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Patterns of Variation in the rDNA Cistron Within and Among World Populations of a Mosquito, *Aedes albopictus* (Skuse)

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ABSTRACT

A restriction map was constructed of the ribosomal cistron in a mosquito, *Aedes albopictus* (Skuse). The 18s, 28s and nontranscribed spacer (NTS) regions were subcloned and used to probe for intraspecific variation. Seventeen populations were examined throughout the world range of the species. No variation was detected in the coding regions but extensive and continuous variation existed in the NTS. The NTS consisted of two nonhomologous regions. The first region contained multiple 190-bp *Alu*I repeats nested within larger *Xho*I repeats of various sizes. There was a large number of length variants in the *Alu*I repeat region of the NTS. No repeats were found in the second region and it gave rise to relatively fewer variants. An analysis of NTS diversity in individual mosquitoes indicated that most of the diversity arose at the population level. Discriminant analysis was performed on spacer types in individual mosquitoes and demonstrated that individuals within a population carried a unqiue set of spacers. In contrast with studies of the NTS in Drosophila populations, there seems to be little conservation of spacers in a population. The importance of molecular drive relative to drift and selection in the generation of local population differentiation is discussed.

MOLECULAR genetic surveys of a variety of multigene families have shown that members of these families exhibit a high degree of homogeneity within species. This homogeneity is inconsistent with an hypothesis of independent evolution among members and rather suggests that some mechanism constrains them to evolve in concert. This phenomenon and the mechanism used to explain it have been termed "concerted evolution" and "molecular drive," respectively (DOVER 1982).

Molecular drive is seen by some as a mechanism which operates independently of traditional evolutionary forces, selection and genetic drift, in shaping the extent of genetic divergence among populations. The rate with which molecular drive homogenizes a gene family is theoretically governed by the size of the family, effective population size, rates of mutation, unequal crossover and gene conversion (DOVER 1982; OHTA 1983; OHTA and DOVER 1983, 1984; NAGYLAKI 1984). Homogeneity theoretically decreases as mutation rate, population size and repeat numbers increase. Unequal crossover *within loci* will generate heterogeneity whereas unequal crossover *among loci* can alter the number of copies of a family member and lead to fixation of variants. Gene conversion will drive variants to fixation gradually under models of unbiased gene conversion or rapidly under biased conversion.

Overlying molecular mechanisms of concerted evolution, selection and genetic drift may also act to maintain or eliminate intraspecific diversity in multigene families. While it is not possible to monitor these many processes in field populations, observations on the degree of homogenization among family members in an individual, in a population or in a species can indicate at which levels heterogeneity is generated. A practical way of identifying sources of variation in field populations is through nested spatial sampling. Such studies can estimate the amount of variation found within individuals, among individuals in a population, among local populations and among distantly separated geographic populations.

In this paper we report the results of a worldwide nested spatial survey of variation in a multigene family, the ribosomal RNA gene family, in a mosquito species, *Aedes albopictus*. Of the many multigene families, the family coding for ribosomal RNA (rDNA) has received the most attention in field studies. This family consists of many copies (100–500 copies in animals or 3,000–10,000 copies in plants) of genes coding for the 18s, 5.8s and 28s RNA components of the ribosome. Each copy is connected by a nontranscribed spacer region (NTS). While the coding region tends to be conserved within species, the NTS typically displays much intraspecific variation.

A. albopictus is a container breeding mosquito which

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oviposits predominantly in tires in North America but in a variety of natural containers in its native habitats. The native habitat extends from Madagascar north throughout the Indomalayan and Oriental regions, China and Japan (Ho, Chan and Chan 1973; Huang 1972; Knight and Stone 1977). It became established in Hawaii between 1830 and 1896 (Hardy 1960; Joyce 1961) and within the past 20 yr has colonized the Solomon and Santa Cruz Islands in the South Pacific (Belkin 1962; Elliott 1980; Pashley and Pashley 1983). During the summer of 1985 the species was discovered throughout Harris County, Texas (Sprenger and Wulthiranyagool 1986). Since that time it has become widely distributed in the United States, as far west as San Antonio, Texas, east to Jacksonville, Florida, and north as far as Chicago, Illinois, and Baltimore, Maryland. Comparison of allozyme frequencies (W. C. Black, S. Kambhampati and K. S. Rai, unpublished data) and diapause data (Hawley *et al.* 1987) from U.S. populations with populations collected throughout the world range of *A. albopictus* suggests that the U.S. populations originated in Japan.

We have completed a series of breeding structure studies of *A. albopictus* in the United States and Malaysia (Black *et al.* 1988, 1989) using allozyme markers. Multiple collections were made within several cities. Variance in allele frequencies among all collections was partitioned into the variance among cities, and collections within cities. This analysis revealed that the amount of differentiation among collections within a city was 4–5 times as large as the variance among cities indicating an extensive amount of local differentiation of populations. This suggested that much genetic drift may accompany colonization of containers in this species.

The purpose of the current study was to compare patterns of genetic differentiation derived with isozyme analysis with those defined by analysis of variation in the rDNA multigene family. Specifically, we were interested in (1) quantifying the amount of variation in rDNA at 4 levels; in individuals, in local populations, in city populations and in countries; (2) estimating the amount of divergence in rDNA types among newly established U.S. populations; and (3) determining if patterns of variation in the rDNA family could also be used to identify the origin of the U.S. populations of *A. albopictus*. As with the allozyme analysis, variation in the rDNA family was measured at a local level to determine what role genetic drift can play in generating variation in multigene families. Variation was examined at the individual level by analyzing restriction digests of DNA isolated from individual mosquitoes. Several mosquitoes were examined within a collection, numerous collections were made within cities, and several cities were sampled within and among six countries throughout the world range of *A. albopictus*.

MATERIALS AND METHODS

Strain descriptions: The origin of each *A. albopictus* strain used in this study is listed in Table 1. The number of generations spent in the laboratory prior to use is also indicated. Material was sent as eggs collected on papers or wooden paddles. These were hatched upon arrival, adults blood fed on mice, and a series of large ovipositions collected. The starting size of laboratory colonies varied greatly but was never less than 50 adults.

DNA isolation: DNA was isolated from larvae of the Mauritius strain of *A. albopictus* according to the method of Black and Rai (1988). This DNA was used in the construction of a genomic library. In all other strains, DNA was isolated according to the same method but without final purification on a CsCl gradient. DNA from single adult female mosquitoes was isolated following the method of Coen, Thoday and Dover (1982).

DNA digestion, blotting and hybridization: All restriction enzyme digestions were performed according to manufacturer instructions (Fisher Promega Biotech). DNA was typically digested for 16 hr at 37° at a concentration of approximately 10 units/μg DNA. For visualization and analysis of fragments greater than 1000 bp in length, digested DNA and size standards (*Hin*DIII digested lamba DNA) were loaded onto a 0.75% TBE (Maniatis, Fritsch and Sambrook 1982) agarose gel (DNA grade—Bio-Rad Laboratories) and run for 16 hr at 40 V. Gels were removed, standards measured, and irradiated on a ultraviolet transilluminator for 15 min. DNA was denatured, neutralized, transferred and baked onto nitrocellulose filters (Micron Separations Inc.) following standard protocols (Southern 1975). The same procedures were used to isolate smaller fragments (<1000 bp); however, 1.5% agarose gels were used and fragments were transferred to Zeta-probe filters (Bio-Rad Laboratories) using alkaline transfer procedures (Bio-Rad Technical Bulletin 1398).

Filters were hybridized at 42° in 50% formamide following standard protocols (Maniatis, Fritsch and Sambrook 1982). Following hybridization, the filter was washed under high stringency conditions (Anderson and Young 1987), dried, loaded into an X-ray cassette with film and exposed overnight.

Genomic library construction, screening and subcloning: A partial genomic library of *A. albopictus* was constructed with *Sau*3AI digested fragments cloned into EMBL3 (Stratagene Cloning Systems) following Maniatis, Fritsch and Sambrook (1982). Recombinant phage was plated on lawns of *Escherichia coli* strain P2392, lifted to nitrocellulose circles, and screened with 18s and 28s rDNA probes from *Calliphora erythrocephala* following Maniatis, Fritsch and Sambrook (1982). Viral clones were amplified and their DNA purified according to Maniatis, Fritsch and Sambrook (1982). This DNA was digested, subcloned into pUC118 and transformed into *E. coli* strain DH5*a*.

Statistical analyses: Sizes of digested fragments were determined by entering the mobilities and molecular weights of standards into the program of Schaffer and Sederoff (1981) to derive a least squares regression line.

Genetic distances were calculated using the formula:

$$D = 1 - (2N_{AB}/(N_A + N_B))$$

where N_A and N_B are the number of spacer types in populations A and B, respectively, and N_{AB} are the numbers of

TABLE 1

Strains of *Aedes albopictus*

Source	Collector(s)	Date	Generations in colony
Candos, Mauritius	C. COURTOIS	April, 1970	~100
Cariacica City, Brazil	F. ANTUNANO	October, 1986	3
Chicago, Illinois	B. BLACK	September, 1987	0
Ebina City, Japan	T. TADANO	August, 1986	3
Evansville, Indiana	V. DUNN	September, 1986	3
Galveston Co., Texas	D. SPRENGER	September, 1986	3
Harris County, Texas	D. SPRENGER	June, 1986	3
Indianapolis, Indiana	M. SINSKO	September, 1986	3
Jacksonville, Florida	G. GREGORY and B. PEACOCK	September, 1986	3
Kuala Lumpur, Malaysia	H. YONG	August, 1986	3
Liberty Co., Texas	D. SPRENGER	September, 1986	3
Manoa Valley, Hawaii	D. SHROYER	July, 1984	10
Memphis, Tennessee	B. BLACK and J. FERRARI	June, 1986	3
Milford, Delaware	C. STACHECKI	September, 1987	1
Montgomery Co., Texas	D. SPRENGER	September, 1986	3
New Orleans, Louisiana	M. ANDIS and E. BORDES	June, 1986	3
Peradeniya, Sri Lanka	F. AMERASINGHE	July, 1986	3
Sabah, Borneo	W. HAWLEY	November, 1987	0
Saga City, Japan	M. MOGI	August, 1986	3
Santa Tereza, Brazil	F. ANTUNANO	June, 1987	1
Singapore City	K. CHAN	August, 1986	3
Sonaphur (Assam), India	V. SHARMA	July, 1986	3
Zama City, Japan	T. TADANO	August, 1986	3

spacers that the populations share in common. If both populations have the same set of spacers then $D = 0$, if there are no spacers in common then $D = 1$.

Discriminant analysis was performed on individual mosquitoes using the SPSS-X (SAS, 1988) statistical package. MAHALANOBIS (1936) distances were calculated using PROC CANDISC on SAS.

The diversity of NTS sizes was analyzed using the Shannon Information measure H (LEWONTIN 1972; SCHALL, LEVERICH and NIETO-SOTELO 1987) where:

$$H = p_i \log_2 p_i$$

and p_i is the frequency of a spacer i in a population. If each population contains a unique set of spacers then the amount of diversity contributed at the population level will be 100%. At the other extreme, if all populations share approximately the same sets of spacers then the proportion of diversity contributed by populations will approach zero. By partitioning a population at various levels the amount of diversity contributed by each level to the total diversity of the system can be estimated.

RESULTS

Mapping the rDNA cistron: Three clones of the *A. albopictus* ribosomal cistron were obtained (Figure 1) from the partial genomic library. These were mapped with 10 restriction enzymes: *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I and *Xho*I. 18s and 28s regions were identified by probing SOUTHERN blots of restriction digests of purified lambda DNA with the 18s and 28s rDNA probes from *Calliphora erythrocephala*. The coding regions are identical in each of the clones (Figure 1); however, the NTS varied among all 3 clones. The second clone differed from the first in having a small insert near the 18s gene. The third clone differed in having an approximately 700-bp insert in the middle of the NTS.

To probe for variation in each region, we subcloned the rDNA cistron into 5 regions corresponding with the 18s, the 28s (alpha), the 28s (beta) subunits and the two halves of the nontranscribed spacer (Figure 1). The NTS often contains tandem repeats (BECKINGHAM 1981; GERBI 1985). To test for repeats throughout the spacer region we digested pX8 with *Eco*RI and *Hin*DIII to remove the insert from the plasmid and then digested with *Dra*I to cut the plasmid because pUC118 and the pX8 insert were approximately the same size. Fragments were separated on agarose gels, transferred to nitrocellulose and probed with pX1. No hybridization was detected, indicating a lack of homology between these two regions of the NTS.

In a second attempt to detect repeats we digested pX1 and pX8 with several 4-bp restriction enzymes: *Alu*I, *Hae*III, *Hha*I, *Hpa*II, *Rsa*I and *Sau*3AI. A 190-bp *Alu*I repeat (Figure 1) was found in pX1 but no repeats were detected in pX8. Products of *Alu*I digestion of pX1 were run alongside *Alu*I digested pUC118 on a 2.0% agarose gel. Only a single bright band was unique to the pX1 lane, indicating that most of pX1 consisted of the *Alu*I repeat. Because pX1 is approximately 2.5 kb this suggests that the first half of the

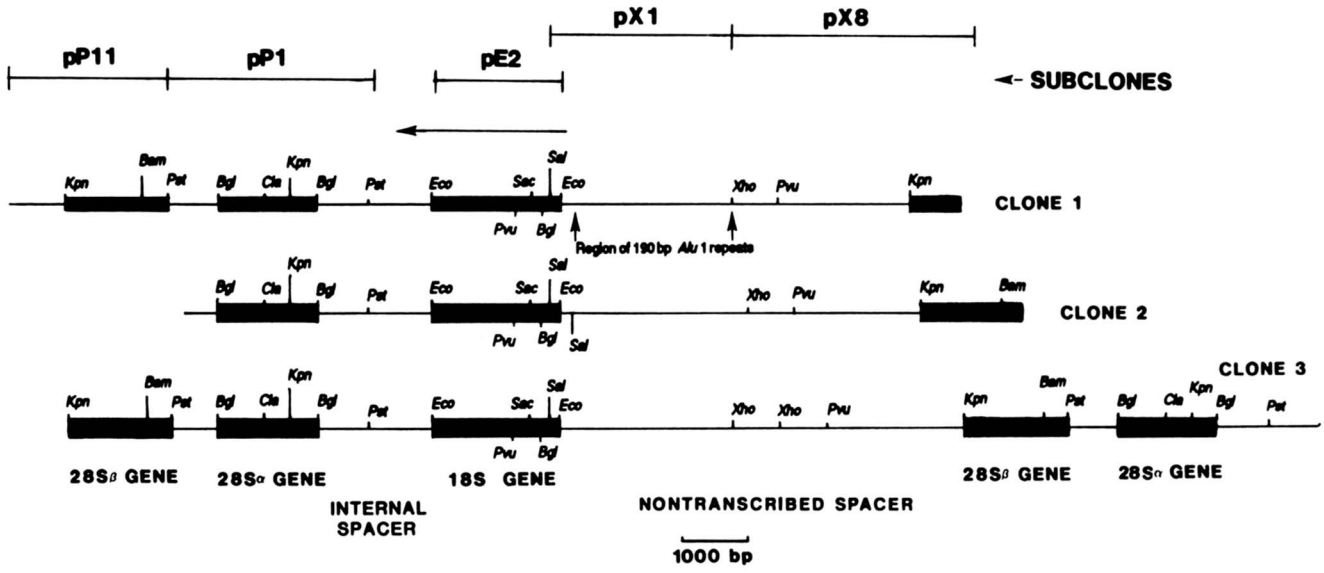

FIGURE 1.—A restriction map of the *A. albopictus* ribosomal cistron. Also indicated are the location of 190 bp *Alu*I repeats in the NTS and the orientation of subclones pP11, pP1, pE2, pX1 and pX8 from clone 1.

NTS consists of 12–13 *Alu*I repeats (Figure 1).

In the process of mapping clone 3 (Figure 1) it was observed that the *Xho*I-*Xho*I insert in the NTS hybridized with pX1, indicating that it arose from duplication with this region of the NTS. The *Xho*I-*Xho*I fragment was subcloned into the *Sal*I site of pUC118. A 190-bp fragment was detected in an *Alu*I digestion of this subclone indicating that the *Xho*I insert consisted of the smaller repeat.

Variation in NTS size among geographic populations of *A. albopictus*: Analysis was performed on DNA extracted from large scale preparations (5,000–10,000 larvae from 200–300 females) of each geographic strain of *A. albopictus*. DNA (5 μg) from each population was digested with *Eco*RI and *Bam*HI to cut the NTS and a portion of the 28s (beta) gene out of the rDNA cistron (Figure 1). These sites were selected because of their location in coding regions and their likelihood of conservation among populations. DNA was transferred to nitrocellulose and probed with a mixture of pX1 and pE2 (Figure 2) or pX1 and pP1 (not shown). These experiments indicated conservation in the size of the 18s and 28s genes among world populations. They also demonstrated blurs representing bands ranging from 3,000 to 10,000 bp in size which hybridized to the NTS probe (Figure 2). This suggests a virtually continuous distribution of spacer sizes in a population. Note, however, that individual bands can be distinguished in this continuum of spacers, suggesting that certain spacers prevail in populations. These are listed by population in Table 2. While certain populations share similar prevalent spacer sizes (*e.g.*, the 4790-bp spacer in Brazil, Jacksonville, and New Orleans populations), no two populations are identical. Even populations collected in

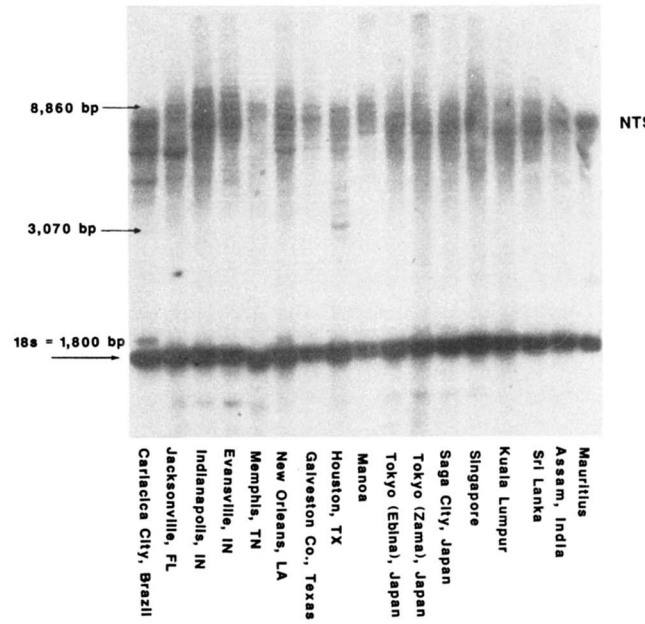

FIGURE 2.—*Eco*RI and *Bam*HI digests of DNA isolated from 17 *A. albopictus* population. Probed with pX1 (NTS probe) and pE2 (18s probe).

proximity (*e.g.*, the Harris and Galveston County populations or the Zama and Ebina populations) vary greatly in their prevalent spacer sizes.

To determine which region of the NTS gave rise to this large amount of variation, DNA from each population was digested with *Eco*RI and *Xho*I and probed with pX1. To further examine variation in this region, DNA was digested with *Alu*I and probed with pX1. Digestion with *Eco*RI and *Xho*I revealed a large number of *Xho*I sites within populations (Figure 3) while the *Alu*I digest indicated that the 190 bp repeat is present and conserved in all populations.

TABLE 2

Prevalent nontranscribed spacer sizes in *A. albopictus* populations

Population	Prevalent spacer size					Range
Mauritius	6,930	6,490	5,420	4,630		2,300
Berhampur	6,490	5,900				590
Sri Lanka	6,490	5,700				790
Kuala Lumpur	7,050	6,400	5,700			1,350
Singapore	6,930	6,100	5,650	4,450		2,480
Saga City, Japan	7,170	5,700				1,470
Tokyo (Zama), Japan	6,490	5,700				790
Tokyo (Ebina), Japan	6,400	6,100				300
Manoa	7,560	7,170	6,930	6,940	5,700	1,860
Houston, Texas	7,050	6,400	6,100	5,500	4,790	
	3,670	3,360	3,070			3,980
Galveston, Co., Texas	10,230	7,170	6,400	4,790		5,440
New Orleans, Louisiana	5,900	4,790				1,110
Memphis, Tennessee	7,170					
Evansville, Indiana	8,460	7,840	7,170	6,400	6,100	2,360
Indianapolis, Indiana	7,170	6,100				1,070
Jacksonville, Florida	4,790					
Cariacica City, Brazil	7,050	6,490	5,700	4,790	2,000	5,050

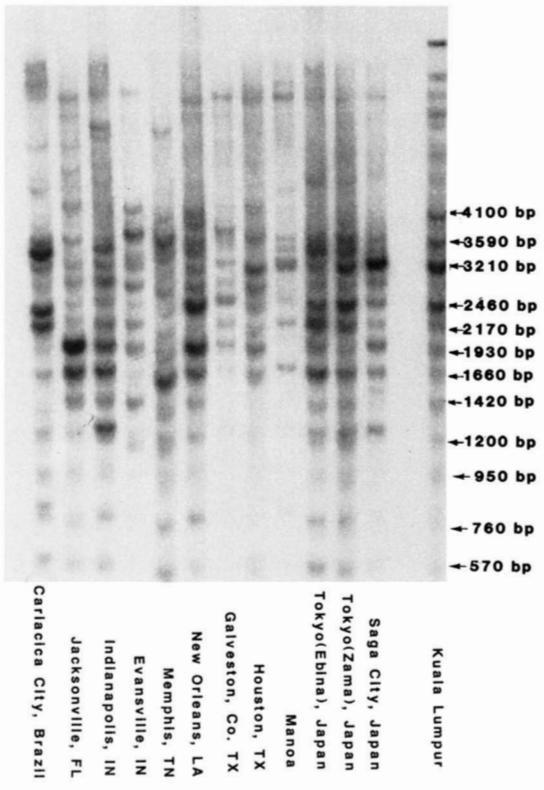

FIGURE 3.—*Eco*RI and *Xho*I digests of DNA isolated from *A. albopictus* populations and probed with pX1 (NTS probe).

This pattern suggests that the extensive variation may result from differences in the numbers of *Alu*I repeats within the larger *Xho*I repeats. To test this hypothesis the molecular weight of each fragment in the *Eco*RI and *Xho*I digest was determined and the size difference of adjacent fragments calculated. The mean difference among adjacent fragments was 282 bp (SE = 10.8 bp, $n = 157$). However, assuming that some fragments might have arisen through duplication of two or more tandem repeats, larger fragments were divided by 2 or 4. Following this correction, the mean difference among adjacent fragments was 229 bp (SE = 3.8 bp, $n = 157$) which is close to the size of the 190-bp *Alu*I repeat.

To examine variation in the other half of the NTS, digestions were performed with *Bam*HI and *Pvu*II (Figure 1) and probed with pP11. This revealed some variation among sites but also conservation of two spacer types (5760 bp and 2860 bp) among all populations (Figure 4).

The relationship between geographic distance and genetic distance in *A. albopictus*: To analyze the relationship between prevalent spacer size and geographic proximity, we calculated the correlation between genetic (D) and geographic distances. If populations in proximity to one another are more closely related than populations at a distance then there will be a positive correlation between genetic and geographic distance. Alternatively no correlation will exist if genetic distances are independent of geographic proximities.

Correlations were calculated using genetic distances based on (1) the length of the entire NTS, (2) the sizes of *Eco*RI/*Xo*I repeats in the NTS and (3) the sizes of *Pvu*II/*Bam*HI fragments of the NTS. A significant positive correlation ($r = 0.20$, $n = 105$, $P \leq 0.05$) was detected using distance based on the entire NTS; however, plotting the points revealed that the correlation was an artifact of the bimodal clustering of points around one and zero. No correlation was detected using the *Eco*RI/*Xho*I region ($r = -0.12$, $n = 66$, $P \geq 0.05$). However, a slightly significant positive correlation was observed using the *Pvu*II/*Bam*HI region ($r = 0.44$, $n = 105$, $P \leq 0.01$).

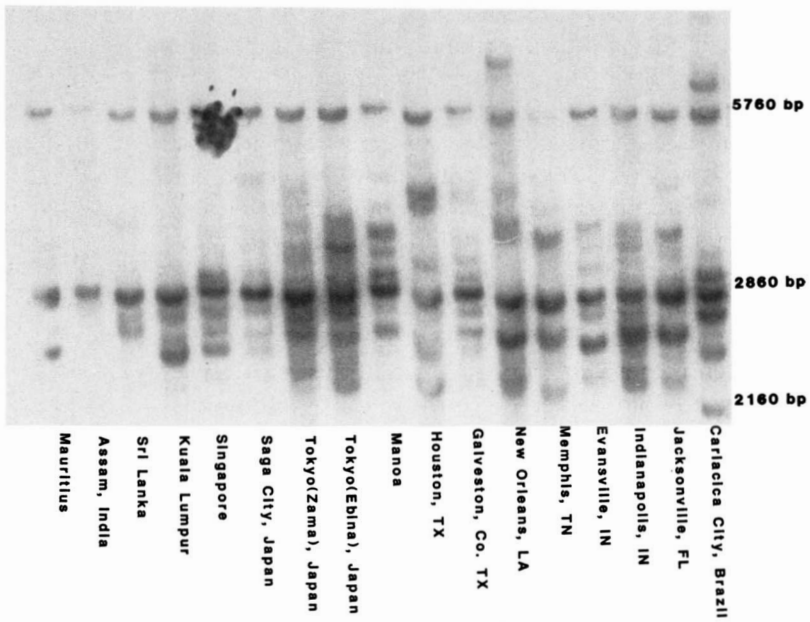

FIGURE 4.—*Bam*HI and *Pvu*II digests of DNA isolated from *A. albopictus* populations and probed with pP11 (28s (beta) probe).

Because *A. albopictus* was only recently established in the United States, the genetic distances might reflect random patterns of differentiation associated with founders effects and genetic drift. The same should not hold true for native habitats of *A. albopictus*. The correlations between genetic and geographic distances were reanalyzed after removing U.S. and Brazilian populations. The correlation based on the entire NTS decreased to -0.16 ($n = 21$) and was not significantly different from zero. In contrast, correlations derived using either one of the other two regions of the NTS increased ($r = 0.93$, $n = 6$, $P \leq 0.01$ (*Eco*RI/*Xho*I region) and $r = 0.57$ (*Pvu*II/*Bam*HI region), $n = 21$, $P \leq 0.01$) indicating a positive correlation between genetic and geographic distance. Despite finding significant correlations between geographic and genetic distance, populations from the United States were not consistently similar to any one of the foreign populations examined.

Variation in NTS size within geographic populations of *A. albopictus*: *Eco*RI and *Bam*HI digests were performed on DNA extracted from individual adult mosquitoes. Within a population, 17–19 individuals were examined and a total of 17 populations were analyzed. For each population, molecular weights were calculated using *Hin*dIII digested lambda standards in the program of SCHAFFER and SEDEROFF (1981) and each blot was probed with pE2 to act as an internal size standard (Figure 5, A–C). Each spacer detected in individual mosquitoes was placed in one of 49 two hundred bp size classes (Table 3).

Three patterns of variation were detected in populations. In populations such as Singapore City (Figure 5A), there was a large number of spacers sizes, individuals shared some spacers in common but no two individuals possessed the same set of spacers. In populations such as Assam, India (Figure 5B), there were few spacers and individuals seemed to share most of these in common. In the Memphis population (Figure 5C), there were several spacer sizes and almost all individuals shared these in common (*i.e.*, possessed the same genotype).

The number of spacer types detected in each population and the mean number of spacer types per mosquito are listed in Table 4. Discriminant analysis was used to classify individuals according to their spacers. If individuals in a population carry a unique set of spacers relative to the total population then there will be a correlation among spacers and the analysis will accurately assign individuals to a particular population. Alternatively, if no correlations exists among spacers carried by an individual then spacers are randomly distributed and the analysis cannot assign individuals unambiguously to populations. Use of this approach is appropriate to analyzing the population genetics of multigene families because if concerted evolution occurs among family members then spacers in populations should evolve as sets rather than independently.

Of the 302 individuals examined from the 17 populations, 76.5% were identified in the analysis as being unique to their population. Alternatively, 23.5% were ambiguous and could have arisen in one or more of the other populations examined in the study. We considered the possibility that these large differences among populations might reflect differences in individual blots. To check for this, five populations were analyzed in continuously overlapping blots. Among those populations, the percentage of individuals correctly classified was 92.1%. The discriminant analysis

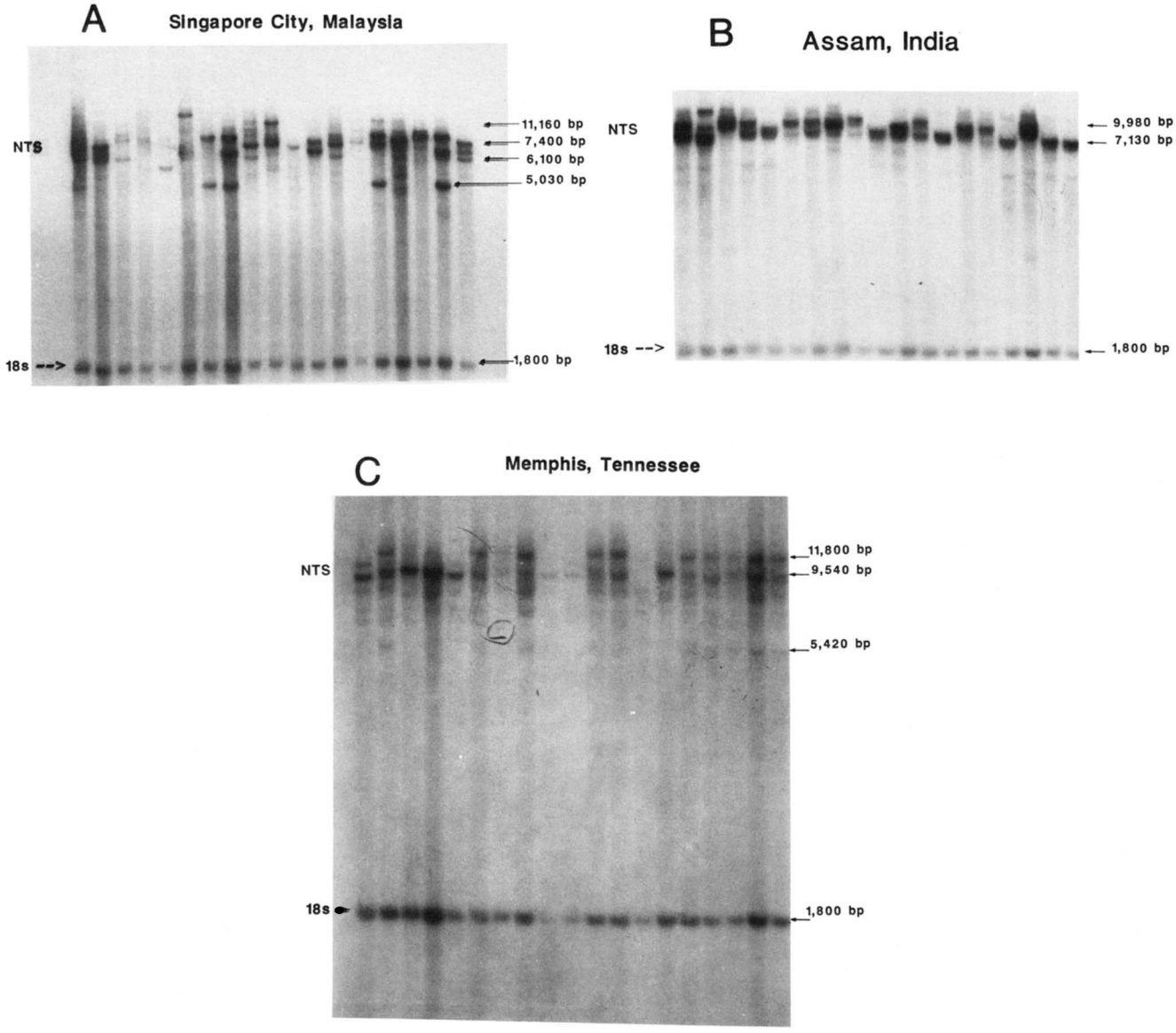

FIGURE 5.—DNA isolated from single mosquitoes, digested with *Eco*RI and *Bam*HI and probed with pX1 (NTS probe) and pE2 (18s probe). (A) Mosquitoes from Singapore City, (B) Assam, India, and (C) Memphis, Tennessee.

then indicates that individuals within a population carried a unique set of spacers and that there was only a small amount of overlap among populations. This suggests concerted evolution plays an important role in establishing sets of spacers in populations.

In discriminant analysis, groups of similar individuals are clustered in ellipses (PIMENTEL 1979). The strong correspondence between ellipses and populations in our analysis suggested that this method might also be used as a statistical measure of the relatedness of populations. The center of each ellipse is termed the centroid. MAHALANOBIS (1936) distance is measure of distances among centroids. Following the same rationale as before, we attempted to correlate Mahalanobis distances with geographic distances to determine if the overlap among populations was related to geographic proximity. We assumed that migration among populations in closer proximity to one another might make them more genetically similar than populations at a distance. There was a slightly negative relationship between geographic and Mahalanobis distance for all populations and a positive but nonsignificant correlation ($r = 0.427$, d.f. = 9, $P > 0.05$) among population in the native habitat. There was no relationship between statistical and geographic distance.

Analysis of spacer diversity in populations: To determine at which level the greatest diversity of spacer types arises, we partitioned the amount of variation in rDNA at four levels; in individuals, in local populations, in city populations and in countries (Table 5). Individual samples (I) were nested within cities (CT), cities were nested within countries (CN), and countries were nested among all populations (T).

TABLE 3

Frequency of spacer types in each of 17 populations throughout the world range of *A. albopictus*

Size class (kbp)	Population																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	All
3.0–3.2	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
3.6–3.8	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1
4.0–4.2	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	1	–	2
4.2–4.4	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	3	–	5
4.4–4.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1
4.6–4.8	–	5	–	–	–	–	–	2	–	–	–	–	–	–	4	2	–	13
4.8–5.0	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1
5.0–5.2	–	–	–	–	–	–	–	–	–	–	5	–	–	–	1	1	–	7
5.2–5.4	–	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1	3
5.4–5.6	–	1	–	–	–	–	–	1	–	16	1	4	–	1	–	5	2	31
5.6–5.8	–	2	–	1	–	–	–	4	–	–	7	–	–	5	2	2	–	23
5.8–6.0	–	4	–	–	–	–	8	2	2	–	–	2	14	3	1	–	2	38
6.0–6.2	–	–	–	–	3	–	–	1	–	–	1	–	3	2	–	5	4	19
6.2–6.4	–	–	–	17	4	–	18	5	2	–	1	6	–	–	7	3	4	67
6.4–6.6	–	–	4	–	–	7	5	5	–	–	8	–	1	4	–	1	5	40
6.6–6.8	–	–	5	4	2	–	–	4	–	–	1	1	–	3	6	5	4	35
6.8–7.0	–	4	–	–	–	–	–	–	10	16	2	–	13	4	2	1	7	59
7.0–7.2	12	–	3	3	12	2	–	2	2	–	9	–	–	9	–	4	4	62
7.2–7.4	–	9	–	4	–	–	7	–	3	–	1	–	13	1	8	4	–	50
7.4–7.6	–	–	–	–	–	8	–	3	–	–	3	8	–	1	–	–	4	27
7.6–7.8	–	4	–	5	4	–	7	4	7	–	–	–	–	–	4	–	–	35
7.8–8.0	–	–	3	2	–	–	–	2	2	16	–	3	–	–	8	8	1	45
8.0–8.2	–	1	–	6	3	–	–	1	–	–	9	10	16	2	–	–	7	55
8.2–8.4	–	7	–	–	5	1	–	1	4	–	2	–	–	–	5	1	5	31
8.4–8.6	–	–	12	–	–	–	–	–	–	–	6	–	1	–	–	3	–	22
8.6–8.8	–	–	–	4	2	–	10	2	–	–	–	–	–	1	–	–	–	19
8.8–9.0	–	–	–	–	–	5	–	3	–	–	1	–	–	4	–	1	1	15
9.0–9.2	–	–	–	–	–	–	–	1	6	–	–	–	11	–	4	–	–	22
9.2–9.4	7	–	–	–	6	–	–	–	–	–	–	–	–	3	–	2	1	19
9.4–9.6	–	3	–	5	3	–	8	1	–	18	–	–	–	–	–	–	–	38
9.6–9.8	–	–	10	–	4	12	–	–	–	–	–	1	10	–	–	–	3	40
9.8–10.0	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	3
10.0–10.2	–	–	–	6	–	–	–	–	–	–	–	–	–	–	4	–	–	10
10.2–10.4	11	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	12
10.4–10.6	–	–	–	–	7	–	–	–	3	–	–	–	–	–	–	–	–	10
10.6–10.8	–	1	–	–	–	5	–	–	–	–	–	–	3	–	–	–	–	9
10.8–11.0	–	–	–	–	7	–	–	–	–	4	–	–	–	–	–	–	1	12
11.0–11.2	–	–	1	3	–	–	–	–	–	–	2	–	–	–	–	–	–	6
11.2–11.4	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1
11.4–11.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
11.6–11.8	–	–	5	–	–	–	–	–	–	–	–	–	5	–	–	–	–	10
11.8–12.0	–	–	–	–	–	–	–	–	–	12	–	–	–	–	–	–	–	12
12.0–12.2	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	3
12.2–12.4	1	–	–	–	–	–	–	–	–	–	–	3	–	–	1	–	–	5
12.4–12.6	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1
12.6–12.8	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	3
12.8–13.0	–	–	–	–	–	–	–	1	–	–	–	–	10	–	–	–	–	11
13.0–13.2	–	–	–	1	–	2	–	–	–	–	–	–	–	–	–	–	–	3
13.2–13.4	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	2
13.6–13.8	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3

Key to population numbers: 1 = Sonaphur (Assam), India; 2 = Cariacica City, Brazil; 3 = Chicago, Illinois; 4 = Ebina City, Japan; 5 = Evansville, Indiana; 6 = Galveston Co., Texas; 7 = Harris County, Texas; 8 = Indianapolis, Indiana; 9 = Liberty Co., Texas; 10 = Memphis, Tennessee; 11 = Milford, Delaware; 12 = Montgomery Co., Texas; 13 = New Orleans, Louisiana; 14 = Sabah, Borneo; 15 = Singapore City; 16 = Santa Tereza, Brazil; 17 = Zama City, Japan. – = spacer type absent.

The average diversity among individuals within samples (H_I) accounted for 65% (H_S/H_T) of total diversity in the species. Differences among samples within cities ($(H_{CT} - H_I)/H_T$) accounted for 5% and differences among cities within a country ($(H_{CN} - H_{CT})/H_T$) for 4% of total diversity. The final level, differences among countries ($(H_T - H_{CN})/H_T$) accounted for 25% of total diversity.

DISCUSSION

The *A. albopictus* ribosomal cistron consists of a coding region which was found to be invariant among

TABLE 4

Number of spacer types and the mean number of spacer types per individual mosquito (± standard error) in *A. albopictus* populations

Population	No. spacer types: Sample size	No. spacer types	Spacer types/ mosquito
Assam	19	4	1.63 ± 0.14
Singapore	18	15	3.22 ± 0.33
Sabah	18	16	2.50 ± 0.34
Japan			
Tokyo			
Ebina	18	13	3.39 ± 0.30
Zama	18	16	2.89 ± 0.20
Brazil			
Rio de Janiero			
Cariacica City	17	12	2.47 ± 0.30
Santa Tereza	18	19	3.11 ± 0.36
United States			
Houston			
Harris County	18	7	3.50 ± 0.29
Montgomery County	17	9	2.23 ± 0.36
Galveston County	17	8	2.47 ± 0.21
Liberty County	16	11	2.69 ± 0.24
New Orleans	19	12	5.26 ± 0.44
Memphis	19	6	4.32 ± 0.27
Evansville	17	15	4.00 ± 0.34
Indianapolis	18	24	2.83 ± 0.29
Chicago	17	11	2.82 ± 0.38
Milford	18	17	3.44 ± 0.23
A. albopictus	302	49	3.12 ± 0.08

TABLE 5

Shannon information measured (*H*) in populations, populations within cities and cities within countries

Population	Populations	Cities	Countries	
Assam	1.705	1.705	1.705	
Singapore	3.603	3.603	3.603	
Sabah	3.661	3.661	3.661	
Japan			4.096	
Tokyo		4.096		
Ebina	3.320			
Zama	3.714			
Brazil			4.297	
Rio		4.297		
Cariacica	3.236			
Tereza	3.966			
United States			4.906	
Houston		4.195		
Harris	2.689			
Montgomery	2.821			
Galveston	2.681			
Liberty	3.196			
New Orleans	3.253	3.253		
Memphis	2.479	2.479		
Evansville	3.717	3.717		
Indianapolis	4.325	4.325		
Chicago	3.049	3.049		
Milford	3.642	3.642		
	3.239	3.502	3.711	4.97
	H (pop)	*H* (city)	*H* (count)	*H* (all)

Partitioning of total diversity:		
Within populations:	*H*(pop)/*H*(all)	65.2%
Among population within cities:	*H*(city)-*H*(pop)/*H*(all)	5.3%
Among cities within countries:	*H*(count)-*H*(city)/*H*(all)	4.2%
Among countries:	*H*(all)-*H*(count)/*H*(all)	25.3%
		100.0%

populations worldwide and a NTS which varied continuously among and within populations. The NTS was shown to consist of two, nonhomologous regions. The first region was bounded by an *Eco*RI site in the 18s gene and an *Xho*I site in the NTS and consisted of multiple *Alu*I repeats nested within larger *Xho*I repeats. No repeat structure was detected in the second half of the NTS, a region bounded by a *Pvu*II site in the NTS and a *Bam*HI site in the 28s (beta) gene.

These two regions differed in the type of variability they generated among populations. There was a large number of variants in the *Eco*RI/*Xho*I region of the NTS. Differences in sizes among spacers in this region suggested that they might have arisen through unequal exchange between the small 190-bp *Alu*I repeats as well as the larger *Xho*I repeats. The *Bam*HI/*Pvu*II region gave rise to relatively fewer variants and two variants were found to be common to and abundant in all populations examined. This type of NTS organization is atypical of most insect species (mostly *Drosophila*) in which the NTS has been examined (BECKINGHAM 1981). However, a similar NTS organization has been recently noted in one species *Drosophila buschii* (SLAVICEK and KRIDER 1987) which contains a central region consisting of 160 bp *Hinc*II repeats and an adjacent region variable in length within the species but without an obvious repeat structure.

The results of our analysis of prevalent spacer sizes in populations indicates that there is no relationship between the size of the entire NTS and geographic proximity of populations. However, when we examined the two regions of the NTS separately we found a positive correlation between geographic distances and genetic distances based on the length of the *Bam*HI/*Pvu*II region. No correlation was detected in the *Eco*RI/*Xho*I region, suggesting that this region evolved at a more rapid rate possibly because of the nested internal repeat structure giving rise to more unequal crossing over or gene conversion. When the newly established United States and Brazilian populations were removed from the analysis, the correlations increased indicating a relationship, albeit weak, between genetic and geographic distance. This and the diversity of bands found in U.S. populations suggests that there may have been a rapid change in the diversity of NTS during the colonization of U.S. populations.

An implicit assumption in the discriminant and Shannon diversity analyses is that similar length variants represent similar sequences. This assumption may be false given the continuum of spacers sizes

detected in the *A. albopictus* population. If so, then we have underestimated the degree of population separation in the discriminant analysis and similarly underestimated the amount of diversity added at the population level. In either case, our analysis of NTS diversity in individual mosquitoes indicated that most of the diversity arose at the population level. Furthermore, the discriminant analysis demonstrated that individuals within a population carry a unique set of spacers and that there was only a small amount of overlap among populations. Analysis of geographic distances with regards to genetic distances or Mahalanobis distances indicated that for the most part, the relatedness of populations was independent of geographic proximity and therefore diversity arises independently in adjacent populations. If most of the diversity in NTS size arises in populations, then populations even in adjacent regions become rapidly differentiated and genetic distances will be independent of geographic distances. This limits the utility of multigene families in defining the relationship among existing populations and in tracing the origins of new populations. This contrasts sharply with correlations between genetic and geographic distance detected with allozyme analysis in which we found a highly significant correlation ($r = 0.89$, $n = 21$, $P \leq 0.001$).

The most notable finding of this study is that there seems to be little conservation of spacers in an *A. albopictus* population. Except for the two *Bam*HI/*Pvu*2 bands and the *Alu*I repeat there was no dominant spacer type in the *A. albopictus* population worldwide. This contrasts markedly with other studies of NTS spacer types in Drosophila populations. COEN, STRACHAN and DOVER (1982) working with the rDNA cistron in Drosophila species, found a high degree of polymorphism for the length and copy number of family members in Drosophila species suggesting high rates of mutation and unequal sister chromatid exchange. A consistent pattern discovered in their work and other work on Drosophila field populations [*Drosophila melanogaster*—(COEN, THODAY and DOVER 1982; WILLIAMS *et al.* 1987) and *Drosophila mercatorum* (WILLIAMS, DESALLE and STROBECK 1985)] is that polymorphisms are found to exist mainly *among* rather than *within* populations. Most individuals within a single population or clusters of local populations shared a common spacer. This suggests that homogenization within populatins may occur very rapidly. In contrast, analysis of rDNA variation among and within plant populations has demonstrated that most of the variation lies *within* rather than *among* populations (LEARN and SCHALL 1987; SCHALL, LEVERICH and NIETO-SOTELO 1987; APPELS and DVORAK 1982). SCHALL, LEVERICH and NIETO-SOTELO (1987), for example, found that, as with the *A. albopictus* population, 61% of diversity was found among individuals of a population. A recent study of broad bean, *Vicia faba*, found copy number changes even among different tissues in a plant (ROGERS and BENDICH 1987). The difference between plant and animal populations may be related to the relatively high number of copies of rDNA in plants but hardly serves to explain the differences between patterns of variation in the *A. albopictus* and Drosophila populations. Of the 17 populations examined in this study, only the Assam, India population would seem to follow the Drosophila pattern.

The rDNA multigene family (Nucleolar Organizing Region) in *D. melanogaster* is located on the *X* and *Y* chromosomes. COEN and DOVER (1983) demonstrated unequal crossovers between the rDNA sequences on these chromosomes albeit at low frequencies ($1.5–8.0 \times 10^{-4}$ crossovers/generation). In all aedine mosquitoes examined, including *A. albopictus*, the NOR is located on chromosome *3* (MCDONALD and RAI 1970; DEV and RAI 1984). This, in contrast with *D. melanogaster*, would allow for homologous recombination and could increase the rate of unequal exchange.

Forces, acting either at the population or molecular level, have generated an enormous amount of NTS diversity within local *A. albopictus* populations. And yet, in seeming contradiction, prevalent spacers were detected in these populations and individual mosquitoes exhibited discrete spacers. This suggests that molecular drive was operating in populations to generate concerted evolution among spacers. In the absence of molecular drive, individuals would have appeared as a continuum of spacers (a blur) and population would have exhibited no prevalent spacer.

Breeding structure studies of *A. albopictus* in the United States and Malaysia (BLACK *et al.* 1988b, c) using allozyme markers suggested that much genetic drift accompanies the establishment of local populations. The large amount of local differentiation in NTS sizes may also reflect the effects of genetic drift. Subsequently, molecular drive might act rapidly, probably within a few generations, to homogenize spacers. COEN, THODAY and DOVER (1982) and BONCINELLI *et al.* (1983) were unable to detect a rapid mechanism in *D. melanogaster*. However, it may be that the unique form of repeat structure in the *A. albopictus* NTS and the location of NORs on homologous chromosomes could generate a more rapid rate of unequal chromatid exchange or gene conversion. Experiments are currently underway to determine rates of unequal crossing over in laboratory *A. albopictus* populations (S. KAMBHAMPATI, unpublished data).

The recent description of molecular drive (DOVER 1986) as a major force in evolutionary genetics has generated much interest in describing this phenomena in field populations. Often the more conventional

sources of intraspecific variation, selection and genetic drift, have been ignored. And yet they must also govern the extent of intraspecific variation in multigene families. Allozyme studies provide a tool for estimating selection and genetic drift in field populations. Patterns of allozyme differentiation have been established in a large variety of animal species. Patterns of differentiation in multigene families have been described in only a few. By correlating patterns of differentiation in multigene families with patterns established in allozyme analysis, future research may allow us to evaluate the relative importance of selection, genetic drift and molecular drive in generating and stabilizing variation in members of multigene families.

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LITERATURE CITED

APPELS, R., and J. DVORAK, 1982 The wheat ribosomal DNA spacer region: its structure and variation in populations and among species. Theor. Appl. Genet. **63:** 337–348.

ANDERSON, M. L. M., and B. D. YOUNG, 1987 Quantitative filter hybridization, pp. 73–111 in *Nucleic Acid Hybridisation: A Practical Approach*, edited by B. D. HAMES and S. J. HIGGINS. IRL Press, Oxford.

BECKINGHAM, K., 1981 Insect rDNA, pp. 205–269 in *The Cell Nucleus*. Vol. X. Part A, edited by H. BUSCH and L. ROTHBLUM. Academic Press, New York.

BELKIN, J. N., 1962 *Mosquitoes of the South Pacific (Diptera, Culicidae)*, 2 Vols. University of California Press, Berkeley.

BLACK, W. C., IV, and K. S. RAI, 1988 Genome evolution in mosquitoes: intra- and interspecific variation in repetitive DNA amounts and organization. Genet. Res. **51:** 185–196.

BLACK, W. C., IV, J. A. FERRARI, K. S. RAI and D. SPRENGER, 1988 Breeding structure of a colonizing species: *Aedes albopictus* (Skuse) in the United States. Heredity **60:** 173–181.

BLACK, W. C., IV, W. A. HAWLEY, K. S. RAI and G. B. CRAIG, JR., 1989 Breeding structure of a colonizing species: *Aedes albopictus* (Skuse) in Peninsular Malaysia and Borneo. Heredity (in press).

BONCINELLI, E., A. BORGHESE, F. GRAZIANI, G. LA MANTIA, A. MANZI, C. MARIANI and A. SIMEONE, 1983 Inheritance of the rDNA spacer in *D. melanogaster*. Mol. Gen. Genet. **189:** 370–374.

COEN, E. S., and G. DOVER, 1983 Unequal exchanges and the coevolution of X and Y rDNA arrays in *Drosophila melanogaster*. Cell **33:** 849–855.

COEN, E. S., T. STRACHAN and G. DOVER, 1982 Dynamics of concerted evolution of ribosomal DNA and histone gene families in the *melanogaster* species subgroup of *Drosophila*. J. Mol. Biol. **158:** 17–35.

COEN, E. S., J. M. THODAY and G. DOVER, 1982 Rate of turnover of structural variants in the rDNA gene family of *Drosophila melanogaster*. Nature **295:** 564–568.

DEV, V., and K. S. RAI, 1984 Genetics of speciation in the *Aedes (Stegomyia) scutellaris* group (Diptera: Culicidae). V. Chromosomal relationships among five species. Genetica **64:** 83–92.

DOVER, G., 1982 Molecular drive: a cohesive mode of species evolution. Nature **299:** 111–117.

DOVER, G., 1986 Molecular drive in multigene families: how biological novelties arise, spread and are assimilated. Curr. Trends Genet. **8:** 159–165.

ELLIOTT, S. A., 1980 *Aedes albopictus* in the Solomon and Santa Cruz Islands, South Pacific. Trans. R. Soc. Trop. Med. Hyg. **74:** 747–748.

GERBI, S. A., 1985 Evolution of ribosomal DNA, pp. 419–517 in *Molecular Evolutionary Genetics*, edited by R. J. MACINTYRE. Plenum, New York.

HARDY, D. E., 1960 Culicidae, pp. 18–22, 81–90 in *Insects of Hawaii*, Vol. 10, edited by E. C. ZIMMERMAN. University Press of Hawaii, Honolulu.

HAWLEY, W. A., P. REITER, R. S. COPELAND, C. B. PUMPUNI and G. B. CRAIG, 1987 *Aedes albopictus* in North America: probable introduction in used tires from Northern Asia. Science **236:** 1114–1116.

HO, E. C., K. L. CHAN and Y. C. CHAN, 1973 The biology and bionomics of *Aedes albopictus* (Skuse), pp. 125–143 in *Vector Control in Southeast Asia*, edited by Y. C. CHAN, K. L. CHAN and B. C. HO. University of Singapore Press, Singapore.

HUANG, Y. M., 1972 Contributions to the mosquito fauna of Southeast Asia. XIV. The subgenus *Stegomyia* of *Aedes* in Southeast Asia. I. The *scutellaris* groups of species. Contrib. Am. Entomol. Inst. (Ann Arbor) **9:** 1–109.

JOYCE, C. R., 1961 Potentialities for accidental establishment of exotic mosquitoes in Hawaii. Proc. Hawaii. Entomol. Soc. **17:** 403–413.

KNIGHT, K. L., and A. STONE, 1977 *A Catalog of the Mosquitoes of the World (Diptera: Culicidae)*, Vol. 6, Ed. 2, Thomas Say Foundation. Entomological Society of America, College Park, Md.

LEARN, G. L., and B. A. SCHALL, 1987 Population subdivision for rDNA repeat variants in *Clematis fremontil*. Evolution **41:** 433–437.

LEWONTIN, R. C., 1972 The apportionment of human diversity. Evol. Biol. **6:** 381–398.

MAHALANOBIS, P. C., 1936 On the generalized distance in statistics. Proc. Natl. Inst. Sci. India **2:** 49–55.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MCDONALD, P. T., and K. S. RAI, 1970 Correlation of linkage groups with chromosomes in the mosquito, *Aedes aegypti*. Genetics **66:** 475–485.

NAGYLAKI, T., 1984 The evolution of multigene families under intrachromosomal gene conversion. Genetics **106:** 529–548.

OHTA, T., 1983 On the evolution of multigene families. Theor. Popul. Biol. **23:** 216–240.

OHTA, T., and G. DOVER, 1983 Population genetics of multigene families that are dispersed into two or more chromosomes. Proc. Natl. Acad. Sci. USA **80:** 4079–4083.

OHTA, T., and G. DOVER, 1984 The cohesive population genetics of molecular drive. Genetics **108:** 501–521.

PASHLEY, D. N., and D. P. PASHLEY, 1983 Observations on *Aedes (Stegomyia)* mosquitoes in Micronesia and Melanesia. Mosq. Syst. **15:** 41–49.

PIMENTEL, R. A., 1979 *Morphometrics: The Multivariate Analysis of Biological Data*. Kendall/Hunt Press, Dubuque, Iowa.

ROGERS, S. O., and A. J. BENDICH, 1987 Heritability and variability in ribosomal RNA genes of *Vicia faba*. Genetics **117:** 285–295.

SAS, 1988. *SAS Users Guide: Statistics*. SAS, Cary, N.C.

SCHAFFER, H. E., and R. R. SEDEROFF, 1981 Improved estimation

of DNA fragment lengths from agarose gels. Anal. Biochem. **115:** 113–122.

SCHALL, B. A., W. J. LEVERICH and J. NIETO-SOTELO, 1987 Ribosomal DNA variation in the native plant, *Phlox divaricata*. Mol. Biol. Evol. **4:** 611–621.

SLAVICEK, J. M., and H. M. KRIDER, 1987 The organization and composition of the ribosomal RNA gene non-transcribed spacer of *D. busckii* is unique among the drosophilids. Genet. Res. **50:** 173–180.

SOUTHERN, E. M., 1975 Detection of specific DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:** 503–517.

SPRENGER, D., and T. WUITHIRANYAGOOL, 1986 The discovery and distribution of *Aedes albopictus* in Harris County, Texas. J. Am. Mosq. Cont. Assoc. **2:** 217–219.

WILLIAMS, S. M., R. DESALLE and C. STROBECK, 1985 Homogenization of geographical variants at the nontranscribed spacer of rDNA in *Drosophila mercatorum*. Mol. Biol. Evol. **2:** 338–346.

WILLIAMS, S. M., G. R. FURNIER, E. FUOG and C. STROBECK, 1987 Evolution of the ribosomal DNA spacers in *Drosophila melanogaster*: different patterns of variation on *X* and *Y* chromosomes. Genetics **116:** 225–232.

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