For example, band 5 showed a frequency of 71% in the Tibú population and 2 and 0% in the Buenaventura and Tierralta populations, respectively. These differences indicated that although individuals belong to the same population, they may present differences in the RAPD-PCR pattern bands.

TABLE V

Distances among populations (Φ_{ST} between pairs of populations); above diagonal: probability of random distance

Populations	Buenaventura	Tierralta	Tibú
Buenaventura	-	< 0.001	< 0.001
Tierralta	0.0499	-	< 0.001
Tibú	0.1528	0.1326	-

The MCA and UPGMA analyses detected genetic similarity among individuals of the three populations, which may be caused by the genetic flow among the *An. nuneztovari* populations in Colombia. Clustering of individuals from the same population (Buenaventura, Tierralta or Tibú) is further evidence that suggests geographic separation (Fig. 4). UPGMA analysis also provided an appreciation of greater or smaller variations in the band profiles in populations according to the formation of clusters in each one. The northeastern population (Tibú), for instance, was subdivided into seven groups and displayed the highest variation in band profiles in the population in comparison with the western regions of Buenaventura and Tierralta, which displayed lower variation when subdivided in a smaller number of clusters.

The geographic separation estimated in the NTSYS tree for Colombian populations of *An. nuneztovari* was comparable to the geographic separation suggested by Manguin et al. (1999), who analyzed 17 RAPD polymorphic loci of two Brazilian populations of *An. nuneztovari*.

Scarpassa et al. (1999) also detected little genetic structure and low geographic differentiation in populations of *An. nuneztovari* from the Brazilian Amazon using isozyme analysis.

Very few loci were eliminated when the Lynch and Milligan correction was used to calculate heterozygosity. This implies the adequate selection of polymorphic bands and the efficacy of using a great number of oligonucleotides. The heterozygosity values obtained with Zhivotovsky's Bayesian method again confirmed the genetic polymorphism among *An. nuneztovari* populations in Colombia.

The F_{ST} values also confirmed that the Colombian populations of *An. nuneztovari* studied belong to the same species. The θ values obtained in this study for the set of three populations (Table III) was similar to that obtained by Scarpassa et al. (1999) for Brazilian populations of *An. nuneztovari* (F_{ST} = 0.070).

When F_{ST} values between groups of two populations were compared, the values between the western populations were greater than for between each of these populations and the northeastern one. This result suggests that the geographic separation among these populations reduces the genetic flow. Scarpassa et al. (1999) found that the genetic distance between *An. nuneztovari* populations from the Brazilian Amazon area and from Buenaventura (Colombia) were comparable to the genetic distance observed within members of anopheline species complexes. They also concluded that the geographic isolation between them had reduced the genetic flow, resulting in the genetic divergence of the Buenaventura population as compared with the Brazilian populations.

When Yan et al. (1999) compared the F_{ST} values for *Aedes aegypti* obtained with different dominant markers, they attributed the differences to the mutation rate in these loci. If the rate of mutation is relatively high, then the F_{ST} values can be seriously underestimated, especially with RAPD markers. Considering these criteria, it is probable that the F_{ST} values in the present study are underestimated. If this is so, then the rates of migration in one generation would had been overestimated in this study.

The Φ_{ST} value generated by AMOVA was equivalent to the F_{ST} value (Tables III, IV), which demonstrates agreement among the parameters calculated by using different methods and again establishes the significant genetic differentiation among populations of *An. nuneztovari* in Colombia.

The significance of the Φ_{ST} (0.1131) values among the three populations and the significance of the Φ_{ST} values for population pairs (Table V) suggest high genetic differentiation of *An. nuneztovari* in Colombia. Tibú, with respect to the other two populations, had the highest values Φ_{ST}. These results suggest that the degree of differentiation may be related to the geographic separation represented by the Eastern Andean mountain range.

Based on cytological studies, Kitzmiller et al. (1973) demonstrated the existence of two sibling species in *An. nuneztovari*: one from Western Venezuela and Northeastern Colombia and the other from Brazil, separated only by a small inversion (2La, right arm of X chromosome). Delgado and Rubio-Palis (1993) demonstrated that *An.*

nunveztovari from Western Venezuela is a highly variable species. On the other hand, Conn et al. (1993, 1998) showed evidence of a distinctive cytotype in *An. nuneztovari* (informally designated as *An. nuneztovari* C) based on cytogenetic analyses of larval polytene chromosomes from 7 sites – 2 in Colombia (Sitronela and Zabaletas located at sea level near Buenaventura in the Cauca Valley) and 5 in Venezuela. They found cytotype C in Western Colombia and Venezuela, Northwest of the Colombian Andean mountain range, and cytotype B in Western Venezuela, to the Southeast of this mountain range. Based on the foregoing studies, it is possible that these Colombian populations of *An. nuneztovari* from Buenaventura, Tierralta and Tibú correspond to cytotype C.

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