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# GENETIC ISOLATION BY DISTANCE AMONG *AEDES AEGYPTI* POPULATIONS ALONG THE NORTHEASTERN COAST OF MEXICO

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*Abstract.* A population genetic analysis of gene flow was conducted among 10 *Aedes aegypti* collections from seven cities along the northeastern coast of Mexico. Four collections were made from Monterrey to examine local patterns of gene flow. Markers included 60 random amplified polymorphic DNA (RAPD) loci amplified by the polymerase chain reaction and single strand conformation polymorphism analysis of variation in a 387-basepair region of the NADH dehydrogenase subunit 4 from the mitochondrial DNA (mtDNA). Seven mitochondrial haplotypes were detected and phylogenetic analysis identified two well-supported clades. Regression analysis of geographic distances and pairwise $F_{ST}$ estimated from RAPD markers indicated that populations are isolated by distance and that free gene flow occurs among collections within 90–250 km. Isolation by distance was not detected using mtDNA haplotypes. The Nuevo Laredo collection had unique RAPD and mtDNA haplotype frequencies and reduced heterozygosity suggesting that few mosquitoes established this population.

The mosquito *Aedes aegypti* is the primary urban vector of dengue and yellow fever viruses. Serotypes 1–4 of dengue virus are a major public health problem for many tropical regions of the world and thousands of cases of dengue fever and dengue hemorrhagic fever are reported worldwide annually.[1-3] Understanding the dispersal patterns of the vector is important for the development of effective control strategies.

*Aedes aegypti* dispersal occurs through adult flight[4] and through transport of eggs, larvae, and adults in containers (e.g. discarded bottles, cans, appliances, tires, and cargo containers) along commerce routes.[5] Early population genetics work defined genetic relationships throughout the worldwide range of *Ae. aegypti*,[6-11] while more recent studies have focused on local patterns of dispersal.[4,12-14]

Control strategies for *Ae. aegypti* during urban outbreaks of dengue fever or yellow fever have assumed that mosquitoes have a lifetime flight range of 50–100 meters[15] and this belief has dictated focal applications of insecticides to disrupt transmission. However, dispersal distances up to 580 meters were observed in the southeastern United States using genetically marked strains.[16] Furthermore, *Ae. aegypti* distributes its eggs among several oviposition sites,[12] and it has been proposed that dispersal may be driven by the search for oviposition sites.[4,12] Adults were fed blood containing rubidium, released from a central location in San Juan, Puerto Rico, and for several days eggs were collected in oviposition traps surrounding the release point.[4] Eggs containing rubidium were detected within at least 840 meters from the release site. However, more recent mark-release-recapture studies have shown that dispersal rates and distances are inversely correlated with the abundance of oviposition sites.[14] All of these studies warn that campaigns to reduce *Ae. aegypti* larval sites during dengue epidemics could have the undesirable effect of increasing the dispersal of infected females.

The results of early isozyme studies showed that *Ae. aegypti* collections cluster with respect to continents and specific countries.[6-11] This pattern suggests that gene flow among populations decreases with increasing geographic distances, a pattern referred to as isolation by distance in population genetics. However, the minimal geographic distance at which gene flow in *Ae. aegypti* becomes limited has yet to be determined. Only two studies, both conducted in Puerto Rico, have examined local patterns of gene flow in *Ae. aegypti*. Examination of allozyme frequencies at 11 isozyme loci among collections covering ~100 km indicate continuous gene flow.[10] A similar result was obtained when examining variation at 57 random amplified polymorphic DNA (RAPD) markers.[13]

In the present study, we examined local patterns of gene flow in *Ae. aegypti* at an expanded geographic scale by performing a nested spatial analysis of gene flow over a distance of 735 km among cities along the northeastern coast of Mexico (Figure 1). Gene flow was also examined among four collections within 45–70 km of one another in Monterrey to determine if the high rates of gene flow detected in Puerto Rico also occur in Mexico. As in our earlier studies, genetic variation was examined in no fewer than 60 individuals.[13] Genetic variation was examined at 60 RAPD loci and in the mitochondrial genome using single strand conformation polymorphism (SSCP) analysis. We analyzed two measures of gene flow in the current study: the effective migration rate (*Nm*) and the variance effective population size (*Ne*). *Nm* is defined as the number of migrating reproductive individuals among populations. In theory, an $Nm \sim 1$ is sufficient to maintain continuous gene flow among populations. *Ne* is measured as a change in the variance of allele frequencies among populations and is defined as the harmonic average of the successfully reproducing adult population over a unit area.

Analysis of the mitochondrial genome was included because of the high mutation rates observed in RAPD markers.[13,17-20] A high mutation rate in RAPDs is problematic for population genetic studies because frequent point mutations are likely to cause the independent gain or loss of RAPD bands in different populations. The RAPDs can therefore underestimate genetic distances and overestimate rates of gene flow.

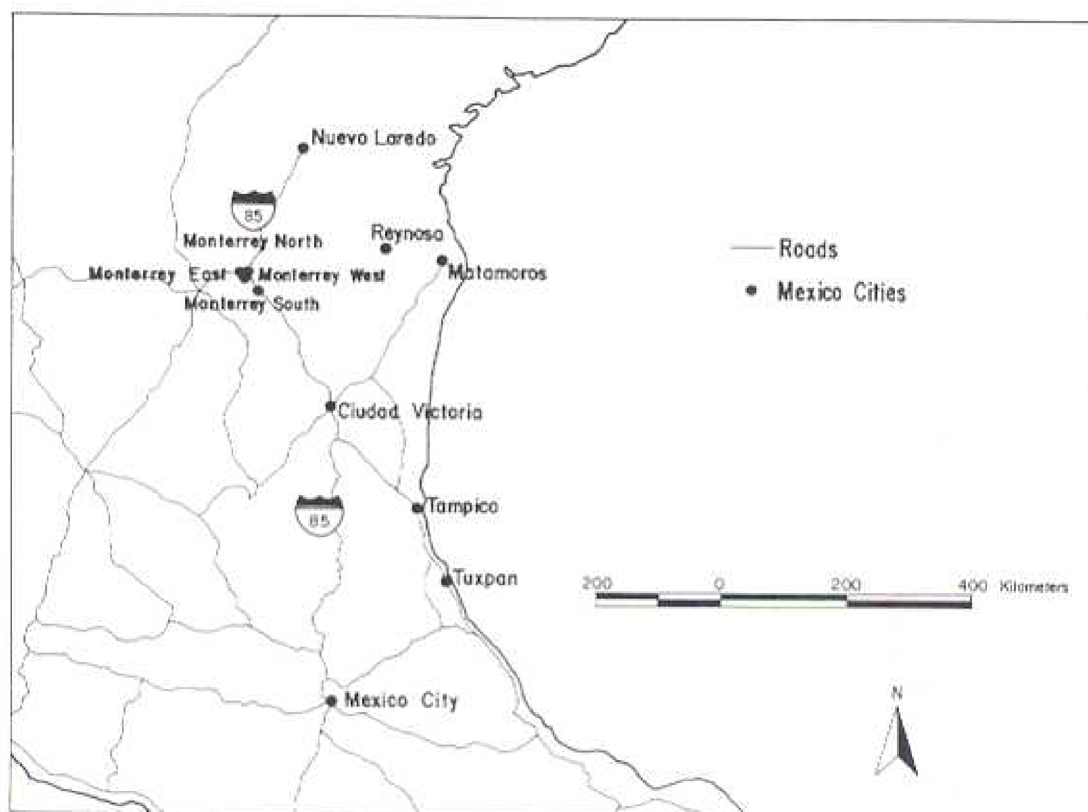


FIGURE 1. Map of the northeastern coast of Mexico showing the locations of *Aedes aegypti* collections.

Mitochondrial DNA (mtDNA) is maternally inherited and does not recombine.[21] When sequence data are collected, these properties allow for phylogenetic analysis of maternal lineages. In addition, patterns of variation in mtDNA haplotype frequencies can be used to estimate rates of gene flow among populations. For these reasons, mtDNA has been used in studies of phylogenetic relationships among and within the four species of the *Anopheles quadrimaculatus* complex[22], among populations of *An. aquasalis, An. rangeli, An. trinkae, An. nuneztovari,*[23–25] and *An. albimanus,*[26] and among and within the African malaria vectors *An. gambiae* and *An. arabiensis.*[27–29]

This is the first study to use mtDNA to study patterns of gene flow in a culicine mosquito. Following earlier studies of mtDNA in our laboratory[30], we used SSCP analysis[31] as a quick, sensitive, and inexpensive means to screen for variation among mitochondrial genes amplified from individual mosquitoes. We then sequenced the most common haplotypes to test the sensitivity and reproducibility of the SSCP technique and to gather data with which to assess phylogenetic relationships among haplotypes.

## MATERIALS AND METHODS

**Mosquito collections and extraction of DNA.** The locations and sample sizes of *Ae. aegypti* larvae collected in each city are listed in Table 1 and the geographic locations of all sampling sites are shown in Figure 1. Collections were obtained from four regions of Monterrey. This design allowed us to analyze gene flow at two levels: among collections within a city and among cities. Mosquito larvae were reared to adults in the laboratory and adults were then stored at −70°C awaiting analysis. The DNA was obtained from individual mosquitoes by salt extraction[32] and suspended in 500 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The DNA was divided into 5–100-μl aliquots and stored at −70°C.

**Random amplified polymorphic DNA–polymerase chain (PCR) reaction.** The RAPD-PCR was completed in 50-μl reaction volumes using 1 μl of template DNA.[32] Amplification was completed in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA). Each set of PCRs was checked for contamination using a negative control (all reagents included except template DNA). All PCR products in a set of amplifications were discarded when any DNA appeared in the negative control. Oligonucleotide primers were C13 and C16 (Operon Technologies, Inc., Alameda, CA.) and R12 (5′-TCG GTC ATA G-3′) and R22 (5′-GAT CAT AGC C-3′) (Nucleic Acid Unity at the Centro de Investigacion y Estudios Avanzados de Instituto Politecnico Nacional). Amplified products were size-fractionated using electrophoresis on large (38 × 50 cm), thin (0.4 mm), glycerol (7%), polyacrylamide (5%, 0.2% cross-linking) gels. Electrophoresis proceeded at constant voltage (350 V) at room temperature for 16 hr (overnight), and the gels were silver stained[32] to visualize DNA fragments. Sharktooth combs (4 mm) were used to load 5–6 μl of sample.

**Mitochondrial gene amplification.** Primers used to amplify the NADH dehydrogenase subunit 4 (ND4) gene were ND4+ (5′-GTD YAT TTA TGA TTR CCT AA-3′) and

TABLE 1

The locations, dates of collections, global positioning system coordinates, and sample sizes of *Aedes aegypti* collections in the northeastern coast of Mexico

| State | Cities | Region | Date | Latitude | Longitude | Number of individuals analyzed |
|---|---|---|---|---|---|---|
| Nuevo Leon | Monterrey | North | 7/9/96 | 25.7 | 100.3 | 57 |
| | | South | 7/17/96 | 25.5 | 100.2 | 58 |
| | | East | 7/24/96 | 25.7 | 100.4 | 58 |
| | | West | 7/24/96 | 25.5 | 100.1 | 58 |
| Tamaulipas | Nuevo Laredo | | 8/10/97 | 27.5 | 99.5 | 48 |
| | Reynosa | | 7/20/97 | 26.2 | 98.2 | 59 |
| | Matamoros | | 7/29/96 | 26.3 | 97.5 | 59 |
| | Ciudad Victoria | | 8/3/96 | 23.7 | 99.3 | 59 |
| | Tampico | | 8/4/96 | 23.7 | 97.9 | 59 |
| Veracruz | Tuxpan | | 8/24/96 | 21.2 | 97.4 | 59 |
| Total | 7 | 4 | | | | 574 |

ND4- (5′-CTT CGD CTT CCW ADW CGT TC-3′). The tubes were heated to 95°C for 5 min and cooled to 80°C prior to the addition of 1 unit of *Taq* DNA polymerase. The program consisted of 10 cycles of 1 min at 92°C, 1 min at 48°C, and 2 min at 72°C. This was followed by 32 cycles of 1 min at 92°C, 35 sec at 52°C, and 2 min at 72°C. A final extension reaction was carried out for 7 min at 72°C and the samples were then cooled overnight at 4°C. Negative controls were as described above for RAPDs.

The amplified regions correspond with nucleotides 8,457–8,846 in *An. quadrimaculatus* (GenBank #L04272) and nucleotides 8,466–8,854 in *An. gambiae* (GenBank #L20934). The PCR product (0.5 μl of 50 μl), was mixed with 5.5 μl of loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol), centrifuged, and heated to 95°C for 4 min on a thermal cycler. It was then plunged directly into ice and all contents were loaded directly onto an SSCP gel. Electrophoresis conditions were as described above for RAPDs except that the gel contained 4% glycerol.

**DNA sequences.** The ND4 PCR products from 27 individuals representing each of the seven haplotypes were sequenced along both strands. The ND4+ and ND4− PCR primers were used in double-stranded sequencing on the ABI synthesizer at Macromolecular Resources at Colorado State University.

**Statistical analysis of RAPD-PCR markers.** The RAPD-PCR bands were analyzed as genetic markers with the following assumptions: 1) RAPD markers segregate in a Mendelian fashion, 2) genotype frequencies at RAPD loci are in Hardy-Weinberg proportions, 3) recessive "band absent" alleles are identical among and within individuals, and 4) dominant "band present" alleles are identical among and within individuals. Statistical methods and equations[13,33] are programmed into a statistical package RAPDPLOT (available from web4@lamar.colostate.edu).

Variation in RAPD allele frequencies within and among cities was examined using a hierarchical analysis[34] in BIOSYS-2[35] after data transformation by RAPDBIOS.[33] RAPDDIST and RAPDFST[13] were used to compute pairwise $F_{ST}$, a standardized measure of variation in haplotype frequencies,[36] among all populations. Effective migration rates ($Nm$) were estimated from $F_{ST}$ [37] (equation 6). Pairwise $F_{ST}$ values were transformed to $F_{ST}/(1 - F_{ST})$ and used to construct a dendrogram among all collections using unweighted pair-group method with arithmetic averaging analysis[38] in the NEIGHBOR procedure in PHYLIP3.5C.[39] RAPDDIST[13] tested the consistency with which the RAPD dataset supported each cluster using a bootstrap analysis with 1,000 replications.

Geographic distances were obtained by Geographic Information Systems on ARC-INFO software (Environmental Systems Research Institute, Inc., Redlands, CA). The $F_{ST}/(1 - F_{ST})$ distances were regressed on pairwise geographic distances among populations to determine if geographic distance among populations serves as a barrier to gene flow.[37] This regression was repeated using a natural logarithm transformation of geographic distance.[40] Transformations, regression analysis, and the Mantel test[41] were performed using MANTEL (available from web4@lamar.colostate.edu). The reciprocal of the slope estimated by this regression provides an estimate of the average effective population size.[40]

**Statistical analysis of mitochondrial haplotype frequencies.** Variation in haplotype frequencies within and among cities was examined using the Analysis of Molecular Variance (AMOVA).[42] Arlequin 1.1 was used to estimate pairwise $F_{ST}$ values and $F_{ST}/(1 - F_{ST})$ among populations and to compute the significance of the variance components associated with each level of genetic structure using a nonparametric permutation test.[42]

For each collection, the nucleotide sequence and the frequency of each haplotype were entered into DnaSP.[43] We estimated the number of polymorphic sites, the average number of nucleotide differences (k) (equation A3),[44] the nucleotide diversity ($\pi$) (equation 10.5),[45] and the nucleotide diversity with Jukes and Cantor correction ($\pi_J$) (equations 10.19 and 5.3).[45] Pairwise genetic distances were computed using $N_{ST}$,[46] which is similar to $F_{ST}$ but incorporates sequence divergence among haplotypes into the overall distance estimate. Effective migration rates ($Nm$) were estimated from $N_{ST}$ [37] (equation 6). As with $F_{ST}$, pairwise $N_{ST}$ values were transformed to $N_{ST}/(1 - N_{ST})$ and regressed on pairwise geographic distances and on a natural logarithm transformation of geographic distance.[40]

**Phylogenetic relationships among ND4 haplotypes.** Phylogenetic relationships among haplotypes were estimated with PAUP4B41 using maximum likelihood,[47] maximum

TABLE 2

Partitioning of variation in the frequency of genetic markers among *Aedes aegypti* collections in Mexico

A Hierarchical analysis of random amplified polymorphic DNA allele frequencies among collections within and among cities

| Source of variation | Variance components | % variation | $F_{st}$ |
|---|---|---|---|
| Among collections | | | |
| In Monterrey | 0.569 | 39.7% | 0.027 (Nm = 9.01) |
| Without Nuevo Laredo | 0.938 | 90.0% | 0.044 (Nm = 5.43) |
| Among cities | 0.863 | 60.3% | 0.040 |
| Without Nuevo Laredo | 0.104 | 10.0% | 0.005 |
| Total | 1.432 | 100.0% | 0.066 |
| Without Nuevo Laredo | 1.042 | 100.0% | 0.049 |

B Analysis of variance in mitochondrial haplotype frequencies among collections within and among cities*

| Source of variation | Degrees of freedom | Sum of squares | Variance components | % variation |
|---|---|---|---|---|
| Among collections | | | | |
| In Monterrey | 3 | 2.957 | 0.012† | 3.31 |
| Without Nuevo Laredo | 3 | 2.957 | 0.012‡ | 3.55 |
| Among collections | | | | |
| Within cities | 6 | 38.890 | 0.074§ | 20.09 |
| Without Nuevo Laredo | 5 | 14.658 | 0.025¶ | 7.37 |
| Within collection | 564 | 158.909 | 0.282# | 76.59 |
| Without Nuevo Laredo | 517 | 154.346 | 0.299** | 89.08 |
| Total | 573 | 200.756 | 0.368 | |
| Without Nuevo Laredo | 525 | 171.962 | 0.335 | |
| Fixation indices | | | Without Nuevo Laredo | |
| F (within Monterrey) | 0.041† (Nm = 11.7) | | 0.035‡ (Nm = 13.8) | |
| F (among cities) | 0.200§ (0.074)¶ | | | |
| F (all collections) | 0.234# (0.109)** | | | |

* Probability (random value ≥ observed value) (100,172 permutations).
† 0.00240 ± 0.00014.
‡ 0.07891 ± 0.00091.
§ 0.04364 ± 0.00060.
¶ 0.00208 ± 0.00014.
# 0.00000 ± 0.00000.
** 0.00000 ± 0.00000.

parsimony, and distance/neighbor joining analyses.[48,49] The consistency with which the dataset supported each branch in the resolved phylogeny was estimated using a bootstrap analysis with 1,000 replications.

## RESULTS

**Random amplified polymorphic DNA allele frequencies among collections.** The frequencies of the dominant RAPD allele at each of the 60 loci (frequency data matrix available from wcb4@lamar.colostate.edu) were subject to a hierarchical analysis to estimate the variance among allele frequencies in Monterrey relative to those among cities (Table 2). Approximately 40% of the total variation in RAPD allele frequencies occurred among collections within Monterrey, while 60% of the variation arose among collections from different cities.

Cluster analysis of pairwise $F_{ST}/(1 - F_{ST})$ among collections (Figure 2) indicated that all Monterrey collections appear within a common cluster that includes nearby Matamoros, albeit with low bootstrap support. However, these collections did not cluster with collections from nearby Reynosa and Nuevo Laredo. Nuevo Laredo differed from all populations in the frequencies of 38 of the 60 RAPD loci.

Pairwise $F_{ST}/(1 - F_{ST})$ among collections were regressed against geographic distance (Figure 3A) and the natural logarithm of the geographic distances (Figure 3B) to determine if gene flow among collections is correlated with geographic distance (i.e., to test for isolation by distance). This analysis indicates a significant correlation between genetic and geographic distances. Because of the large differences in allele frequencies between Nuevo Laredo and all of the other collections, regression analysis was repeated excluding Nuevo Laredo (Table 3A). Exclusion of Nuevo Laredo caused a decrease in Mantel probabilities; however, the regression with transformed geographic distances remained significant. Genetic distances remained small at geographic distances <4.5 km and became large at 5.5 km (Figure 3B). This indicated that populations become reproductively isolated at distances of 90–250 km ($e^{4.5}$–$e^{5.5}$). The average effective population size ranged from 74 to 132 mosquitoes/km.

Estimating effective migration rates ($Nm$) from $F_{ST}$ assumes that populations fit the assumptions of the Island Model of Wright.[34] A major assumption of this model is that migration rates are equal among all populations. Our regression analysis indicated that this assumption was false among collections from different cities. However, a regression analysis was repeated among Monterrey collections and genetic and geographic distances were independent (slope = −0.00308, $R^2$ = 0.08, Mantel probability = 0.259). We therefore made pairwise estimates of $Nm$ among collections within Monterrey. Using the $F_{ST}$ of Wright in the hierarchical analysis, $Nm$ ranged from 5.4 to 9.0 individuals (Table 2A). Using the method of Lynch and Milligan,[33] $Nm$ ranged from

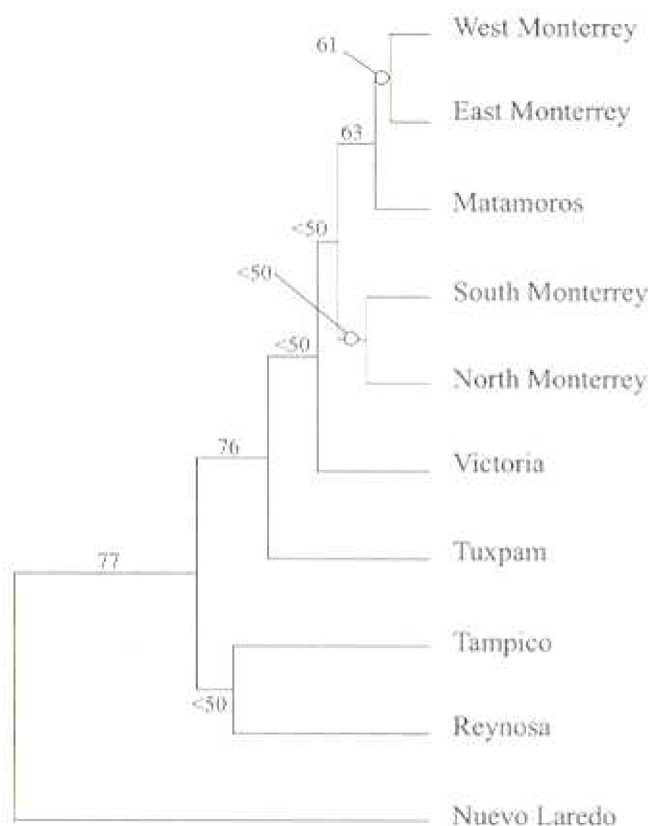


FIGURE 2. Unweighted pair-group method with arithmetic averaging cluster analysis of pairwise $F_{ST}/(1 - F_{ST})$ relationship between collections. Analysis was done using the random amplified polymorphic DNA markers.

5.9 to 19 individuals among pairwise comparison of collections and was 7.6 among all collections (Table 3B).

**Mitochondrial haplotype frequencies among collections.** The ND4 gene was amplified and surveyed for variation using SSCP among all 574 mosquitoes. All unique haplotypes were then compared on a single SSCP gel. We attempted to sequence PCR products from at least a pair of mosquitoes with identical SSCP patterns, but haplotype 6 was only sequenced once. In addition, we sequenced PCR products from an additional 14 individuals in which the identity or uniqueness of the SSCP patterns were in question. These 25 sequences are available on GenBank (#AF203344-#AF203368).

This process established identity among haplotypes that were initially incorrectly identified as unique and occasionally identified unique haplotypes that were initially thought to be identical when run on different gels. The process of re-screening haplotype variation resulted in the identity of ~1% of haplotypes being re-designated in the final dataset. Sequences of mosquitoes with identical SSCP patterns were identical within each haplotype. Sequence analysis therefore confirmed that the SSCP technique was specific and reproducible among mosquitoes. Sequences also confirmed that the SSCP technique is sensitive to single substitutions. The SSCP haplotypes 1 and 2 differed by a single C ⇔ T transition at position 162 and haplotypes 3 and 4 differed by a single C ⇔ T transition at position 17.

A total of 7 different ND4 haplotypes were detected among the 574 *Ae. aegypti* examined in this study. The frequencies of the 7 haplotypes are displayed within Monterrey (Figure 4A) and among the other collections (Figure 4B). The frequencies of each haplotype in each collection are available upon request from wcb4@lamar.colostate.edu. Haplotype number designations correspond to their relative frequencies in this study. As with the RAPD markers, it is clear that mosquitoes in the Nuevo Laredo collection are genetically distinct in mtDNA haplotype frequencies from mosquitoes in all other collections. The number of polymorphic sites and the diversity indices for each collection and for all mosquitoes are listed in Table 3C. Note that the average number of nucleotide differences and the nucleotide diversity among mosquitoes within Nuevo Laredo are at least an order of magnitude lower than all other collections. In addition, haplotype 7 was unique to Nuevo Laredo.

Haplotype frequencies were compared among collections within Monterrey and among all collections using AMOVA.[42] Most (~77%) of the variation in haplotype frequencies arose among individuals in a collection, while ~20% of the variation arose among cities and only 3.3% arose among sites within Monterrey. Haplotype frequency patterns are similar within Monterrey (Figure 4A) but different among cities (Figure 4B). AMOVA analysis was repeated excluding Nuevo Laredo. The amount of variation among cities decreased to ~7.4%, while the variation among sites within Monterrey remained approximately the same.

As with RAPD markers, pairwise $F_{ST}/(1 - F_{ST})$ comparisons among collections were regressed against geographic distance and the natural logarithm of the geographic distances (Table 3A) to test for isolation by distance. These regression analyses were repeated using $N_{ST}$ to incorporate sequence diversity into estimate of genetic distances. As above, regression analysis was repeated with Nuevo Laredo excluded. The result of the Mantel test was not significant in any of the 8 regressions performed (Table 3A).

Regression analysis was repeated among Monterrey collections and genetic and geographic distances were independent (slope = −0.00594, $R^2$ = 0.02, Mantel probability = 0.475). When $F_{ST}$ in the AMOVA was used, *Nm* was 11.7 individuals (Table 2B). When $N_{ST}$[36] was used, *Nm* ranged from 3.5 to 11.6 individuals among pairwise comparisons of collections and was 8.6 among all collections (Table 3B).

The Mantel test was also used to compare pairwise $F_{ST}/(1 - F_{ST})$ between RAPD and mitochondrial markers (Figure 3C). The test result approached significance (Table 3A) and there was a large correlation ($R^2$ = 0.49); however, this correlation arose entirely because of the large genetic distance between the Nuevo Laredo collection and all other collections. Once the Nuevo Laredo collection was excluded from regression analysis the Mantel test result was insignificant and $R^2$ = 0.01.

**Phylogenetic relationships among individual haplotypes.** The 387 basepairs of the mitochondrial ND4 haplotypes of *Ae. aegypti* were manually aligned with the homologous regions of *An. gambiae* and *An. quadrimaculatus* and no gaps were required for optimal alignment. Phylogenetic analysis indicated the existence of two historical mitochondrial lineages among the seven haplotypes (Figure 5). However, with the exception of haplotype 7, which was unique to the Nuevo Laredo collection, the two mitochon-

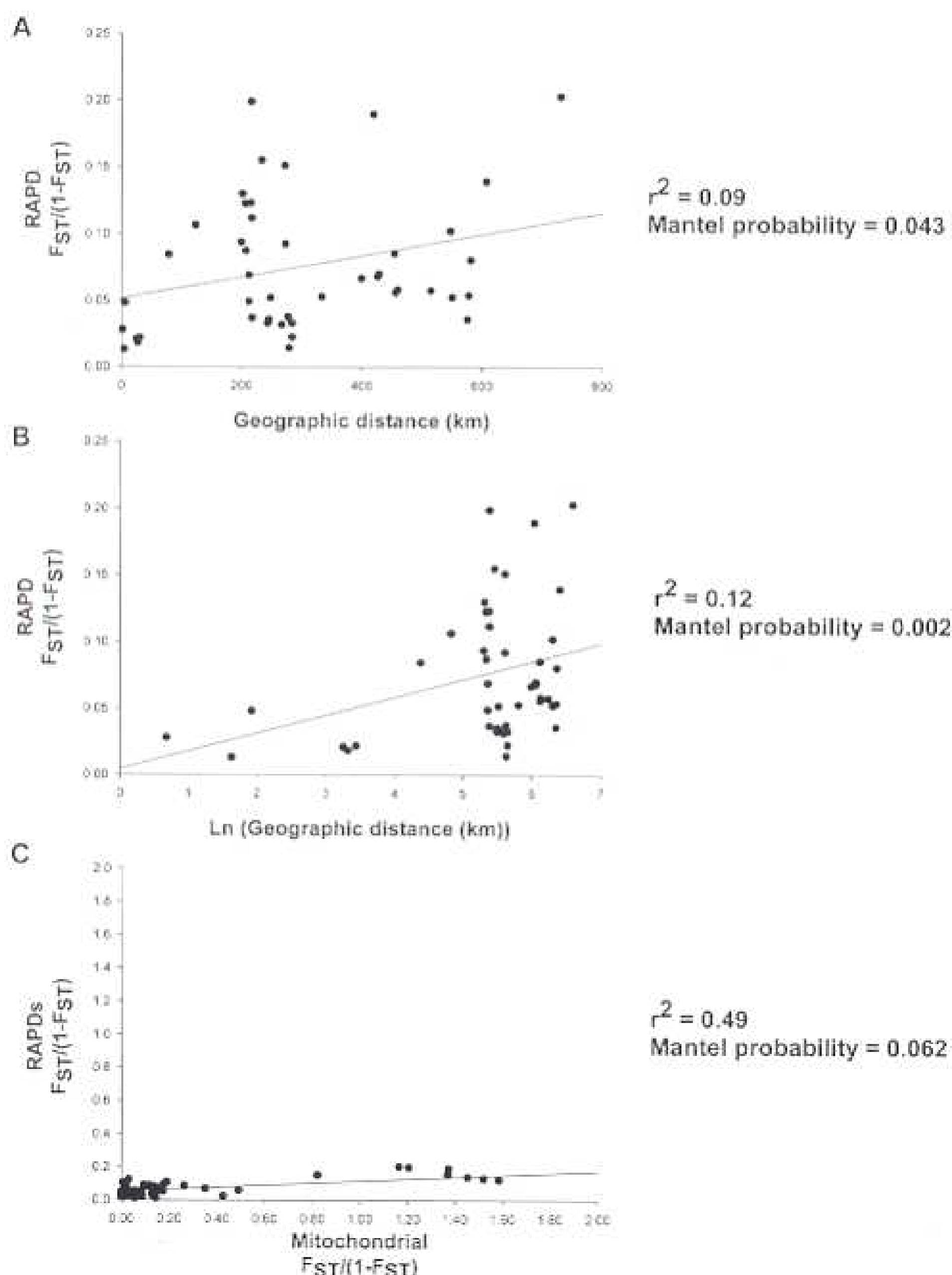


FIGURE 3. Regression analysis of **A**, pairwise $F_{ST}/(1 - F_{ST})$ (random amplified polymorphic DNA [RAPD] markers) regressed on pairwise geographic distances between collections, **B**, pairwise $F_{ST}/(1 - F_{ST})$ (RAPD markers) regressed on pairwise natural logarithm transformed geographic distances, and **C**, pairwise $F_{ST}/(1 - F_{ST})$ (RAPD markers) regressed on pairwise $F_{ST}/(1 - F_{ST})$ (mitochondrial DNA markers)

drial clades occurred in similar frequencies among collections.

## DISCUSSION

The average genic heterozygosity among the 60 RAPD loci ($H = 0.339$) was similar to that obtained among 57 RAPD loci in an analysis in Puerto Rico ($H = 0.354$)[13] and was approximately twice that among 11 allozyme loci in an earlier survey in Puerto Rico ($H = 0.163$)[10] or in a survey of 23 allozyme loci in *Ae. aegypti* populations worldwide ($H = 0.152$).[5] The higher variability in RAPD markers probably reflects the types of mutations that give rise to RAPD polymorphisms. The RAPD-PCR uses a 10-oligonucleotide primer, with a minimum GC content of 60% that at 37°C anneals to many arbitrary regions of a genome during the PCR.[50] Many polymorphic loci are simultaneously amplified during the RAPD-PCR. Most mutations in RAPD loci appear as the presence or absence of an amplified DNA fragment suggesting that mutation(s) at the RAPD locus disrupt the PCR either by preventing primer annealing or by acquiring insertions that increase the distance between annealing sites beyond a size that can be amplified by a conventional PCR.

The nucleotide diversity ($\pi_1$) in the mitochondrial ND4 gene in *Ae. aegypti* is 3–6 times greater than $\pi_1$ within *An.*

TABLE 3

**A**, Regression of $F_{ST}$ or $N_{ST}$ for both random amplified polymorphic DNA (RAPD) and mitochondrial (mt) markers on geographic (geo.) distances; **B**, Estimates of $F_{ST}$, $N_{ST}$, and Nm for both RAPD and mitochondrial markers among collections in North, South, East, and West Monterrey collections; **C**, Estimates of variability in the mitochondrial genome among *Aedes aegypti* collections

A Regressions

| All collections / Slope | Intercept | $R^2$ | Mantel probability | 4Dme |
|---|---|---|---|---|
| RAPD | | | | |
| $F_{ST}/(1 - F_{ST}) = 0.00008 \times$ geo. distance | 0.05132 | 0.09 | 0.0430 | |
| $F_{ST}/(1 - F_{ST}) = 0.01350 \times \ln$(geo. distance) | 0.00405 | 0.12 | 0.0020 | 74 individuals |
| RAPD without Nuevo Laredo | | | | |
| $F_{ST}/(1 - F_{ST}) = 0.00004 \times$ geo. distance | 0.04263 | 0.08 | 0.1279 | |
| $F_{ST}/(1 - F_{ST}) = 0.00755 \times \ln$(geo. distance) | 0.01619 | 0.14 | 0.0500 | 132 individuals |
| Mitochondrial DNA | | | | |
| $F_{ST}/(1 - F_{ST}) = 0.00033 \times$ geo. distance | 0.28284 | 0.01 | 0.1439 | |
| $F_{ST}/(1 - F_{ST}) = 0.08077 \times \ln$(geo. distance) | −0.04653 | 0.03 | 0.1239 | 12 individuals |
| $N_{ST}/(1 - N_{ST}) = 0.00120 \times$ geo. distance | 0.41155 | 0.02 | 0.1638 | |
| $N_{ST}/(1 - N_{ST}) = 0.20907 \times \ln$(geo. distance) | −0.33448 | 0.02 | 0.1838 | 5 individuals |
| Mitochondrial DNA without Nuevo Laredo | | | | |
| $F_{ST}/(1 - F_{ST}) = 0.00008 \times$ geo. distance | 0.09090 | 0.02 | 0.2897 | |
| $F_{ST}/(1 - F_{ST}) = 0.01588 \times \ln$(geo. distance) | 0.03165 | 0.04 | 0.1778 | 63 individuals |
| $N_{ST}/(1 - N_{ST}) = 0.00001 \times$ geo. distance | 0.08389 | 0.00 | 0.3067 | |
| $N_{ST}/(1 - N_{ST}) = 0.00094 \times \ln$(geo. distance) | 0.06292 | 0.00 | 0.4476 | 1,064 individuals |
| RAPD vs. mitochondrial DNA | | | | |
| $F_{ST}/(1 - F_{ST})_{RAPD} = 0.06016 \times F_{ST}/(1 - F_{ST})_{mtDNA}$ | 0.05245 | 0.49 | 0.0619 | |
| RAPD vs. mitochondrial DNA without Nuevo Laredo | | | | |
| $F_{ST}/(1 - F_{ST})_{RAPD} = 0.02486 \times F_{ST}/(1 - F_{ST})_{mtDNA}$ | 0.05239 | 0.01 | 0.3946 | |

B

| | N | Polymorphic sites | k | π | RAPD $F_{ST}$ (LM) | Mitochondrial $N_{ST}$ | Mitochondrial Nm |
|---|---|---|---|---|---|---|---|
| North vs. South | 115 | 13 | 5.246 | 0.01355 | 0.021 (11.7) | 0.000 | (∞)* |
| North vs. East | 115 | 13 | 4.184 | 0.01081 | 0.046 (5.9) | 0.126 | (3.5) |
| North vs. West | 115 | 13 | 4.343 | 0.01122 | 0.027 (9.0) | 0.085 | (5.4) |
| South vs. East | 116 | 13 | 3.766 | 0.00973 | 0.021 (11.7) | 0.075 | (6.2) |
| South vs. West | 116 | 13 | 3.949 | 0.01020 | 0.018 (13.6) | 0.041 | (11.6) |
| East vs. West | 116 | 13 | 2.503 | 0.00647 | 0.013 (19.0) | 0.000 | (∞)* |
| All | | | | | 0.032 (7.6) | 0.055 | (8.6) |

* ∞ = 1/0.

C

| | N | Polymorphic sites | k | π | $\pi_1$ |
|---|---|---|---|---|---|
| Nuevo Laredo | 48 | 14 | 0.881 | 0.00228 | 0.00232 |
| Reynosa | 59 | 13 | 6.291 | 0.01626 | 0.01662 |
| Monterrey South | 58 | 13 | 4.978 | 0.01286 | 0.01314 |
| Monterrey North | 57 | 13 | 5.569 | 0.01439 | 0.01470 |
| Monterrey East | 58 | 13 | 2.258 | 0.00583 | 0.00596 |
| Monterrey West | 58 | 13 | 2.752 | 0.00711 | 0.00725 |
| Ciudad Victoria | 59 | 14 | 2.286 | 0.00591 | 0.00600 |
| Matamoros | 59 | 14 | 5.953 | 0.01538 | 0.01572 |
| Tampico | 59 | 14 | 2.927 | 0.00756 | 0.00770 |
| Tuxpam | 59 | 14 | 6.060 | 0.01566 | 0.01599 |
| All sites | 574 | 15 | 5.549 | 0.01434 | 0.01465 |
| Clade 1 (1, 2, 6) | 412 | 12 | 0.943 | 0.00244 | 0.00247 |
| Clade 2 (3, 4, 5, 7) | 162 | 13 | 2.013 | 0.00520 | 0.00529 |

*gambiae* (0.0038), *An. arabiensis* (0.0023–0.0051), and *An. albimanus* (0.0045–0.0051).[24–29] This difference is curious given that 45 haplotypes of the ND5 gene were identified in Guatemala,[26] while we have found only seven haplotypes for the ND4 gene at an equivalent geographic scale in Mexico. These differences may arise due to higher constraints on the mutation rate (i.e., balancing selection) on the ND5 gene. Alternatively the higher rate in the ND4 gene may be due to the existence of two well-supported clades in *Ae. aegypti*, while no well-supported clades were detected in any of the mtDNA studies in the *Anopheles* species. To test the latter hypothesis, we estimated $\pi_1$ within each of the two *Ae. aegypti* mtDNA clades separately (Table 3C), and $\pi_1$ values were more similar to values estimated in *Anopheles* species.

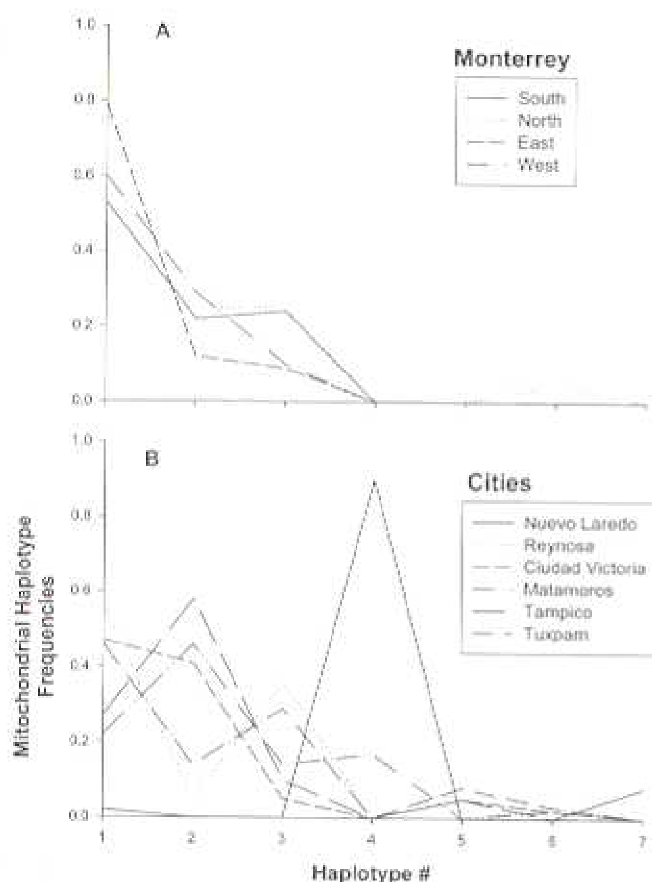


FIGURE 4. **A**, relative frequencies of the seven NADH dehydrogenase subuint 4 (ND4) haplotypes among collections in Monterrey. **B**, relative frequencies of ND4 haplotypes among cities.

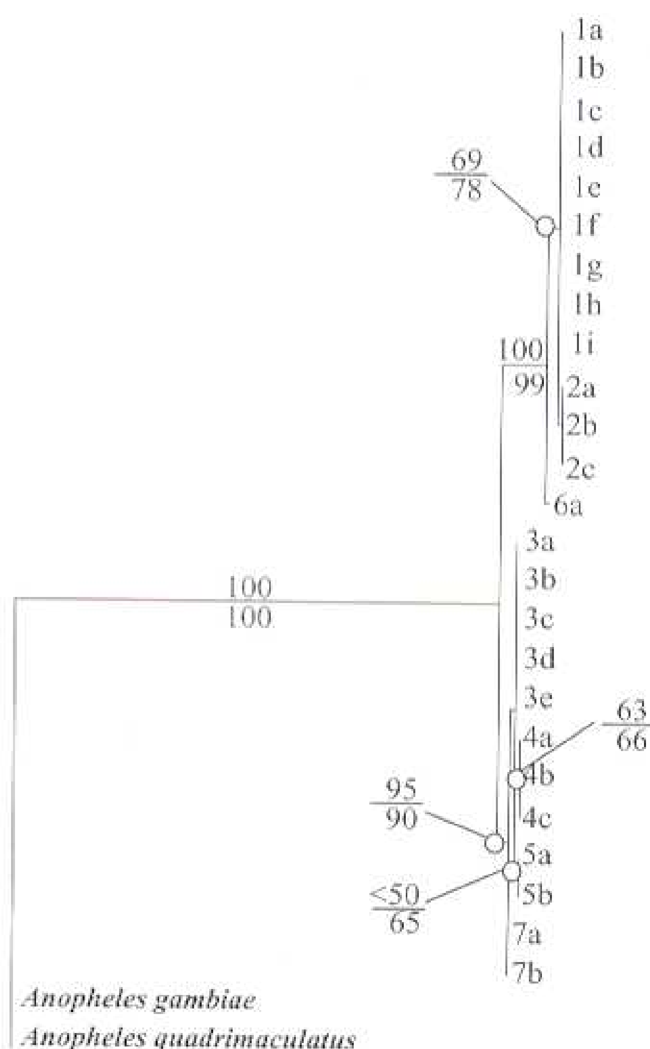


FIGURE 5. Maximum likelihood tree showing phylogenetic relationships among individual haplotypes. Bootstrap support using maximum parsimony analysis appears above each branch while bootstrap support using Tamura-Nei genetic distance/neighbor joining appears below each branch.

The existence of distinct mitochondrial lineages in *Ae. aegypti* probably reflects the existence of distinct, historic, matriarchal lineages within the species. These probably arose through historical, prolonged separation of populations. A similar explanation has been proposed to explain the existence of two distinct subspecies (*Ae. aegypti aegypti* and *Ae. aegypti formosus*) within Africa.[5] These results suggest either that both mitochondrial lineages were introduced simultaneously into Mexico or represent a recent introgression of a new maternal lineage. These possibilities cannot be distinguished without a worldwide survey of mtDNA in *Ae. aegypti*.

The high mutation rate in RAPDs is problematic in population genetic studies because point mutations in the RAPD locus cause the gain or loss of a RAPD band independently in different populations, leading to underestimation of genetic differences among populations and overestimation of migration rates. This bias occurs because *Nm* is estimated from the equation $F_{ST} = (1/4N(m + \mu) + 1)$ where $\mu$ is the forward mutation rate. When using allozymes and RFLP markers, $\mu$ has been estimated at $10^{-5}$–$10^{-6}$ and is rounded to ~0. However, if $\mu \geq m$, this will increase the denominator and reduce estimates of $F_{ST}$ and increase estimates of *Nm*. The *Nm* estimated from RAPDs in this study range from 5.4 to 19 migrants/generation, with an average of 7.6 among all collections. This is very close to the estimate of $N_m = 9.7$ from the Puerto Rico study.[13] Most importantly, estimates of *Nm* from the mitochondrial DNA are similar to the estimates of *Nm* from RAPDs, ranging from 3.5 to 11.7, with an average of 8.6 among all collections. This suggests that mutation rates are probably similar between RAPDs and mtDNA and that effective migration rates in *Ae. aegypti* remain some of the highest ever reported among insects. The question remains as to whether this high rate arises through transport of mosquitoes through human commerce or natural dispersal through adult flight.

Despite estimating similar *Nm* values for RAPDs and mtDNA, the results of the regression analyses differed greatly between the two markers. Genetic and geographic distances were correlated when using RAPD markers while no correlation was detected with mtDNA. There are several possible explanations for this result. The RAPD analysis estimates $F_{ST}$, *Nm*, and *Ne* across markers from throughout the entire genome, while the mtDNA provides only a single estimate. Examination of additional inherited cytoplasmic fac-

tors, or perhaps additional mitochondrial genes, might therefore provide more accurate estimates of $F_{ST}$, $Nm$, and $Ne$. It is also possible that the ND4 gene is under strong balancing selection at the nucleotide level (i.e., any nucleotide substitutions reduce fitness even in third codon positions). This would explain why we detected 45 haplotypes among *An. albimanus* populations in Guatemala and a highly significant correlation between genetic and geographic distances when over a similar distance in Mexico only seven haplotypes were found. The only probable means to differentiate among these possible explanations will be to examine an additional mtDNA gene, probably ND5, among *Ae. aegypti* populations.

The Nuevo Laredo population was genetically distinct from all other populations in this study. The low $\pi_i$ (Table 3C) for mitochondrial markers, the presence of a unique haplotype 7, as well as the high frequency of the otherwise uncommon haplotype 4 are consistent with the hypothesis that a substantial population bottleneck occurred during or after the founding of this population. Either few individuals established the population or the effective population size is regularly severely reduced either by insecticide applications or extended periods of dry weather. In addition, if the density of oviposition sites is related to female dispersal then we might expect females to actively disperse when sites are abundant but to remain in one area when sites are scarce. At least two of these conditions apply to Nuevo Laredo; the 48 mosquitoes used in this study were collected from the same location and were all that could be found in Nuevo Laredo. Furthermore, Nuevo Laredo has an extremely dry climate. A similar result was obtained when examining heterozygosity among *Ae. albopictus* populations that had been recently introduced into the southeast United States.[51] Populations that had just been detected or were being actively eradicated had significantly reduced heterozygosity.

These results have a number of implications for reduction of *Ae. aegypti* populations. The large $Nm$ estimated in this study confirms results from earlier studies and suggests that over distances of 90–250 km, populations remain genetically uniform. We would therefore expect these populations to be genetically similar for genes that control vector competence for dengue and possibly genes that confer insecticide resistance. However, at distances >250 km, populations are likely to differ in both the frequency and types of alleles at loci that impact dengue transmission. The effective population sizes in this study ranged from 74 to 132 individuals. These are relatively large estimates and suggest that extensive reduction in population size will be necessary to reduce gene flow. Results from the Nuevo Laredo population indicate that *Ae. aegypti* populations existing in isolated or extreme habitats may shift in genetic composition. It will be very interesting to compare dengue vector competence and insecticide resistance phenotypes in this population with other populations in Mexico.

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