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A NEW CYTOTYPE OF *ANOPHELES NUNEZTOVARI* FROM WESTERN VENEZUELA AND COLOMBIA

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ABSTRACT. Cytogenetic analysis of the larval polytene chromosomes of *Anopheles nuneztovari* from 5 collection sites in Táchira and Zulia states northwest of the Andean Cordillera in western Venezuela and from 2 sites in the Department of Valle, western Colombia, revealed what appears to be a distinctive cytotype informally designated as *An. nuneztovari* C. Its chromosomes are homosequential with those of *An. nuneztovari* B from western Venezuela southeast of the Cordillera but differ in the presence of a well-defined chromocenter and unique inversion polymorphisms. The large complex inversion in western Venezuela, 2Lb, is present at a frequency of 0.263 and deviates significantly from Hardy-Weinberg equilibrium in 3 of the 5 sites. Two smaller inversions (2Lc and 2Ld) that are included in 2Lb are present in the Colombian samples at a frequency of 0.300.

INTRODUCTION

The distribution of the malaria vector *Anopheles nuneztovari* Gabaldón includes an extensive portion of northern South America (Fig. 1) as well as eastern Panama (Faran 1980). Early behavioral and ecological observations (Elliott 1972) as well as cytological differences (Kitzmiller et al. 1973) suggested that *An. nuneztovari* consisted of 2 geographically distinctive populations, one a nonvector in the Amazonian drainage basin and the other a vector in western Venezuela and northern Colombia. These populations have been designated informally as A (Amazonian) and B (Colombian/Venezuelan) (Conn 1990) and can be distinguished cytologically by a fixed inversion in the XR arm (Kitzmiller et al. 1973). Unfortunately, no voucher specimens from the collection sites of Kitzmiller et al. (1973) were saved to determine whether concomitant morphological differentiation existed. The results of an electrophoretic study (Steiner et al. 1980) comparing samples from western Venezuela with Suriname showed allele frequency differences at the Est-5 locus that might be considered diagnostic. The authors suggested, however, that additional sites and larger samples should be analyzed. Within Venezuela a comparative study of male genitalia of *An. nuneztovari* from 2 sites, one to the north and the other to the south of the Andean Cordillera, suggested that these represent 2 distinct populations (Avila Nuñez 1989[4]).

Anopheles nuneztovari has been incriminated as the main vector of the malaria parasite, *Plasmodium vivax* (Grassi and Feletti), in northern Colombia and western Venezuela (Gabaldón and Guerrero 1959, Gabaldón et al. 1963). More recently, results of monoclonal antibody surveys of CS proteins and/or salivary gland dissections for sporozoites have implicated *An. nuneztovari* in the transmission of *P. vivax* in Pará State, Brasil (de Arruda et al. 1986), eastern Peru (Hayes et al. 1987) and Amapá State, Brasil (Tadei et al. 1991), as well as in the transmission of *Plasmodium falciparum* in Amapá State (Tadei et al. 1991). These findings confound the earlier, simpler hypothesis of vector and nonvector status of *An. nuneztovari* based primarily on geographical distribution (Kitzmiller et al. 1973).

In this report, we present evidence for a distinctive cytotype, C, of *An. nuneztovari* from western Colombia and Venezuela northwest of the Andean Cordillera, and we document the existence of deviation from Hardy-Weinberg equilibrium for the complex inversion 2Lb in Venezuela.

MATERIALS AND METHODS

In Venezuela, larvae and adult females (the latter for progeny rearings) were collected at 5 sites: Caño Macho (8°22′0″N, 72°21′0″W), Guaramito (8°13′48″N, 72°19′59″W) and Moravia (8°11′45″N, 72°21′14″W) in Táchira State; and Casigua (8°50′43″N, 72°30′10″W) and Río Socuavó (8°54′0″N, 72°38′0″W) in Zulia State (Figs. 2 and 3). This area is just northwest of the Andean Cordillera and southwest of Lake Maracaibo. The average rainfall in the Maracaibo Basin varies from 1,800 to 3,800 mm annually, and the average temperature is 27°C. The mean altitude is 90 m above sea level (Ewel et al. 1976). The Colombian collection sites of Sitronela and

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[4] Avila Nuñez, J. L. 1989. Estudio bionomico de dos poblaciones de *Anopheles nuñeztovari* Gabaldón, 1940 (Diptera: Culicidae) separadas geograficamente en el Occidente de Venezuela. Lic. Thesis. Univ. de los Andes, Mérida, Venezuela.

Fig. 1. The presumed distribution of the 3 cytotypes (A, B and C) of the *Anopheles nuneztovari* species complex.

Zabaletas are located at sea level near Buenaventura in the Cauca Valley (Fig. 4). This valley is part of the marshy coastal lowland with a mean temperature of 27°C and an average annual rainfall of 7,400 mm (Box 1990).

Details of larval rearing, salivary gland dissection and polytene chromosome slide preparation can be found in Conn (1990). From the Venezuelan sites, chromosomes of 177 larvae were examined. Of these, 59 larvae were field collected and 118 were F_1 progeny (1–2 larvae per female from a total of 72 mothers). The large, complex inversion polymorphism initially described by Kitzmiller et al. (1973) in the 2L chromosome arm has been named 2Lb following the convention of Coluzzi et al. (1973). The breakpoints of the new inversion polymorphisms described here are based on the standard *An. nuneztovari* chromosome map (Kitzmiller et al. 1973) and on the photomap of Conn (1990). In the Department of Valle, Colombia, both larvae (n = 5) and F_1 progeny (n = 8) from 5 adult females were analyzed from Zabaletas, and F_1 progeny (n = 2) from one adult female from Sitronela. Link-reared voucher specimens have been deposited at the National Museum of Natural History, Smithsonian Institution, Washington, DC.

RESULTS

All larvae analyzed (Table 1) had identical polytene chromosome banding patterns for all chromosomes. The chromosomal banding pat-

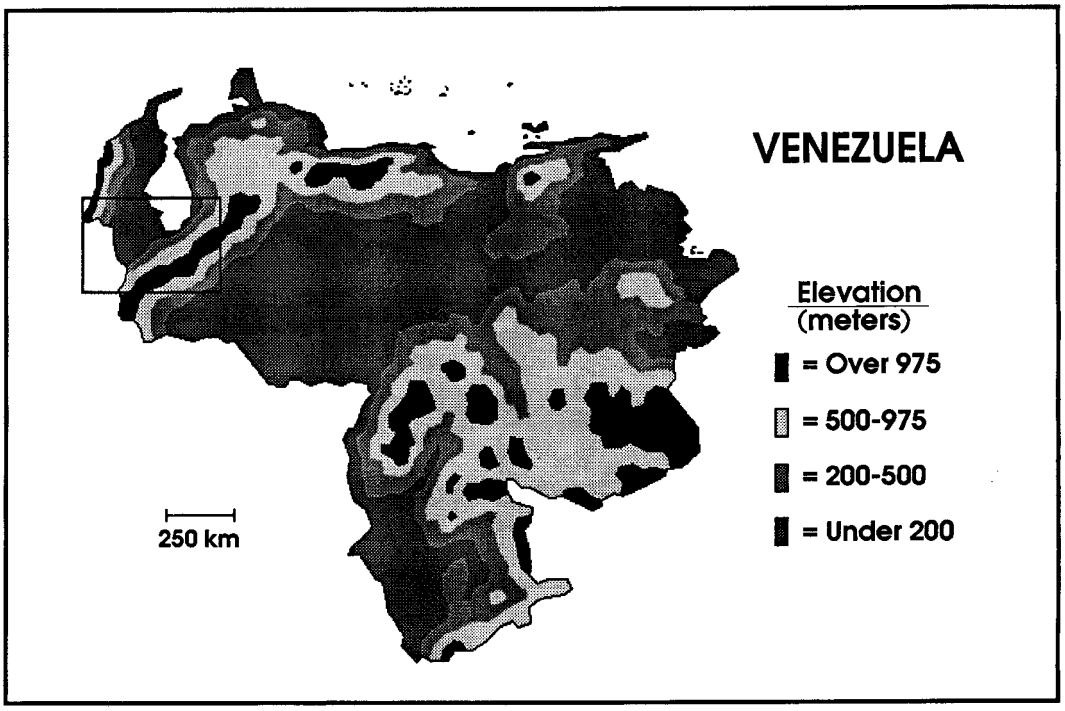

Fig. 2. Venezuela. The rectangle is the study area that is enlarged in Fig. 3.

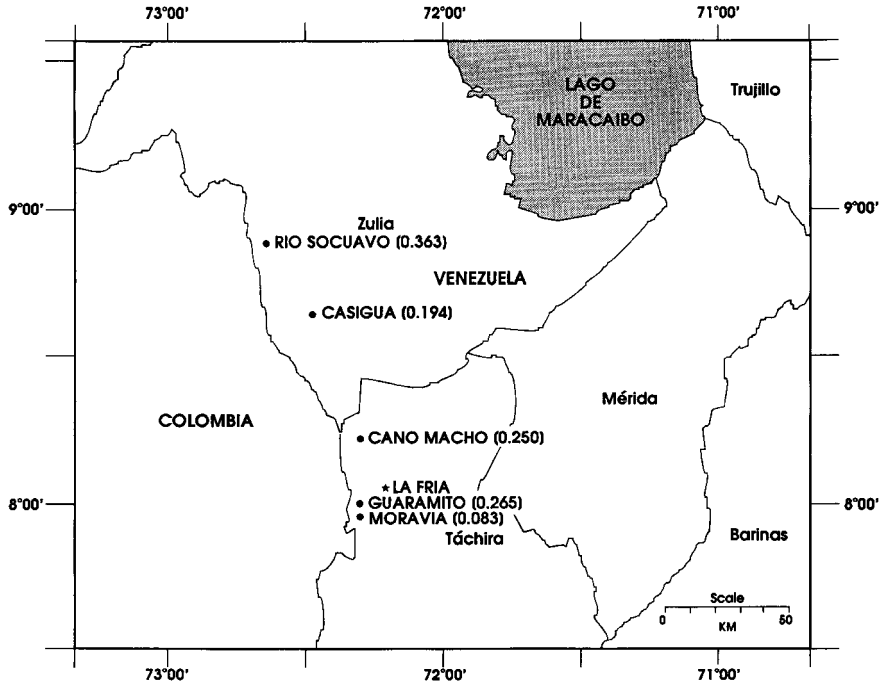

Fig. 3. The study area in western Venezuela. The 5 collection sites are Río Socuavó, Casigua, Caño Macho, Guaramito and Moravia. The frequency of the inverted constituent (b) in the complex inversion 2Lb is found in brackets after each site.

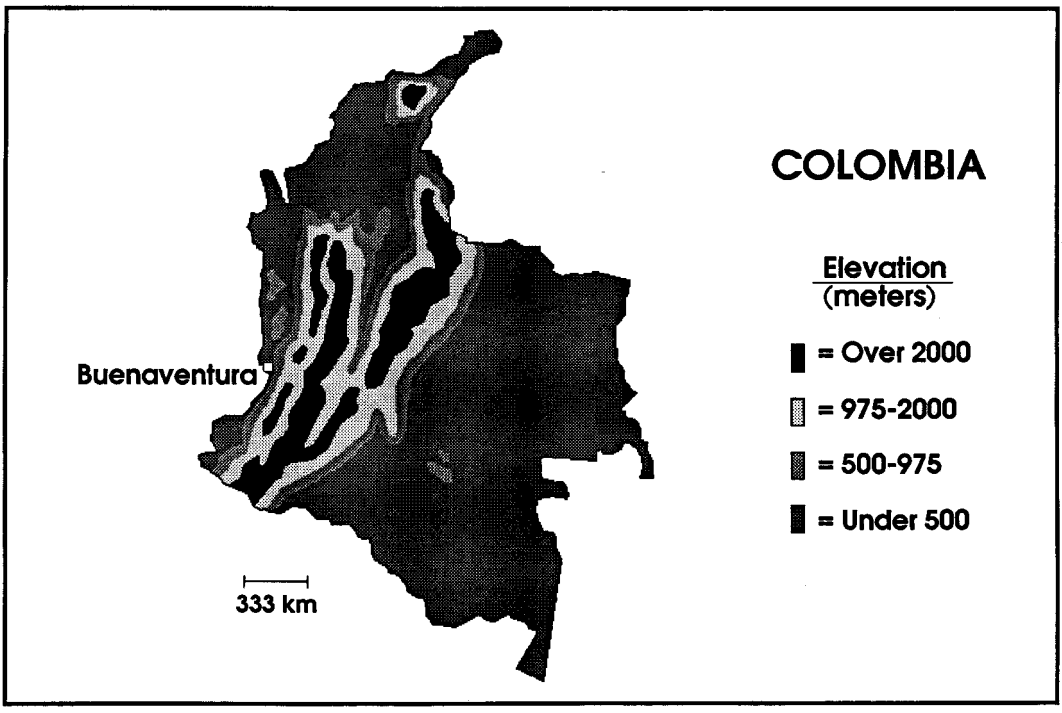

Fig. 4. Colombia. Buenaventura is the nearest town to the collection sites of Sitronela and Zabaletas.

tern of *An. nuneztovari* C is also identical (i.e., homosequential) to that of *An. nuneztovari* B from Barinas, Venezuela (Kitzmiller et al. 1973, Conn 1990). However, one striking difference is the presence of a marked chromocenter in each polytene cell of the *An. nuneztovari* C mosquitoes (Fig. 5).

Several previously unreported inversion polymorphisms were observed. Inversion 2Ra (10A/B–12A/B) was encountered in one larva from Casigua. A single larva from Guaramito displayed inversion 3Ra (31B/C–34B). Inversion 3Rb (28A/B-33B) was observed in one larva from Caño Macho. A very small inversion loop involving 2 sets of double bands in the 3L arm (3Lb, 38A/B–38C), was seen as a small "knot" and found in 2 larvae from Caño Macho. Asynapsis for the band in section 15A, just proximal to the section limit 14/15 in the 2R arm (Fig. 5), was detected in 3 larvae from Casigua, in 4 from Caño Macho and in 2 from Guaramito.

The complex inversion (2Lb) involving much of the 2L arm was first described by Kitzmiller et al. (1973, Fig. 8) based on the analysis of 4 larvae collected from Casigua. This same inversion was found from all 5 collection sites in Venezuela in the present study. A test for homogeneity of genotype frequencies between the 5 localities showed no significant differences for 2Lb ($\chi^2 = 5.90$, 4 df, $P < 0.30$). For the most part we agree with Kitzmiller et al. (1973) on the designation of the major breakpoints (from the centromere to the limit of 22/23; Fig. 6), but the larger synaptic region within this complex inversion appears to extend from 19/20 to 21A/B rather than regions 21B, 21C and 20A. We also determined that the included region from 16A to 18C actually appears to extend farther, from 16A to 19B/C. We agree that the small inversion 2La (15/16 to 16A/B) in *An. nuneztovari* B (Kitzmiller et al. 1973, Conn 1990) is involved in the complex 2Lb inversion, as suggested by Kitzmiller et al. (1973). Simple inspection of the data on frequency of the 2Lb inversion indicated that several of the populations analyzed were not in Hardy-Weinberg equilibrium, primarily because there were no inversion homozygotes. Because the frequencies of the 2Lb heterokaryotype in field-collected larvae (26 of 59) and F_1 progeny (66 of 118) were not significantly different ($\chi^2 = 1.77$, 1 df, $P < 0.20$), these 2 types of samples were combined for each collection site for calculation of χ^2 for the fit to a Hardy-Weinberg equilibrium. When analyzed by geographic location, 3 sites, Caño Macho, Guaramito and Río Socuavó, deviated significantly from the Hardy-Weinberg equilibrium for inversion 2Lb (Table 2).

In the Colombian samples, 9 of the 15 larvae had 2 inversions, 2Lc and 2Ld (Fig. 6), that appear to be included within the complex 2Lb inversion from Venezuela. Inversion 2Lc commences at the centromere (breakpoint 15/16) and ends at 18B/C. This inversion polymorphism has the proximal breakpoint coincident both with the small inversion 2La (found in *An. nuneztovari* B from Venezuela southeast of the Cordillera) and the large complex inversion 2Lb from the Maracaibo Basin, Venezuela. Inversion 2Ld (19B/C–21B, between the 2nd and 3rd heavy dark bands; Fig. 6) is also included within inversion 2Lb, and is presumably linked to 2Lc, as they were always found together. The remaining 6 larvae from Colombia all displayed the standard 2L sequence. Inversion frequencies of 2Lc + 2Ld did not deviate from Hardy-Weinberg equilibrium ($\chi^2 = 2.76$, 1 df, $P < 0.10$).

Table 1. Collections of *An. nuneztovari* C from Venezuela and Colombia used for cytogenetic analysis.

Location	Collection date[1]	Number analyzed
1. Larval collections		
Venezuela		
Caño Macho	July 18, 1989	2
	Nov. 8, 1989	4
	June 6, 1990	29
Guaramito	July 19, 1989	6
Casigua	June 7, 1990	18
Colombia		
Zabaletas	April 25, 1992	5
2. F_1 progeny from adult female collections		
Venezuela		
Caño Macho	July 17, 1989	8
	Nov. 7, 1989	9
	June 5, 1990	4
Guaramito	Aug. 19, 1989	9
	Nov. 8, 1989	19
Moravia	June 6, 1990	18
Río Socuavó	July 8, 1991	14
	July 9, 1991	9
	July 10, 1991	12
	Sept. 22, 1992	16
Colombia		
Sitronela	April 24, 1992	2
Zabaletas	April 26, 1992	8
	Total	192

[1] Date for F_1 progeny is the date adults were caught.

DISCUSSION

Although homosequential species as first described for *Drosophila* showed significant mor-

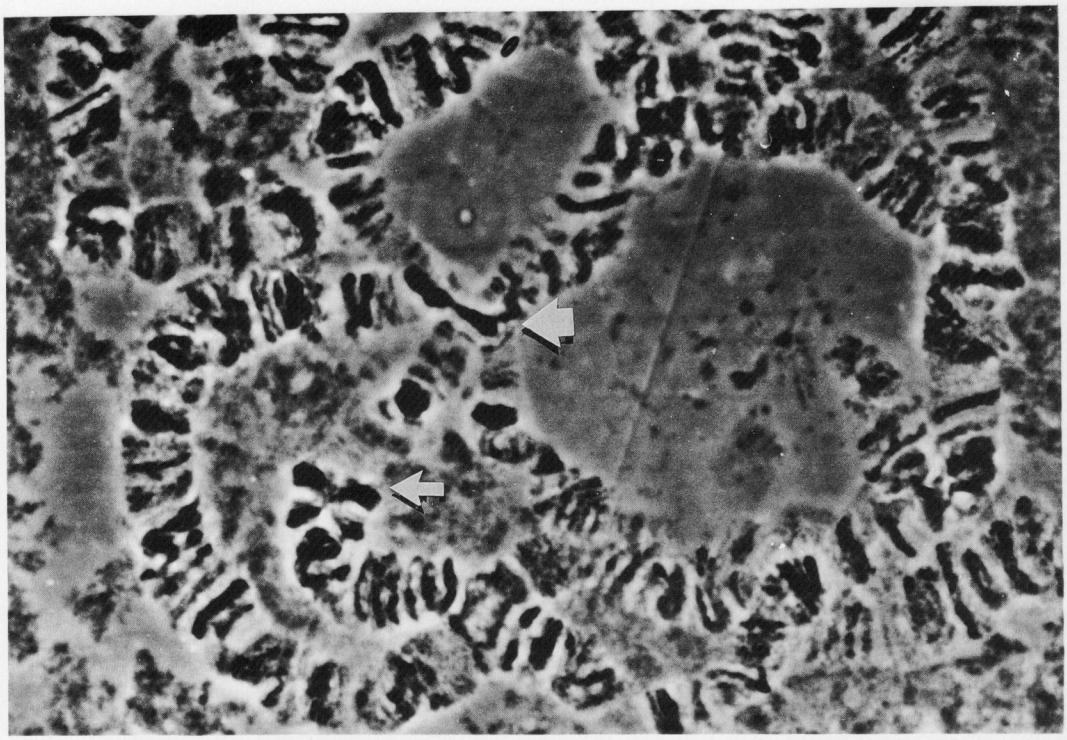

Fig. 5. The chromocenter (large arrowhead) and the asynaptic region (section 15A) in chromosome arm 2L (small arrowhead) in *Anopheles nuneztovari,* population C.

phological differences (Carson et al. 1967), the existing examples documented for anopheline mosquitoes are not easily distinguished morphologically. In the homosequential species *An. funestus* Giles and *An. aruni*? (subgenus *Cellia*), the morphological differences are considered "slight and formerly would never have been considered sufficient to recognize the specific distinctness of these taxa" (Green and Hunt 1980). For *An. freeborni* Aitken and *An. hermsi* Barr and Guptavanij (subgenus *Anopheles*), Fritz et al. (1991) state that there are no reliable morphological characters in any life stage to distinguish these 2 homosequential species. Although *An. nuneztovari* B and C have not yet been examined in all life stages for evidence of morphological differences, Avila Nuñez (1989) found a significant difference in the mean lengths of the mesosomal leaves of the male genitalia of *An. nuneztovari* from opposite sides of the Andean Cordillera in Venezuela, corresponding to *An. nuneztovari* C vs. B. However, this character is believed to be quite variable within *An. nuneztovari* (Faran 1980).

A marked chromocenter (a site of highly repetitive DNA) has been described in only 2 other species in the *Nyssorhynchus* subgenus: *An. albimanus* Wiedemann (Keppler et al. 1973) and

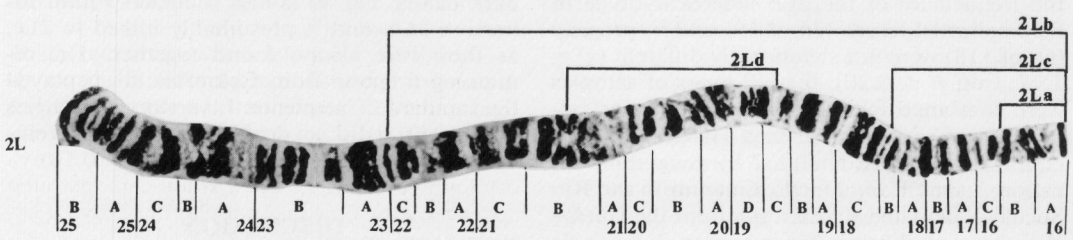

Fig. 6. The 2L larval polytene chromosome arm of *Anopheles nuneztovari* showing the break points of inversion polymorphisms 2La, 2Lb, 2Lc and 2Ld.

Table 2. Frequency of inversion 2Lb by site.

Site		Genotype +/+	Genotype +/b	Genotype b/b	Freq. inv. const.[1]	χ^2	*P*
Caño Macho	Obs.	28	28	0			
(n = 56)	Exp.	31.500	21.000	3.500	0.250	6.22	0.01
Guaramito	Obs.	16	18	0			
(n = 34)	Exp.	18.368	13.245	2.387	0.265	4.40	0.01
Moravia	Obs.	15	3	0			
(n = 18)	Exp.	15.136	2.740	0.124	0.083	0.15	0.70
Casigua	Obs.	11	7	0			
(n = 18)	Exp.	11.693	5.629	0.677	0.194	1.05	0.31
R. Socuavó	Obs.	14	37	0			
(n = 51)	Exp.	20.694	23.586	6.720	0.363	16.51	<0.001

[1] Freq. inv. const.: frequency inverted constituent (b).

An. aquasalis Curry (Frizzi and Ricciardi 1955, Kitzmiller and Chow 1971). All polytene chromosome preparations examined for these species show distinct chromocenters. In the *An. nuneztovari* complex, none of the 602 individuals from population B in western Venezuela had a chromocenter (Conn 1990) nor, surprisingly, did the 4 individuals examined from Casigua by Kitzmiller et al. (1973). We consider the chromocenter to be a prominent cytological marker. It has been used to identify 2 out of 12 cytotypes in the *Eusimulium vernum* (Macquart) black fly species complex (Brockhouse 1985).

The presence of the chromocenter, in addition to the complex inversion (2Lb), as well as the other inversions suggests that *An. nuneztovari* C is genetically differentiated from B. Lack of the 2Lb homozygote indicates either complete lethality or a very low fitness. We cannot determine which of these explanations is more likely because our Venezuelan sample size (n = 177) might fail to detect the homozygote if its fitness is very low. The persistence of this inversion at a frequency of 0.263 is probably due to some form of hybrid superiority. A similar case of hybrid superiority has been documented for species A and B of the *An. quadrimaculatus* complex (Seawright et al. 1991). The left arm of chromosome 3 occurs in 2 forms, $3L_1$ and $3L_2$ (Kaiser et al. 1988), and high frequencies (0.723 and 0.733 in species A and B, respectively) of the heterokaryotype ($3L_1/3L_2$) were observed for field collections (Seawright et al. 1991).

Normally, with a complex inversion such as 2Lb, there are several additional genotypic classes present that result in simple inversion loops (such as 2Lc and 2Ld in the Colombian samples) or inverted homozygotes. Presumably in the Maracaibo Basin these other genotypes are being selected against at some level.

Possible explanations for the discrepancies in Hardy-Weinberg equilibrium in the 5 samples from the Maracaibo Basin in western Venezuela include environmental heterogeneities, subdivided population structure, clinal variation or small sample sizes. In both *An. gambiae* Giles and *An. arabiensis* Patton significant differences in heterokaryotype frequencies in villages less than 10 km apart were attributed to environmental heterogeneities (Coluzzi et al. 1979). In particular, in *An. gambiae*, inversion 2Rd varied from 0.430 to 0.590; in *An. arabiensis*, the 2Ra arrangement ranged from 0.400 to 0.667. One hypothesis suggested by these authors to account for these different frequencies involved the occurrence of population bottlenecks during the dry season accompanied by genetic drift. The abundance of *An. nuneztovari* at El Vigía (8°37'N, 71°39'W), Mérida State, western Venezuela (presumably also C) is extremely low (i.e., less than 25 adult females collected per month [Scorza et al. 1981]) during the dry season (August–October and January–March). Because the 5 *An. nuneztovari* C sites in Táchira and Zulia states have similar precipitation patterns (Atlas de Venezuela 1979) compared to those found in El Vigía, each may be subject to annual bottleneck effects and subsequent genetic drift. However, the effects may differ from site to site if inversion 2Lb is strongly influenced by microhabitat differentiation. This may explain, in part, the deviation from Hardy-Weinberg in samples from Caño Macho, Guaramito and Río Socuavó and not from the other 2 sites (Table 2).

A subdivided population structure may result in part from poor adult dispersal with a concomitant reduction in gene flow, as was suggested to explain the distribution of intergenic spacer fragments in *An. gambiae* from western Kenya (McLain et al. 1989). Their study demonstrated restricted gene flow in 2 populations only 10 km apart based on significant differences in the frequencies of 4 ribosomal DNA array types. Although adult dispersal and flight range have not

been systematically measured in *An. nuneztovari*, in *An. albimanus* (also in the subgenus *Nyssorhynchus*), reports range from 1,800 m for wild populations in Panama (Zetek 1915) to 3 km for laboratory-reared mosquitoes released in El Salvador (Hobbs et al. 1974).

There is no strong evidence suggesting clinal variation in the frequency of inversion 2Lb in *An. nuneztovari* C from western Venezuela, although this phenomenon is common in well-studied anophelines (e.g., *An. gambiae* [Coluzzi et al. 1979, 1985], *An. quadrimaculatus* Say [Kaiser et al. 1988]). The collection sites in western Venezuela (Fig. 3) are within 1 degree of each other, both latitudinally and longitudinally. The mean altitude is 90 m (Ewel et al. 1976), and they are all in the same ecological zone (humid tropical forest [Ewel et al. 1976]) in the Maracaibo Basin, formed during the Pleistocene (Atlas de Venezuela 1979) and surrounded by the Andean Cordillera to the southeast and the Serranía de Perijá to the west.

A power test for contingency tables (Glantz 1992) revealed that the statistical confidence in detecting a difference ($P < 0.05$) for inversion 2Lb from Hardy-Weinberg equilibrium is less than 80% based on the sample sizes at each of the 5 sites in the Maracaibo Basin. This strongly suggests that additional collections should be made in this area to further evaluate the significance of the frequencies of the 3 genotypic classes of 2Lb in cytotype C.

Because we have collected cytotype C from Buenaventura, western Colombia, we hypothesize that its distribution includes Colombia north and west of the Andean Cordillera (Fig. 4) as well as the Maracaibo Basin in western Venezuela (Fig. 2). However, this hypothesis can only be tested with additional samples from this region. Cytotype B occurs in the lower montane areas of western Venezuela (Kitzmiller et al. 1973, Conn 1990) but it is also likely to be found in the Venezuelan savannahs (between the Andean Cordillera and the Orinoco River basin) and in contiguous eastern Colombia (Fig. 1). The range of cytotype A is believed to include the whole Amazon Basin (Kitzmiller et al. 1973). The Orinoco River in Venezuela (Fig. 1) marks a well-defined geological boundary between the savannah region and the Guiana Shield (considered to be part of the Amazon Basin) and appears to serve as a species boundary between cytotypes A and B. Irritability to DDT of *An. nuneztovari* from Uraba (in the Atlantic region of Colombia near Panama, presumably cytotype C) and from Oriente (in the Colombian Llanos east of the Andean Cordillera along the Venezuelan border, presumably cytotype B) was significantly different ($P < 0.01$ [Quiñones and Suarez 1989]), and may be due in part to the genetic differentiation we have documented.

In order to clarify the genetic structure of the *An. nuneztovari* complex, we and several colleagues are currently involved in analyzing the isozymes, mitochondrial DNA and morphological characteristics of field populations from across the range of this species. For 8 restriction enzymes surveyed, estimates of mtDNA sequence divergence between 5 populations of B and C for 81 individual mosquitoes (0.001–0.010 [Conn et al., unpublished data]), is in the range of intraspecific distances calculated for members of other anopheline species complexes (*An. quadrimaculatus* [Mitchell et al. 1992], *An. aquasalis* [Conn et al. 1993]), suggesting that although B and C may be separable cytologically, they are very closely related. In concordance with these data, an electrophoretic analysis of 28 enzyme loci of samples from either side of the Andes did not show any discriminatory or diagnostic locus differences (Nei's unbiased genetic distance < 0.001 [G. Fritz, unpublished data]). The morphological data have not been completely analyzed. No crosses have been made between members of the complex, because attempts at forced copulation have failed. There are no laboratory colonies of members of this complex, and there is a general dearth of formal genetic information. Therefore, most of the insights into genetic structure will necessarily come from biochemical and cytogenetic analysis.

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