Sequence Analysis of the Ribosomal DNA Internal Transcribed Spacer 2 from Populations of *Anopheles nuneztovari* (Diptera: Culicidae)

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Sequence variation of the ribosomal DNA internal transcribed spacer 2 (ITS2) was examined for populations of the malaria vector *Anopheles nuneztovari* collected in Colombia, Venezuela, Bolivia, Suriname, and Brazil. Mosquitoes from Colombia and Venezuela had identical ITS2 sequences and were distinguished from sequences in other populations by three insertion/deletion events (indels) and by one transversion. The length of the ITS2 was 363–369 bp, and it had a G+C content of 55.3%–55.7%. Variation in the length of the ITS2 between and within populations was due to indels in simple repeats. ITS2 consensus sequences were similar or identical for samples from the following three groups: (1) Colombia, Bolivia, and Venezuela; (2) Suriname and northern Brazil; and (3) eastern and central Brazil. The presence of two different consensus sequences from a single location near Manaus, Brazil, suggests that populations from eastern Brazil and those from Suriname converge in this region of the Amazon Basin. These data show that putative cryptic species of *An. nuneztovari* are distinguished by very minor differences in DNA sequence of the ITS2 region.

Introduction

Eukaryotic ribosomal DNA (rDNA) has several properties that make it useful for studying genetic variability and divergence within and between species: tandemly repeated genes, secondary structure of transcribed regions, differential rates of evolution between spacers and coding regions, and concerted evolution (Arnheim 1983; Gerbi 1985; Tautz et al. 1987). rDNA is composed of tandemly repeated transcriptional units separated from each other by intergenic spacers. Each transcriptional unit, in turn, includes spacers and regions coding for the ribosomal subunits. Unlike single-copy genes, the repeated units of rDNA do not evolve independently but are relatively homogenous in sequence within a species. Molecular processes such as unequal crossing-over, gene conversion, and transposition have been proposed as mechanisms that not only generate variation in multigene arrays on which natural selection can operate, but also as mechanisms by which mutations spread through a multigene family (homogenization) and become fixed in a population/species (molecular drive) (Fogel and Mortimer 1969; Smith 1976; Dover 1982; Walsh 1986; Williams 1990).

Key words: *Anopheles nuneztovari*, *Nyssorhynchus*, ribosomal DNA, internal transcribed spacer 2, cryptic species.

The structure of the rDNA of mosquitoes has been described through restriction-enzyme analysis for *Anopheles albimanus* (Beach et al. 1989), the *An. gambiae* species complex (Collins et al. 1989), two species of *Aedes* (Black et al. 1989; Gale and Crampton 1989; Fallon et al. 1991), and three species in the North American *maculipennis* complex (Collins et al. 1990). As in other eukaryotes (Long and Dawid 1980), the rDNA of mosquitoes is composed of tandemly repeated transcriptional units separated by intergenic spacers. Within each transcriptional unit, two spacers separate the 18S, 5.8S, and 28S rDNA subunits, the internal transcribed spacer 1 (ITS1) and 2 (ITS2), respectively.

Because of the relatively rapid rate at which new mutants are fixed in the rDNA spacers, these regions may distinguish closely related species that otherwise show little genetic divergence (Brown et al. 1972; Furlong and Maden 1983; Tautz et al. 1987; Porter and Collins 1991) and may even differ between populations as well (Williams et al. 1985). Rapid sequence divergence between transcribed spacers was first demonstrated by Brown et al. (1972) for the frog sister species *Xenopus laevis* and *X. mulleri*; whereas their coding regions were virtually identical, the transcribed spacers were highly divergent. In mosquitoes, species-specific sequences of the ITS2 have been used to distinguish closely related species (Porter and Collins 1991) and to infer phylogenetic relationships (Wesson et al. 1992).
Anopheles (Nyssorhynchus) nuneztovari Gabaldón is a member of one of five major human malaria-vector complexes in the New World (Goriup and Pull 1988) and has a broad distribution spanning most of northern South America (fig. 1) (Faran 1980). For widely distributed species, especially those with allopatric populations separated by major geographic barriers (e.g., the Amazon River, Andes Mountains, etc., for An. nuneztovari), increased geographic distance is often associated with greater genetic divergence, though this relationship is not always correlated. Because such "species" may actually include a number of races, cryptic species (morphologically indistinguishable), sibling species (phylogenetically closely related), and ecotypes, they are of particular interest in the study of intraspecific versus interspecific variation. In the case of an important disease vector such as An. nuneztovari, genetic divergence and its underlying causes are particularly important in malariaology, where vector capacity may be differentially expressed among mosquito strains or among closely related species (Coluzzi 1970).

Studies on genetic variation of Anopheles species have described examples of incipient speciation and parapatric ecotypes (Coluzzi et al. 1979, 1985; Fritz et al. 1991) and show that this genus contains many cryptic species (see reviews by Reid 1970; Kitzmiller 1976). Both the investigation of genetic variability and the discovery and identification of cryptic species have relied primarily on cytotaxonomy, crossing experiments, and the use of molecular tools such as enzyme electrophoresis or DNA probes (Kitzmiller 1976; Mahon et al. 1976; Narang et al. 1989; Booth et al. 1991).

Dobzhansky (1937) was the first to emphasize that the process of species formation, in contrast to race formation, involves the development of reproductive isolating mechanisms. Hybrid sterility in anopheline mosquitoes seems to be among the first isolating mechanisms to develop during speciation and appears to be a highly reliable guide to the existence of reproductive isolation (Kitzmiller et al. 1967; Coluzzi 1970; Kitzmiller 1976). Unfortunately, postcopulatory reproductive isolation has not been tested successfully with any species of Nyssorhynchus, as most species will not mate in captivity and none have been force-copulated successfully. Thus, inferences on the presence and delineation of cryptic species in this subgenus have, at present, relied on various parameters that measure genetic divergence. Although An. nuneztovari is not yet formally recognized as a species complex, various reports have described chromosomal (Kitzmiller et al. 1973; Conn et al. 1993), behavioral (see reviews by Elliott 1968, 1972), allozyme (Steiner et al. 1980), and morphological differences (Rozeboom and Gabaldón 1941) between allopatric populations, differences that suggest significant intraspecific genetic divergence or a species complex.

Differentiation of the ITS2 between populations and species depends on many factors, including genetic drift, the relative number and size of repeats, rates of unequal crossover, gene conversion, immigration, number of loci, and mating systems (Smith 1973; Black and Gibson 1974; Arnheim 1983; Levinson and Gutman 1987). The degree of differentiation observed within a species, therefore, is a balance struck between those processes that generate variability and those that lead to homogenization and fixation. The purpose of this investigation was to describe and compare the consensus sequence of the rDNA ITS2 of the mosquito An. nuneztovari throughout its distribution, including samples from the various geographic races or putative cryptic species reported in the literature.

Material and Methods
Sample Collections
Mosquitoes were obtained during 1991-92 from various locations throughout the distribution of Anopheles nuneztovari in South America (fig. 1 and table 1). Prospective collecting sites were determined either by reviewing published collection records or on the advice of mosquito- and malaria-control personnel in host countries and of other scientists presently working with South American anopheline mosquitoes.

Both larvae and adult females were collected, the former by sampling in various larval habitats and the
latter by animal or human bait. Isofemale lines were established from field-caught female mosquitoes, and several individuals from each line were linked-reared in order to obtain larval and pupal exuviae and pinned adults for species identification. Species were identified using the keys by Faran (1980) and Faran and Linthicum (1981) and were confirmed by the Walter Reed Biosystematics Unit, Smithsonian Institution, Washington, D.C.

Polymerase Chain Amplification

The rDNA ITS2 region was amplified by the polymerase chain reaction (PCR), according to specifications outlined by GeneAmp DNA amplification kits (Perkin Elmer Cetus). Individual mosquitoes were macerated in 100 μl of a buffer containing 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetate, 50 mM NaCl, at pH 8.2. Samples were then boiled for 5 min, and 4–5 μl were used in each 100-μl amplification reaction. The PCR temperature profile consisted of 25 cycles of 1 min at 94°C, 2 min at 37°C, and 2 min at 72°C. The two primers used (28 nt each) annealed to conserved regions of the 5.8s and 28s rDNA subunits that flank the ITS2 (5'-GTGGATCTGGTAACGACCATG-3' and 5'-GTGAATTCATGCTTAAATTTAGGGGTA-3', respectively) (Porter and Collins 1991). Amplified fragments were concentrated to a volume of 10 μl by either ethanol precipitation or centrifugation in Ultrafree R MC filter units made of regenerated cellulose (Millipore). Each amplification product was electrophoresed in a 0.6% agarose gel, and the ITS2 fragment was excised. DNA was recovered from each gel slice by either electroelution or phenol extraction followed by precipitation with ethanol.

DNA Sequencing

The ITS2 of three individuals from separate family lines was sequenced for each collection site except Bolivia, where only one specimen was available. Consensus sequences of the ITS2 were obtained by direct sequencing of double-stranded PCR products by using the fmol DNA sequencing system (Promega). Sequencing reactions employed either α-32P-dATP (1,500 Ci/mM) incorporation or end-labeled primers (γ-33P-ATP [3,000 Ci/mM]), and reaction products for both strands of the ITS2 were run on 6% gradient acrylamide gels.

DNA Sequence Analysis

DNA sequences were analyzed using version 6.0 of the Sequence Analysis Software Package of the Genetics Computer Group (Devereux et al. 1984). The secondary structure of the ITS2 regions was generated by the FOLD program (Freier et al. 1986), which finds a best-fit secondary structure with minimum free energy, by the method of Zuker and Stiegler (1981). The sequence analyzed by the FOLD program included flanking regions of the 5.8s and 28s coding regions as suggested by Yeh and Lee (1990). The ITS2 sequence of anopheline mosquitoes is known for only three closely related species in the subgenus *maculipennis* (Porter and Collins 1991). Thus, the secondary structure of *An. nuneztovari* was compared with that of one of these species, *An. hermsi*, based on the sequence reported by Porter and Collins (1991). The GenBank accession number of the ITS2 of *An. nuneztovari* is L22462.

Results

DNA Sequence Analysis

Sequence was obtained from within 3–10 bp of the primers that annealed to the 5.8s and 28s regions flanking the ITS2. Boundaries of the 5.8s and 28s regions were estimated by comparison with those determined by Porter and Collins (1991), for three species of mosquitoes in the *Anopheles maculipennis* complex, and by secondary-structure considerations. The ITS2 of *An. nuneztovari* (fig. 2) begins at approximately position 67 and ends at position 440. The ITS2 sequences were 363–

### Table 1

Collection Sites of *Anopheles nuneztovari* in South America

<table>
<thead>
<tr>
<th>Country</th>
<th>State</th>
<th>Location (abbreviation)</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bolivia</td>
<td>. . . .</td>
<td>Guayaramerin (GU)</td>
<td>10°51'S, 65°21'W</td>
</tr>
<tr>
<td>Brazil</td>
<td>Roraima</td>
<td>Boa Vista (BV)</td>
<td>2°49'11&quot;N, 60°40'24&quot;W</td>
</tr>
<tr>
<td>Brazil</td>
<td>Amazonas</td>
<td>Puraquequara, Manaus (PR)</td>
<td>3°6'7&quot;S, 60°130'W</td>
</tr>
<tr>
<td>Brazil</td>
<td>Pará</td>
<td>Utinga, Belém (BL)</td>
<td>1°24'36&quot;S, 48°26'58&quot;W</td>
</tr>
<tr>
<td>Brazil</td>
<td>Pará</td>
<td>Velha Timbóteua, Capanema (CP)</td>
<td>1°17'S, 47°34'W</td>
</tr>
<tr>
<td>Suriname</td>
<td>Brokopondo</td>
<td>Victoria, Brokopondo (VC)</td>
<td>5°5'N, 54°58'W</td>
</tr>
<tr>
<td>Venezuela</td>
<td>Barinas</td>
<td>Animas, C. Bolivar (AN)</td>
<td>8°20'54&quot;N, 70°45'W</td>
</tr>
<tr>
<td>Venezuela</td>
<td>Zulia</td>
<td>Rio Socuavo (RS)</td>
<td>8°54'0&quot;N, 72°38'0&quot;W</td>
</tr>
<tr>
<td>Colombia</td>
<td>Valle</td>
<td>Sitronela, Buenaventura (SI)</td>
<td>3°49'N, 77°4'W</td>
</tr>
</tbody>
</table>
Simple tandem repeats were present at various locations along the ITS2. For example, GA repeats of varying lengths occur at positions 340 (four to eight repeats) and 416 (three to five repeats), TGATT is rep...
peated at position 112, CCTC at position 300, and GGTG at position 159. Other repeats were within 1–8 bases from each other: e.g., CGCGGA at positions 285 and 293 and ACCAAA at positions 326, 333, and 355.

**Intra-individual Variation**

Since direct sequencing of PCR products produces a consensus sequence, variation between copies of the ITS2 within individuals would only be detected if a particular variant was relatively common (e.g., a common sequence length variant may produce a shifted "shadow" of bands on a sequencing autoradiogram). There was no indication that common variants in ITS2 sequence were present in three individuals from Colombia (SI), six individuals from both sides of the Andes Mountains in Venezuela (RS and AN), and nine individuals from the Atlantic Coast of Brazil (BL and CP) (fig. 2). However, additional bands on sequence autoradiograms of all nine individuals sampled in Boa Vista and Puraquequara, Brazil, and in Suriname would be consistent with both a transition variant at position 334 (C-T) and variation in the number of GA repeats (approximately four to eight) beginning at position 340.

**Intrapopulation Variation**

Mosquitoes within each sampling site had identical ITS2 sequences, except in Puraquequara, Brazil (PR), where two different consensus sequences were present. One individual had the sequence that is present in eastern Brazil (CP and BL), except for a repeat of seven instead of eight GAs at position 340–355 (fig. 2). Two other individuals had sequences that were identical to those found in Boa Vista, Brazil (BV), and Suriname (VC).

**Interpopulation Variation**

Differences in the length and sequence of the ITS2 between populations were due to three variable regions of simple repeats, at positions 328, 348, and 422 (fig. 2). Samples from Colombia (SI) and both sides of the Andes Mountains in Venezuela (RS and AN) had identical ITS2 sequences, whereas the sequence from the single individual from Bolivia (GU) differed by an extra GA at position 348. The consensus sequences from these regions differed from that of the Atlantic Coast of Brazil by an indel of two adenines at position 328, by having four versus eight GA repeats at position 340, by having five versus three GA repeats at positions 416, and by a guanine instead of a thymine at position 426 (fig. 2). Six samples from Boa Vista (Brazil) and Suriname were identical to one another but differed from the samples in eastern Brazil by the absence of two adenines in position 328 and by four instead of eight GA repeats at position 340.

**Secondary Structure**

Wesson et al. (1992) published sequences of the internal transcribed spacers for three genera of mosquitoes and used conserved blocks of sequence and compensatory changes to predict pairing constraints. The sequence of the ITS2 of anopheline mosquitoes has only been published for three species in the subgenus Anopheles (Porter and Collins 1991). These sequences are highly divergent (~62% identity) from that found in An. nuneztovari, and there are no delineated large blocks of conserved sequence such as those found between Aedes species, Psorophora ferox (von Humboldt), and Haemagogus mesodentatus Komp and Kumm (Wesson et al. 1992). Nevertheless, FOLD predicts secondary-structure models for An. hermsi and An. nuneztovari that are quite similar (fig. 3). The 5' and 3' ends of the ITS2 begin at or near a cruciform junction demarcating stem-loop regions and followed by a long stem, which subsequently splits into two stem loops.

**Discussion**

**Secondary Structure**

The FOLD program of Zuker and Stiegler (1981) produces an rRNA secondary-structure model of minimum free energy, on the basis of published values of stacking and loop-destabilizing energies (fig. 3). Although the minimum free energy is optimized with FOLD, the real secondary structure may, on the basis of base-pairing constraints, have a different free-energy value (Zuker and Stiegler 1981). Yeh and Lee (1990) used enzymatic and chemical structure probes to estimate the secondary-structure models of various eukaryotes. Their data were more consistent with secondary-structure models in which the 3' end of the 5.8S sequence was base-paired with the 5' end of the 25S sequence, bringing the ends of the ITS2 sequence together.

For both Anopheles hermsi and An. nuneztovari, the FOLD program paired the 5.8S and 28S sequences to each other in the same manner as has been shown for various eukaryotes as well as for mosquitoes in other genera (Yeh and Lee 1990; Wesson et al. 1992) (fig. 3). The 5.8S pairs with itself to form a stem-loop region and is followed by a stem formed by pairing with the 5' end of the 28S. Unlike the situation in other mosquito genera (Wesson et al. 1992), however, the juncture of the ITS2 with the 5.8S and 28S sequences in these two anopheline species occurs at or near a cruciform area that demarcates one or more stem-loop regions. In this respect, the secondary-structure models of An. hermsi and An. nuneztovari are more similar to the models proposed for eukaryotes other than mosquitoes (Yeh and Lee 1990).

Although there is little sequence similarity between An. hermsi and An. nuneztovari, they retain folding fea-
FIG. 3.—Secondary structure of the ITS2 of *Anopheles nuneztovari* (A) and *An. hermsi* (B). Sequences include ~66 bases from the 5.8S and 17–23 bases from the 28S rDNA coding regions. Arrows delineate the ITS2 region. Free-energy values (i.e., ΔG) were −146.1 for *An. hermsi* and −118.3 for *An. nuneztovari*. Sequences for *An. hermsi* were obtained from Porter and Collins (1991).
folding features is consistent with studies that have demonstrated common conformational motifs among even distantly related organisms (plants, fungi, invertebrates, and vertebrates) (Hancock et al. 1988; Yeh and Lee 1990). These studies suggest that the three-dimensional structure of the ITS2 is an important part of its function, which has been substantiated by deletion studies (Musters et al. 1990; Van der Sand et al. 1992). The latter have demonstrated that the ITS2 is essential for the correct and efficient processing and maturation of certain ribosomal units. Furthermore, information for the efficient removal of ITS2 from its RNA precursor is dispersed throughout the entire ITS2 region, and indels that affect secondary structure differentially alter rRNA processing.

In their study of three related mosquito genera (Aedes, Psorophora, and Haemogogus), Wesson et al. (1992) found that intraspace variable regions appear to coevolve and that ITS2 variation is constrained to some extent by its secondary structure. As with the sequence variants for Ae. aegypti and Ae. simpsoni reported by Wesson et al. (1992), the sequence variation observed among samples of An. nuneztovari simply added to stem length or occurred in unpaired loop regions, thus maintaining secondary-structure integrity. Nevertheless, the secondary structures of Aedes, Psorophora, and Haemogogus (Wesson et al. 1992) have more in common with each other than any has to the anopheline motif. Although Wesson et al. (1992) describe conserved blocks of sequence within the ITS2 among these three genera, such blocks were not evident between An. nuneztovari and An. hermsi. This observation suggests that genetic differentiation within the genus Anopheles may be greater than that found between other genera in the family Culicidae.

**ITS2 Sequence**

As in other anophelines (Porter and Collins 1991), an A-T rich spacer does not separate a distinct 2S region from the 5.8S in An. nuneztovari. Such a spacer has been reported for another species in the suborder Nematocera, Sciara coprophila (Jordan et al. 1980), and in the suborder Cyclorrhapha, Drosophila melanogaster (Tautz et al. 1988). The conserved 5.8S and 28S coding regions flanking the ITS2 of An. nuneztovari were nearly identical to those reported for anophelines and mosquitoes in other genera (Porter and Collins 1991; Wesson et al. 1992). This similarity in the rDNA coding regions contrasts with the significant divergence of the ITS2 between An. hermsi and An. nuneztovari; the ITS2 of An. nuneztovari is ~60 bp longer and has only a 62% identity with An. hermsi. Since these two species are in separate subgenera, however, the sequence divergence between them is not necessarily indicative of sequence divergence between species in the subgenus Nyssorhynchus.

The sequence divergence of the ITS2 that may be “typical” between closely related species of anopheline mosquitoes is not known. There is only one published comparison of the ITS2 sequences of cryptic species of mosquitoes, a study by Porter and Collins (1991) on *An. freeborni* and *An. hermsi*. To date, these two species are, by some measures of genetic divergence, the most closely related species of anophelines known. Both species are found in the western United States, may be parapatric, and are indistinguishable by polytene chromosome banding patterns, enzyme loci, mitochondrial restriction-fragment-length polymorphisms, and morphology (Barr and Guptavanij 1988; Collins et al. 1990; Fritz et al. 1991). Hybrid females are fertile, whereas males are partially fertile in one backcross and completely sterile in the reciprocal backcross (Fritz et al. 1991). By all these measures of divergence (except for crossing studies, which have not been successful in *An. nuneztovari*), *An. freeborni* and *An. hermsi* are more similar to each other than are the different geographic populations of *An. nuneztovari* sampled in this study. Yet, Porter and Collins (1991) report that, in the ITS2 sequence, the divergence between *An. hermsi* and *An. freeborni* is much greater than that found between populations of *An. nuneztovari* thousands of kilometers apart: differences at 11 positions, including 8 single-nucleotide mismatches, 2 indels (1 in a repeat), and 1 3-bp mismatch.

Differences in the ITS2 sequence between geographically distant samples of *An. nuneztovari* are small and are confined to only three regions of single-base repeats and simple repeat motifs. This sequence divergence is similar, in amount and degree, to that reported within and between individuals of *Ae. aegypti* and *Ae. simpsoni* from a single locarc (Wesson et al. 1992). Although Wesson et al. (1992) cloned the ITS2, the nonrandom association between specific variants along the ITS2 suggests that most of the variation observed was not an artifact of cloning. The studies by Black et al. (1989), Kambhampati and Rai (1991), and Wesson et al. (1992) demonstrate that the internal transcribed and nontranscribed spacers of aedine mosquitoes are not conserved in populations or individuals. In effect, the variance-to-mean ratio of spacer variants (see Ohta and Dover 1984) in populations and species of *Aedes* appears to be relatively high.

Although the presence of spacer variants in individuals was not ascertainable within the scope of this study, prior studies suggest that intraspecific and intrapopulational variants are rare in anopheline mosquitoes (Fritz et al. 1991; Porter and Collins 1991; Scott et al.
1993). Porter and Collins (1991) sequenced 13 clones of *An. hermsi* and *An. freeborni* and found only four single-base-pair substitutions, which they ascribed to polymerase misincorporation. Fritz et al. (1991) analyzed, by Southern blotting, the rDNA internal and external spacer regions of 45 *An. hermsi* and *An. freeborni* collected in Washington, Oregon, and various locations in California and found no indication of polymorphism. Scott et al. (1993) concluded that there was little or no sequence heterogeneity among copies of the intergenic spacer, either within individuals or between conspecifics of the *An. gambiae* species complex. In the present study, except for the sample from Puraquequara, Brazil, consensus ITS2 sequences were identical among individuals of *An. nuneztovari* collected from single locations and were identical between samples from some sites separated by geographic barriers and great distance (e.g., the Pacific Coast of Colombia (SI) and either side of the Andes Mountains in Venezuela [RS and AN]). Thus, the presence of a single consensus sequence at these locations is consistent with other studies demonstrating limited spacer heterogeneity in the genus *Anopheles*.

Alone, the minimal geographic differences, in spacer sequence, among *An. nuneztovari* do not argue strongly for a cryptic-species complex. However, prior studies on the behavior (Gabaldón and Guerrero 1959; Gabaldón et al. 1963; Elliott 1968, 1972), morphology (Rozeboom and Gabaldón 1941), cytogenetics (Kitzmiller et al. 1973), and genetic distances estimated from data on mtDNA restriction sites (J. Conn, unpublished data) and allozyme frequencies (G. N. Fritz, unpublished data) suggest at least two cryptic species: one in Venezuela and Colombia northwest of the Orinoco River and an Amazonian species. This distinction is consistent with differences in the ITS2 sequence; all samples from Colombia and Venezuela were identical but were different from those obtained from the Brazilian Amazon. In addition, the ITS2 sequences obtained from Suriname and eastern Brazil were different both from each other and from those obtained from mosquitoes in the Amazonian interior. These data are also consistent with genetic distances generated from mtDNA restriction sites (J. Conn, unpublished data) and allozyme frequencies (G. N. Fritz, unpublished data).

If *An. nuneztovari* is shown definitively to be a separate species in all of these regions, then ITS2 sequence divergence between them has been minimal. Indels in regions of single-base repeats and simple repeat motifs account for most of the sequence variation observed and suggest their role as a major cause of divergence in the evolution of this spacer. In *An. nuneztovari*, sequence repeats, which may be subject to slipped-strand mispairing (SSM), are found at various locations along the length of the ITS2. Levinson and Gutman (1987) state that simple repeats such as these are hot spots for SSM events and that these events, in concert with unequal crossing-over, can account for widespread simple repetitive DNA sequences. They propose that the process of SSM is more likely to be a major factor in the initial expansion of short repeat motifs, which are subsequently predisposed to further expansion by unequal crossing-over. A rapid rate of fixation of such mutations in tandemly repeated genes may subsequently distinguish closely related species. In addition, natural selection may play a role in establishing and maintaining spacer sequence similarity both between distant populations and among closely related species (Williams et al. 1987; Williams 1990). Williams et al. (1987) analyzed the overall length and organization of the X- and Y-linked rDNA nontranscribed spacers of *D. melanogaster* obtained from five continents and provided strong evidence that the X-linked rDNA arrays were under selective constraints.

The disparity between the genera *Aedes* and *Anopheles*, in the degree of intraindividual/intraspecific spacer heterogeneity, may reflect a difference in the way in which their genomes are organized and processed. Many of the genomic characteristics that have been described as conducive to faster rates of homogenization and fixation (Smith 1973; Black and Gibson 1974; Dover 1982; Arnheim 1983; Ohta and Dover 1984; Levinson and Gutman 1987; Williams et al. 1987) are more characteristic of anopheline mosquitoes than they are of *Aedes* species. Genome size can be quite variable within species of *Aedes* (it varies threefold between strains of *Ae. albopictus*) and is approximately three to five times the size of that found in the genus *Anopheles* (Black and Rai 1988; Kumar and Rai 1990b). Anopheline mosquitoes have the least amount of repetitive DNA of any mosquito analyzed to date, while aedine mosquitoes have the most (Black and Rai 1988; Cockburn and Mitchell 1989). The number of rDNA repeat units varies considerably between *Aedes* species (Kumar and Rai 1990a), whereas copy number appears to be more uniform among *Anopheles* species (Collins et al. 1989). Although the number of rDNA repeat units is not known for species in the subgenus *Nyssorhynchus*, Collins et al. (1989) estimate that there are ~700 copies (diploid number) for species in the *An. gambiae* complex (subgenus *Cellia*). Whereas the rDNA of most anopheline species is X linked, the rDNA of aedine mosquitoes can occur as at least two loci on separate chromosomes with a copy number that may vary threefold between developmental stages (Park and Fallon 1990) and fivefold within a species (Kumar and Rai 1990a).

The possibility that even small differences in the ITS2 consensus sequence distinguish closely related spe-
cies in the genus *Anopheles* is suggested by the sympatry of two consensus sequences in Puraquequara, Brazil. Two individuals in Puraquequara had the sequence that is found in Suriname and Boa Vista, whereas a third individual had a sequence almost identical to that found in eastern Brazil. Since the sequencing protocol followed in this study generates the consensus sequence of the hundreds of ITS2 copies present in an individual, the two sequence types present in Puraquequara, Brazil, represent individuals that differ substantially in their overall ITS2 array. Ohta and Dover (1984) have shown that, under realistic rates of gene conversion, unequal exchange, and transposition, fixation of a mutant copy in a multigene family proceeds without a large variance at any given generation. This small variance is due, in part, to the much slower rates at which these molecular events occur, relative to the rate at which chromosomes distribute at each generation, though variance is affected by many factors, including mating systems, the frequency of mutation, the relative frequencies of intrachromosomal versus interchromosomal events, immigration, and natural selection (Ohta and Dover 1984; Williams 1990). To date, all analyses of rDNA spacers in anophelines indicate that variants are absent or rare. In addition, identical ITS2 consensus sequences seem to be maintained over broad areas, between populations separated by great distances and geographic barriers (e.g., the Pacific Coast of Colombia with both sides of the Venezuelan Andes and Suriname with Boa Vista, Brazil). It would seem unlikely, therefore, that immigrant conspecifics from adjacent areas have a different ITS2 consensus sequence and account for the presence of two ITS2 types in Puraquequara.

The results of analysis of the ITS2 consensus sequence of *An. nuneztovari* are consistent with those of other studies that indicate low frequency and variance of spacer mutants in the genus *Anopheles*. Identical consensus sequences are maintained even for populations that inhabit different ecological zones, are separated by major geographic barriers, and are quite distant from each other. When geographically distant populations are found to differ in sequence, then the divergence is minimal and limited to regions that are prone to high rates of mutation. Finally, the rate of evolution in the rDNA spacers of species in the genus *Anopheles* apparently varies considerably with that observed in the genus *Aedes*.

**Acknowledgments**

We thank L. P. Lounibos, S. E. Mitchell, G. Hall, and O. P. Perera for reviews and assistance. We thank the Mosquito Pathology Unit in Gainesville for the use of their quarantine facility, and we appreciate the cooperation of E. L. Peyton, R. Wilkerson, and R. Harbach of the Walter Reed Biosystematics Unit, Washington, D.C. We are also grateful for the cooperation of various persons associated with malaria control and research programs in South America, including A. Anselmi, R. Sifontes, J. Berti, E. Borges, C. Moreno, R. Alvarado, N. Castillo, and P. Morel (Dirección de Malariología y Saneamiento Ambiental, Venezuela); H. Pérez, C. Brancho, and M. de la Rosa (Instituto Venezolano de Investigaciones Científicas); J. Scorza, E. Rojas, and E. Brown (Universidad de los Andes, Trujillo, Venezuela); R. Zimmermann (Organización Panamericana de la Salud, Venezuela); R. Dussan, C. Iveros, and L. Villarreal (Servicio de la Erradicación de Malaria, Colombia); R. Vargas (Ministerio de Previsión Social y Salud Pública, Bolivia); H. Bermudez (Universidad Mayor de San Simón, Cochabamba, Bolivia); J. Cuba and C. Tófffen (Unidad Sanitaria, Riberalta, Bolivia); H. Momen, M. G. Rosa-Freitas, R. Lourenco-de-Oliveira, T. Fernandez da Silva, and L. B. Luz (Fundacao Oswaldo Cruz, Brasil); A. Cruz Marques, J. Fonseca Sandoval, R. Da Luz Lacerda, A. Wanderley, and G. Calderon (Fundacao Nacional de Saude, Brasil); M. Marins Póvoa (Instituto Evandro Chagas); and L. Resida and C. Limon (Bureau of Public Health, Paramaribo, Suriname). This research was supported by the National Institutes of Health grant AI-31034.

**LITERATURE CITED**


