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GEOGRAPHIC VARIATION OF MALE GENITALIA OF *ANOPHELES NUNEZTOVARI* (DIPTERA: CULICIDAE)

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ABSTRACT. The structure of the male genitalia of three known cytotypes (A, B, and C) of *Anopheles nuneztovari* varies geographically. Morphometric analyses of 437 specimens revealed significant variation for collection sites within and among cytotypes. Most genitalic characters failed to distinguish cytotypes. Four characters distinguish cytotype B males of the other two cytotypes. The aedeagal leaflets are longer and more heavily sclerotized, the parabasal tubercle is shorter, and the larger accessory seta is shorter among cytotype B males. Specimens lacking leaflets on both sides of the aedeagus were observed only among cytotype A. Within cytotype A progeny broods, 6.7–21% of specimens lacked both leaflets. Considerable overlap of characters exists between the male genitalia of cytotypes A and C. The results of morphological analyses are contrasted with findings from recent molecular studies. Characters of the male genitalia appear to be of limited utility for delimiting the probable relationships among cytotypes of *Anopheles nuneztovari sensu lato.*

INTRODUCTION

Male genitalic characters are important in the taxonomy of *Anopheles* (*Nyssorhynchus*) Blanchard (Gabaldón 1940, Galvao 1943, Levi-Castillo 1949, Faran 1980, Faran and Linthicum 1981, Linthicum 1988). *Anopheles nuneztovari* Gabaldón was first described by Gabaldón (1940) on the basis of morphology of male genitalia from specimens collected in the Venezuelan state of Cojedes. One year later, Rozeboom and Gabaldón (1941) described *An. goeldii* Rozeboom and Gabaldón, a closely related species purportedly differing in male genitalia, from specimens collected at Boa Vista, Brazil; in this same paper, Gabaldón claimed to have collected *An. goeldii* from La Ceiba, Trujillo State, Venezuela. Although Floch and Abonnenc (1946) synonymized *An. goeldii* with *An. nuneztovari* based on specimens from French Guiana, Gabaldón (1981) still considered *An. nuneztovari* and *An. goeldii* to be separate species. *Anopheles nuneztovari* has been redescribed several times since its original description (Sutil 1976, Faran 1980, Savage 1986), somewhat confusing the issue. Sutil (1976) based his redescription on specimens from western Venezuela, including some from San Carlos, the type locality of *An. nuneztovari.* Specimens from across northern South America, including material fitting the description of *An. goeldii,* were examined by Faran (1980). Savage (1986) reexamined the holotype and presumed paratypes of *An. nuneztovari.* In Venezuela, differences in length of aedeagal leaflets and degree of sclerotization of leaflets between specimens collected in the states of Táchira (probably cytotype C) and Barinas (probably cytotype B) were encountered by Avila Nuñez (1989). Kitzmiller et al. (1973) found geographic variation of cytologic characters of *An. nuneztovari* and determined that two forms were present, which Conn (1990) called A (Amazonian) and B (western Venezuela southeast of the Andes). Recently, Conn et al. (1993) described a third cytotype, C, of *An. nuneztovari* from Colombia and western Venezuela northwest of the Andes. The ranges of cytotypes B and C in Venezuela are separated by the Andes Mountains. The purpose of this study was to determine whether the morphology of male genitalia can be used

Table 1. Summary of *An. nuneztovari* cytotypes, collection sites, geographic coordinates, and number of individuals per collection site.

Cytotype	Country	Collection site	Locality and state	Geographic coordinates	Sample size
A	Brazil	BL	Belém, Pará	1° 24′ 36″ S, 48° 26′ 58″ W	9
A	Brazil	PR	Puraquequara, Amazonas	3° 6′ 7″ S, 60° 1′ 30″ W	135
A	Brazil	UR	Urucuri, Pará	1° 17′ S, 47° 34′ W	12
A	Suriname	VC	Victoria, Brokopondo	5° 5′ N, 54° 58′ W	50
B	Venezuela	CA	Caño Amarillo, Apure	7° 21′ N, 71° 52′ W	150
B	Venezuela	EN	El Nula, Apure	7° 21′ N, 71° 52′ W	10
B	Venezuela	SO	Solano, Táchira	7° 32′ 6″ N, 71° 50′ 10″ W	33
C	Colombia	SI	Sitronela, Valle	3° 49′ N, 77° 4′ W	22
C	Venezuela	RS	Río Socuavó, Zulia	8° 54′ 0″ N, 72° 38′ 0″ W	16

to distinguish among the three cytotypes of *An. nuneztovari.*

MATERIALS AND METHODS

Procurement of specimens. Host-seeking females were collected from three sites in Brazil, one in Colombia, one in Suriname, and four in Venezuela (Table 1). Blood-fed females were returned to the laboratory and allowed to oviposit. Isofemale progeny broods were reared at 26°C. The specimens from PR (see Table 1 for collection site abbreviations) were comprised of four groups: progeny broods of three females, viz., numbers 5, 83, and 87; and a group of unrelated specimens from several rearing lines. Males from CA were reared from 12 isofemale lines. The specimens from BL, EN, RS, SI, SO, and UR were mixed collections of progeny from several isofemale rearing lines.

Material examined. In all, 437 genitalia were examined from the following collecting localities: Cytotype A—BRAZIL: BL, n = 9; PR 5, n = 33; PR 83, n = 60; PR 87, n = 28; PR, n = 14; UR, n = 12; SURINAME: VC, n = 50. Cytotype B—VENEZUELA: CA 0, n = 4; CA 20, n = 15; CA 25, n = 6; CA 30, n = 21; CA 33, n = 9; CA 37, n = 4; CA 40, n = 33; CA 48, n = 15; CA 49, n = 5; CA 50, n = 12; CA 54, n = 7; CA 63, n = 19; EN, n = 10; SO, n = 33. Cytotype C—COLOMBIA: SI, n = 22; VENEZUELA: RS, n = 16. Subtotals by cytotype are A, n = 206; B, n = 193; C, n = 38.

Preparation of specimens. Genitalia were clipped from the abdomen, cleared in 5% NaOH, washed with 1% acetic acid, dehydrated in a graduated alcohol series (70%, 90%, 95% EtOH) and essence of Euparal, and mounted on microscope slides in Euparal. Some specimens were placed into Essig's fluid (Essig 1948) and stained with Wilkey's stain (Wilkey 1962) between the acetic acid wash and dehydration in order to stain lightly sclerotized areas. The majority of specimens were partially dissected during the mounting process, i.e., the proctiger was removed so that the aedeagus and ventral lobes were seen more easily.

Mensuration of specimens. Specimens were examined and illustrations made by using a phase-contrast microscope fitted with a drawing tube. Measurements were made from illustrations by using a Summagraphics® digitizing tablet and SigmaScan® software. Twenty characters were digitized for each specimen, 14 direct measurements (Fig. 1) and six ratios of two variables. Direct measurements taken (in micrometers) were lengths of the gonocoxa (GC), gonostylus (GS), gonostylar claw (GSC), subapical seta (SAS), aedeagus (AEL), left leaflet of aedeagus (LLL), right leaflet of aedeagus (RLL), basal apodeme (BAD), parabasal seta (PBS), parabasal tubercle (PBL), internal seta (INSET), and the

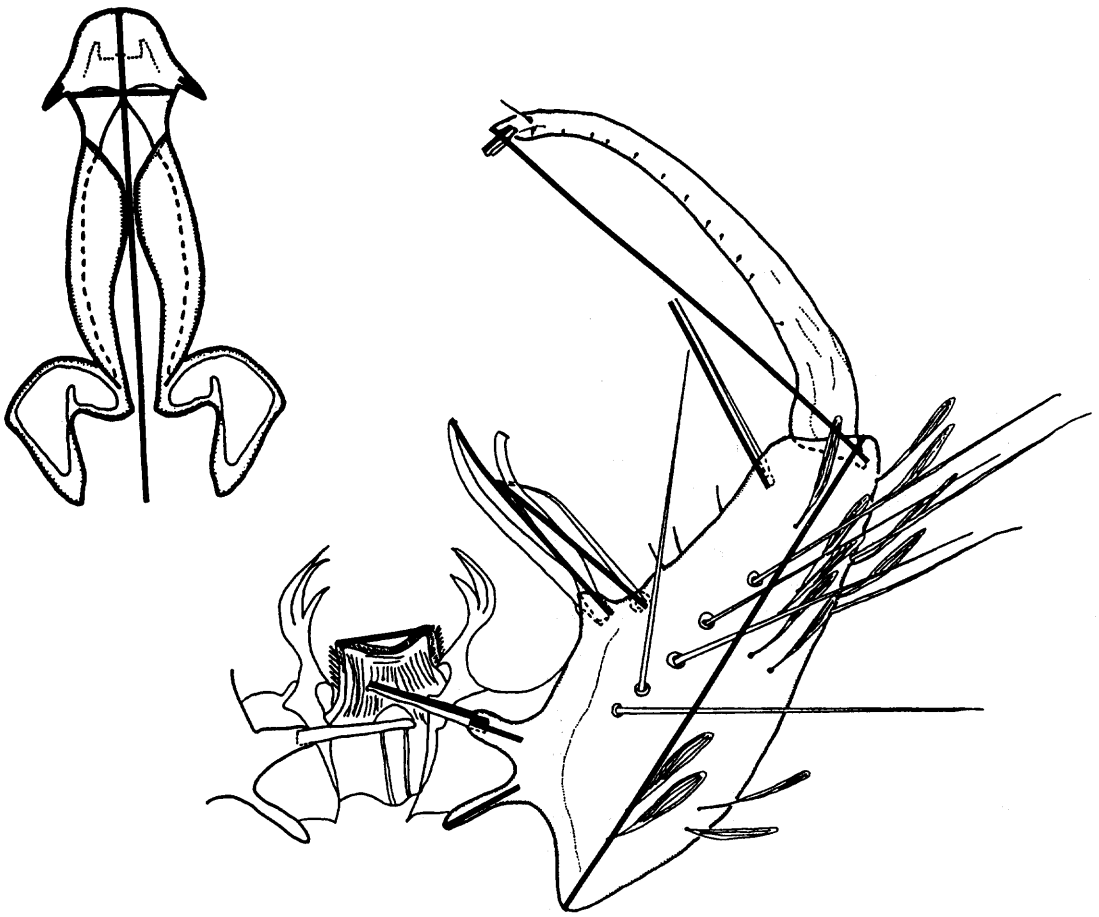

Fig. 1. Male genitalic characters measured for *An. nuneztovari* in this study: lengths of gonocoxa (GC), gonostylus (GS), gonostylar claw (GSC), subapical seta (SAS), aedeagus (AEL), left leaflet of aedeagus (LLL), right leaflet of aedeagus (RLL), basal apodeme (BAD), parabasal seta (PBS), parabasal tubercle (PBL), internal seta (INSET), and the larger of the two accessory setae (ACCSET); widths of ventral lobes at apex (VLW) and aedeagus (AEW). Heavy lines indicate axes along which measurements were made.

larger of the two accessory setae (ACCSET) and widths of ventral lobes at apex (VLW) and of aedeagus (AEW). Ratios calculated were width of ventral lobe to length of gonocoxa (VLW/GC), length of subapical seta to length of gonocoxa (SAS/GC), length of gonocoxa to length of gonostylus (GC/GS), length of gonocoxa to length of basal apodeme (GC/BAD), length of aedeagus to length of parabasal seta (AEL/PBS), and width of aedeagus to length of parabasal seta (AEW/PBS). In addition, the degree of sclerotization of aedeagal leaflets (LEAFSCLE) was scored as a binary datum, 0 signifying light sclerotization and 1 indicating heavy sclerotization.

Statistical analysis. Prior to analyses, direct measurements were transformed by $X' = \log(X + 1)$ and ratios were transformed by $X' = (X + 3/8)^{1/2}$ (Zar 1984). In order to examine variation within cytotypes, a subset of the entire dataset was analyzed. The following groups were chosen for analysis because they were composed of numerous individuals and were geographically dispersed: cytotype A—BL, PR 5, PR 83, PR 87, UR, VC (n = 192); cytotype B—CA 20, CA 30, CA 40, CA 63, EN, SO (n = 131); cytotype C—RS, SI (n = 38). Differences among collection sites were analyzed by using multivariate analysis of variance (MANOVA). Wilk's lambda was

calculated to test for overall effect of collection site (Pimentel 1979). Mean separations were performed by the Ryan-Einot-Gabriel-Welsch multiple range test.

Analysis of differences among the three cytotypes was conducted on the entire dataset. MANOVA with collection site nested within cytotype was conducted on the transformed data to test differences among cytotypes and to test for differences among collection sites within cytotypes. Wilk's lambda was calculated to determine whether differences existed among cytotypes and among sites within cytotypes (Pimentel 1979). Those variables that differed among cytotypes were subjected to discriminant analysis after backward elimination of redundant variables (Flury and Riedwyl 1988). Relative importance of variables in distinguishing males of the three cytotypes was determined by ranking standardized canonical coefficients (Strickman and Pratt 1989). MANOVA and discriminant analysis were used to avoid problems arising from correlation of characters (Atchley and Martin 1971) and overlap of characters when considered individually (Jolicoeur 1959). Use of binary data in discriminant analysis is supported by previous studies (Krzanowski 1975, Vlachonikolis and Marriott 1982, Wilson et al. 1993). All analyses were conducted with a statistical computer software package (SAS Institute 1985). Results of all analyses are reported as untransformed means ± SEM.

RESULTS

Differences within cytotypes. Significant morphological variation was present within all cytotypes. Site effect as indicated by Wilk's lambda was significant for cytotypes A and B (cytotype A, Wilk's lambda = 0.0411, $P < 0.001$; cytotype B, Wilk's lambda = 0.0475, $P < 0.001$) but not for cytotype C (Wilk's lambda = 0.0026, $P < 0.18$).

Among cytotype A males, the following characters differed among collection sites: GC, GS, GSC, VLW, AEL, AEW, LLL, RLL, VLW/GC, SAS/GC, and AEW/PBS (Table 2). PR 5 males had significantly longer GC than other males. Males from PR 5, PR 83, and VC had longer GS than did males from BL, whereas males from PR 87 and UR did not. Males from BL had longer GSC than did males from UR. UR males had narrower VLW than all other males except PR 87. PR 5 males had longer AEL than did UR males, but males from neither of these sites were different from males from any other sites. Similarly, males from UR and VC differed in AEW, but males from neither of these sites were different from males of any other site. Males from BL had longer LLL than any other males except those from VC; BL males had longer RLL than any other males. Significant differences among sites were found for the following ratios: VLW/GC, SAS/GC, GC/GS, and AEW/PBS. No statistically significant variation was found for the following eight characters: SAS, BAD, PBS, PBL, INSET, ACCSET, GC/BAD, and AEL/PBS. All aedeagal leaflets were lightly sclerotized. All groups had specimens lacking leaflets on one or both sides of the aedeagus; however, some specimens that appeared to lack leaflets were found to have very small leaflets that were folded or appressed to the aedeagus. Within the three progeny broods from PR, the number of specimens lacking leaflets on both sides was 21% for PR 5 (7/33), 6.7% for PR 83 (4/60), and 18% for PR 87 (5/28).

Among cytotype B males, 13 characters varied among collection sites: GS, GSC, SAS, VLW, AEL, AEW, BAD, PBS, PBL, ACCSET, SAS/GC, AEL/PBS, and AEW/PBS (Table 3). Males from EN had longer GS than did males from SO, but neither of these groups of males was significantly different in GS length from other groups. Males from CA 40 and EN had significantly longer GSC than did males only from SO. Males from SO had significantly smaller SAS than did any other males except those from CA 63. Only CA 63 and EN males differed from each other in VLW. The males from CA 30, CA 63, and EN had larger AEL than did males from CA 20, but no other sites or families differed in this character. No sites or families differed in length of BAD except EN and SO. Only SO had smaller ACCSET than other males. The following ratios differed among collection sites

Table 2. Genitalic characters for male cytotype A *Anopheles nuneztovari* at different collection sites.[1,2]

Character[3]	Collection sites[4]					
	BL (n = 9)	PR 5 (n = 33)	PR 83 (n = 60)	PR 87 (n = 28)	UR (n = 12)	VC (n = 50)
GC	327.7 ± 6.1b	392.9 ± 12.9a	313.3 ± 3.6b	316.1 ± 1.9b	317.4 ± 4.5b	318.2 ± 3.0b
GS	273.3 ± 10.3c	305.9 ± 1.5a	295.3 ± 1.7ab	287.9 ± 2.6bc	285.1 ± 3.8bc	291.8 ± 1.5ab
GSC	20.2 ± 1.0b	22.5 ± 0.5ab	21.6 ± 0.2ab	22.6 ± 0.3ab	23.8 ± 1.0a	22.3 ± 0.3ab
SAS	112.7 ± 6.6a	112.1 ± 3.7a	103.9 ± 1.8a	110.3 ± 2.5a	123.0 ± 3.1a	117.9 ± 2.1a
VLW	64.6 ± 5.8ab	67.8 ± 2.0a	63.5 ± 1.1ab	58.3 ± 1.4bc	53.5 ± 2.4c	70.4 ± 1.8a
AEL	178.7 ± 4.4ab	192.0 ± 1.7a	181.3 ± 1.5ab	183.9 ± 1.6ab	177.7 ± 3.3b	186.3 ± 2.2ab
AEW	39.3 ± 1.7ab	40.8 ± 0.7ab	40.7 ± 0.5ab	41.2 ± 0.8ab	37.2 ± 0.7b	44.6 ± 0.8a
LLL	4.8 ± 1.9a	4.1 ± 1.7c	1.3 ± 0.3bc	1.3 ± 0.3bc	0.4 ± 0.3c	3.5 ± 0.5ab
RLL	5.0 ± 2.5a	3.1 ± 1.3b	0.8 ± 0.2b	1.5 ± 0.4b	0.5 ± 0.3b	3.2 ± 0.4b
BAD	54.0 ± 3.4a	55.0 ± 2.0a	52.9 ± 0.9a	50.4 ± 1.2a	51.7 ± 0.2a	54.1 ± 1.7a
PBS	64.6 ± 2.8a	72.1 ± 0.9a	66.0 ± 0.7a	71.2 ± 0.8a	73.6 ± 0.2a	69.2 ± 1.6a
PBL	36.7 ± 3.0a	42.9 ± 2.0a	46.1 ± 1.1a	39.5 ± 1.6a	44.7 ± 0.2a	41.1 ± 1.5a
INSET	107.7 ± 4.2a	113.1 ± 1.7a	101.5 ± 1.7a	107.7 ± 2.1a	106.9 ± 3.3a	109.1 ± 2.4a
ACCSET	143.2 ± 4.0a	145.0 ± 1.6a	143.7 ± 0.8a	148.4 ± 1.3a	152.3 ± 2.2a	146.8 ± 2.8a
VLW/GC	0.195 ± 0.014ab	0.185 ± 0.012ab	0.203 ± 0.004ab	0.185 ± 0.005b	0.171 ± 0.007b	0.221 ± 0.006a
SAS/GC	0.345 ± 0.022ab	0.299 ± 0.018ab	0.333 ± 0.006ab	0.348 ± 0.008ab	0.388 ± 0.009a	0.371 ± 0.006ab
GC/GS	1.216 ± 0.054a	1.289 ± 0.044a	1.061 ± 0.012b	1.100 ± 0.011ab	1.115 ± 0.017ab	1.092 ± 0.010b
GC/BAD	6.271 ± 0.423a	7.765 ± 0.635a	6.021 ± 0.118a	6.351 ± 0.132a	6.225 ± 0.241a	6.376 ± 0.398a
AEL/PBS	2.724 ± 0.096a	2.689 ± 0.041a	2.769 ± 0.036a	2.610 ± 0.034a	2.443 ± 0.054a	2.791 ± 0.095a
AEW/PBS	0.612 ± 0.026ab	0.576 ± 0.013ab	0.621 ± 0.009a	0.584 ± 0.014ab	0.514 ± 0.019b	0.661 ± 0.021a

[1] Means ± SE in the same row followed by the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch multiple range test).
[2] Direct measurements in μm, ratios are unitless values.
[3] GC, gonocoxal length; GS, gonostylar length; GSC, gonostylar claw length; SAS, subapical seta length; VLW, ventral lobe width; AEL, aedeagal length; AEW, aedeagal width; LLL, left leaflet length; RLL, right leaflet length; BAD, basal apodeme length; PBS, parabasal seta length; PBL, parabasal tubercle length; INSET, internal seta length; ACCSET, accessory seta length; VLW/GC, ventral lobe width/gonocoxa; SAS/GC, subapical seta/gonocoxa; GC/BAD, gonocoxa/basal apodeme; AEL/PBS, aedeagal length/parabasal seta; AEW/PBS, aedeagal width/parabasal seta.
[4] BL, Belém, Brazil; PR5, Puraquequara 5, Brazil; PR83, Puraquequara 83, Brazil; PR87, Puraquequara 87; UR, Urucuri, Brazil; VC, Victoria, Suriname.

Table 3. Genitalic characters for male cytotype B *Anopheles nuneztovari* at different collection sites.[1,2]

Character[3]	Collection sites[4]					
	CA 20 (n = 4)	CA 30 (n = 21)	CA 40 (n = 33)	CA 63 (n = 19)	EN (n = 10)	SO (n = 33)
GC	309.1 ± 5.0a	318.7 ± 3.2a	309.6 ± 2.9a	317.5 ± 5.7a	324.7 ± 4.0a	317.1 ± 3.5a
GS	275.5 ± 3.6ab	274.1 ± 2.6ab	274.4 ± 2.8ab	276.3 ± 4.7ab	284.3 ± 4.6a	270.8 ± 3.0b
GSC	22.7 ± 0.3ab	22.6 ± 0.3ab	23.3 ± 0.3a	22.9 ± 0.7ab	23.3 ± 1.5a	21.2 ± 0.6b
SAS	119.5 ± 2.3ab	123.0 ± 2.9ab	118.6 ± 2.3ab	110.8 ± 2.9bc	122.1 ± 3.3a	104.5 ± 3.4c
VLW	63.8 ± 1.5ab	65.2 ± 1.9ab	58.0 ± 1.2ab	56.3 ± 3.1b	65.4 ± 2.7a	65.0 ± 1.8ab
AEL	176.1 ± 4.8b	181.3 ± 3.3a	180.3 ± 2.1ab	183.5 ± 3.3a	189.6 ± 3.1a	177.7 ± 2.4ab
AEW	37.9 ± 1.0b	37.6 ± 0.7b	38.7 ± 0.6ab	39.7 ± 0.9ab	42.0 ± 1.1a	38.3 ± 0.7ab
LLL	10.6 ± 1.0a	15.2 ± 1.2a	13.8 ± 0.6a	12.0 ± 0.8a	11.7 ± 1.1a	14.2 ± 1.1a
RLL	10.6 ± 0.8a	14.9 ± 0.8a	13.5 ± 0.6a	11.8 ± 0.5a	12.9 ± 1.31a	12.0 ± 1.0a
BAD	51.9 ± 1.6ab	55.4 ± 1.7ab	52.0 ± 1.1ab	54.3 ± 1.4ab	59.6 ± 2.2a	50.0 ± 1.7b
PBS	66.3 ± 0.8ab	71.8 ± 0.7a	66.3 ± 1.1ab	69.5 ± 1.5a	69.2 ± 1.3a	61.3 ± 2.0b
PBL	41.9 ± 1.5bc	39.1 ± 0.9c	41.2 ± 1.1c	42.8 ± 1.9ab	47.7 ± 1.5a	34.7 ± 1.8c
INSET	100.1 ± 1.8a	104.1 ± 1.3a	100.9 ± 1.9a	101.4 ± 1.7a	97.8 ± 3.9a	91.2 ± 3.0a
ACCSET	136.8 ± 1.5a	136.1 ± 1.2a	137.8 ± 1.1a	136.7 ± 1.8a	139.8 ± 1.7a	118.2 ± 3.4b
VLW/GC	0.207 ± 0.006a	0.208 ± 0.007a	0.187 ± 0.004a	0.176 ± 0.010a	0.203 ± 0.008a	0.204 ± 0.006a
SAS/GC	0.390 ± 0.007a	0.386 ± 0.007a	0.384 ± 0.007a	0.350 ± 0.010ab	0.377 ± 0.012a	0.331 ± 0.011b
GC/GS	1.122 ± 0.012a	1.164 ± 0.009a	1.131 ± 0.012a	1.153 ± 0.023a	1.145 ± 0.024a	1.175 ± 0.017a
GC/BAD	6.038 ± 0.221a	5.853 ± 0.188a	6.045 ± 0.147a	5.906 ± 0.159a	5.485 ± 0.146a	6.536 ± 0.220a
AEL/PBS	2.660 ± 0.075b	2.533 ± 0.058ab	2.737 ± 0.044ab	2.677 ± 0.083ab	2.754 ± 0.075ab	3.009 ± 0.110a
AEW/PBS	0.571 ± 0.015ab	0.526 ± 0.013b	0.588 ± 0.011ab	0.576 ± 0.011ab	0.610 ± 0.020ab	0.638 ± 0.027a

[1] Means ± SE in the same row followed by the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch multiple range test).
[2] Direct measurements in μm, ratios are unitless values.
[3] Abbreviations as in Table 1.
[4] All Venezuelan localities: CA 20, Caño Amarillo 20; CA 30, Caño Amarillo 30; CA 40, Caño Amarillo 40; CA 63, Caño Amarillo 63; EN, El Nula; SO, Solano.

Table 4. Genitalic characters for male cytotype C *Anopheles nuneztovari* at different collection sites.[1,2]

Character[3]	Collection sites[4] RS (n = 16)	SI (n = 22)
GC	325.1 ± 4.4a	322.7 ± 4.0a
GS	294.6 ± 3.2a	279.8 ± 4.1b
GSC	22.0 ± 0.6b	23.7 ± 0.5a
SAS	103.7 ± 6.0a	104.5 ± 3.0a
VLW	62.9 ± 3.2a	67.6 ± 1.7a
AEL	189.3 ± 3.0a	174.1 ± 3.9b
AEW	40.2 ± 0.6a	41.3 ± 1.0a
LLL	2.0 ± 0.5b	4.0 ± 0.5a
RLL	2.2 ± 0.5a	4.1 ± 0.7a
BAD	53.2 ± 1.4a	49.1 ± 1.8a
PBS	70.1 ± 1.6a	66.0 ± 1.3b
PBL	40.4 ± 1.6a	35.5 ± 2.0a
INSET	107.1 ± 2.1a	96.1 ± 3.8a
ACCSET	145.4 ± 2.0a	137.7 ± 3.6a
VLW/GC	0.196 ± 0.009a	0.211 ± 0.005a
SAS/GC	0.321 ± 0.019a	0.323 ± 0.008a
GC/GS	1.105 ± 0.018b	1.155 ± 0.009a
GC/BAD	6.182 ± 0.208a	6.780 ± 0.281a
AEL/PBS	2.749 ± 0.089a	2.657 ± 0.081a
AEW/PBS	0.565 ± 0.017b	0.637 ± 0.021a

[1] Means ± SE in the same row followed by the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch multiple range test).
[2] Direct measurements in μm, ratios are unitless values.
[3] Abbreviations as in Table 1.
[4] RS, Río Socuavó, Venezuela; SI, Sitronela, Colombia.

and families: SAS/GC, AEL/PBS, AEW/PBS. Seven characters did not vary among collection sites: GC, LLL, RLL, INSET, VLW/GC, GC/GS, and GC/BAD. All specimens but one had heavily sclerotized aedeagal leaflets.

Seven characters differed between the RS and SI collection sites for cytotype C males: GS, GSC, AEL, LLL, PBS, GC/GS, and AEW/PBS (Table 4). Venezuelan cytotype C males, i.e., RS, had significantly larger AEL and PBS. Males from SI had significantly larger GS, GSC, LLL, GC/GS, and AEW/PBS. All specimens had lightly sclerotized leaflets.

Differences among cytotypes. Overall, when all characters were considered together, morphological differences were detected among cytotypes (Wilk's lambda = 0.0006, $P < 0.015$) and for collection sites and families within cytotypes (Wilk's lambda = 0.0288, $P < 0.001$). Eleven variables varied significantly among cytotypes, and 17 characters varied among collection sites and families within cytotypes (Table 5).

The backward elimination process removed seven variables from the analysis. Four variables, RLL, PBL, INSET, and LEAFSCLE, were retained for discriminant analysis. The majority of specimens of cytotype A (97.75%) and cytotype B (99.44%) were classified correctly to cytotype. Most cytotype C specimens (84.85%) were misclassified into cytotype A. Four cytotype A specimens were misclassified into cytotype C, and one cytotype B male was misclassified into cytotype A. One discriminant function accounted for the differences among males of cytotype B *vs.* cytotypes A + C: Z = 9.75 (LEAFSCLE) + 0.17 (RLL) − 0.06 (PBL) − 0.002 (INSET).

The most obvious differences among the specimens examined were those of the leaflets of the aedeagus, which for cytotype B specimens were visibly more sclerotized and usu-

ally discernably longer than were those of cytotype A and C males (Fig. 2). The aedeagal leaflets of these males appeared to be wider at their bases than were those of cytotype A and C males. The aedeagal leaflets of cytotype B males in general appeared to be longer and straighter than those of cytotype A and C males.

Two forms of gonostylar claw were observed. One form was long and thin, whereas the other form was shorter and thicker. Both forms may be observed on specimens belonging to a single cytotype and even within a locality (Fig. 3). Several specimens were found that had a long, thin gonostyler claw on one gonostylus and a short, thick gonostylar claw on the other gonostylus.

DISCUSSION

Characters analyzed in this study present a bewildering range of variation and overlap among the cytotypes. In fact, variation among collection sites within a cytotype was as great as or greater than variation among cytotypes. This potentially confounding nested variation is important taxonomically because it would be easy to select morphological characters that appear to distinguish among cytotypes but in reality serve only to identify collection sites. However, use of discriminant analysis techniques revealed that cytotype B males were different from cytotypes A and C.

The most readily apparent difference between cytotype B males and males of other cytotypes is the form of the aedeagal leaflets. Faran (1980) described *An. nuneztovari* as ". . . with or without very small, membranous . . ." leaflets. Sutil (1976) redescribed *An. nuneztovari* based on his collections from western Venezuela and made no mention of specimens lacking leaflets. Savage (1986) illustrated the holotype of *An. nuneztovari.* This specimen, as well as three presumed paratypes, was collected from San Carlos, Cojedes, well within the range of cytotype B. Savage (1986) further states, ". . . I believe that leaflets are always present and are diagnostic for *nuneztovari.*" This discrepancy between Faran (1980) and Savage (1986) most probably is due to Faran's material being a mixed collection of cytotypes A, B, and C, whereas Savage examined and illustrated the type material of *An. nuneztovari,* which apparently is cytotype B. There also may have been some specimens of *An. dunhami* Causey in Faran's material (Peyton 1993). Avila Nuñez (1989) demonstrated that male *An. nuneztovari* from Caño Macho, in Táchira state, have aedeagal leaflets that are significantly shorter than those of males from the La Lengüeta region of Barinas state. The Caño Macho site is within the range of *An. nuneztovari* cytotype C, whereas La Lengüeta is within the range of cytotype B (Conn 1990). In the current study, the aedeagal leaflets of cytotype B males were found to be longer and visibly more heavily sclerotized compared with those of cytotypes A

Table 5. *F*-values for tests of differences among cytotypes and among collection sites within cytotypes for male *Anopheles nuneztovari* (degrees of freedom: cytotype, 2; site within cytotype, 22; error, 251).

Character	*F*-values: Among cytotypes A, B, & C	*F*-values: Sites within a cytotype
GC	2.36^{ns}	3.69****
GS	2.72^{ns}	3.97****
GSC	2.92^{ns}	3.25****
SAS	6.26**	3.34****
VLW	3.28*	4.06****
AEL	5.84**	3.65****
AEW	0.60^{ns}	3.27****
LLL	213.72****	2.49***
RLL	239.02****	3.24****
BAD	2.08^{ns}	1.57^{ns}
PBS	0.22^{ns}	2.78****
PBL	7.34***	2.59***
INSET	6.60**	2.03**
ACCSET	8.24***	2.79****
VLW/GC	4.32*	2.93****
SAS/GC	7.36***	3.13****
GC/GS	0.30^{ns}	2.57***
GC/BAD	2.83^{ns}	0.82^{ns}
AEL/PBS	3.15*	1.50^{ns}
AEW/PBS	0.09^{ns}	2.53***

* Significant at 0.05 level.
** Significant at 0.01 level.
*** Significant at 0.001 level.
**** Significant at 0.0001 level.
^{ns} Not significant.

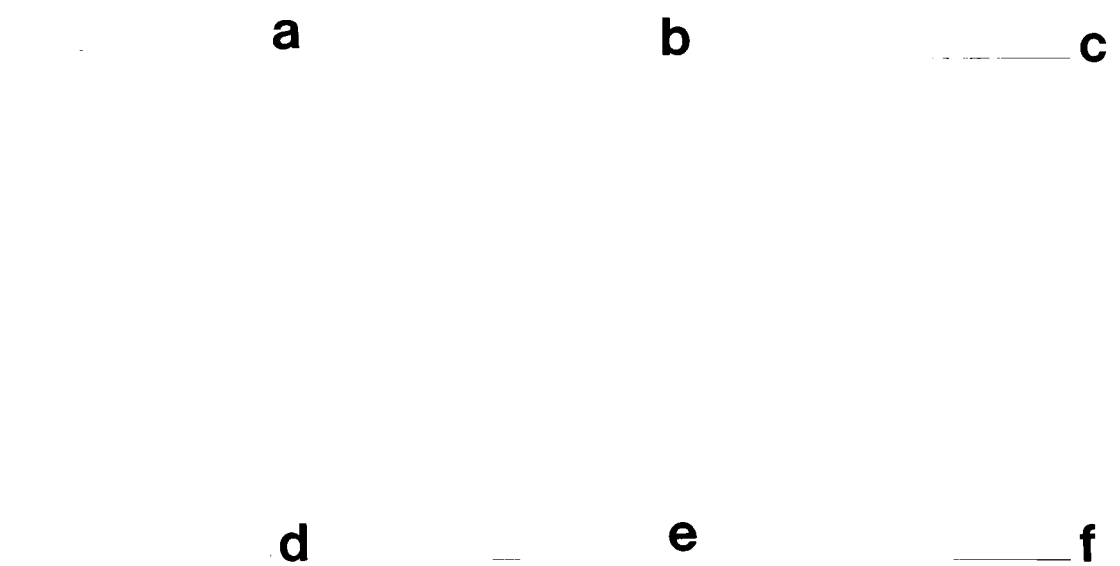

Fig. 2. Aedeagal leaflets of male *Anopheles nuneztovari* reared from field-collected females. a,b, SI, cytotype C; c,d, PR, cytotype A; e, VC, cytotype A; f, SO, cytotype B.

and C. Interestingly, this difference of length and sclerotization of aedeagal leaflets has been illustrated in the past. In his monograph of Venezuelan mosquitoes, Cova-Garcia (1961) depicted the aedeagus of *An. nuneztovari* showing long leaflets with wide bases. Forattini (1962), however, illustrated the male genitalia of *An. nuneztovari* depicting only one small leaflet. Forattini's (1962) diagnosis refers to the presence of "pequenos espículos" (i.e., small spines). Forattini (1962) illustrated a Brazilian specimen, collected at Santana, Amapa state, Brazil (*fide* letter from O.P. Forattini). Therefore, comparative illustrations of cytotype A and B males have been available for over 30 years. Additionally, several cytotype A males lacked leaflets. Causey (1945) illustrated the genitalia of *An. goeldii,* which in his photograph lacks leaflets, greatly resembling *An. nuneztovari* cytotype A.

In their description of *An. goeldii,* Rozeboom and Gabaldón (1941) relied in part on the length of the aedeagal leaflets to distinguish among *An. goeldii, An. nuneztovari,* and *An. rangeli* Gabaldón, Cova-Garcia, and Lopez. They also provided some other characters to separate *An. goeldii* and *An. nunez-*

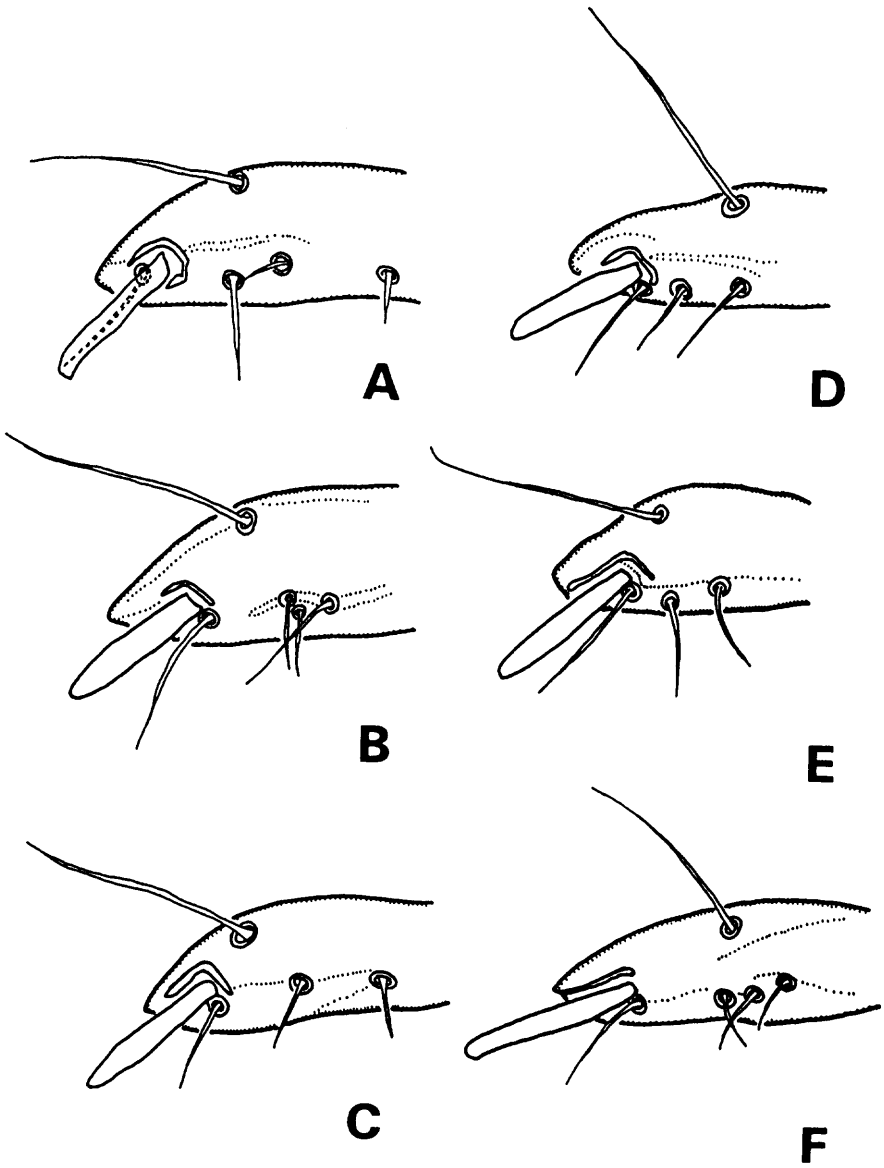

Fig. 3. Gonostylar claws of *An. nuneztovari* males. A, Cytotype A (VC); B, cytotype A (PR); C, cytotype B (SO); D, cytotype B (EN); E, cytotype B (EN); F, cytotype C (SI).

tovari, these being the presence or absence of the refringent structure, the relative size and degree of pigmentation of the preapical plate, the width of the apex of the fused ventral lobes relative to the width of the aedeagus, and the degree of sharpness of outer corners and steepness of the lateral slopes of the fused ventral lobes. Faran (1980) attributed many of these differences to the angle of observation. Based on an examination of over 100 specimens, Gabaldón (1981) stated that *An. nuneztovari* has leaflets that are always well sclerotized, although they may be short, medium, or long in length, whereas this is not true for *An. goeldii.* Floch and Abonnenc (1946), after examining the male genitalia of both species, stated that they believed the two specific epithets referred to the same species.

Based on historical records and the known geographic distributions of the cytotypes of *An. nuneztovari* (Conn, personal communication), it is likely that the specimens upon which the description of *An. goeldii* was based were *An. nuneztovari* cytotype A.

Gabaldón (1981) defended the validity of the species *An. goeldii,* admitting, however, that the two nominal species were very similar in appearance; he insisted that the major difference between them was the length and sclerotization of aedeagal leaflets. Gabaldón (1981) also admits that he had misidentified some mosquitoes from Trujillo, Venezuela, as *An. goeldii* when they were actually *An. nuneztovari* and that his misidentification may have contributed to the confusion surrounding the two species. Work is being conducted currently on the biology and ecology of *An. nuneztovari,* and any further statements concerning the validity of *An. goeldii* would be premature at best.

Analysis of mitochondrial DNA indicates that cytotypes B and C are more closely related to each other than to cytotype A (Conn, personal communication). Morphology of male genitalia indicates that cytotypes A and C are more similar to each other than to cytotype B. This discrepancy presents a problem if male genitalia are used to classify members of the *An. nuneztovari* species complex. It is possible to separate cytotype B males from cytotype A and C males on the basis of genitalic characters but not to separate cytotype A and C males. However, morphometry of male genitalia has proven useful in the identification of members of other anopheline species complexes, e.g., *An. balabacensis* Baisas and *An. dirus* Peyton and Harrison (Sucharit and Choochote 1983).

This study indicates that male genitalia can be used to distinguish cytotype B males from males of cytotypes A and C. The length, width at base, and degree of sclerotization of aedeagal leaflets are the most visible differences between cytotype B males and males of other cytotypes, permitting separation of specimens into B and "not B" groups. There are other, statistically significant differences between cytotype B males and males of cytotypes A and C, but these characters are useful only in combination.

It is clear that cytotype B males from western Venezuela are different from males from other parts of the South American continent. Differences also have been documented by studies of mitochondrial DNA (Conn, personal communication), ribosomal DNA (Fritz et al. 1994), isozymes (Fritz, personal communication), and morphology of eggs (Linley, personal communication). The concordant geographic variation of male genitalia, cytology, and isozymes between cytotypes B and C supports their status as forms of a single species with the Andes Mountains as a geographic barrier (Mayr 1942), particularly if there is some genetic exchange between the two populations, as Conn (personal communication) suggested. The similarity of male genitalia between cytotypes A and C may be the result of convergent evolution if these cytotypes truly are allopatric. On the other hand, Conn (personal communication) hypothesized that the cytotype C populations may have arisen from a recent colonization by Amazonian populations, which would account for the similarity of cytotype A and C male genitalia. Although detectable morphological variations concordant with other data would further support the view of cytotypes B and C as subspecies of a single species, distinct and different from cytotype A, the lack of such differences in male genitalia between cytotypes A and C does not negate this view. It would be a mistake to attach too much weight to similarity of genital morphology, especially when the preponderance of evidence suggests otherwise (Rensch 1934, Mayr 1942).

Whether or not these differences in morphology of genitalia indicate reproductive isolation must be verified experimentally (Goulson 1993). This confirmation is not likely to be achieved soon because, with the exception of *An. albimanus* Wiedemann (Rozeboom 1936) and *An. deaneorum* Rosa-Freitas (Kline et al. 1990), the species in the subgenus *Nyssorhynchus* are not colonized and efforts at forced mating have so far been unsuccessful. Verification or refutation of

separate species status will have to come from other sources, viz., cytology, studies of mitochondrial DNA, sequence analysis, and morphology of other life stages. However, this study does indicate that the cytotype B males of *An. nuneztovari* can be distinguished morphologically from males of cytotypes A and C using a suite of genitalic characters.

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