A GENETIC STUDY OF THE MALARIA VECTOR *ANOPHELES NUNEZTOVARI* FROM WESTERN VENEZUELA

JAN CONN[1]

Instituto de Zoologia Tropical, Universidad Central de Venezuela, Caracas, Venezuela

ABSTRACT. A photomap of larval polytene chromosomes of *Anopheles nuneztovari* (population B) from western Venezuela is presented. Samples of *An. nuneztovari* B taken from 4 study sites in the states of Barinas and Táchira comprise a single panmictic population, which is chromosomally identical to the cytotype identified by Kitzmiller et al. (1973) from western Venezuela and Colombia. The frequency of polymorphism 2La has increased significantly in the 16 years that have elapsed since the 1973 study. In addition, inversion 2La does not conform to Hardy-Weinberg equilibrium. These 2 changes are most likely due to environmental alterations which have resulted in the heterozygous condition becoming more locally adaptive.

INTRODUCTION

Any serious study of a malaria vector must initially address taxonomic problems in order to ensure accurate identification of the vector species, and to determine the full extent of its distribution. *Anopheles nuneztovari* Gabaldon, like several other malaria vectors, is believed to be a complex of sibling or cryptic species (Elliott 1968, 1972). Its range extends from eastern Panama through northern Colombia and Venezuela, the Guyanas and much of the Amazon basin. There are no reliable records for the southern or western limits of its distribution (Faran 1980, Faran and Linthicum 1981). Within Venezuela, it is thought to be restricted to the western part of the country (Sutil 1976; Fig. 1). It is the major regional vector of *Plasmodium vivax* (Grassi and Feletti) in western Venezuela and northern Colombia (Gabaldon and Guerrero 1959, Gabaldon et al. 1963). Recently, *An. nuneztovari* was implicated as a vector of *P. vivax* in Pará State, Brazil (Arruda et al. 1986), as well as in eastern Peru (Hayes et al. 1987).

A major problem exists in studies of *An. nuneztovari* in western Venezuela. Nearly all identifications in the past have been based on the adult female, although Faran's (1980) description and keys to the Albimanus Section of the subgenus *Nyssorhynchus* (which includes *An. nuneztovari* and other closely related members of the Oswaldoi Complex) stresses that this stage is the most unreliable for identification due to wide variability in morphological characters. The need for alternative, more dependable methods of identification is obvious.

Early ecological and behavioral observations suggested that *An. nuneztovari* consisted of 2 distinct forms separated geographically. One of these, found in Brazil, Suriname and Ecuador, bit at sunset, was mainly exophagic and was considered to be primarily zoophilic (Elliott 1972). The other, studied in western Venezuela and northern Colombia, fed at 2200 h, was primarily endophagic and was a vector of *P. vivax* (Renjifo and de Zulueta 1952, Elliott 1972). Cytological studies by Kitzmiller et al. (1973) demonstrated the existence of 2 sibling species of *An. nuneztovari*, one in western Venezuela and northern Colombia, and the other in Amazonian Brazil. These sibling species could be separated by a fixed inversion difference in the XR chromosome arm (sections 1B/C-2C/3), and on differences in inversion polymorphisms in the 2L and 3L arms. Steiner et al. (1980) compared isozyme profiles of *An. nuneztovari* from western Venezuela (Barinas) and from Suriname (near Brownsweg, Brokopondo). They found high levels of genetic variation in both samples, indicating the possibility of relatively recent divergence. They suggested that the Est-5 locus could be diagnostic for the 2 populations but cautioned that samples from additional localities should be analyzed to confirm their results.

This study examines *An. nuneztovari* from Barinas and Táchira states in western Venezuela 16 years after the research undertaken by Kitzmiller et al. (1973) and provides a chromosome photomap of this population.

MATERIALS AND METHODS

The study took place in an area where Apure, Barinas and Táchira states meet in western Venezuela (Fig. 1). This area is approximately 760 km^2 at a mean altitude of 160 m above sea level. Rainfall is high (2,000–4,000 mm annually), and the average temperature is 24°C. This region is subject to profound intervention by human activities such as deforestation for agriculture and cattle rearing.

The 4 collection sites were Los Jabillos (JAB; 7°32′10″N, 71°40′4″W), Caño Lindo de Piscuri (CLP; 7°33′33″N, 71°51′30″W), Guaquitas

[1] Present address: U.S. Department of Agriculture, Insects Affecting Man and Animals Laboratory, P.O. Box 14565, Gainesville, FL 32604.

Fig. 1. The distribution in Venezuela of *Anopheles nuneztovari* (Sutil 1976). The small rectangle at the junction of Táchira, Barinas and Apure states is the study site which is enlarged in Fig. 2.

(GUA; 7°32′6″N, 71°50′10″W) and El Achote (7°36′28″N, 71°54′19″W) (Fig. 2). The maximum distance between sites is Los Jabillos to El Achote (approximately 55 km). There are no major geographical barriers in the region of the 4 collection sites and little, if any, ecological differentiation. Two types of collections were made. The first involved field-collected larvae that were hand-carried alive to the laboratory at the Universidad Central de Venezuela (UCV), Caracas, to be dissected and examined. The second type of collection was to establish isofemale lines. Adult females were captured and blood-fed either inside or outside small huts between 2000–2400 h during monthly trips to the study site from November 1988 to November 1989. Female adults were identified within 24 h of collection at the field station at Solano. Eclosion and rearing took place in the insectary at UCV at 12L:12D photoperiod, 26–28°C and 70–85% RH. Just prior to pupation the salivary glands from 1 to 3 fourth instar F_1 larvae per family were dissected and stained as described by Munstermann and Marchi (1986) with the following modifications: salivary glands were fixed in 45% acetic acid for approximately 30 sec, stained for 5 sec in 0.5% aceto-lactic-orcein, then flooded with propionic acid. Slides were preserved by ringing the cover-slip with lactic acid and storing at −30°C. An Olympus phase contrast microscope (Model BH-2) was used for analysis and the best preparations were photographed with Kodak Technical Pan 2415® film. Ovarian nurse cells from blood-fed adult females

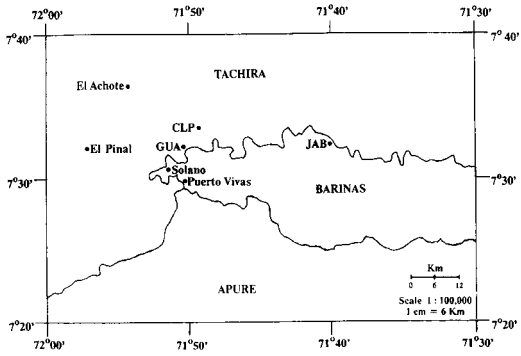

Fig. 2. The study site in western Venezuela. The 4 collection sites are JAB, CLP, GUA and El Achote.

were routinely poor and could not be used for cytogenetic analysis. Examination of the chromosomes of progeny-reared fourth instar larvae also served to confirm the provisional identification of the mother. Associated specimens from representative families were preserved for morphological examination. The identification of a small sample of *An. nuneztovari* adults from the study site was confirmed by E. L. Peyton.

Kitzmiller et al. (1973) based their chromosome map on the population of *An. nuneztovari* from Manaus, Brazil, chosen arbitrarily as the standard, which I refer to as *An. nuneztovari* A. The population from western Venezuela used for this study is referred to as *An. nuneztovari* B. Section limits for the *An. nuneztovari* B photomap (Fig. 3) are based on the map and

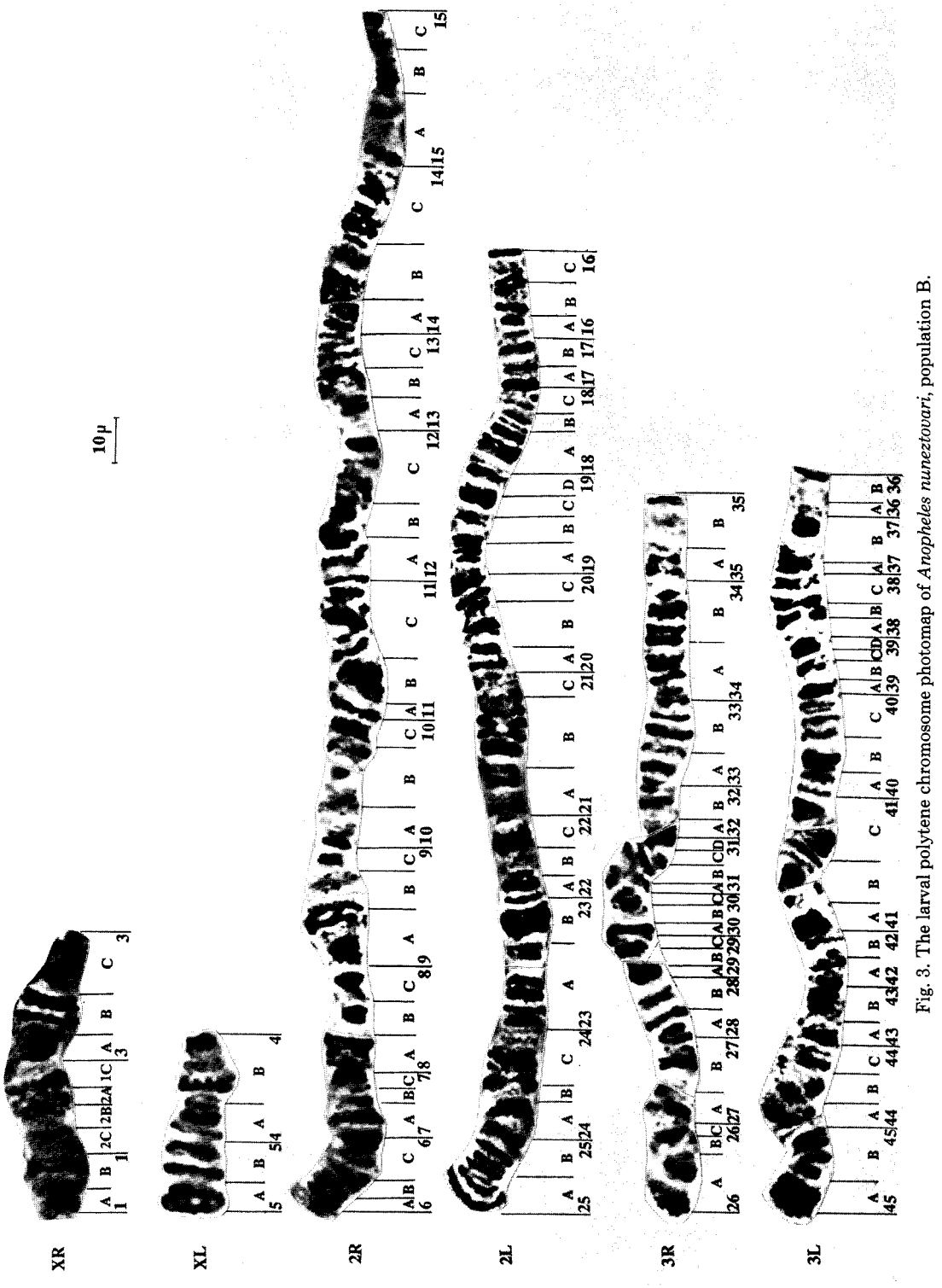

Fig. 3. The larval polytene chromosome photomap of *Anopheles nuneztovari*, population B.

drawings of Kitzmiller et al. (1973). The XR arm of *An. nuneztovari* B is not standard and is labeled accordingly. The common inversion polymorphism in the 2L arm in section 16 (initially described by Kitzmiller et al. (1973), Fig. 7) has been named 2La following nomenclature of Coluzzi et al. (1973). In descriptions of inversion 2La, the following nomenclature is used to depict the genotypes: standard sequence, +/+; heterozygote, +/a; homozygous inversion, a/a.

RESULTS

No significant differences were detected in the chromosome banding pattern of *An. nuneztovari* B from Barinas and Táchira states compared with the population of *An. nuneztovari* described from Barinas by Kitzmiller et al. (1973). However, 2 asynaptic regions differed. Kitzmiller et al. (1973) stated that section 13A in the 2R arm was "invariably asynaptic" in their description of the polytene chromosome standard. In the present study the paired homologs in region 13A were found in very close synapsis in all 602 individual larvae examined. Also, section 16 (the site of inversion 2La) was "variable, sometimes asynaptic" (*ibid.*) although in the present study, this region was not asynaptic, but either standard (+/+) or else heterozygous (+/−). Asynaptic regions (involving one or more bands) detected in this study but not mentioned in the work of Kitzmiller et al. (1973) are listed below, followed by the frequency of each in parentheses: 1B (proximal band, 0.06), 1C (0.16), XR arm; 4A (single distal band, 0.35), 5B (proximal band, 0.17), XL arm; 8B (both heavy bands involved, 0.11), 11C (proximal band, 0.10), 12A (single distal band 0.07), 2R arm; 17A (proximal band, 0.05), 18C (distal band, 0.08), 23A (2nd band from distal end, 0.09), 24B (0.08), 24C (both proximal grey bands involved, 0.09), 2L arm; 27A (distal band, 0.12), 27B (proximal dark band, 0.06), 28B (distal band, 0.09), 29C (0.06), 30A (0.06), 3R arm; and 38B (0.11), 40A (proximal band, 0.16), 44B (proximal pair of bands, 0.23), 44A (0.07), 3L arm.

Larvae examined from the 4 different sites (Fig. 2) were identical in all aspects of their banding patterns, including the frequency of inversion 2La (Table 1) and are therefore considered to belong to a single panmictic population. The total number of larvae examined (n = 602) consisted of 164 field-collected larvae and 438 F_1 progeny. The 2La inversion frequencies of heterozygous field-collected larvae (58/164) and F_1 progeny (159/438) were not significantly different (chi-squared = 0.045, 1df, P = 0.832), and therefore the total was used for calculating the Hardy-Weinberg equilibrium (Table 2).

All adult females identified as *An. nuneztovari* (n = 260) in the field were confirmed as *An. nuneztovari* B by chromosome analysis of the larval salivary glands of their progeny. Of 18 adult females identified as either *An. rangeli* Gabaldon, Cova Garcia and Lopez or *An. oswaldoi* (Peryassu) (both members of the Oswaldoi Complex), 13 were indisputably *An. nuneztovari* B by cytological criteria (i.e., all bands on all arms including the X chromosome were identical). The remaining 5 made poor preparations and their identification could not be determined.

Examination of adult females from our study area suggested there was a distinct morphological variant which appeared very close to *An. nuneztovari* (Y. Rubio and N. Delgado, personal communication). The 2 were distinguished by a difference in wing pattern in the adult female i.e., in *An. nuneztovari*, the humeral pale spot was less than 2.0× length of the prehumeral dark spot, while in the variant, the humeral pale spot was equal to or greater than 2.0× length of the prehumeral dark spot. E. L. Peyton identified the variant as *An. nuneztovari.* Chromosomal analysis of 107 F_1 progeny from 60 field-collected females confirmed that this morphological variant was identical to *An. nuneztovari* B (all bands, including those in the X chromosome, were identical; inversion 2La was present at the same frequency). Therefore, these individuals were included in Table 1 as *An. nuneztovari* B since there was no evidence to show they comprised a distinctive genetic population. The progeny of another morphological variant found infrequently in the study site which con-

Table 1. Test for the independence of inversion 2La in field-collected and F_1 larvae of *Anopheles nuneztovari* B at 4 sites in western Venezuela.

Site	Total no. larvae	Genotype +/+	Genotype +/a	Frequency inverted constituent
ACH	51	39	12	0.118
GUA	187	117	70	0.187
CLP	176	107	69	0.196
JAB	188	123	65	0.173

χ^2 = 5.167, 3 df, P = 0.160.

Table 2. Test of inversion 2La in *Anopheles nuneztovari* B (n = 602) for conformity to Hardy-Weinberg equilibrium.

	+/+	+/a	a/a	χ^2
Observed	386	216	0	28.84*
Expected	405.38	177.25	19.38	

Note: n, total number of larvae; frequencies of sequences are as follows: std. hom. (+) = 0.821; inv. hom. (a) = 0.179.

* Significant deviation from Hardy-Weinberg equilibrium; $P < 0.001$, 1 df.

sistently differed from *An. nuneztovari* (N. Delgado, personal communication) were also examined chromosomally. This variant had the humeral pale spot on the wing smaller than the prehumeral dark spot (a morphological character which is normally restricted to *An. triannulatus* (Neiva and Pinto) (Faran 1980). Cytologically, the 22 larvae analyzed (progeny of 12 female adults collected in July and August 1989) were not different from *An. nuneztovari* B and are also included in Table 1.

The frequency of inversion 2La increased significantly in the 16-year interval since the study of Kitzmiller et al. (1973): in the same area in western Venezuela, 9 of 226 larvae were heterozygous for the 2La inversion in 1973, compared with 216 of 602 in 1989 (chi-square = 54.55, 1df, $P < 0.001$). In addition, this inversion did not conform to Hardy-Weinberg equilibrium (Table 2). The high number of heterozygous individuals observed ($n = 216$) compared with the number expected ($n = 177.25$) is probably due to marginal overdominance. The inversion frequency was examined over time, at monthly intervals, to determine whether it varied with dry and rainy seasons (Table 3). The data indicate no statistical variation in polymorphism frequency.

DISCUSSION AND CONCLUSIONS

The cytological determination of the 2 adult female variants of *An. nuneztovari* as *An. nuneztovari* B is additional evidence for the apparently high level of morphological variation within *An. nuneztovari* from western Venezuela. Chromosomal examination also provides a means of differentiating intra- from interpopulation variability among closely related members of the Oswaldoi Complex. The asynapsis in the homosequential section 16 in the 2L arm of the individuals of *An. nuneztovari* not displaying the 2La inversion noted by Kitzmiller et al. (1973) may have indicated a genetic difference independent of the banding pattern (Kitzmiller et al. 1967) such as a difference in the amount of AT-rich DNA in *Chironomus* (Schmidt 1980) or a lack of DNA homology in *Drosophila* (Riede and Renz 1983). The increase in the number of heterozygous (+/a) individuals for polymorphism 2La during the 16-year interval between the 2 studies may be due to one or a combination of several factors: genetic changes within the 2La inversion; environmental changes; within or between year seasonal variations. A detailed examination of changes in inversion frequencies over time in *D. pseudoobscura* could not differentiate between 2 hypotheses: environmental alterations eliciting genetic changes; and the rise and spread of more adaptive gene complexes (Anderson et al. 1975). It may be in the case of 2La in *An. nuneztovari* B that a change within the inversion has altered the fitness relationships of the alternate gene arrangements (+/+ and +/a) such that the +/a genotype has become more adaptive, at least in the study site. Alternatively, due to the profound changes in the environment (including deforestation and loss of breeding sites), sufficient selection pressure may have resulted in the increase in the +/a genotype over time. Studies of changes in chromosome frequencies over time in populations of *D. subobscura* gave varying results. For example, no significant differences in any chromosome frequencies were found from 2 samples taken 10 years apart in Ribarroja, Spain (Prevosti 1964, De Frutos and Prevosti 1984). On the other hand, significant differences were found in all chromosomes except J in samples at Tibidabo, Spain, collected 21 years apart (De Frutos and Prevosti 1984). These apparently contradictory results may be explained if chromosome polymorphism is strongly influenced by microdifferentiation which would result in the collection of heterogeneous samples (*ibid.*). The data for 2La in *An. nuneztovari* show no heterogeneity for 4 different sites (Table 1) for a 10-month sampling period (Table 3). It seems unlikely that polymorphism 2La is affected by microdifferentiation at least in this study. Seasonal fluctuations are known to occur in many chromosomal arrangements. In populations of *An. gambiae* Giles from Kaduna, Nigeria, the *bcd* arrangement in the 2R chromosome arm was found significantly more frequently in mosquitoes resting indoors during the dry season (Coluzzi et al. 1979). Since samples from the rainy and dry seasons in western Venezuela showed no change in the frequency of 2La, it is assumed that this inversion is not sensitive to this particular type of seasonal change.

Table 3. Test for independence of polymorphism 2La in *Anopheles nuneztovari* B during wet and rainy seasons in western Venezuela.

Month	Total no. larvae	Genotype +/+	Genotype +/a	Frequency inverted constituent
Jan	25	15	10	0.200
Feb	32	22	10	0.156
March	44	28	16	0.182
April	39	25	14	0.179
May	63	34	29	0.230
June	106	68	38	0.179
July	118	83	35	0.148
August	81	47	34	0.210
September	29	11	16	0.276
October	50	27	23	0.230

Note: Jan–May represent the dry season; June–Oct represent the rainy season. $\chi^2 = 13.151$, 9 df, $P = 0.107$.

ACKNOWLEDGMENTS

This study was supported by the U.S. National Academy of Sciences BOSTID grant MVR-72 (Y. Rubio, PI). Acknowledgment is made to Y. Rubio for initiating and organizing this project, and for access to the field station at Solano, Barinas. For technical and logistical support in Venezuela I thank R. Alvarado, J. Amson, J. Arrivillaga, R. Barrera, N. Delgado, the Division of Malariology, C. Jiminez, C. Machado-Allison, D. Ortiz Garcia, L. P. Lounibos, L. E. Munstermann, the Pan American Health Organization, E. L. Peyton, S. Ramos, Y. Rangel, D. Rodriguez, The Tropical Zoology Institute at the Central University of Venezuela, G. Vele and R. Zimmermann. J. Kitzmiller donated valuable chromosome reference material and R. Baker and the administrative staff at the Florida Medical Entomology Laboratory, Vero Beach, provided space and technical help. Special thanks to L. E. Munstermann for the basic details of the study site map (Fig. 2).

REFERENCES CITED

Anderson, W., Th. Dobzhansky, O. Pavlovsky, J. Powell and D. Yardley. 1975. Genetics of natural populations XLII. Three decades of genetic change in *Drosophila pseudoobscura*. Evolution 29:24–36.

de Arruda, M., M. B. Carvalho, R. S. Nussenzweig, M. Maracic, A. W. Ferreira and A. H. Cochrane. 1986. Potential vectors of malaria and their different susceptibility to *Plasmodium falciparum* and *Plasmodium vivax* in northern Brazil identified by immunoassay. Am. J. Trop. Med. Hyg. 35:873–881.

Coluzzi, M., M. Di Deco and G. Cancrini. 1973. Chromosomal inversions in *Anopheles stephensi*. Parassitologia 15:129–136.

Coluzzi, M., A. Sabatini, V. Petrarca and M. A. Di Deco. 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans. R. Soc. Trop. Med. Hyg. 73:483–497.

De Frutos, R. and A. Prevosti. 1984. Temporal changes of chromosomal polymorphism in natural populations of *Drosophila subobscura*. Genetica 63:181–187.

Elliott, R. 1968. Studies on man-vector contact in some malarious areas in Colombia. Bull. W.H.O. 38:239–253.

Elliott, R. 1972. The influence of vector behavior on malaria transmission. Am. J. Trop. Med. Hyg. 21:755–763.

Faran, M. E. 1980. Mosquito Studies (Diptera, Culicidae) XXXIV. A revision of the Albimanus Section of the subgenus *Nyssorhynchus* of *Anopheles*. Contrib. Am. Entomol. Inst. (Ann Arbor) 15:1–215.

Faran, M. E. and K. J. Linthicum. 1981. A handbook of the Amazonian species of *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae). Mosq. Syst. 13:1–81.

Gabaldon, A. and L. Guerrero. 1959. An attempt to eradicate malaria by weekly administration of pyrimethamine in areas of out-of-doors transmission in Venezuela. Am. J. Trop. Med. Hyg. 8:433–439.

Gabaldon, A., L. Guerrero and G. G. Martin. 1963. Malaria refractaria en el occidente de Venezuela. Rev. Venez. Sanid. 14:513–530.

Hayes, J., G. Calderon, R. Falcón and V. Zambrano. 1987. Newly incriminated anopheline vectors of human malaria parasites in Junin Department, Peru. J. Am. Mosq. Control Assoc. 3:418–422.

Kitzmiller, J. B., G. Frizzi and R. H. Baker. 1967. Evolution and speciation within the Maculipennis complex of the genus *Anopheles*, pp. 151–210. *In*: Wright, J. W. and R. Pal. (eds.), Genetics of insect vectors of disease, Elsevier, Amsterdam.

Kitzmiller, J. B., R. D. Kreutzer and E. Tallaferro. 1973. Chromosomal differences in populations of *Anopheles nuneztovari*. Bull. W.H.O. 48:435–455.

Munstermann, L. E. and A. Marchi. 1986. Cytogenetic and isozyme profile of *Sabethes cyaneus*. J. Hered. 77:241–248.

Prevosti, A. 1964. Chromosomal polymorphism in *D. subobscura* populations from Barcelona (Spain). Genet. Res. 5:27–38.

Renjifo, S. and J. de Zulueta. 1952. Five years' observations of rural malaria in eastern Colombia. Am. J. Trop. Med. Hyg. 1:598–611.

Riede, I. and M. Renz. 1983. Study on the somatic pairing of polytene chromosomes. Chromosoma 88:116–123.

Schmidt, E. R. 1980. Chromosomenevolution und die Vermehrung repetitiver AT-reicher DNA-Sequenzen bei Chironomiden. Verh. Dtsch. Zool. Ges. 1980:365.

Steiner, W. W. M., J. B. Kitzmiller and D. L. Osterbur. 1980. Gene differentiation in chromosome races of *Anopheles nuneztovari* Gabaldon. Mosq. Syst. 12:306–319.

Sutil, E. 1976. Redescripción de la especie *Anopheles* (*Nyssorhynchus*) *nuneztovari* Gabaldon 1940 y su distribucion geográfica en Venezuela. Bol. Dir. Malariol. Sanam. Amb. 16:33–45.