Comparisons of Genetic Variability and Genome Structure Among Mosquito Strains Selected for Refractoriness to a Malaria Parasite

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Restriction fragment length polymorphism (RFLP) markers were used to evaluate *Aedes aegypti* genome structure and genetic variability within and between substrains selected for different levels of refractoriness to the malaria parasite, *Plasmodium gallinaceum*. The MOYO-R substrain was previously selected for complete refractoriness and the MOYO-IS substrain for intermediate susceptibility from the Moyo-In-Dry (MOYO) strain by selective inbreeding ($F = 0.5$). Eighteen mapped RFLP markers were used to provide coverage of the mosquito genome. The two substrains showed reduced genetic diversity compared with the MOYO strain, including significant reductions in mean heterozygosity, number of alleles per locus, and proportion of polymorphic loci. Genetic differentiation between the two substrains was statistically significant, as reflected by differences in allele frequencies. Significant pairwise linkage disequilibrium among the RFLP loci was detected in all three strains, most evidently in the MOYO strain. This is surprising because the RFLP loci examined are separated by large map distances, and therefore linkage disequilibrium should decay to zero after many generations of laboratory culture. Our hypothesis to explain this phenomena is that lack of recombination, or low recombination rates in some regions of the *A. aegypti* genome, is a result of chromosome inversions. Finally, we used graphical genotyping, wherein whole genome genotypic information for individual mosquitoes is represented in a simple graphic format, to illustrate genome structure and allelic variation within and among the mosquito strains. Our analysis revealed an apparent chromosomal deletion on chromosome 3 for some individuals in the MOYO strain and MOYO-IS substrain.

Malaria, one of the most important parasitic diseases today, infects about 400 million people worldwide and results in about 2 million deaths per year, primarily in children from tropical Africa (World Health Organization 1995). The emergence of pesticide resistance in mosquito vectors and antimalarial drug resistance in *Plasmodium* has significantly limited malaria control programs. Novel control strategies based on genetic disruption of mosquito vector competence have been proposed (Collins and Besansky 1994; James 1992). Knowledge of genome complexity and structure is essential for the success of mosquito genetic manipulation. The development of DNA-based genetic markers provides the technology to elucidate the genetic mechanisms of vector competence and to evaluate mosquito genome structure at the molecular level (Severson 1994).

The *Aedes aegypti–Plasmodium gallinaceum* system is an excellent model system to investigate the genetic basis of mosquito vector competence (Kilama and Craig 1969; Severson et al. 1995b; Thathy et al. 1994; Ward 1963). This host-parasite system is relatively easy to manipulate in the laboratory, and a wealth of genetic information is available for the host species. *P. gallinaceum* also shares a close phylogenetic relationship with *P. falciparum*, the most serious malaria parasite infecting humans (Waters et al. 1993). In addition, chromosomal conservation among mosquito species both within and across subfamilies has been suggested by several studies, using either isozyme markers (Matthews and Munstermann 1994) or DNA markers (Kumar and Rai 1993; Severson et al. 1994b); therefore, rapid advances in the molecular genetics of *A. aegypti* could facilitate our understanding of the genetic relationship between *P. falciparum* and its *Anopheles* vectors. A genetic linkage map based on restriction fragment length polymorphism (RFLP) markers has been constructed for *A. aegypti* (Severson et al. 1993). Using these RFLP markers,

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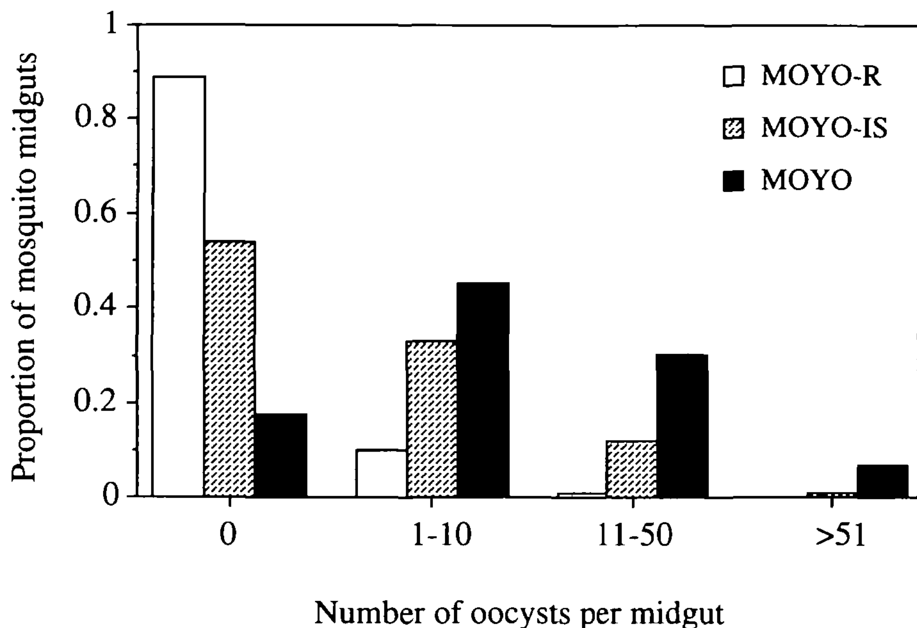


Figure 1. Oocyst distribution among the three *Aedes aegypti* strains exposed to *Plasmodium gallinaceum*. Sample size is 102 female individuals for the MOYO strain, 177 for the substrain selected for refractoriness (MOYO-R), and 124 for the substrain selected for intermediate susceptibility (MOYO-IS).

two putative quantitative trait loci (QTL) that significantly affect the susceptibility of *A. aegypti* to *P. gallinaceum* subsequently were identified (Severson et al. 1995b). Successful development of a physical map for *A. aegypti* (Brown et al. 1995) and construction of saturated linkage maps for QTL genome regions should facilitate map-based cloning of the genes conferring refractoriness and/or susceptibility to *P. gallinaceum*.

The mosquito genome consists of single or low-copy DNA sequences and repetitive DNA with short-period interspersion (Black and Rai 1988). Intraspecific and interspecific variation in abundance and distribution of repetitive DNA has received considerable attention (Black and Rai 1988; Kumar and Rai 1991; McLain et al. 1986, 1987). In this study we focused on allelic variations of single- or low-copy DNA sequences (i.e., RFLP markers developed from cDNAs) among *A. aegypti* strains and then used these markers to examine mosquito genome structure. Unlike *Drosophila* and *Anopheles* species, the lack of well-developed polytene chromosomes in *A. aegypti* obstructs detection of chromosomal inversions, deletions, duplications, and translocations (Sharma et al. 1978). Information on genome organization and complexity is valuable for our understanding of the population genetics of genes conferring refractoriness. For example, significant linkage disequilibrium (defined as nonrandom association between alleles of different genes) between refractory genes and other genes could well affect the behavior of the refractory genes in a population if other genes are also subject to natural selection or artificial selection (e.g., insecticides).

The use of RFLP markers to genotype individual mosquitoes within a population at many loci distributed across the entire genome may provide indirect evidence supporting putative QTL locations and reveal potential chromosomal abnormalities. These genotypic data can be represented in a format termed graphical genotyping (Severson and Kassner 1995; Young and Tanksley 1989), and therefore whole-genome genotypic information for individual mosquitoes can be easily visualized. In this study we used graphical genotyping to illustrate allelic variation along the whole genome in two *A. aegypti* substrains selected for different levels of susceptibility to *P. gallinaceum*, and to examine mosquito genome structural changes associated with selective inbreeding. We also identified a chromosomal deletion at one locus and found significant linkage disequilibrium among several loci in these laboratory mosquito strains.

Materials and Methods

Mosquito Strains

Two substrains (MOYO-R and MOYO-IS) and a laboratory stock strain (Moyo-In-Dry; MOYO) of *A. aegypti* were used in this study. The MOYO strain was originally collected from Shauri Moyo Village, Mombasa, Kenya, in 1974. We estimate that the MOYO strain has been maintained in the laboratory for more than 150 generations. The MOYO-R and MOYO-IS were selected for complete refractoriness to *P. gallinaceum* (i.e., number of oocysts per mosquito midgut = 0) and intermediate susceptibility (number of oocysts per midgut ranges from 0–10) from the MOYO strain by four generations of recurrent sib mating (see Thathy et al. 1994). Therefore the two substrains have a theoretical inbreeding coefficient (F) of 0.5 (Crow and Kimura 1970). Details for the selection of these substrains are provided by Thathy et al. (1994). Mosquitoes were reared as previously described (Christensen and Sutherland 1984). Following selection, each substrain has been maintained as a random mating colony with a minimum population size of 800. We examined generation 31 following initial selection from the MOYO strain. The relative susceptibilities of the three strains to *P. gallinaceum* were periodically examined using the method described by Thathy et al. (1994), and all strains received the same exposure to the parasite. Prevalence and mean infection intensities were calculated based on six independent samples.

RFLP and Probe Selection

DNA extraction from individual mosquitoes, digestion with *Eco*RI, Southern blotting, and hybridization were as previously described (Severson and Kassner 1995; Severson et al. 1993). Eighteen mapped RFLP markers (Mosquito Genomics 1996) were selected to provide coverage for the entire *A. aegypti* genome, with an average resolution of 8.8 cM (Figure 1). All clones used were random cDNA clones, except that *MalI* is a known gene (James et al. 1989). We examined a total of 50 females and 50 males each for MOYO and MOYO-IS, and 88 females and 74 males for MOYO-R.

Data Analysis

Among-strain variation in infection intensity was analyzed using the Wilcoxon rank-sum test, and prevalence was analyzed using the G test (Sokal and Rohlf 1981). Infection intensity is defined as the number of oocysts per infected individual, and prevalence as the percentage of infected individuals. RFLP genotype data were analyzed using the GENEPOP computer program (Raymond and Rousset 1995). Analyses included computation of allele frequencies, observed and expected

Table 1. Allele frequency, observed (H_{obs}) heterozygosity, and F_{IS} estimates revealed by RFLP in *Aedes aegypti* strains susceptible (MOYO), refractory (MOYO-R), and intermediate in susceptibility (MOYO-IS) to *Plasmodium gallinaceum*

Chromosome	Locus	Strain[a]	Alleles 1	2	3	4	5	6	H_{obs}	F_{IS}[b]
1	LF90	MOYO	0.365	0.615	0.020				0.510	−0.045
		MOYO-R	0.780	0.220	—				0.379	−0.105
	LF230	MOYO-IS	0.338	0.662	—				0.495	−0.105
		MOYO	0.778	0.222					0.323	0.064
		MOYO-R*	0.332	0.668					0.363	0.182*
		MOYO-IS	0.800	0.200					0.300	0.062
	LF198	MOYO	0.310	0.410	0.280				0.680	−0.035
		MOYO-R	0.637	0.363	—				0.477	−0.032
		MOYO-IS	0.606	0.394	—				0.525	−0.098
	LF178	MOYO	0.505	0.145	0.205	0.115	0.030		0.650	0.027
		MOYO-R	0.391	0.609	—	—	—		0.474	0.004
		MOYO-IS*	0.374	0 626	—	—	—		0.586	−0.252
	TY7	MOYO	0.275	0.725					0.350	0.123
		MOYO-R	—	1.000					0.000	—
		MOYO-IS	—	1 000					0.000	—
2	ARC1	MOYO	0.106	0.394	0.343	0.157			0.717	−0.038
		MOYO-R	—	1.000	—	—			0.000	—
		MOYO-IS	—	1 000	—	—			0.000	—
	LF138	MOYO	0.413	0.582	0.005				0.469	0.047
		MOYO-R	1.000	—	—				0.000	—
		MOYO-IS	0.732	0.268	—				0.414	−0.056
	LF124	MOYO**	0.191	0.057	0.340	0 412			0.536	0.206**
		MOYO-R	—	1.000	—	—			0.000	—
		MOYO-IS	0.278	0.576	—	0.146			0.576	−0.011
	LF282	MOYO	0.685	0.030	0.230	0.010	0.005	0.040	0.450	0.053
		MOYO-R	0.760	0.240	—	—	—		0.425	−0.164
		MOYO-IS	0.379	0.424	0.197	—	—		0 677	−0.061
	LF98	MOYO	0.110	0.410	0.480				0.580	0.015
		MOYO-R	0.327	0.673	—				0 432	0.018
		MOYO-IS	0.105	0.895	—				0.210	−0.117
	LF250	MOYO	0.258	0.692	0.051				0 495	−0.095
		MOYO-R	0.439	0.561	—				0.481	0.024
		MOYO-IS	0.081	0.919	—				0.162	−0.087
	LF115	MOYO*	0.600	0.375	0.025				0.400	0.198
		MOYO-R	0.661	0.335	0.003				0.437	0.031
		MOYO-IS	0.105	0.895	—				0.170	0.096
3	LF352	MOYO	0.365	0.360	0.200	0.065	0.010		0.670	0.033
		MOYO-R	0.697	0.303	—	—	—		0.478	−0.133
		MOYO-IS	1.000	—	—	—	—		0.000	—
	LF261	MOYO	0.192	0.505	0.263	0.040			0.626	0.017
		MOYO-R	—	1 000	—	—			0.000	—
		MOYO-IS	—	1.000	—	—			0.000	—
	LF168	MOYO	0.005	0 525	0 460	0.010			0.600	−0.170
		MOYO-R	0.392	0.608	—	—			0.426	0.107
		MOYO-IS	0.460	0.540	—	—			0.556	−0.119
	MalI	MOYO***	0.105	0.395	0.450	0.050			0.440	0.299***
		MOYO-R	1.000	—	—	—			0.000	—
		MOYO-IS	1.000	—	—	—			0.000	—
	LF347	MOYO	0.247	0.485	0.134	0.088	0.046		0.588	0.130
		MOYO-R	0.413	0.587	—	—	—		0.427	0.120
		MOYO-IS	0.505	0.495	—	—	—		0.424	0.150

* $P < .05$; ** $P < .01$; *** $P < .001$.

[a] Strain marked with asterisks indicates that its allele frequencies at that locus do not conform to Hardy–Weinberg expectations.

[b] F_{IS} was computed as Weir and Cockerham (1984). A significant, positive F_{IS} estimate indicates heterozygote deficiency; negative F_{IS} indicates excess of heterozygosity.

heterozygosity per locus, and a test for conformance with the Hardy–Weinberg equilibrium at each locus, using the exact Hardy–Weinberg test (Guo and Thompson 1992; Haldane 1954; Weir 1990). We further tested whether distortion from Hardy–Weinberg equilibrium resulted from deficient or excessive heterozygosity (F_{IS}), using the method described by Rousset and Raymond (1995). F_{IS} is defined as [1 − (observed heterozygosity/expected heterozygosity)] (Weir and Cockerham 1984). The among-strain variation in heterozygosity was analyzed following the method of Weir (1990). Briefly, individuals were scored at each locus as heterozygous or homozygous with a 1 or 0, respectively. These data were analyzed with ANOVA using strains, individuals, loci, and interactions of loci and individuals as factors. All factors were treated as random effects except loci, because the same loci were repeatedly scored (Weir 1990). The denominators for each effect 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).

Genetic differentiation between MOYO-R and MOYO-IS was estimated with Wright's F statistics, based on the method of Weir and Cockerham (1984), and using the FSTAT computer software (Goudet 1995). The significance of the F statistics was tested by jackknifing 1000 replicates per locus (Goudet 1995; Weir 1990).

Because multiple alleles were found for some loci, the normalized total linkage disequilibrium D' for each pair of loci was calculated (Hedrick 1987; Lewinton and Kojima 1960). D' for locus A with k alleles and locus B with l alleles is

$$D' = \sum_{i=1}^{k} \sum_{j=1}^{l} p_i q_j |D_{ij}/D_{max}|, \qquad (1)$$

where $D_{ij} = P_{ij} - p_i q_j$; $D_{max} = \min[p_i q_j, (1 - p_i)(1 - q_j)]$ when $D_{ij} < 0$, or $D_{max} = \min[p_i(1 - q_j), (1 - p_i)q_j]$ when $D_{ij} > 0$; p_i and q_j are the frequencies of alleles Ai and Bj at loci A and B. P_{ij} is the frequency of a gamete with alleles Ai and Bj. D_{ij} was estimated from the genotypic data using the maximum likelihood method (Weir 1990). Significance of the genotypic disequilibrium estimate was tested by chi-square statistics using the LINKDIS computer program provided by Garnier-Gere and Dillmann (1992). D' values range from 0 to 1 and are independent of allele frequency (Hedrick 1987). Therefore comparison of D' among strains or pairs of loci was possible. We performed linear regression between D' and map distance to test for the distance effect. RFLP data for the LF227 locus were excluded from the above analysis because a chromosomal deletion at this locus was detected in MOYO and MOYO-IS (see below), and consequently the heterozygote genotype (e.g., *Aa*) could not be differentiated from the homozygote genotype (e.g., *AA*).

Finally, graphical genotypes for all individuals were prepared as described by Young and Tanksley (1989), and graphs for 15 individuals randomly selected from each of the three strains are presented.

Results

Variation in Mosquito Susceptibility to Parasitism

The relative susceptibility of the three *A. aegypti* strains to *P. gallinaceum* is shown

Table 2. Proportion of polymorphic loci and population mean heterozygosity ($\bar{H}_{obs}$) for the three *Aedes aegypti* strains

		MOYO (n = 100)			MOYO-R (n = 162)			MOYO-IS (n = 100)		
Chromosome	Number of RFLP loci examined	Number of alleles per locus	Proportion of polymorphic loci	$\bar{H}_{obs}$ (SD)	Number of alleles per locus	Proportion of polymorphic loci	$\bar{H}_{obs}$ (SD)	Number of alleles per locus	Proportion of polymorphic loci	$\bar{H}_{obs}$ (SD)
1	5	3.0	1.00	0.503 (0.165)	1.8	0.80	0.339 (0.196)	1.8	0.80	0.381 (0.238)
2	7	3 7	1.00	0.521 (0.104)	1.7	0.57	0.254 (0.238)	2.0	0.86	0.316 (0.246)
3[a]	5	4.4	1.00	0.585 (0.087)	1.6	0.60	0.266 (0.244)	1.4	0.40	0.196 (0 272)
Overall loci	17	3.7	1.00	0.534 (0.118)	1.7	0.65	0.282 (0.217)	1.8	0 71	0.299 (0.247)

[a] RFLP marker LF227 was not included in the analysis. See text for details.

(Table 1), the proportion of polymorphic loci, and the observed average heterozygosity (Table 2). A total of 63 unique alleles were identified for the 17 RFLP loci analyzed. Only 27 alleles (43%) were common to the three strains, and 30 alleles were unique to the MOYO strain (Table 1). An average of 3.7 alleles per locus was observed in the MOYO strain, but the MOYO-R and MOYO-IS substrains had only an average of 1.7 and 1.8 alleles per locus, respectively (Table 2). The genotypes at several loci were in Hardy–Weinberg disequilibrium, apparently due to heterozygote deficiencies, because significant positive F_{IS} was found at these loci (Table 1).

in Figure 1. The MOYO strain showed a prevalence of 82.3%, significantly greater than either the MOYO-IS (45.9%; G = 33.1, df = 1, P < .001) or MOYO-R (11.3%; G = 148.6, df = 1, P < .001) substrains. The mean intensity of the MOYO strain (16.1 oocysts/midgut) was also significantly higher than MOYO-IS (8.6; χ^2 = 4.7, df = 1, P < .05) or MOYO-R (3.8; χ^2 = 13.2, df = 1, P < .001). The mean infection intensity and prevalence in MOYO-IS were significantly greater than the MOYO-R (χ^2 = 5.0, df = 1, P < .05 for intensity; G = 33.1, df = 1, P < .001 for prevalence). The within-strain variation in infection intensity was considerably higher in the MOYO strain (SD = 20.6, n = 84) than for MOYO-R (SD = 3.7, n = 20) or MOYO-IS (SD = 10.2, n = 57; Figure 1).

Genetic Variation

Within- and among-strain genetic variability can be quantified by allele frequencies

Table 3. F-statistics analysis of polymorphic loci for the MOYO-R and MOYO-IS substrains

Chromosome	Locus	F_{IS}[a]	F_{IT}	F_{ST}
1	LF90	−0 100	0.268**	0.334**
	LF230	0.146*	0.449**	0.354**
	LF198	−0.055	−0.057	−0.002
	LF178	−0.090	−0.094	−0.003
2	LF282	0.024	0.203**	0.184**
	LF98	−0.006	0.117*	0.122**
	LF250	0.010	0.268**	0.260**
	LF115	0.047	0.495**	0.470**
3	LF168	0.023	0.028	0.005
	LF347	0.137*	0.147**	0.012[b]

[a] F_{IS} = fixation indices of individuals relative to the total subpopulations; F_{IT} = fixation indices of individuals relative to the total populations. It takes into account both the effects of nonrandom mating within subpopulations (F_{IS}) and the effects of population subdivision (F_{ST}); F_{ST} = coefficient of population differentiation, and it measures the between-population component of standard genetic variance.

[b] P = 0.052.

* P < .05; ** P < .01; *** P < .001.

Table 4. Pairwise total linkage disequilibrium (D') among the three *Aedes aegypti* strains for the RFLP loci

Chromosome 1		LF90	LF230	LF198	LF178		
MOYO	LF230	0.261					
	LF198	0.049	0.196				
	LF178	0.449	0.235	0.273			
	TY7	0.389	0.158	0.121	0.223		
MOYO-R	LF230	0.479***					
	LF198	0 036	0.275				
	LF178	0.031	0.095	0.055			
	TY7	—[b]	—	—	—		
MOYO-IS	LF230	0.435**					
	LF198	0.054	0.644**				
	LF178	0.245	0.641*	0.308**			
	TY7	—	—	—	—		
Chromosome 2		**ARC1**	**LF138**	**LF124**	**LF282**	**LF98**	**LF250**
MOYO	LF138	0.204					
	LF124	0.173	0.236**				
	LF282	0.181	0.087***	0.267**			
	LF98	0.109	0.179	0.249	0.179		
	LF250	0.172	0.467**	0.249	0.194***	0.167	
	LF115	0.143	0.157	0.109	0.324	0.069*	0.239*
MOYO-R	LF138	—					
	LF124	—	—				
	LF282	—	—	—			
	LF98	—	—	—	0.147		
	LF250	—	—	—	0.059	0.252*	
	LF115	—	—	—	0.113	0.012	0.240
MOYO-IS	LF138	—	—				
	LF124	—	0.229				
	LF282	—	0.129	0.340**			
	LF98	—	0.316	0.323	0.397		
	LF250	—	0.209	0.359	0.629*	0.395***	
	LF115	—	0.117	0.438	0.634*	0.032	0.113
Chromosome 3		**LF352**	**LF261**	**LF168**	***MalI***		
MOYO	LF261	0.152*					
	LF168	0.076	0.362**				
	MalI	0.492*	0.549*	0.119**			
	LF347	0.198	0.241**	0.182**	0.262***		
MOYO-R	LF261	—					
	LF168	0.014	—				
	MalI	—	—	—			
	LF347	0.018	—	0.050	—		
MOYO-IS	LF261	—					
	LF168	—	—				
	MalI	—	—	—			
	LF347	—	—	0.047	—		

[a] Indicates linkage disequilibrium estimate is significantly different from 0 at P < .05, ** P < .01, and *** P < .001.

[b] Linkage disequilibrium estimate is not available due to fixation of a locus in a population.

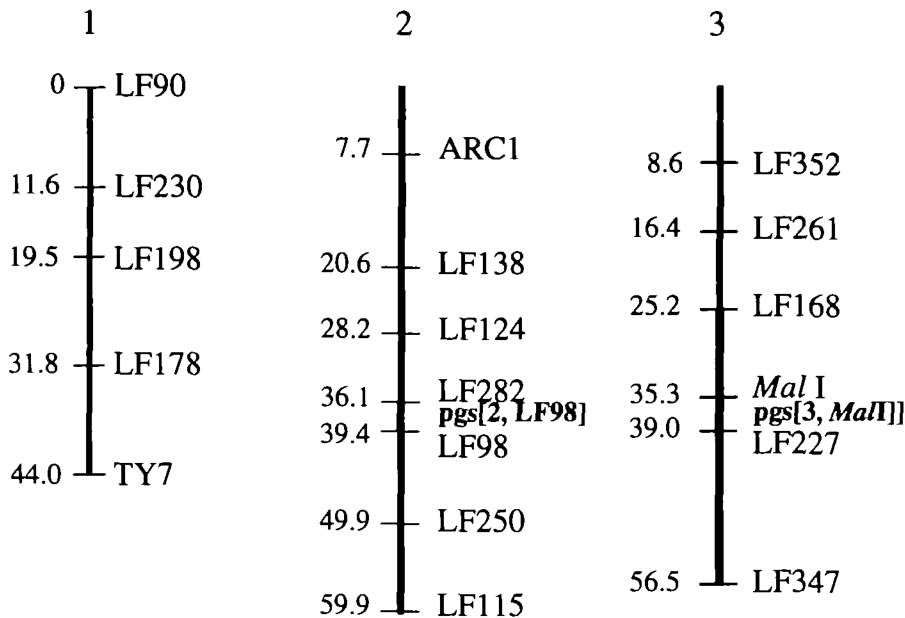


Figure 2. Relative map position of the 18 RFLP markers used in this study. Map distances are in Kosambi centimorgans. The RFLP markers best defining the two quantitative trait loci for the susceptibility of *Aedes aegypti* to *Plasmodium gallinaceum* are indicated (pgs[2, LF98] and pgs[3, *MalI*]; Severson et al. 1995b)

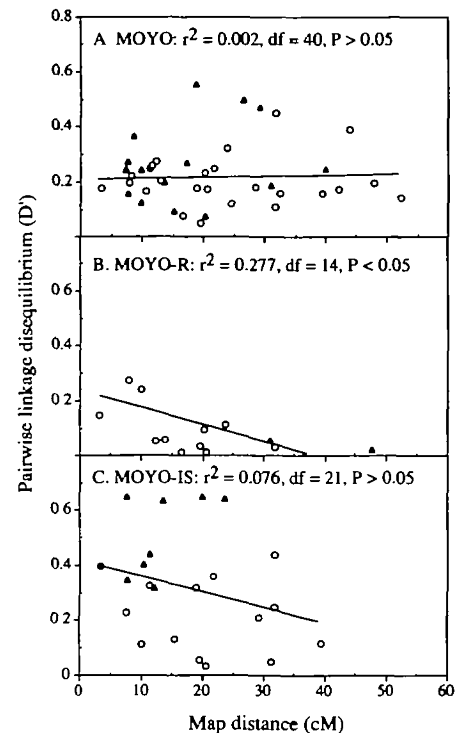


Figure 3. Correlation between pairwise linkage disequilibrium (D') and map distance for the three *Aedes aegypti* strains. The D' estimates were obtained from Table 3 and plotted against the map distance obtained from Figure 1. Statistically significant disequilibria are shown as solid triangles and nonsignificant disequilibria as open circles.

All loci were polymorphic in the MOYO strain, but 35% and 29% of the loci were fixed in the MOYO-R and MOYO-IS substrains, respectively. The mean heterozygosity of the MOYO-R and MOYO-IS substrains was significantly reduced by 47% and 44%, respectively, compared to the MOYO strain (Table 2; ANOVA, $F = 10.38$, df = 2, 32, $P < .001$). This is apparently due to fixation of alleles at loci on chromosomes 2 and 3 and the reduced number of alleles at other loci. The reduction in mean heterozygosity in the two substrains was in accordance with the theoretical expectation of 50% (Crow and Kimura 1970).

Although no significant difference was detected in the mean heterozygosity between MOYO-R and MOYO-IS (Table 2; ANOVA, $F = 0.149$, df = 1, 16, $P > .05$), the two substrains exhibited significant differences in allele frequency at most of the polymorphic loci (Table 1). Significant F_{ST} estimates at those loci provide further support for these results (Table 3). Genetic differentiation between the MOYO substrains may have resulted from genetic drift, or selection, or both.

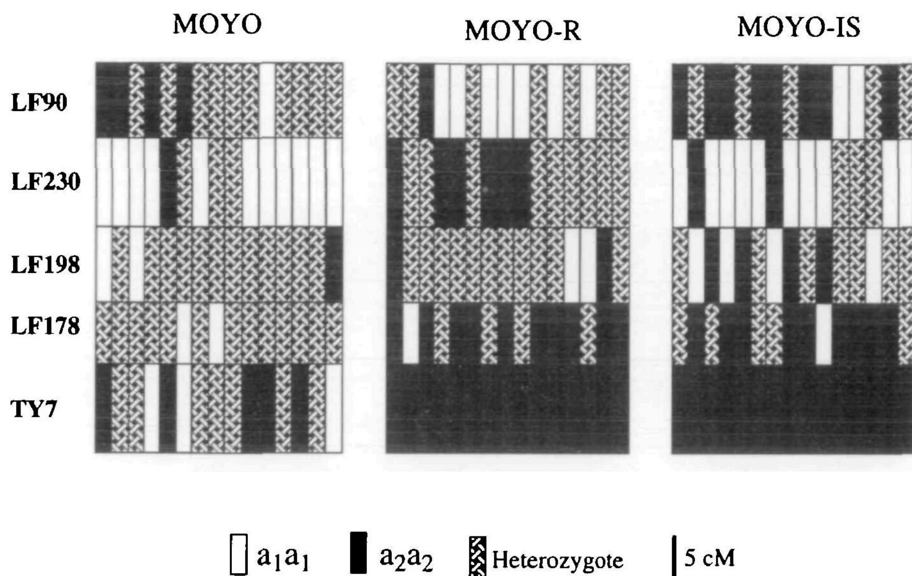
Genotypic Linkage Disequilibrium

Genotypic linkage disequilibrium among pairs of polymorphic loci was evaluated for each of the three strains. The results are presented in Table 4, with marker loci arranged in chromosomal order. Significant pairwise linkage disequilibrium was detected in each strain, and it was remarkably evident for the loci on chromosomes 2 and 3 of the MOYO strain (Table 4). Significant linkage disequilibrium (D') was not expected in any of these strains. In theory the decay of linkage disequilibrium in t generations relative to its initial value can be estimated as $(1 - r)^t$ for a random mating population, where r is the recombination frequency between a pair of loci (Hedrick 1987). Thus D' should decay to zero for pairs of loci with fairly large recombination frequencies (e.g., the smallest recombination frequency, r, among all pairs of loci studied is 0.0612 for LF282-LF98; see Figure 2) after long-term laboratory culture (e.g., $t > 150$ generations for MOYO). To test for the effect of recombination on the disruption of allelic associations among those RFLP loci, linear correlations between D' estimates and their corresponding map distances were determined for each of the three strains. We found significant correlations with the MOYO-R substrain, but not with MOYO and MOYO-IS (Figure 3), suggesting that recombination did not effectively break down nonrandom allelic associations in MOYO and MOYO-IS. Time-dependent decay in linkage disequilibrium probably occurred in MOYO-R. This suggestion was supported by the data that fewer pairs of loci were in linkage disequilibrium for MOYO-R and by generally smaller D' estimates (Table 4).

Graphical Genotyping

Graphical genotypes for 15 randomly selected individuals from each of the three strains are shown in Figure 4. We found that a chromosomal deletion event occurred around the LF227 locus in 10.9% of the individuals in the MOYO strain and 16.2% in the MOYO-IS substrain (Figure 4C). Southern blots of *Eco*RI-digested DNA from those individuals showed no hybridization to the LF227 clone, while hybridization was observed with all individuals with all other markers examined (Figure 4C). Therefore, absence of hybridization to LF227 with those individuals was not due to incomplete DNA digestion or poor probe conditions.

We have previously identified two RFLP markers, LF98 on chromosome 2 and *MalI* on chromosome 3, which best define the QTL for *A. aegypti* susceptibility to *P. gallinaceum* (Severson et al. 1995b). The MOYO strain exhibited high polymorphism with both markers, and most individuals were heterozygotes (Figure 4). The high level of genetic diversity of this strain may reflect large variation in susceptibility to the malaria parasite (Figure 1). Marker *MalI* was fixed in the MOYO-R and MOYO-IS substrains (Figure 4). Most individuals of the two substrains were homozygous for the same allele at the LF98 locus (46% for MOYO-R and 79% for



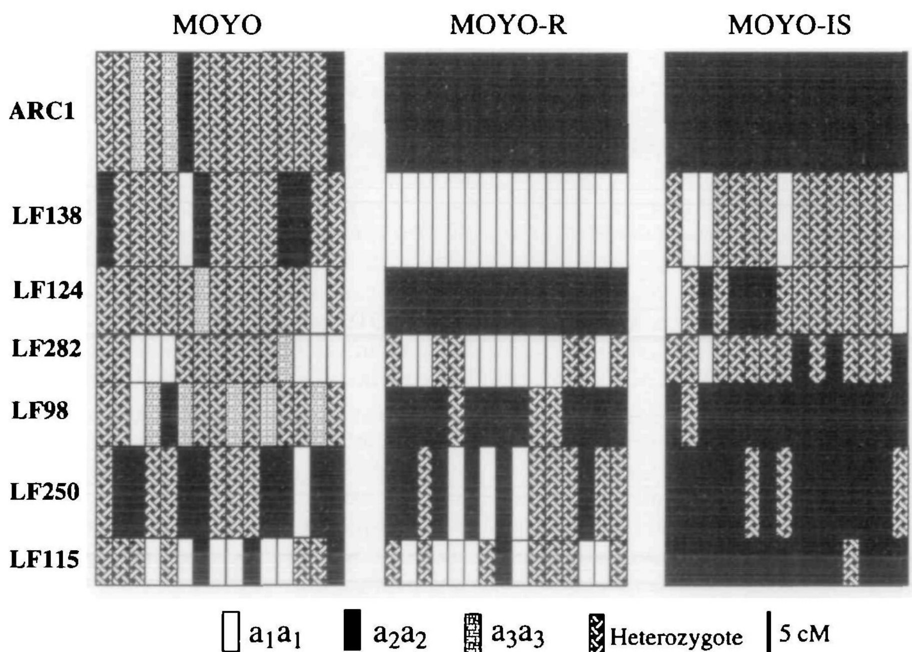


Figure 4. Graphical genotyping for 15 individual mosquitoes representing the MOYO, MOYO-R, and MOYO-IS strains. For each panel each column represents the chromosomal organization for a single mosquito. Individual markers reflect the half-recombinational distance between adjacent markers or between an adjacent marker and the end of the linkage group.

MOYO-IS), although a small proportion was heterozygous (43% for MOYO-R and 21% for MOYO-IS) or homozygous for a second allele (11% for the MOYO-R and 0% for MOYO-IS substrains).

Discussion

This study examined genetic variation within and among *A. aegypti* strains selected for refractoriness to *P. gallinaceum*, and evaluated mosquito genome structure, particularly in response to selection, using RFLP markers developed from *A. aegypti* (Severson et al. 1993). *A. aegypti* population genetic variation has previously been studied with isozymes (Tabachnick 1982; Tabachnick et al. 1985) and random-amplified polymorphic DNA (RAPD) markers (Ballinger-Crabtree et al. 1992). Isozyme and RAPD techniques have the advantage of relative simplicity; however, RFLP markers offer several advantages. First, by using RFLP markers we were able to detect high levels of polymorphism in the three *A. aegypti* strains (Table 2). Because RFLP markers segregate as codominant markers, heterozygotes can be scored directly and population allele frequencies can be accurately estimated (Table 1), and therefore the RFLP markers are valuable for studying population genetic structure (Table 3). The RFLP markers used here detected significantly higher mean heterozygosity in our *A. aegypti* populations (see Table 2) than did isozyme markers in other *A. aegypti*, *A. albopictus* (Tabachnick et al. 1985), and *Anopheles* spp. (Estrada-Franco et al. 1993; Fritz et al. 1995; Manguin et al. 1995) populations or other Diptera groups (Graur 1985). Second, although chromosomal inversions and translocations undoubtedly occurred during mosquito evolution (Matthews and Munstermann 1994), these authors and other studies (e.g., Severson et al. 1994b) suggest that the basic genome structure may be largely conserved for mosquito species within the subfamilies Culicinae and Anophelinae. Support for this suggestion of genome structure conservation was provided when Severson et al. (1995a) demonstrated complete conservation of linkage group and linear order in *A. albopictus* for 18 RFLP markers developed from *A. aegypti*. For these species, the same RFLP loci may be examined for genetic variation for populations within a species or between these species. In addition, the RFLP markers provide an opportunity for sampling the whole mosquito genome because their relative map position is known (Figure 2; see also Mosquito Genomics 1996). Therefore RFLP technology provides a powerful tool to study mosquito population genetic variation. Finally, because our RFLP markers are single- or low-copy cDNA sequences, it is possible to use them to reveal genomic anomalies such as chromosomal duplications and deletions. Our data suggest a chromosomal deletion around locus LF227 for some individuals of the MOYO and MOYO-IS strains (Figure 4).

We observed significant linkage disequilibrium between loci separated by large map distances (i.e., 7.6–40.1 cM; see Figure 3). Severson and Kassner (1995) also reported similar results in other populations of *A. aegypti*. In *Drosophila melanogaster*, very little linkage disequilibrium is observed between genes that are more

C. Chromosome 3

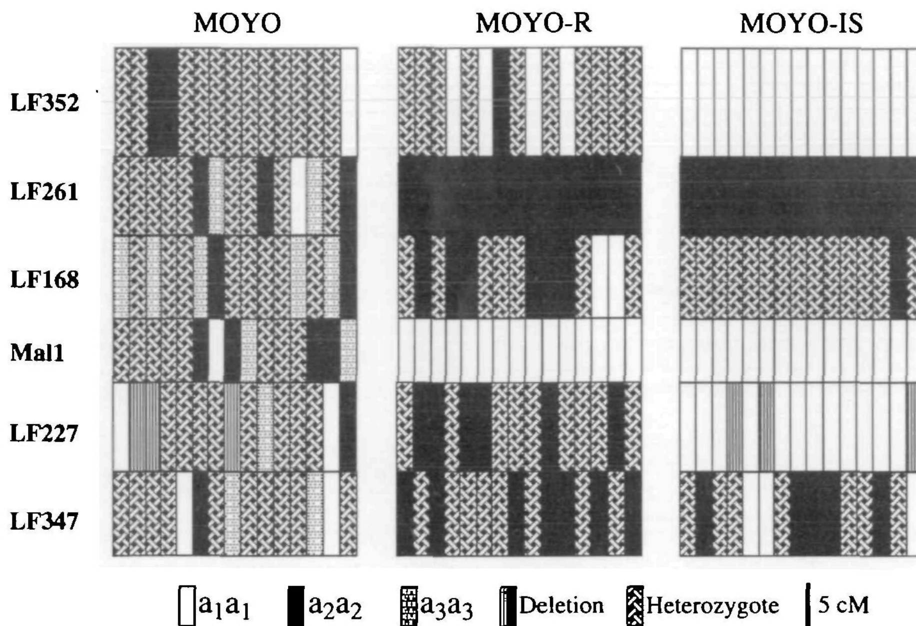


Figure 4. *Continued.*

than 10 cM apart (Langley et al. 1977, 1978). Linkage disequilibrium is more likely to be found for closely linked genes, usually in genome regions less than 100 kb (e.g., Aquadro et al. 1992; Langley et al. 1988; Macpherson et al. 1990; Schaeffer and Miller 1993; Smit-McBride et al. 1988), or for functionally related genes (e.g., Baker 1975; Bech-Hansen et al. 1983; Van der Loo et al. 1987). In a large, random-mating population, linkage disequilibrium should be nearly zero without epistatic selection or other genetic mechanisms. If a population is founded by a small number of individuals in which significant linkage disequilibrium exists between a given pair of loci, this linkage disequilibrium should decrease with each successive generation at a rate inversely proportional to the recombination rate between them. Therefore our mosquito populations were expected to be at linkage equilibrium at all RFLP loci examined in this study.

Linkage disequilibrium may result from various genetic mechanisms other than epistatic selection, such as low recombination rate, genetic drift, population subdivision, migration, and hitchhiking among the linked genes (Hedrick et al. 1978; Hill and Robertson 1968; Lewinton 1974; Nei 1987; Ohta and Kimura 1969). The rationale for epistatic selection is that natural selection may favor particular combinations of alleles at different loci (coadapted gene complex), therefore the frequencies of these allelic combinations in the gametes are higher than expected from the random combinations of their frequencies (Smit-McBride et al. 1988). Population subdivision and migration are not applicable to our mosquito populations. Genetic drift resulted in significant genetic differentiation between the MOYO-R and MOYO-IS substrains (see Table 3); however, it is not likely to be an important mechanism for the observed linkage disequilibrium in these populations because genetic drift alone is not likely to create substantial linkage disequilibrium in a population with rapid growth (Slatkin 1994). We also argue that epistatic selection and genetic hitchhiking are not major mechanisms because (1) the RFLP markers are random cDNA sequences whose putative functions are clearly not related (Severson and Zhang 1996), and (2) in the MOYO strain, linkage disequilibrium was detected in 48% of the pairs of loci on chromosomes 2 and 3, although they are not tightly linked (Table 4). We hypothesize that the lack of recombination or low recombination rates in some regions of the *A. aegypti* genome is probably a result of chromosomal inversions. Nonsignificant correlation between linkage disequilibrium and map distance in some mosquito strains supports this hypothesis (e.g., MOYO and MOYO-IS; see Figure 3). Examples of linkage disequilibrium resulting from paracentric inversions have been documented in *Drosophila* (Prakash 1977; Prakash and Lewinton 1968; Voelker et al. 1978). It is well known that chromosomal inversion polymorphisms occur in the mosquito *An. gambiae* (Coluzzi et al. 1985), and the presence of naturally occurring inversions in *A. aegypti* have been suggested (Macdonald and Sheppard 1965; Severson and Kassner 1995). Direct cytologic evidence or genetic assay with single pair matings is required for verification of this hypothesis.

Finally, we used graphical genotyping to evaluate the *A. aegypti* genome. The reduction in allelic variation resulting from selective inbreeding in the MOYO-R and MOYO-IS substrains, including reduced heterozygosity, the proportion of polymorphic loci, and loss of alleles, can be easily visualized from Figure 4. Using this method, Severson and Kassner (1995) found that different alleles were fixed in loci across a large distance in two *A. aegypti* strains refractory and susceptible to the filarial worm *Brugia malayi*, including chromosomal regions influencing filarial worm susceptibility (Severson et al. 1994a) and intensity (Beerntsen et al. 1995). The large variation in the susceptibility to *P. gallinaceum* in the MOYO strain is reflected by elevated genetic heterogeneity at the markers flanking the QTL on chromosomes 2 and 3 (Figures 1 and 4). One allele for the marker *MalI* most closely linked to the QTL on chromosome 3 was fixed in the MOYO-R and MOYO-IS substrains. The chromosomal region containing the QTL on chromosome 2 was polymorphic in the two substrains (Figure 4). In addition, these two substrains exhibited allelic variations in other genome regions. Whether such allelic variations affect mosquito fitness and vector competence remains unknown. Graphical genotyping, therefore, provided us a method to easily reveal genetic changes due to past evolutionary history, examine within- and among-population genetic variation, and formulate hypotheses about the genetic basis of vector competence.

References

Aquadro CF, Jennings RMJ, Bland MM, Laurie CC, and Langley CH, 1992. Patterns of naturally occurring restriction map variation, dopa decarboxylase activity variation and linkage disequilibrium in the *Ddc* gene region of *Drosophila melanogaster*. Genetics 132:443–452.

Baker WK, 1975. Linkage disequilibrium over space and time in natural populations of *Drosophila montana*. Proc Natl Acad Sci USA 72:4095–4099.

Ballinger-Crabtree ME, Black WC, and Miller BR, 1992. Use of genetic polymorphisms detected by the random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for differentiation and identification of *Ae-*

des aegypti subspecies and populations. Am J Trop Med Hyg 47:893–901.

Bech-Hansen NT, Linsley PS, and Cox DW, 1983. Restriction fragment length polymorphisms associated with immunoglobulin C genes reveal linkage disequilibrium and genomic organization. Proc Natl Acad Sci USA 80: 6952–6956.

Beerntsen BT, Severson DW, Klinkhammer JA, Kassner VA, and Christensen BM, 1995. *Aedes aegypti*: a quantitative trait locus (QTL) influencing filarial worm intensity is linked to QTL for susceptibility to other mosquito-borne pathogens. Exp Parasitol 81:355–362.

Black WC and Rai KS, 1988. Genome evolution in mosquitoes: intraspecific and interspecific variation in repetitive DNA amounts and organization. Genet Res 51: 185–196.

Brown SE, Menninger J, Difillipantonio M, Beaty BJ, Ward DC, and Knudson DL, 1995. Toward a physical map of *Aedes aegypti*. Insect Mol Biol 4:161–167.

Christensen BM and Sutherland DR, 1984. *Brugia pahangi*: exsheathment and midgut penetration in *Aedes aegypti*. Trans Am Microsc Soc 103:423–433.

Collins FH and Besansky NJ, 1994. Vector biology and the control of malaria in Africa. Science 264:1874–1875.

Coluzzi M, Petrarca V, and Di Deco MA, 1985. Chromosomal inversion integradation and incipient speciation in *Anopheles gambiae*. Boll Zool 52:45–63.

Crow JF and Kimura M, 1970. An introduction to population genetics theory. New York: Harper & Row.

Estrada-Franco JG, Ma MC, Gwadz RW, Sakai R, Lanzaro GC, Laughinghouse A, Galvan-Sanchez C, Cespedes JL, and Vargas-Sagarnaga R, 1993. Evidence through crossmating experiments of a species complex in *Anopheles pseudopunctipennis* sensu lato: a primary malaria vector of the American continent. Am J Trop Med Hyg 49: 735–745.

Fritz GN, Bermudez H, and Seawright JA, 1995. Genetic differentiation and diagnostic loci of *Anopheles nuneztovari*, *An. trinkae*, and *An. rangeli* (Diptera. Culicidae). J Med Entomol 32:663–672.

Garnier-Gere P and Dillmann C, 1992. A computer program for testing pairwise linkage disequilibria in subdivided populations. J Hered 83:239.

Goudet J, 1995. FSTAT version 1.2: a computer program to calculate *F*-statistics. J Hered 86:485–486.

Graur D, 1985. Gene diversity in Hymenoptera. Evolution 39:190–199.

Guo SW and Thompson EA, 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics 48:361–372.

Haldane JBS, 1954. An exact test for randomness of mating. J Genet 52:631–635.

Hedrick PW, 1987. Gametic disequilibrium measures: proceed with caution. Genetics 117:331–341.

Hedrick PW, Jain S, and Holden L, 1978. Multilocus systems in evolution. Evol Biol 11:101–182.

Hill WG and Robertson A, 1968. Linkage disequilibrium in finite populations. Theor Appl Genet 38:226–231.

James AA, 1992. Mosquito molecular genetics: the hands that feed bite back. Science 257:37–38.

James AA, Blackmer K, and Racioppi JV, 1989. A salivary gland-specific maltase-like gene of the vector mosquito, *Aedes aegypti*. Gene 75:73–83.

Kilama WL and Craig GB Jr, 1969. Monofactorial inheritance of susceptibility to *Plasmodium gallinaceum* in *Aedes aegypti*. Ann Trop Med Parasitol 63:419–432.

Kumar A and Rai KS, 1991. Organization of a cloned repetitive DNA fragment in mosquito genomes (Diptera: Culicidae). Genome 34:998–1006.

Kumar A and Rai KS, 1993. Molecular organization and evolution of mosquito genome. Comp Biochem Physiol 106B:495–504.

Langley CH, Ito K, and Voelker RA, 1977. Linkage disequilibrium in natural populations of *Drosophila melanogaster*: seasonal variation. Genetics 86:447–454.

Langley CH, Shrimpton AE, Yamazaki T, Miyashita N, Matsuo Y, and Aquadro CF, 1988. Naturally-occurring variation in the restriction map of the *Amy* region of *Drosophila melanogaster*. Genetics 119:619–629.

Langley CH, Smith DB, and Johnson FM, 1978 Analysis of linkage disequilibria between allozyme loci in natural populations of *Drosophila melanogaster*. Genet Res 32:215–230.

Lewinton RC, 1974. The genetics basis of evolutionary change. New York: Columbia University Press.

Lewinton RC and Kojima K-I, 1960. The evolutionary dynamics of complex polymorphisms. Evolution 14: 116–129.

Macdonald WW and Sheppard PM, 1965. Cross-over values in the sex chromosomes of the mosquito *Aedes aegypti* and evidence of the presence of inversions. Ann Trop Med Parasitol 59:74–87.

Macpherson JN, Weir BS, and Brown AJL, 1990. Extensive linkage disequilibrium in the *achaete-scute* complex of *Drosophila melanogaster*. Genetics 126:121–129.

Manguin S, Roberts DR, Peyton EL, Fernandez-Salas I, Barreto M, Loayza RF, Spinola RE, Granaou RM, and Rodriguez MH, 1995. Biochemical systematics and population genetic structure of *Anopheles pseudopunctipennis*, vector of malaria in Central and South America. Am J Trop Med Hyg 53:362–377.

Matthews TC and Munstermann LE, 1994. Chromosomal repatterning and linkage group conservation in mosquito karyotypic evolution. Evolution 48:146–154.

McLain DK, Rai KS, and Fraser MJ, 1986. Interspecific variation in the abundance of highly repeated DNA sequences in the *Aedes* scutellaris (Diptera: Culicidae) subgroup. Ann Entomol Soc Am 79:787–791.

McLain DK, Rai KS, and Fraser MJ, 1987. Intraspecific and interspecific variation in the sequence and abundance of highly repeated DNA among mosquitoes of the *Aedes albopictus* subgroup. Heredity (Edinburgh) 58:373–381

Mosquito Genomics, 1996. The *Aedes aegypti* database available from the World Wide Web at URL http://klab agsci.colostate.edu.

Nei M, 1987. Molecular evolutionary genetics. New York: Columbia University Press.

Ohta T and Kimura M, 1969. Linkage disequilibrium at a steady state determined by random genetic drift and recurrent mutation. Genetics 63:229–238.

Prakash S, 1977. Further studies on gene polymorphism in the mainbody and geographically isolated populations of *Drosophila pseudoobscura*. Genetics 85: 713–719.

Prakash S and Lewinton RC, 1978. A molecular approach to the study of genic heterozygosity III. Direct evidence of coadaptation in gene arrangements of *Drosophila*. Proc Natl Acad Sci USA 59:398–405

Raymond M and Rousset F, 1995. GENEPOP (version 1.2): a population genetics software for exact tests and ecumenicism. J Hered 86:248–249.

Rousset F and Raymond M, 1995. Testing heterozygote excess and deficiency. Genetics 140:1413–1419.

SAS, 1994 JMP® user's guide. Cary, North Carolina: SAS Institute.

Schaeffer SW and Miller EL, 1993. Estimates of linkage disequilibrium and the recombination parameter determined from segregating nucleotide sites in the alcohol dehydrogenase region of *Drosophila pseudoobscura*. Genetics 135:541–552.

Severson DW, 1994. Applications of molecular marker analysis to mosquito vector competence. Parasitol Today 10:336–340.

Severson DW and Kassner VA, 1995. Analysis of mosquito genome structure using graphical genotyping. Insect Mol Biol 4:279–286.

Severson DW, Mori A, Zhang Y, and Christensen BM, 1993. Linkage map for *Aedes aegypti* using restriction fragment length polymorphisms. J Hered 84:241–247.

Severson DW, Mori A, Zhang Y, and Christensen BM, 1994a. Chromosomal mapping of two loci affecting filarial worm susceptibility in *Aedes aegypti* Insect Mol Biol 3:67–72.

Severson DW, Mori A, Zhang Y, and Christensen BM, 1994b. The suitability of RFLP markers for evaluating genetic diversity among and synteny between mosquito species. Am J Trop Med Hyg 50:425–432.

Severson DW, Mori A, Kassner VA, and Christensen BM, 1995a. Comparative linkage maps for the mosquitoes, *Aedes albopictus* and *Ae. aegypti*, based on common RFLP loci. Insect Mol Biol 4:41–45.

Severson DW, Thathy V, Mori A, Zhang Y, and Christensen BM, 1995b. Restriction fragment length polymorphism mapping of quantitative trait loci for malaria parasite susceptibility in the mosquito *Aedes aegypti*. Genetics 139:1711–1717.

Severson DW and Zhang Y, 1996. Generation of expressed sequence tags and sequence-tagged sites as physical landmarks in the mosquito, *Aedes aegypti*, genome Genome 39:224–229.

Sharma GP, Mittal OP, Chaudhry S, and Pal V, 1978. A preliminary map of the salivary gland chromosomes of *Aedes* (Stegomyia) *aegypti* (Culicadea, Diptera). Cytobios 22:169–178.

Slatkin M, 1994. Linkage disequilibrium in growing and stable populations. Genetics 137:331–336.

Smit-McBride Z, Moya A and Ayala FJ, 1988 Linkage disequilibrium in natural and experimental populations of *Drosophila melanogaster*. Genetics 120:1043–1051.

Sokal RR and Rohlf FJ, 1981. Biometry. San Francisco: W. H. Freeman.

Tabachnick WJ, 1982. Geographic and temporal patterns of genetic variation of *Aedes aegypti* in New Orleans. Am J Trop Med Hyg 31:849–853.

Tabachnick WJ, Wallis GP, Aitken TH, Miller BR, Amato GD, Lorenz L, Powell JR, and Beaty BJ, 1985. Oral infection of *Aedes aegypti* with yellow fever virus: geographic variation and genetic considerations. Am J Trop Med Hyg 34.1219–1224.

Thathy V, Severson DW, and Christensen BM, 1994. Reinterpretation of the genetics of susceptibility of *Aedes aegypti* to *Plasmodium gallinaceum*. J Parasitol 80:705–712.

Van der Loo W, Arthur CP, Richardson BJ, Wallace-Drees M, and Hamers R, 1987. Non-random allele associations between unlinked protein loci: are the polymorphisms of the immunoglobulin constant regions adaptive? Proc Natl Acad Sci USA 84:3075–3079.

Voelker RA, Cockerham CC, Johnson FM, Schaffer HE, Mukai T, and Mettler LE, 1978. Inversions fail to account for allozyme clines. Genetics 88:515–527.

Ward RA, 1963 Genetic aspects of the susceptibility of mosquitoes to malaria infection. Exp Parasitol 13:328–341.

Waters AP, Higgins DG, and McCutchan TF, 1993. Evolutionary relatedness of some primate models of *Plasmodium*. Mol Biol Evol 10:914–923.

Weir BS, 1990. Genetic data analysis. Sunderland, Massachusetts: Sinauer.

Weir BS and Cockerham CC, 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370.

World Health Organization, 1995. World Health Report 1995—executive summary. Available on the World Wide Web at URL http://www.who.ch.

Young ND and Tanksley SD, 1989. Restriction fragment length polymorphism maps and the concept of graphical genotypes. Theor Appl Genet 77:95–101.

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