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# Population genetics of the yellow fever mosquito in Trinidad: comparisons of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers

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## Abstract

**Recent development of DNA markers provides powerful tools for population genetic analyses. Amplified fragment length polymorphism (AFLP) markers result from a polymerase chain reaction (PCR)-based DNA fingerprinting technique that can detect multiple restriction fragments in a single polyacrylamide gel, and thus are potentially useful for population genetic studies. Because AFLP markers have to be analysed as dominant loci in order to estimate population genetic diversity and genetic structure parameters, one must assume that dominant (amplified) alleles are identical in state, recessive (unamplified) alleles are identical in state, AFLP fragments segregate according to Mendelian expectations and that the genotypes of an AFLP locus are in Hardy–Weinberg equilibrium (HWE). The HWE assumption is untestable for natural populations using dominant markers. Restriction fragment length polymorphism (RFLP) markers segregate as codominant alleles, and can therefore be used to test the HWE assumption that is critical for analysing AFLP data. This study examined whether the dominant AFLP markers could provide accurate estimates of genetic variability for the *Aedes aegypti* mosquito populations of Trinidad, West Indies, by comparing genetic structure parameters using AFLP and RFLP markers. For AFLP markers, we tested a total of five primer combinations and scored 137 putative loci. For RFLP, we examined a total of eight mapped markers that provide a broad coverage of mosquito genome. The estimated average heterozygosity with AFLP markers was similar among the populations (0.39), and the observed average heterozygosity with RFLP markers varied from 0.44 to 0.58. The average $F_{ST}$ (standardized among-population genetic variance) estimates were 0.033 for AFLP and 0.063 for RFLP markers. The genotypes at several RFLP loci were not in HWE, suggesting that the assumption critical for analysing AFLP data was invalid for some loci of the mosquito populations in Trinidad. Therefore, the results suggest that, compared with dominant molecular markers, codominant DNA markers provide better estimates of population genetic variability, and offer more statistical power for detecting population genetic structure.**

*Keywords: Aedes aegypti*, AFLP, genetic diversity, population genetic structure, RFLP

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## Introduction

Estimating genetic variation and genetic structure of natural populations relies on genotyping individual specimens. Amplified fragment length polymorphism (AFLP) markers result from a polymerase chain reaction (PCR)-based DNA fingerprinting technique that can detect

multiple restriction fragments in a single polyacrylamide gel, and thus are potentially useful for population genetic studies (Vos *et al*. 1995). The AFLP technique involves restriction digestion of genomic DNA with a rare cutter (e.g. the six-base recognition restriction enzyme *Eco*RI), and a frequent cutter (e.g. the four-base recognition restriction enzyme *Mse*I). Following adapter ligations, restriction fragments from a total digest of genomic DNA are selectively amplified, and separated by gel electrophoresis. Thus, AFLP polymorphisms identify variations in the restriction site or the primer-extension site, or insertions/deletions in the body of the DNA fragment. Some AFLP loci may segregate as codominant markers, but they have to be analysed as dominant markers because the inheritance patterns of the amplified fragments are very difficult to resolve when multiple loci are genotyped in a single, gel electrophoresis. Therefore, in order to estimate population genetic diversity and differentiation, one must make the following assumptions (Apostol *et al*. 1996). First, AFLP fragments segregate according to Mendelian expectations. Second, amplified fragments of the same size (dominant alleles) are identical in state among and within populations. Third, unamplified fragments (recessive alleles) of a locus are identical in state among and within populations. Finally, genotypes at all AFLP loci are in Hardy–Weinberg equilibrium (HWE).

The assumption that amplified fragments segregate in a Mendelian fashion is unknown without preliminary pedigree analysis (Lynch & Milligan 1994). Because the AFLP PCR products are separated by polyacrylamide gel electrophoresis that distinguishes DNA fragments of 1-bp size difference, the possibility that products of different loci have the same molecular weight, and thus comigrate to the same position on a gel, is probably very small. Therefore, dominant alleles of the same molecular size are probably identical in state among and within populations. However, whether dominant alleles of the same molecular size are identical by descent can only be resolved by sequence analysis, but this is beyond the scope of the present study. The assumption that genotypes at all AFLP loci are in HWE is untestable using only AFLP markers. The assumption that all recessive AFLP alleles at a given locus are identical in state within and between natural populations is unlikely to be true, given that many different sequence polymorphisms can result in the same phenotype: absence of amplification.

Restriction fragment length polymorphism (RFLP) markers are presumably neutral, highly polymorphic and segregate as codominant markers. Thus, the HWE assumption, critical for analysing dominant DNA marker data, can be tested by genotyping the same individuals and the same populations using both AFLP and RFLP markers. RFLP markers are relatively short DNA fragments generated by incubating genomic DNA samples with restriction endonucleases. These enzymes cleave the DNA at specific nucleotide recognition sites that are palindromic. The resulting restriction fragments are visualized after electrophoretic size fractionization through agarose gels and hybridization to a radiolabelled DNA sequence. A genetic linkage map, largely based on cDNA sequences, has been constructed for *Aedes aegypti* (Severson *et al*. 1993). Thus, mosquito population genetic variability can be examined for many loci that cover the whole genome.

The aim of this study was to use natural populations of the yellow fever mosquito, *A. aegypti*, to test the HWE assumption required for population genetic analysis with dominant molecular markers. *A. aegypti* is an important vector of yellow fever and dengue fever viruses in many tropical countries, including Trinidad and Tobago (West Indies). Control efforts for *A. aegypti* have focused primarily on habitat reduction and chemical treatment, which is based on the destruction of breeding sites and the use of insecticides, including DDT in the 1950s and several organophosphates (OPs) since the 1960s. These control measurements not only cause periodic reductions in mosquito population size, which may have genome-wide consequences on genetic diversity (Kreitman & Akashi 1995), but also lead to increased population resistance to insecticides (Gilkes *et al*. 1956; Rawlins & Wan 1995). Selection by insecticides may reduce population genetic diversity at the loci conferring insecticide resistance and other surrounding loci through the genetic hitchhiking effect (Yan *et al*. 1998). Because *A. aegypti* populations in Trinidad are subject to systematic and potentially severe disturbance, dominant markers may not have the power to detect the underlying genetic changes. Dominant markers can also increase the sampling variance and lead to a greater bias in parameter estimation as a consequence of lack of complete genotypic information. Unbiased estimates of genetic variability within and between populations are critical for understanding the evolutionary history of mosquito populations and disease epidemiology.

The issues addressed in the present study are also important for population genetic analysis using other dominant molecular markers. The random amplified polymorphic DNA (RAPD)–PCR technique has been used for various organisms (e.g. Haig *et al*. 1994; Peakall *et al*. 1995; Yeh *et al*. 1995), including *A. aegypti* mosquitoes (Apostol *et al*. 1996). It involves amplification of random segments of genomic DNA using a single, randomly constructed nucleotide sequence as the primer. The PCR product can be separated by size on a standard agarose gel and visualized by ethidium bromide staining. The technique is fast, easy to perform, requires no prior sequence information and reveals large numbers of polymorphisms. The AFLP and the RAPD–PCR techniques share several features. Both are PCR based and require no prior sequence

information, products of multiple loci appear on the same gel and the amplified fragments show simple Mendelian dominance in segregating populations. Therefore, the assumptions required for analysing the AFLP genotypic data are also applicable to RAPD data.

## Materials and methods

### *Collection of samples*

The mosquito samples used in this study were a subset of samples that were previously studied (Yan *et al*. 1998). Three geographically distinct villages from Trinidad were sampled in April 1995 (Fig. 1). For each village, 100 ovitraps were distributed and about one-half of the village's residential area was covered (≈ two traps every five houses). Each ovitrap consisted of a black plastic container (height 12 cm, length 6 cm, width 5 cm, and volume 400 mL) with ≈ 250 mL of water, into which a 12.5 × 2.5 cm hardboard paddle was placed in an upright manner. Female *Aedes aegypti* mosquitoes will readily oviposit on the paddle, near the water interface. After 2–3 days, the hardboard paddles were removed and transported to the laboratory. The eggs from the paddles collected in one area were hatched in one container and reared into adults. All adults were identified as *A. aegypti* by microscopic examination; female adults were frozen for subsequent DNA analysis. Overall, 98% of hardboard paddles contained *A. aegypti* eggs. The average number of eggs was 14.5 (SE = 1.9; range, 1 to 120 per ovitrap; median, 8.0). Previous studies with *A. aegypti* in Puerto Rico estimated that the mean number of families represented per ovitrap was 4.7 (Apostol *et al*. 1994). That is, several females oviposited in the same container during the sampling period. Therefore, the likelihood was small that siblings within a subpopulation were genotyped in the subsequent analysis.

### *AFLP and primer selection*

DNA was extracted from individual mosquitoes as described in Severson *et al*. (1993), and resuspended in 12 μL of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Ten per cent of the total genomic DNA from single mosquitoes

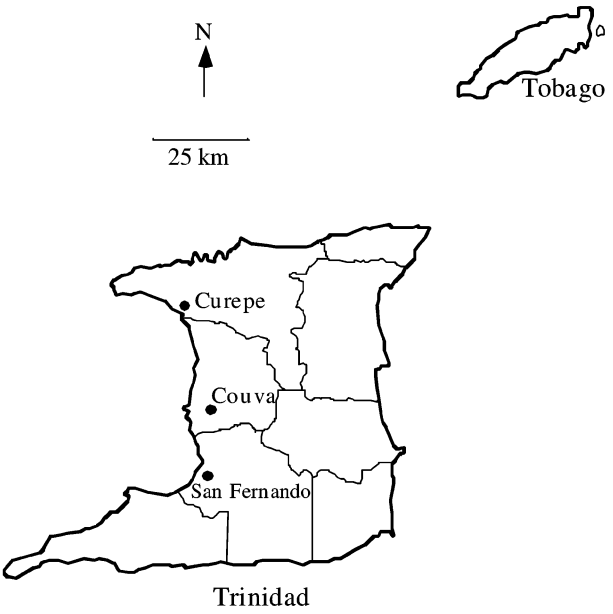


**Fig. 1** Map of Trinidad. Three samples were collected from Trinidad: Curepe (10°38.62′N; 61°24.23′W), Couva (10°26.12′N; 61°28.19′W) and San Fernando (10°18.11′N; 61°28.21′W).

(≈ 100 ng) was used for AFLP analysis, and the remaining DNA (≈ 900 ng) for RFLP analysis. AFLP analysis was performed as recommended by the manufacturer (Perkin-Elmer). Briefly, AFLP involves restriction digestion, adapter ligation, PCR amplification and gel electrophoresis. The genomic DNA was double digested with *Eco*RI and *Mse*I. The DNA fragments were ligated with *Eco*RI and *Mse*I adaptors, generating template DNA for PCR amplification. Two primers used for PCR amplification were designed, based on the adaptor sequences and restriction site sequences. Selective nucleotide sequences were added to the 3′ end of each primer. The PCR amplification was conducted in two steps: preselective amplification and selective amplification. The preselective amplification used a single nucleotide as the selective sequence in the primer design, and the primer sequences were 5′-GACTGCGTACCAATTC + A-3′ (*Eco*RI primer), and 5′-GATGAGTCCTGAGTAA + C-3′ (*Mse*I primer). For selective amplification, a total of five primer combinations were used and primer sequences are presented in Table 1. The *Eco*RI and *Mse*I primers in the selective

**Table 1** Primer sequences used in amplified fragment length (AFLP) selective amplification

| Primer combination | *Eco*RI primer (5′–3′) | *Mse*I primer (5′–3′) | No. of loci scored |
|---|---|---|---|
| 1 | GACTGCGTACCAATTC\ACA | GATGAGTCCTGAGTAA\CAC | 41 |
| 2 | GACTGCGTACCAATTC\ACA | GATGAGTCCTGAGTAA\CAT | 25 |
| 3 | GACTGCGTACCAATTC\ACG | GATGAGTCCTGAGTAA\CTA | 29 |
| 4 | GACTGCGTACCAATTC\AGG | GATGAGTCCTGAGTAA\CTG | 18 |
| 5 | GACTGCGTACCAATTC\AGG | GATGAGTCCTGAGTAA\CTT | 24 |
| Total | | | 137 |

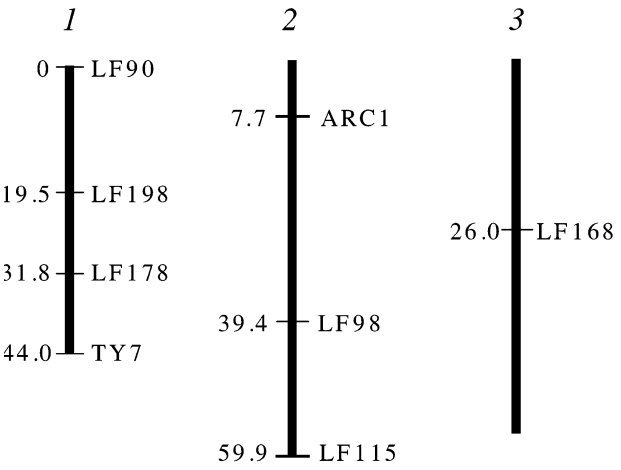


**Fig. 2** Partial linkage map of *Aedes aegypti* ($2N = 6$) to show the relative map positions of the eight restriction fragment length polymorphism (RFLP) loci used in the study. Chromosome numbers are in italics. Numbers on the left side of each chromosome represent cumulative recombination distances in Kosambi centimorgans (cM). All markers are random cDNA.

amplification used three nucleotides; therefore each primer combination amplified different subsets of all the fragments in the total digest.

PCR products were then mixed with 20 µL of sequencing loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% Bromophenol Blue). The mixtures were heated for 3 min at 90 °C, and then quickly cooled in ice. Two microlitres of each sample was electrophoresed in a 5% polyacrylamide sequencing gel at 60 W for ≈ 2.5 h. After electrophoresis, the gels were fixed in 10% acetic acid and dried, and exposed to a Molecular Dynamics phosphoimager screen. The AFLP banding patterns were visualized using the Molecular Dynamics phosphoimager analysis system. The bands were scored using Keygene software.

Using the AFLP method, we genotyped a total of 116 mosquitoes for the three Trinidad populations (41 for Curepe, 37 for Couva and 38 for San Fernando).

### *RFLP and probe selection*

The same individuals used for AFLP analysis were also genotyped using RFLP markers. Genomic DNA digestion with *Eco*RI, Southern blotting and hybridization were performed as previously described (Severson *et al*. 1993). Eight mapped cDNA sequences were selected as RFLP probes, and their mapping position is shown in Fig. 2. All clones used were random cDNA clones.

### *Data analysis*

*DNA polymorphism and HWE tests* DNA polymorphisms may be measured as the proportion of polymorphic loci, number of alleles and heterozygosity. For RFLP data, conformance with HWE was tested using the probability test for each locus and each population, using GENEPOP (Raymond & Rousset 1995). If distortion from HWE was found for a locus in a subpopulation, we further tested whether it resulted from deficient or excessive heterozygosity, using Wright's $F_{IS}$ statistics and the method of Raymond & Rousset (1995). $F_{IS}$ is defined as:

$$[1 - (\text{observed heterozygosity}/\text{expected heterozygosity) from HWE}] \quad (1)$$

The significance of the average $F_{IS}$ over all loci was tested using the method of Robertson & Hill (1984). The among-population variation in heterozygosity was analysed following the method of Weir (1990). Briefly, individuals were scored at each locus as heterozygous or homozygous, using 1 or 0, respectively. These data were analysed with analysis of variance (ANOVA) using populations, individuals nested within populations, loci and interactions of loci and populations as factors (Weir 1990). All factors were treated as random effects, except the populations. The denominators for each factor in the model were synthesized by the SAS, JMP statistical program from linear combinations of the appropriate mean squares and have the same expectation as the effect to be tested under the null hypothesis. The degrees of freedom were adjusted for imbalance in the experiment by Satterthwaite's method (see SAS 1994).

For AFLP data, only polymorphic loci were scored. Because AFLP loci segregate as dominant markers, to estimate allele frequency and population heterozygosity, the HWE assumption was made. The allele frequency of the null recessive allele ($q$) at locus $i$ was estimated using the method of Lynch & Milligan (1994):

$$q(i) = \sqrt{x(i)}\left(1 - \frac{Var[x(i)]}{8[x(i)]^2}\right)^{-1} \quad (2)$$

where $x(i)$ is the frequency of null recessive homozygotes at locus $i$, and $Var[x(i)] = x(i)[1 - x(i)]/N$, where $N$ is the sample size. The heterozygosity at locus $i$, was:

$$H(i) = 2q(i)\,[1 - q(i)] + 2\,Var[q(i)] \quad (3)$$

where $Var[q(i)] = [1 - x(i)]/(4N)$. Variation in heterozygosity among populations and loci was analysed using ANOVA with the populations, primers and loci nested with the primers as factors. Primers and loci were treated as random effects.

### *Linkage disequilibrium between RFLP loci*

Because heterozygotes cannot be distinguished from the dominant homozygotes for AFLP loci, and the observed

heterozygosity is a critical parameter for estimation of linkage disequilibrium, linkage disequilibrium between pairs of AFLP loci was therefore not calculated. However, linkage disequilibrium was tested for all pairs of RFLP loci to evaluate the independence of genotypes at one locus from other loci. The correlation coefficient between alleles of different loci was used to express the magnitude of linkage disequilibrium, and was computed based on the procedure of Weir & Cockerham (1984), using the LINKDOS program described by Garnier-Gere & Dillmann (1992) and based on Black & Krafsur (1985). A $\chi^2$-statistic can be used to test the significance of linkage disequilibrium, but it can be seriously inflated by the presence of rare alleles. We used the Fisher exact test, which is available in GENEPOP (Raymond & Rousset 1995). The test uses the Markov-chain method based on the contingency tables for all pairs of loci in each population. The number of possible contingency tables for each population is $n(n-1)/2$, where $n$ is the number of loci examined. The statistical power of the Fisher exact test to detect significant linkage disequilibrium depends on the frequencies of alleles and genotypes, magnitude of disequilibrium and sample size (Fu & Arnold 1992).

Genetic drift and epistatic selection are two major genetic mechanisms that create and maintain linkage disequilibrium in natural populations (Ohta & Kimura 1969; Lewinton 1974). Genetic drift causes random fluctuation of gamete frequencies in a population, and thus increases the variance of the linkage disequilibrium. Epistatic selection maintains favourable combinations of alleles and reduces the variance of the linkage disequilibrium because favourable combinations of alleles should appear consistently among populations. Theoretical considerations of Ohta (1982), based on partitioning total variance components ($D_{IT}$) of disequilibrium into within-population ($D_{ST}$ and $D_{ST}$) and between-population ($D_{ST}$ and $D_{ST}$) components provided a statistical approach to discriminate the two mechanisms. Comparisons between $D_{IS}$ and $D_{ST}$, $D_{IS}$ and $D_{ST}$ would allow discrimination of the two hypotheses (Ohta 1982). We used the LINKDOS program described by Garnier-Gere & Dillman (1992), and available in GENEPOP, to analyse variance of linkage disequilibrium.

### *Population genetic structure and gene flow*

Population genetic structure was examined using Wright's *F*-statistics. For RFLP data, $F_{ST}$ was calculated based on the procedure of Weir & Cockerham (1984) and using FSTAT (Goudet 1995). Standard deviations (SD) of *F*-statistics were obtained for each locus by a jackknife procedure over the alleles, and were used to test the significance of the *F*-statistics. For AFLP data, $F_{ST}$ was estimated from Wright (1951), without information on heterozygosity frequencies:

$$F_{ST} = \frac{Var[q(i)]}{a(i)[1-q(i)]} \quad (4)$$

where $Var[q(i)]$ is the variance in the frequency of an AFLP allele among subpopulations and $q(i)$ is the weighted average frequency among all subpopulations. Wright's $F_{ST}$ and a less biased estimate of $F_{ST}$ for dominant markers described by Lynch & Milligan (1994) was calculated using RAPDFST, written by W. C. Black IV (Apostol *et al*. 1996), and its significance was tested using a $\chi^2$-contingency test. Both Wright's $F_{ST}$ and Lynch & Milligan's $F_{ST}$ were reported.

Gene flow ($Nm$) was estimated from the $F_{ST}$ statistics of each locus using the relationship where $N$ is the effective population size of a deme and $m$ is the rate of gene flow (Wright 1951). This equation assumes the infinite-island model of population structure and gene flow. Few populations probably conform to this assumption, but it provides a useful approximation of the relative magnitude of gene flow.

## Results

### *RFLP analysis*

*DNA polymorphisms and HWE tests* All eight cDNA markers examined in this study were polymorphic. Figure 3 shows a representative RFLP autoradiograph using the cDNA clone LF98 as a probe. A total of 45 alleles were identified, and 37 alleles (82.2%) were common to all four populations. The average number of alleles per locus varied from 4.6 to 5.1 among the populations (Table 2).

The observed heterozygosity varied significantly among loci ($P < 0.001$). In particular, the heterozygosity at the LF90 locus was significantly lower than other loci examined (Table 2). Heterozygosity was positively correlated with the number of alleles at a locus ($r^2 = 0.57$, d.f. = 23, $P < 0.001$). Average heterozygosity, however, did not vary among the three populations ($P > 0.05$).

The genotype frequencies of several loci did not conform to HWE (Table 2). Overall, one-third of the loci tested exhibited deviation from HWE. For the Curepe and Couva populations, significant positive $F_{IS}$ statistics were observed for all loci not in HWE, suggesting a deficiency of heterozygosity. However, both positive and negative $F_{IS}$ were found for the San Fernando population.

### *Linkage disequilibrium*

Genotypic linkage disequilibrium among pairs of polymorphic loci was evaluated for each of the three populations. The genotypic data included a total of eight loci for each of the three populations. The analysis tested a total of 84 pairs of loci [$(8 \times 7) \div 2 = 28$] for each population;

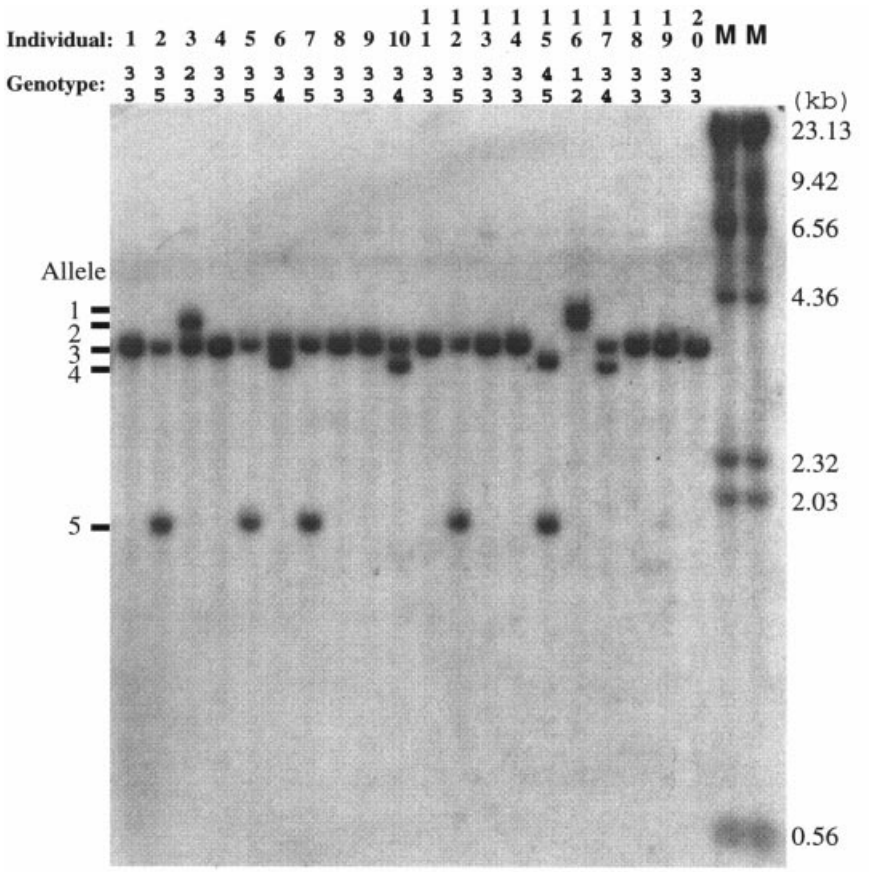


**Fig. 3** A representative Southern blot autoradiograph of *Aedes aegypti*. Each lane represents a single female mosquito collected in Curepe, Trinidad. The mosquito genomic DNA was digested with *Eco*RI and probed with a cDNA clone, LF98. Mosquito identification number and genotype is shown. M indicates DNA size marker. The molecular sizes of alleles are: 3.97 kb (allele 1), 3.83 kb (allele 2), 3.59 kb (allele 3), 3.42 kb (allele 4) and 1.67 kb (allele 5).

**Table 2** RFLP polymorphisms of three *Aedes aegypti* populations from Trinidad measured by observed heterozygosity ($H_O$) and the no. of alleles ($n$), and the estimated heterozygosity at amplified fragment length polymorphism (AFLP) loci

| | Curepe | | | Couva | | | San Fernando | | |
|---|---|---|---|---|---|---|---|---|---|
| Chromosome | $n$ | $H_O$ | $F_{IS}$† | $n$ | $H_O$ | $F_{IS}$ | $n$ | $H_O$ | $F_{IS}$ |
| RFLP markers | | | | | | | | | |
| Locus | | | | | | | | | |
| LF90 | 2 | 0.073 | −0.026 | 2 | 0.028 | 0.000 | 4 | 0.184 | 0.065 |
| LF198 | 5 | 0.512 | 0.264*** | 4 | 0.514 | 0.105* | 5 | 0.735 | −0.007 |
| LF178 | 6 | 0.656 | 0.186* | 6 | 0.771 | 0.010 | 7 | 0.941 | −0.179* |
| TY7 | 4 | 0.878 | −0.198 | 4 | 0.448 | 0.093 | 4 | 0.459 | 0.089 |
| Average over chromosome 1 | 4.3 | 0.530 | | 4.0 | 0.440 | | 5 | 0.579 | |
| Locus | | | | | | | | | |
| ARC1 | 5 | 0.780 | −0.082 | 5 | 0.472 | 0.229** | 5 | 0.711 | −0.048 |
| LF98 | 6 | 0.659 | 0.053 | 5 | 0.622 | −0.022 | 6 | 0.737 | −0.136* |
| LF115 | 4 | 0.293 | −0.031 | 4 | 0.400 | 0.075 | 4 | 0.432 | 0.028 |
| Average over chromosome 2 | 5.0 | 0.577 | | 4.7 | 0.498 | | 5.0 | | 0.627 |
| Locus | | | | | | | | | |
| LF168 | 6 | 0.756 | 0.011 | 7 | 0.514 | 0.214*** | 6 | 0.632 | 0.160* |
| Average over all eight loci | 4.8 | 0.576 | | 4.6 | 0.471 | | 5.1 | 0.604 | |
| AFLP markers | | | | | | | | | |
| Average over 137 loci | | 0.392 | | | 0.390 | | | 0.394 | |
| (SD) | | (0.120) | | | (0.115) | | | (0.115) | |

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

†Significant $F_{IS}$ indicates distortion from Hardy–Weinberg equilibrium (HWE). Positive $F_{IS}$ indicates a heterozygosity deficit from the HWE expectation, negative $F_{IS}$ indicates an excess of heterozygosity.

**Table 3** Correlation coefficients expressing linkage disequilibrium between alleles of different pairs of restriction fragment length polymorphism (RFLP) loci for *Aeges aegypti* populations from Trinidad

| | LF90 | LF198 | LF178 | TY7 | ARC1 | LF98 | LF115 |
|---|---|---|---|---|---|---|---|
| LF198 | 0.235 | | | | | | |
| | 0.127 | | | | | | |
| | 0.176 | | | | | | |
| LF178 | 0.150 | 0.180** | | | | | |
| | 0.149 | 0.183 | | | | | |
| | 0.155 | 0.218* | | | | | |
| TY7 | 0.182*** | 0.133* | 0.185 | | | | |
| | 0.307 | 0.201 | 0.226 | | | | |
| | 0.218 | 0.243*** | 0.237** | | | | |
| ARC1 | 0.084 | 0.203*** | 0.175*** | 0.129 | | | |
| | 0.099 | 0.144 | 0.165 | 0.192 | | | |
| | 0.197* | 0.190 | 0.181 | 0.092 | | | |
| LF98 | 0.100 | 0.165 | 0.148 | 0.159 | 0.194 | | |
| | 0.111 | 0.162 | 0.233*** | 0.128 | 0.167 | | |
| | 0.083 | 0.156 | 0.195 | 0.134 | 0.198* | | |
| LF115 | 0.117 | 0.129 | 0.141 | 0.107 | 0.124 | 0.159 | |
| | 0.096 | 0.240* | 0.238 | 0.148 | 0.157 | 0.188 | |
| | 0.131 | 0.185 | 0.136 | 0.159 | 0.191 | 0.100 | |
| LF168 | 0.151 | 0.144 | 0.140 | 0.087 | 0.124 | 0.090 | 0.170 |
| | 0.040 | 0.099 | 0.158** | 0.179 | 0.135*** | 0.134 | 0.187 |
| | 0.115* | 0.216* | 0.196 | 0.156 | 0.158 | 0.149 | 0.148 |

Note: upper value refers to the Curepe population, middle value to Couva, and lower value to San Fernando populations, respectively. The map distance (Kosambi centimorgans) between loci is shown in Fig. 2.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

27 pairs for loci within a chromosome and 57 pairs between chromosomes. Generally, significant linkage disequilibrium would not be expected in natural populations, particularly for pairs of loci with fairly large recombination distance (e.g. the smallest recombination distance among all pairs of loci studied is 12.3 centimorgans (cM) for LF198–LF178; see Fig. 2). However, a large number of pairs of loci were in linkage disequilibrium (Table 3). Overall, 19% (16 out of 84) of loci pairs were in linkage disequilibrium: seven pairs of loci within the same chromosome (six for chromosome 1 and one for chromosome 2) and nine pairs of loci between different chromosomes (five between chromosomes 1 and 2, three between chromosomes 1 and 3, and one between chromosomes 2 and 3). Analysis of Ohta's *D*-statistics found $D_{IS} < D_{ST}$ and $D_{IS} > D_{ST}$ for 75% pairs of loci, and $D_{IS} > D_{ST}$ for 25% of pairs (data not shown). This result suggests that genetic drift was the primary mechanism leading to linkage disequilibrium, although nonsystematic selection cannot be excluded (Ohta 1982).

## *Population genetic structure*

Analysis of *F*-statistics for the three Trinidad population found small, but statistically significant, $F_{ST}$ estimates for six loci (Table 4). The $F_{ST}$ estimates at two loci (LF115 and LF168) were not statistically significant. $F_{ST}$ estimates showed a sixfold difference among loci with significant $F_{ST}$ values; the average $F_{ST}$ over all loci was 0.063. Assuming that the populations are at an equilibrium between migration and random drift, the average number of migrants exchanged per generation (*Nm*) among the three populations, based on the $F_{ST}$ estimate, was 3.7 migrants per generation (95% confidence interval (CI): 2.9–4.9).

## *AFLP analysis*

*DNA polymorphisms* Although large numbers of restriction fragments were generated from individual mosquitoes for each primer combination using the AFLP technique (Fig. 4), only fragments that were polymorphic and unambiguously scored were used for the data analysis, regardless of their frequencies within a population. A total of 137 loci was scored for the five primer combinations. Heterozygosity was estimated by assuming that allele frequencies of all loci were in HWE. The average heterozygosity was similar for the three populations ($P > 0.05$). The estimated heterozygosity varied significantly among the AFLP loci ($P < 0.001$); however, the average heterozygosity did not vary among the primers ($P > 0.05$).

**Table 4** $F_{ST}$ statistics of three *Aedes aegypti* populations from Trinidad

| Chromosome | Locus | $F_{ST}$ (SD)* |
|---|---|---|
| RFLP markers | | |
| 1 | LF90 | 0.033 (0.017)* |
| | LF198 | 0.154 (0.058)*** |
| | LF178 | 0.030 (0.031)*** |
| | TY7 | 0.123 (0.085)*** |
| 2 | ARC1 | 0.107 (0.086)*** |
| | LF98 | 0.025 (0.026)** |
| | LF115 | 0.004 (0.017) |
| 3 | LF168 | 0.005 (0.011) |
| Summary over all eight loci | | 0.063 (0.022)*** |
| AFLP markers (average over 137 loci) | | |
| Average Wright's $F_{ST}$ | | 0.033 (0.036)*** |
| Average Lynch & Mulligan's $F_{ST}$ | | 0.037 (0.073)*** |

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

### *Population genetic structure*

$F_{ST}$ was estimated for each of the 137 AFLP loci. Wright's $F_{ST}$ estimates ranged from 0 to 0.194, and the median $F_{ST}$ value was 0.022 (Fig. 5). The average $F_{ST}$ over all AFLP loci was 0.033. The Lynch & Milligan's $F_{ST}$ estimate was similar to the Wright's $F_{ST}$ estimate (mean = 0.037; range, –0.036–0.294; median, 0.037). Using Wright's $F_{ST}$ and the formula of Wright (1951), the gene flow (*Nm*) was estimated at 7.2 migrants per generation (95% CI: 6.2–9.0).

## Discussion

### *Comparison of genetic markers*

The population genetic structure of *Aedes aegypti* has been examined with several genetic markers, including isozyme, RAPD and RFLP markers. Isozyme markers were primarily used in the 1970s and 1980s to examine the mosquito genetic structure on both microgeographical (Tabachnick & Powell 1978; Tabachnick 1982) and macrogeographical (Tabachnick & Powell 1979; Powell *et al*. 1980; Wallis *et al*. 1983, 1984; Tabachnick 1991) scales. Many isozyme markers are monomorphic, and thus few informative loci can be studied. Also, protein electrophoresis usually requires fresh mosquitoes, which may be difficult to obtain for field populations. DNA-based molecular markers, particularly PCR-based DNA fingerprinting and genotyping techniques, can be used to examine many loci and do not require fresh materials, and thus have become commonly used for population genetic studies.

PCR-based genotyping methods can be broadly classified into two categories in terms of allele dominance: dominant (e.g. RAPD–PCR and AFLP) and codominant (e.g. microsatellite) markers. RAPD–PCR has been used to study genetic variation in various organisms (e.g. Kambhampati *et al*. 1992; Apostol *et al*. 1994, 1996; Haig *et al*. 1994; Peakall *et al*. 1995; Yeh *et al*. 1995). Because agarose-gel electrophoresis does not have the ability to resolve DNA fragments of similar sizes, polyacrylamide gels have been used for electrophoresis of RAPD–PCR products (e.g. Antolin *et al*. 1996; Mutebi *et al*. 1997). This technique is more sensitive for identification of a greater number of markers than the standard analysis of RAPD–PCR products on agarose gels. AFLP is a recently developed DNA fingerprinting technique that has been used mostly for plant genetic research (e.g. Becker *et al*. 1995; Meksem *et al*. 1995; Lu *et al*. 1996). The AFLP and RAPD–PCR techniques share some similarities. When AFLPs or RAPDs are electrophoresed on polyacrylamide gels, amplified DNA fragments of 1-bp size differences can be detected. Thus, polyacrylamide gel electrophoresis can minimize the problem that the PCR products of different loci with similar molecular size will comigrate. RAPD–PCR often amplifies repetitive sequences; the AFLP fragments include both single- or low-copy sequences and repetitive sequences, depending on the complexity of the genome. The *A. aegypti* genome consists of single- or low-copy DNA sequences and very abundant repetitive DNA with short-period interspersion (Black & Rai 1988). It is unknown what proportion of polymorphic fragments examined in this study are repetitive sequences. Nonetheless, the AFLP technique has several advantages over the RAPD–PCR technique. First, several hundred loci can be examined in one AFLP polyacrylamide gel. Second, AFLP markers examine the variation in the length of restriction fragments. AFLP has been shown to be a reliable and reproducible marker assay (Lin & Kuo 1995; Vos *et al*. 1995), while reproducibility is sometimes a problem for RAPD–PCR.

Like RAPDs, AFLPs must be analysed as dominant

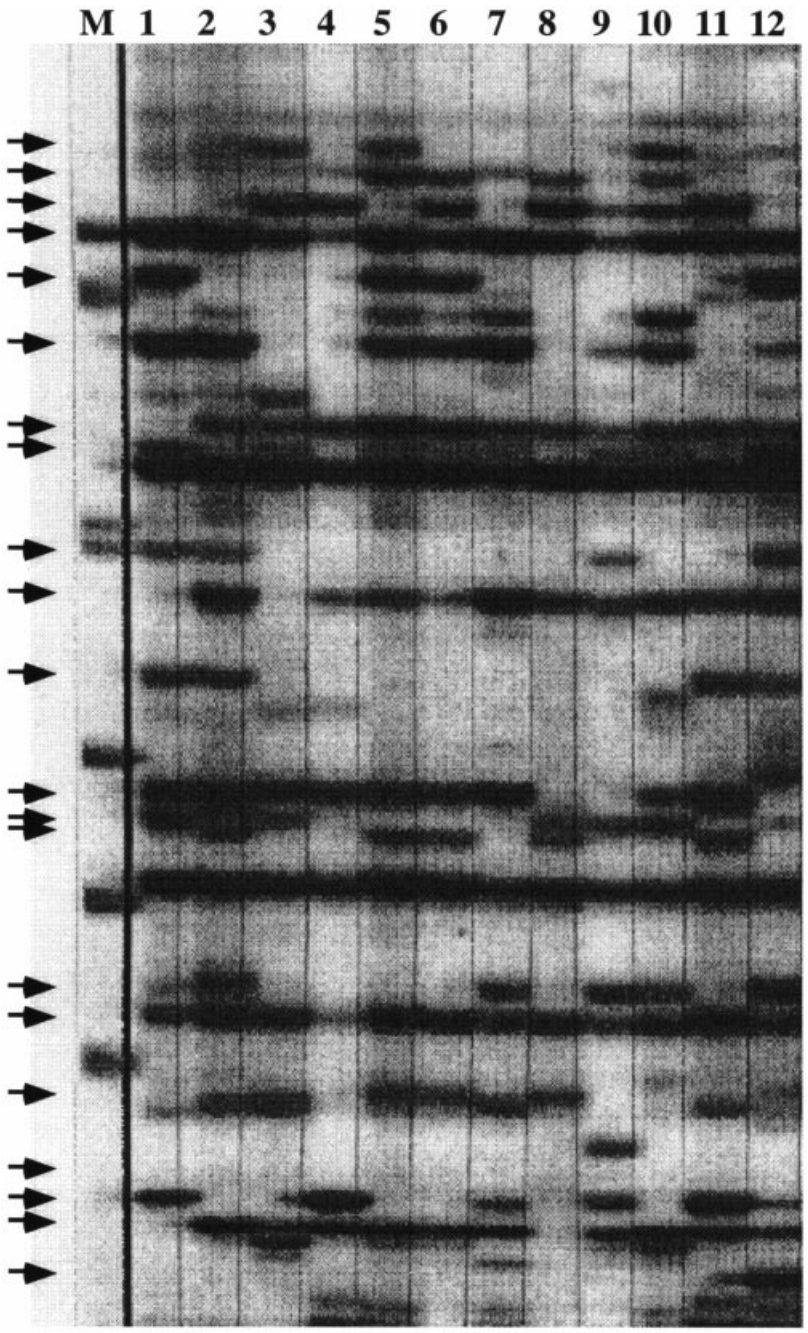


**Fig. 4** A representative amplified fragment length polymorphism (AFLP) autoradiograph for *Aedes aegypti*. The fragments shown in the figure were amplified using the primer combination no. 1 of Table 1. Only a proportion of fragments is shown. Arrows on the left of the figure represent the fragments being scored. Each lane represents a single mosquito. M indicates DNA size marker.

markers because of multilocus amplification. There could be a 'nested' codominance in the amplification patterns (e.g. indels or variations in the number of simple sequence repeats (SSRs)); however, the codomiance loci are very difficult to identify with multilocus fingerprinting techniques in the pedigree analysis. Therefore, amplified fragments have to be analysed as dominant alleles, and assumptions such as HWE must be made in order to calculate allele frequencies. Often the HWE assumptions are untestable and therefore the resulting allele frequency estimates and inferences of population structure, based on dominant markers, may be inaccurate. RFLP markers are codominant, and can be used to test this assumption. The present study also examined whether RFLP and AFLP markers lead to similar estimations of population genetic structure parameters. Because both AFLP and RFLP markers examine the polymorphism of restriction fragments in the mosquito genome, comparisons between the two markers were more appropriate than between RAPD and RFLP. Because all individuals used for AFLP

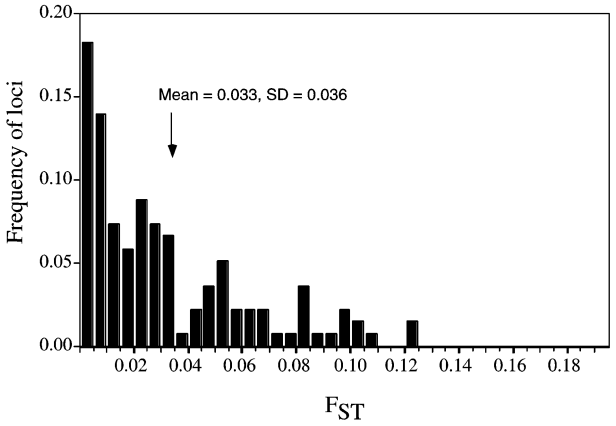


**Fig. 5** Distribution of $F_{ST}$ values for the three *Aedes aegypti* populations of Trinidad at 137 amplified fragment length polymorphism (AFLP) loci.

analysis were also subjected to RFLP analysis, we minimized the possibility that observed differences between the two markers, in estimation of DNA polymorphisms and population structure parameters, were caused by the artefacts of bias resulting from sampling different individuals or from different sample sizes.

### *Comparison of genetic diversity*

The observed heterozygosity ($H_O$) with RFLP markers ($H_O$ ranged from 0.47 to 0.60) was significantly higher than the expected heterozygosity with AFLP markers ($H_E = 0.39$; *t*-test, $P < 0.001$ for the three populations). Such a phenomenon is, at least partly, caused by the fact that the RFLP loci have a greater number of alleles (an average of five) than the AFLP loci (two alleles at any locus). RFLP and AFLP markers revealed much higher heterozygosities than 11 isozyme loci in an earlier survey in Trinidad ($H_O = 0.08$) or in nine Caribbean countries (average $H_O = 0.12$; Wallis *et al*. 1984). It is unknown whether observed polymorphisms in AFLPs and RFLPs result from polymorphic restriction sites or from indels. The expected heterizygosities with AFLP markers in the three Trinidad populations were similar to the Puerto Rico populations examined with RAPD markers ($H_E = 0.35$; Apostol *et al*. 1996), suggesting that the genetic diversity, defined by heterozygosity of the Trinidad populations may be similar to the Puerto Rico populations. Wallis *et al*. (1984) found that *A. aegypti* in larger islands (e.g. Puerto Rico) showed significantly higher heterozygosity at isozyme loci than *A. aegypti* from smaller islands (e.g. Trinidad), and thus the effective population sizes were larger in large islands. However, comparisons of heterozygosity at 18 RFLP loci between the Trinidad and Tobago populations did not support their conclusion: the observed heterozygosity in these two islands was not significantly different, although Tobago is a much smaller island than Trinidad (Yan *et al*. 1998).

We observed that heterozygosity varied substantially among the RFLP loci. In particular, the LF90 locus exhibited much lower heterozygosity than other loci in the genome for the three populations studied. Yan *et al*. (1998) suggested that the polymorphism pattern of the LF90 locus probably reflects the result of a hitchhiking effect associated with periodic insecticide selection. Because all other loci showed relatively high RFLP heterozygosity, they either were not under the direct selection of insecticides, or genetic hitchhiking effects caused by insecticide selection did not occur at these loci.

High RFLP heterozygosities for most loci suggest that the mosquito populations have not experienced population bottlenecks despite intensive insecticide applications. A population bottleneck reduces gene diversity for all loci in the genome, and recovery of gene diversity by mutation could take millions of generations (Nei *et al*. 1975). The observed high heterozygosity suggests that the effective population size of *A. aegypti* in Trinidad has never been small. Heterogeneous habitats may protect some field populations from insecticide exposure. Alternatively, genetic diversity may be introduced and maintained by large gene flow among populations through natural dispersal (Reiter *et al*. 1995) and human-assisted migration via transportation of *A. aegypti* eggs (Chadee 1990).

Because mosquito populations have been repeatedly subjected to insecticide treatment, suitable and easily identifiable niches have often been vacated and soon recolonized by mosquitoes. Thus, the process of population extinction and recolonization creates opportunities for genetic drift. Genetic drift could lead to extensive linkage disequilibrium. Ohta's *D*-statistics suggest that the observed linkage disequilibrium was largely caused by genetic drift.

It is worthwhile to note that some RFLP loci were not in HWE. Departure from HWE could be caused by sampling error, inbreeding and selection. Also, it could result from the RFLP technique itself, for example, null alleles or miscoding restriction fragments. Sample errors could result from small sample size or siblings used in a study. The small sample size used in the study was not an important factor causing departure from HWE because our previous study, using more than 150 individuals and 18 loci per population, found that 37% of loci were not in HWE (Yan *et al*. 1998). If siblings were sampled or population inbreeding had occurred, one would expect a genome-wide departure from HWE, and positive $F_{IS}$ for all loci. Our data found both negative and positive $F_{IS}$ estimates, suggesting that neither inbreeding nor sampling effects were plausible mechanisms. We argue that the null alleles or fragment miscodings are unlikely explanations. Null alleles could arize when DNA fragments

that would hybridize to the cDNA probe are small (normally less than ≈ 250 bp for our technique) and run off the gel. Therefore, subsequent Southern blot analysis would not detect these fragments. In order to detect very small fragments with Southern blot analysis, one would need restriction sites within the clone and other restriction sites very close to the ends of the probe sequence. In our mapping experiments, we screened ≈ 10 strains of *A. aegypti* with more than 70 cDNA probes. The resulting fragments range from 50 bp to upwards of 20 kb, but rarely less than 500 bp (Severson *et al*. 1993). The large Southern fragment size may be related to the size of our probes (400–2000 bp in length). Also, the restriction enzyme used in these studies (*Eco*RI) is a six-base recognition cutter, which should cut at restriction sites present, on average, at every 4 kb of genomic DNA. Therefore, the null allele hypothesis is possible, but unlikely. Fragment miscoding could happen when two different fragments that are homologous or partially homologous to the cDNA probe have the same molecular size, and thus comigrate during gel electrophoresis. In this case, heterozygotes will be miscoded as homozygotes. The pedigree analyses with these cDNA probes in the previous mapping experiments did not find evidence for comigrating fragments with all cDNA clones mapped (Severson *et al*. 1993).

A more plausible explanation for departure from HWE is selection. In Trinidad, an important vector-control method is the use of OP insecticides, i.e. temephos for larval control and malathion as adulticide. Frequent insecticide treatments not only exert intense selection on the treated population, but also provide an opportunity for untreated adjacent populations to migrate into depopulated habitats. Is departure from HWE unique to the Trinidad populations? Tabachnick & Powell (1978) examined the genetic diversity of *A. aegytpi* populations in coastal Kenya in four isozyme loci, and found that the esterase locus exhibited significant deviation from HWE ($F_{IS}$ ranged from 0.19 to 0.34). They hypothesized that null alleles in the esterase locus caused departure from HWE. Residual spraying with DDT and OP insecticides for mosquito-borne disease control was a common practice in coastal Kenya. Increased esterase activities are responsible for resistance to OP insecticides (Mourya *et al*. 1993). It is possible that departure from HWE in the African populations was caused by insecticide selection. Although more data are needed to support the selection hypothesis, the present study suggests that HWE, the critical assumption for the analysis of AFLP data, may not hold for some loci. Thus, estimation of mosquito population heterozygosity based on dominant markers could be biased. Because of the invalidity of the HWE assumption, estimation of linkage disequilibrium using dominant markers also becomes inaccurate. Therefore, dominant markers cannot completely eliminate the problem of bias, particularly for populations of *A. aegypti* mosquitoes that are frequently disturbed by human activities. Because our study examined the *A. aegypti* populations of Trinidad for only one season, the conclusions should not be generalized into other areas or other populations.

### *Comparison of population genetic-structure parameters*

Using RFLP markers we estimated that the average $F_{ST}$ was 0.063, almost twice the value obtained by the AFLP markers (0.033). AFLP markers led to a low average $F_{ST}$ estimate because a large number of AFLP loci had very small $F_{ST}$ values (see Fig. 5). The $F_{ST}$ estimate from the AFLP markers was probably less accurate. Lack of complete genotypic information owing to AFLP allelic dominance increases the sampling variance and could result in biased estimation of population genetic-structure parameters. Lynch & Milligan (1994) recommended pruning loci with a null phenotype frequency less than $3/N$ ($N$ is the sample size) to obtain less biased estimates. The lowest null phenotype frequency was 0.16 (data not shown), far above $3/N$. Therefore, this procedure was not applicable to our AFLP data. However, the problem of obtaining biased $F_{ST}$ estimates could probably be reduced by increasing the sample size (Lynch & Milligan 1994).

Our $F_{ST}$ estimate for the three Trinidad *A. aegypti* populations was larger than the $F_{ST}$ value found in the six Puerto Rico cities using RAPD markers ($F_{ST} = 0.021$; Apostol *et al*. 1996). Because we used different markers and a different number of samples, and the geographical distances among the three populations in the Trinidad populations are also different from those among the Puerto Rico populations, one cannot conclude that the genetic differentiation of *A. aegypti* populations among the Trinidad cities is larger than among the Puerto Rico cities. Owing to a limited number of sampling sites, we were unable to test whether the genetic structure of *A. aegypti* populations in Trinidad fits into the model of isolation-by-distance (Wright 1943). Wallis *et al*. (1984) showed that the Caribbean island populations exhibited high within-region genetic heterogeneity. This study demonstrated high genetic heterogeneity for *A. aegypti* populations within an island.

The differences in $F_{ST}$ estimates between RAPD, AFLP, RFLP and isozyme markers may be a result of differences in the mutation rate of these loci. The effect of mutation rate on $F_{ST}$ estimates can be examined in Wright's $F_{ST}$ formula:

$$F_{ST} = 1/[1 + 4N_e(m + \mu)] \quad (5)$$

where $N_e$ is the effective population size, $m$ is the migration rate and $\mu$ is the mutation rate. $F_{ST}$ could be seriously

underestimated if the mutation rate of the genetic loci is relatively large. RAPD loci tend to have higher mutation rates than AFLP and RFLP loci, and thus $F_{ST}$ could be underestimated with RAPD markers. On the other hand, the mutation rate of isozyme markers is usually lower than DNA markers and therefore one would expect larger $F_{ST}$ estimates to be obtained with isozyme markers. Although isozyme markers have been extensively used to study population genetic structure of *A. aegypti* (see the review by Tabachnick 1991), $F_{ST}$ statistics were unfortunately not reported in the literature. Also, the previous studies examined *A. aegypti* populations from different geographical areas at a different spatial scale. Comparisons of $F_{ST}$ values between the populations of the present study and previous studies may not be appropriate. Because AFLPs and RFLPs examine the length variation in restriction fragments, and the mutation rates of AFLP and RFLP loci are more or less similar, the differences in the $F_{ST}$ estimates reported in this study should not be entirely caused by mutation-rate differences. The smaller $F_{ST}$ estimate with AFLP markers is probably caused by lack of complete genotypic information.

In summary, this study used AFLP and RFLP markers to examine the effects of dominant and codominant markers on the estimates of population genetic structure parameters for *A. aegypti*. We found that the HWE assumption required for analysing dominant markers did not hold for ≈ one-third of the loci tested. The $F_{ST}$ estimate with the dominant AFLP markers was ≈ one-half the $F_{ST}$ value estimated using the codominant RFLP markers. Although AFLP markers are suitable for high-density genome mapping, their use in population genetics could lead to seriously biased estimates of genetic diversity and inaccurate assessments of genetic structure. Polymorphic codominant RFLP markers produce more accurate estimates and yield more genetic information for *A. aegypti*, a species that has been frequently subjected to human disturbance.

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