Malaria Vector *Anopheles* (*Nyssorhynchus*) *nuneztovari* Comprises One Genetic Species in Colombia Based on Homogeneity of Nuclear ITS2 rDNA

DIANA M. SIERRA,[1] IVAN DARIO VELEZ,[1] AND YVONNE-MARIE LINTON[2]

J. Med. Entomol. 41(3): 302–307 (2004)

ABSTRACT Previous studies indicated that two distinct chromosomal forms of *Anopheles nuneztovari* Gabaldón, cytotypes B and C, occurred on the west and east of the Latin American Andes Mountains, respectively. To determine the taxonomic status of *Anopheles* (*Nyssorhynchus*) *nuneztovari* in Colombia, link-reared specimens were collected from four sites: in the departments of Chocó (La Pacurita) and Valle (Sitronella) in the west, and Norte de Santander (Guaramito and Tibú) in the east. Nuclear ITS2 sequences were generated for 46 individuals. Only two specimens (4.4%) showed divergent haplotypes, varying from the consensus by a single-base polymorphism (0.18%). These results suggest that populations of *An. nuneztovari* corresponding to cytotypes (B and C) are conspecific.

KEY WORDS *Anopheles nuneztovari*, cytotype B and C, ITS2, Colombia

Anopheles (*Nyssorhynchus*) *nuneztovari* Gabaldón is widely distributed in eastern Panama and northern South America, including Amazonia. Significant differences in host preference and biting behavior between populations of *An. nuneztovari* in Colombia and Venezuela compared with those in the Brazilian Amazon led Elliott (1972) to propose that these geographical distinct populations of *An. nuneztovari* comprised two allopatric races. Differences in autosomal inversion polymorphisms and a fixed XR chromosome inversion between Venezuelan and Brazilian *An. nuneztovari* (Kitzmiller et al. 1973) corroborated this theory. After additional chromosomal analyses, Conn (1990) designated them cytotype A (Amazonia) and B (Venezuela/Colombia), respectively. Further support for a two-species separation based on cytotype was provided by a taxonomic comparison of the egg structures (Linley et al. 1996), male genitalia (Hribar 1994), isoenzymes (Scarpassa et al. 1996, 1999), and various DNA studies (Fritz et al. 1994, Conn et al. 1998, Scarpassa et al. 2000). Later, a comparative study of chromosomes from western Colombia and Venezuela revealed a third cytotype, differing from cytotypes A and B by the presence of a unique chromocenter and differences in the frequency of a large, complex inversion. This chromosomal form, found in *An. nuneztovari* populations west of the Andes, was designated cytotype C (Conn et al. 1993).

Despite being found infected with both *P. vivax* and *P. falciparum* in the Brazilian Amazon (Arruda et al. 1986), *An. nuneztovari* populations (cytotype A) are predominantly zoophilic, and not implicated as important malaria vectors in this region. Conversely, *An. nuneztovari* populations in Colombia and Venezuela are highly anthropophilic and act as primary malaria vectors (Rey and Renijifo 1950, Gabaldón and Guerrero 1959). Because of the vector significance of cytotype B and C populations in Colombia, the current study endeavored to accumulate molecular evidence to assess the taxonomic status of the two cytotypes.

The Colombian *An. nuneztovari* were collected from four localities in three geographically distinct regions in Colombia, including sites from west and east of the Andes. Nuclear ITS2 sequences were generated to compare the haplotypes with those generated by Fritz et al. (1994). Sequence data tested the null hypothesis that *An. nuneztovari* exists as two separate genetic forms in Colombia, conforming to the cytotypes B and C of Conn (1990) and Conn et al. (1993).

Materials and Methods

Mosquito Samples. Specimens of *An. nuneztovari* were collected from three departments in Colombia: La Pacurita, Chocó (5° 42′ N, 76° 39′ W), Valle, Sitronella (3° 53′ N, 77° 3′ W), and two sites in Norte de Santander, Tibú (8° 38′ N, 72° 44′ W) and Guaramito (9° 41′ N, 68° 39′ W) (Fig. 1). Wild-caught larvae were individually link-reared. The adults were used for DNA extraction, and the associated larval and pupal exuviae were preserved as voucher specimens. Progeny of blood-fed females captured in human bait

[1] Programa de Estudio y Control de Enfermedades Tropicales (PECET), Facultad de Medicina, Universidad de Antioquia, AA.1226 Medellin, Colombia.

[2] Department of Entomology and Biomedical Sciences Theme, The Natural History Museum, Cromwell Rd., London SW7 5BD, England, UK (e-mail: y.linton@nhm.ac.uk).

Fig. 1. Outline map of Colombia, showing collection sites of *An. nuneztovari* used in this study, in relation to the position of the Andes mountain range. The species is considered to comprise two cytotypes: B on the east and C on the west of the Andes (Conn 1990, Conn et al. 1993).

collections were individually reared to obtain voucher specimens of all life stages and sexes. Identifications for the progeny broods and larval collections were confirmed independently at The Natural History Museum (BMNH), London. Voucher specimens comprising associated larval and pupal exuviae and eggs were deposited at the Programa de Estudio y Control de Enfermedades Tropicales (PECET) Laboratories, Universidad de Antioquia, Medellin, Colombia.

Molecular Techniques. DNA was extracted from individual mosquitoes stored in 100% isopropanol, following the phenol–chloroform extraction protocol in Linton et al. (2001). Polymerase chain reaction (PCR) amplification and sequencing in both directions were carried out according to the protocol listed in Linton et al. (2001). Sequences were edited and aligned using Sequencher version 3.1.1 (Genes Codes Corporation, Ann Arbor, MI) and CLUSTAL X software packages. Similarity with those sequences available in GenBank was assessed using FASTA search. After sequencing, template DNA was dried and deposited at −70°C in the Molecular Systematics Laboratory, Department of Entomology, The Natural History Museum, London (BMNH) for future reference.

Results

Variability in ITS2 Sequences. Forty-six sequences comprising the 3′ end of the 5.8S gene, the ITS2, and the 5′ end of the 28S ribosomal gene, were generated for 18 individuals from Chocó, 10 from Sitronella, 13 from Tibú, and 5 from Guaramito. Sequences are available in GenBank under the following accession numbers: Chocó (AY028109-AY028126), Tibú (AY028096-AY028108), Sitronella (AY028087-AY028095, AY028128), and Guaramito (AY028081-AY028085).

The alignment was 549 bp in length (including primers, 43 bp), 53.0% GC rich, and unambiguous. The ITS2 region was 363 bp in length and two haplotypes, varying by a single base polymorphism (G⇔C) at base 365, were revealed (Fig. 2). Haplotype 1 accounted for 44 (95.65%) of the specimens analyzed (GenBank accession nos. AY028081-AY028085, AY028087-AY028124, and AY028128). The two remaining individuals, both from Chocó (GenBank accession nos. AY028125 and AY028126), shared haplotype 2.

Comparison with Other Published ITS2 Sequences. Previous studies that generated ITS2 sequences for *An. nuneztovari* deposited four sequences in GenBank. Danoff-Burg and Conn submitted three *An. nuneztovari* sequences (U92350, U92351, and U92343 of unspecified origin) to GenBank in 1997. One consensus sequence (L22462) of 28 resolved sequences was submitted to GenBank by Fritz et al. (1994). All GenBank and published *An. nuneztovari* ITS2 sequences, were aligned with the Colombian haplotypes generated in the current study (Fig. 2). This alignment comprised samples (including ours) from Colombia (54), Suriname (3), Brazil (6), Bolivia (1), and of unspecified origin (3). The alignment was 373 bp in length, reflecting the 363–373-bp ITS2 region amplified in other studies; only the variable region between bases 264–373 is shown in Fig. 2.

The alignment showed complete homology between our Colombian haplotype 1 and the nine individuals sequenced by Fritz et al. (1994) from Sitronella (3), and Zulia (3) and Brokopondo (3) (L22462) (Fig. 2). The length of the ITS2 region was 363 bp for all Colombian and Venezuelan samples. Haplotype 2 differed by one base from L22462 (Fig. 2). Maximum sequence divergence of 2.9% between our Colombian haplotype 2 and U92343 of unspecified origin was noted.

Sixteen (4.07%) bases were variable among the sequences. Ten were accounted for by three insertion/deletion (indel) events at bases 264–265 (two bases), bases 282–289 (eight bases) and bases 356–360 (four bases) (Fig. 2). At bases 264–265, an AA insert was noted in sequences U92350, U92351, and U92343 (Danoff-Burg and Conn, direct submission 1997). Two additional indels consisted of four GA and two GA microsatellite repeats (Fig. 2). Specimens from Suriname, and Brazil (Fig. 2) differed from the other populations in sharing the GAGA deletion followed by a parsimony informative G⇔T transversion at bases 356–361. The singleton polymorphic base change (G⇔C) at base 365 was unique in the Colombian haplotype 2 sequences generated in the current study (Fig. 2).

Discussion

Concerted evolution of multigene families within species has resulted in rapidly evolving spacer regions, such as the ITS2 rDNA. Sequencing of these regions has been used to effectively determine the species composition of *Anopheles* mosquito complexes (Collins and Paskewitz 1996). Low levels of intraspecific variation in the ITS2 region has proven useful for the design of species-specific primers or PCR-restriction fragment-length polymorphism assays to differentiate members of species complexes and for phylogeny reconstruction in mosquitoes. Previous studies of ITS2 sequences have shown little or no intraspecific variation in sibling species of *Anopheles.* Intraspecific variation in five colony strains of species of the *An. gambiae* complex was reported to range between 0 and 0.43% (Paskewitz et al. 1993). In contrast, Beebe et al. (2001) reported population-specific ITS2 sequences in the *An. bancroftii* group comparing populations from Queensland, Australia, and the western province of Papua New Guinea

The ITS2 data presented in the current study indicate *An. nuneztovari* to be a single genetic species on both sides of the Andes mountain range, despite differences in karyotype morphology described by Conn et al. (1993). Homosequential chromosomes have been documented in other *Anopheles* species complexes, for example, *An. labranchiae* Falleroni and *An. atroparvus* van Thiel of the Maculipennis Complex (Kitzmiller et al. 1967). However, examples such as cytotypes B and C of *An. nuneztovari*, where distinct chromosomal forms comprise one genetic species, are less common. However Mukabayire et al. (1999), who used nuclear ITS2 and mtDNA cytochrome *b* sequences, observed that populations of *An. funestus* Giles exhibiting differing behaviors and karyotypes in East Africa (Kenya) and West Africa (Senegal and Burkina Faso), concluding that all comprised a single species.

The combination of our ITS2 data set with that of Fritz et al. (1994) clearly shows that *An. nuneztovari* in Colombia comprises a single genetic species. The low level of genetic diversity in the ITS2 region denotes intraspecific variation and may even be accounted for by intra-individual variation in the multiple copies of the rDNA subunits in the nuclear genome. By the nature of the polymerase chain reaction (PCR) and direct sequencing of PCR products, obtained sequences comprise a consensus sequence. Hence, intra-individual variation is detected only if variants are relatively common and visualized as shadow bases on a sequencing chromatogram. The two sequences generated for haplotype 2 showed guanine shadow bands at the polymorphic base; this finding suggested that if the ITS2 sequences of these individuals were reamplified, the resulting sequence may equally correspond to the dominant haplotype. For this reason, we believe that the two haplotypes

```
                2222222222222222222222222222222222333333333333333333333333333333
                6666667777777777888888888899999999990000000000111111111122222222223
                4567890123456789012345678901234567890123456789012345678901234567890

COL-CHO (16)    --TACCAAAAGAGAGAGA--------CCAAACGTACCCTGAAGCAACGGATGCGCACACGAGTGTGC
COL-CHO (2)     ...............................................................
COL-TIB (11)    ...............................................................
COL-GUA (5)     ...............................................................
COL-SIT (13)    ...............................................................
FCOL-SIT (3)    ...............................................................
FVEN-BAR (3)    ...............................................................
FVEN-ZUL (3)    ...............................................................
L22462          ...............................................................
FBOL-BEN (1)    ..................GA...........................................
FSUR-BRO (3     ...............................................................
FBRA-ROR (3)    ...............................................................
FBRA-AMA (2)    ...............................................................
FBRA-AMA (1)    AA..............GAGAGA.........................................
FBRA-PAU (3)    AA..............GAGAGAGA.......................................
FBRA-PAV (3)    AA..............GAGAGAGA.......................................
U92350          AA..............................................................
U92351          AA..............GA..............................................
U92343          AA..............GAGAGAGA.......................................


                3333333333333333333333333333333333333333333333
                3333333334444444444555555555566666666667777
                1234567890123456789012345678901234567890123

COL-CHO (16)    AGCTCATCGCGATCAGTAAGAGAGAGAGAGTACCGATCACTAG
COL-CHO (2)     ..................................C........
COL-TIB (11)    ...........................................
COL-GUA (5)     ...........................................
COL-SIT (13)    ...........................................
FCOL-SIT (3)    ...........................................
FVEN-BAR (3)    ...........................................
FVEN-ZUL (3)    ...........................................
L22462          ...........................................
FBOL-BEN (1)    ...........................................
FSUR-BRO (3     ........................----T.............
FBRA-ROR (3)    ........................----T.............
FBRA-AMA (2)    ........................----T.............
FBRA-AMA (1)    ........................----T.............
FBRA-PAU (3)    ........................----T.............
FBRA-PAV (3)    ........................----T.............
U92350          ...........................................
U92351          ...........................................
U92343          ...........................................
```

Fig. 2. A 109 bp fragment (bases 264–373) of the 3′ end of the 373-bp alignment of the ITS2 region for all *An. nuneztovari* specimens sequenced to date. Bases 1–263 (not shown) are completely homologous. Sequences prefixed by F are those in the published alignment of Fritz et al. (1994), but which are not entered in GenBank. Locality abbreviations are as follows: CHO, Chocó; TIB, Tibú; SIT, Sitronella; GUA, Guaramito; BAR, Barinas; ZUL, Zulia; BEN, Beni; BRO, Brokopondo; ROR, Roraima; AMA, Amazonas; PAU, Pará-Utinga; PAV, Pará-Velha). Replicates with the same haplotype from each region are noted in parentheses. The four ITS2 sequences of *An. nuneztovari* available in GenBank are also included: L22462 (Fritz et al. 1994, believed to be a consensus sequence of Colombian and Venezuelan *An. nuneztovari*); and U92343, U92350, and U92351 from *An. nuneztovari* of unspecified origin (Danoff-Burg and Conn, direct submission 1997). Abbreviations for country codes are as follow: COL, Colombia; VEN, Venezuela; SUR, Suriname; BRA, Brazil. Sequence names prefixed only with COL were generated in this study.

from Chocó represent intraspecific gene flow or genetic drift instead of the fixed base differences that typically diagnose cryptic species.

A parallel scanning electron microscopy study of the eggs from progeny broods used in this study, from Chocó, Sitronella, and Tibú (Sierra 2001), corrobo-

rated our molecular evidence. Past studies have concentrated on differentiation of Colombian and Venezuelan cytotypes from the Brazilian cytotype A. Studies that have separated the cytotype B and/or C from the Amazonian *An. nuneztovari* (cytotype A) include those on larval and pupal chaetotaxy (Hribar 1995a), isozymes (Scarpassa et al. 1999), mtDNA restriction fragment-length polymorphism (Conn et al. 1998, Scarpassa et al. 2000), and nuclear ITS2 DNA sequences (Fritz et al. 1994). However, studies attempting to define diagnostic characters for cytotypes B and C found no evidence from comparisons of male genitalia (Hribar 1994) or mtDNA sequences (Perera 1993, Scarpassa et al. 1999). These earlier results are consistent with the findings reported herein that cytotypes B and C are conspecific and that gene flow in *An. nuneztovari* is not restricted by the Andes Mountains.

Homogeneity of *An. nuneztovari* ITS2 haplotypes between populations in Bolivia, Colombia, and Venezuela indicate that one species, corresponding to the polymorphic cytotypes B/C, is present along the western Pacific fringe countries of South America. This species is a highly effective malaria vector and genetically distinct from the Amazonian form (cytotype A) that exhibits low vector capacity. The taxonomic and vectorial status of the other genetic form sympatric with cytotype A in the Brazilian Amazon, identified on the basis of ITS2 sequences by Fritz et al. (1994) and further evidenced by mtDNA restriction fragment-length polymorphism haplotypes (Conn et al. 1998), warrants further study.

Acknowledgments

We are indebted to W. Rojas, E. Pabón, G. Rua, L. Agudelo, F. Ruiz, and L. Velazques for field assistance. We gratefully acknowledge R. E. Harbach (Department of Entomology, The Natural History Museum, London) for morphological confirmation of the material used in this study. We thank R. E. Harbach and Jan Conn (Griffin Laboratory, Wadsworth Center, Slingerlands, NY) for critical review of this manuscript before submission. This study was funded by Programa de Estudio y Control de Enfermedades Tropicales laboratory, University of Antioquia, Medellin, Colombia, and The Mosquitoes Programme, Department of Entomology, The Natural History Museum, London.

References Cited

Arruda, M. de, M. B. Carvalho, R. S. Nussenzweig, 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.

Beebe, N. W., J. Maung, A. F. van der Hurk, J. T. Ellis, and R. D. Cooper. 2001. Ribosomal DNA spacer genotypes of the *Anopheles bancroftii* group (Diptera: Culicidae) from Australia and Papua New Guinea. Insect Mol. Biol 10: 407–413.

Collins, F. H., and S. M. Paskewitz. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. Insect Mol. Biol 5: 1–9.

Conn, J. 1990. A genetic study of the malaria vector *Anopheles nuneztovari* from western Venezuela and Colombia. J. Am. Mosq. Control Assoc. 6: 400–405.

Conn, J., Y. R. Puertas, and J. A. Seawright. 1993. A new cytotype of *Anopheles nuneztovari* from western Venezuela and Colombia. J. Am. Mosq. Control Assoc. 9: 294–301.

Conn, J. E., S. E. Mitchell, and A. F. Cockburn. 1998. Mitochondrial DNA analysis of the Neotropical malaria vector *Anopheles nuneztovari.* Genome 41: 313–327.

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

Fritz, G. N., J. Conn, A. F. Cockburn, and J. A. Seawright. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). Mol. Biol. Evol. 11: 406–416.

Gabaldón, 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.

Hribar, L. 1994. Geographical variation of male genitalia of *Anopheles nuneztovari* Gabaldón. Mosq. Syst. 26: 132–144.

Hribar, L. 1995a. Larval and pupal chaetotaxy of *Anopheles nuneztovari* (Diptera: Culicidae). Trans. Am. Entomol. Soc. 121: 249–257.

Kitzmiller, J. B., G. Frizzi, and R. Baker. 1967. Evolution and speciation within the *maculipennis* complex of the genus *Anopheles,* pp. 151–210. *In* J. W. Wright and R. Pal [eds.], Genetics and vectors of disease. Elsevier, Amsterdam, The Netherlands.

Kitzmiller, J. B., R. D. Kreutzer, and E. Tallaferro. 1973. Chromosomal differences in populations of *Anopheles nuneztovari.* Bull. W.H.O. 48: 435–445.

Linley, J. R., L. P. Lounibos, J. Conn, D. Duzak, and N. Nishimura. 1996. A description and morphometric comparison of eggs from eight geographic populations of the South American malaria vector *Anopheles* (*Nyssorhynchus*) *nuneztovari.* J. Am. Mosq. Control Assoc. 12: 275–292.

Linton, Y.-M., R. E. Harbach, M. S. Chang, T. G. Anthony, and A. Matusop. 2001. Morphological and molecular identity of *Anopheles* (*Cellia*) *sundaicus* (Diptera: Culicidae), the nominotypical member of a malaria vector species complex in Southeast Asia. Syst. Entomol. 26: 357–366.

Mukabayire, O., D. Boccolini, L. Lochouarn, D. Fontenille, and N. J. Besansky. 1999. Mitochondrial and ribosomal internal transcribed spacer (ITS2) diversity of the African malaria vector *Anopheles funestus.* Mol. Ecol. 8: 289–297.

Paskewitz, S. M., D. M. Wesson, and F. H. Collins. 1993. The internal transcribed spacers of ribosomal DNA in five members of the *Anopheles gambiae* species complex. Insect Mol. Biol. 2: 247–257.

Perera, O. P. 1993. Phylogenetic analysis of two mitochondrial genes from several species of the subgenus *Nyssorhynchus* (Culicidae: Anopheles) and the development of species-specific probes for their identification. Ph.D. dissertation, University of Florida, Gainesville.

Rey, H., and J. Renijifo. 1950. *Anopheles* (*Nyssorhynchus*) *nuneztovari* infectado en la naturaleza con *Plasmodium.* Rev. Acad. Colomb. Cienc. Exactas. Fis. Quim. Nat. 7: 534–537.

Scarpassa, V. M., W. P. Tadei, and M. F. Suarez. 1996. Allozyme differentiation among allopatric populations of *Anopheles nuneztovari* (Diptera: Culicidae). Braz. J. Genet. 19: 265–269.

Scarpassa, V. M., W. P. Tadei, and M. F. Suarez. 1999. Population structure and genetic divergence in *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. Am. J. Trop. Med. Hyg. 60: 1010–1018.

Scarpassa, V. M., S. Geurgas, A.M.L. Azeredo-Espin, and W. P. Tadei. 2000. Genetic divergence in mitochondrial DNA of *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. Genet. Mol. Biol. 23: 71–78.

Sierra, D. M. 2001. Caracterización morphológica y molecular de cuatro populaciones del vector de la malaria *Anopheles nuneztovari* Gabaldón (Diptera: Culicidae) en Colombia. M.S. thesis, Universidad de Los Andes, Venezuela.

Received for publication 30 September 2002; accepted 11 December 2003.