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Natural skip oviposition of the mosquito *Aedes aegypti* indicated by codominant genetic markers

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Abstract. This study examines the use of codominant restriction fragment length polymorphism (RFLP) markers to estimate the number of sibling families found within and among oviposition sites used by the mosquito *Aedes aegypti* (L) (Diptera: Culicidae). Estimates were made using pairwise relatedness (r_{xy}) calculations based on alleles shared between individuals. Genotypes for eight laboratory mosquito families were determined at six RFLP loci and the observed allele frequencies were used to generate simulated distributions of r_{xy} from full-sibling and unrelated pairs of individuals. The midpoint (mp) between the means of the pairwise r_{xy} distributions was used to discriminate full-sibling families from unrelated families. Clusters of individuals with r_{xy} values higher than the mp value were grouped as putative sibling families. This method was tested by calculating actual r_{xy} for all pairwise comparisons of the known laboratory full-sibling and paternal half-sibling families, followed by UPGMA cluster analysis to group sibling families. The technique was then used for sibling estimations on wild caught mosquitoes collected at three locations in Trinidad, West Indies. From field populations, 35 families were estimated among 122 individuals tested with an average of 6.2 families per container. Members of 19 predicted families clustered as groups across multiple containers, providing molecular evidence for skip-oviposition behaviour in *Ae. aegypti* females, whereby individual females oviposit in more than one container.

Key words. *Aedes aegypti*, container breeding, dengue vector, mosquito larvae, oviposition behaviour, relatedness, RFLP, skip-oviposition, vector biology, yellow fever mosquito, Trinidad, West Indies.

Introduction

Molecular markers have been used to resolve family relationships among groups of organisms believed to consist of related and unrelated individuals (Schuster & Mitton, 1991; Tegelstrom *et al*., 1991; Apostol *et al*., 1993; Blouin *et al*., 1996). Genetic relatedness (r) within groups is based on the expected proportions of alleles shared between genomes of individuals with highly related individuals sharing more alleles in common than unrelated individuals. For diploid species, $r = 1/2$ for full-siblings and parent offspring pairs, $r = 1/4$ for half-sibs or for an individual with uncles, aunts, grandparents and grandchildren, $r = 1/8$ for first cousins and $r = 0$ for unrelated individuals. Several statistical methods have been developed to estimate coefficients of relatedness (Pamilo & Crozier, 1982; Queller & Goodnight, 1989; Ritland, 1996; Lynch & Ritland, 1999). These techniques are valuable for population studies when parentage and genetic relationships are uncertain. There are, however, limitations in determining individual relatedness among field populations using molecular markers. Unbiased estimates of relatedness cannot be obtained without knowledge of the allelic frequencies of both the local and the background population (Lynch, 1988). Consequently, the number of loci examined and their allelic polymorphism limit accurate relatedness estimates.

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Despite their limitations, estimations of relatedness can be used to separate individuals into similar genetic groups by using suitable genetic markers. Several genetic marker types have been used to study populations of the yellow fever mosquito *Aedes aegypti* including isozymes, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), and restriction fragment length polymorphisms (RFLPs) (Powell *et al*., 1980; Wallis & Tabachnick, 1990; Apostol *et al*., 1996; Yan *et al*., 1998, 1999). RAPD and AFLP analyses are relatively easy to perform and a large number of loci can be examined in individual mosquitoes. However, because both RAPD and AFLP fragments segregate as dominant alleles, several assumptions must be made: (i) the recessive alleles (the null condition) are identical in state, (ii) alleles segregate according to Mendelian expectations and (iii) genotypes are in Hardy–Weinberg equilibrium (HWE). RFLP markers are particularly suitable for population genetic studies (Yan *et al*., 1998, 1999; Dubreil & Charcosset, 1999; Brouwer *et al*., 2000) because they are highly polymorphic, the fragments segregate as codominant alleles, and they can be used to test HWE assumptions with unbiased estimates of genetic parameters.

Aedes aegypti is the primary vector for several important human pathogens, including yellow fever and dengue fever viruses, with a range including subtropical and tropical areas of the world (Christophers, 1960). Attempts at controlling mosquito-borne diseases have been most effective when systematic source reduction of breeding sites and insecticide applications are conducted. However, these efforts have led to the selection of insecticide-resistant strains of mosquitoes. In addition, changes in public health policies and population demographics have contributed to the resurgence of vector-borne diseases. For example, dengue prevalence around the world has increased dramatically in the past decade. In 1998 there were more than 616 000 cases of dengue in the Americas, including 11 000 cases of dengue haemorrhagic fever (WHO, 2001).

At present, mosquito populations are being investigated at the molecular and behavioural levels, with the goal of developing novel control strategies through genetic manipulation of the mosquito vector. Female *Ae. aegypti* deposit their eggs in natural and artificial containers of water in and around human dwellings. Mark–release–recapture studies using *Ae. aegypti* indicate a wide dispersal range, from 35 m to over 800 m, from a point of release (Trpis & Hauserman, 1986; Reiter *et al*., 1995; Trpis *et al*., 1995; Muir & Kay, 1998). In addition a shortage of suitable oviposition sites has been shown to increase the dispersal of adult females (Edman *et al*., 1998). These results suggest that larval habitat reduction may encourage the dispersal of these vectors in their search for oviposition sites and thereby contribute to the spread of disease (Reiter *et al*., 1995; Edman *et al*., 1998).

Gravid females exhibit skip-oviposition behaviour, whereby they deposit eggs across several oviposition sites rather than at a single site (Corbet & Chadee, 1993). Consequently, a single container is likely to contain a mixture of sibling families representing multiple ovipositing females. The ability to estimate the number of families in a single container, as well as within and among surrounding containers, is an important component in understanding *Ae. aegypti* population behaviour. Previous efforts used multilocus RAPD allele matching to estimate the number of sibling families within individual oviposition sites in Puerto Rico (Apostol *et al*., 1994). In this study we explored the utility of a predictive method for sibling analysis, both within and among oviposition sites, based on pairwise relatedness coefficients calculated from alleles at codominant RFLP marker loci. The method was tested using known laboratory-reared sibling families of *Ae. aegypti* and then used to evaluate field collected samples from oviposition sites in Trinidad, West Indies.

Methods

Laboratory strain mosquitoes

To obtain RFLP genotypes from mosquitoes with known sibling relationships, strains of *Ae. aegypti* were outcrossed to create eight full-sibling families (Table 1). Test progeny were derived from pairwise matings of several combinations of five laboratory mosquito strains: Bronze, Formosus, Hamburg, Liverpool and RED; origins of these strains are described elsewhere (Severson *et al*., 1994). The eight full-sibling families included two sets of paternal half-sibling families. The rational for examining matings between related individuals, which provided a mixture of full-sib and half-sib families, is that it would better approximate a highly related local population at a natural breeding site. For each family, 12 full-sibling adults, six male and six female, were randomly chosen for analysis from each outcross (one of the sibling families, F1R5, was limited to six individuals) for a total of 90 individuals. Mosquitoes were reared in an environmental chamber at 26°C and 84% relative humidity and the adults maintained on a 5% sucrose solution. Females were isolated and allowed to oviposit individually and their progeny were reared to adults for genetic analysis. Female mosquitoes were blood fed on anaesthetized rats.

Table 1. Mating scheme for preparing full-sibling and half-sibling families.

Family	Male	Female
1	Bronze (B2)	Liverpool (L4)
2	Formosus (F1)	RED (R1)
3	Formosus (F1)	RED (R2)
4	Formosus (F2)	RED (R6)
5	Hamburg (H1)	RED (R2)
6	Hamburg (H1)	RED (R4)
7	Hamburg (H4)	RED (R3)
8	RED (R2)	Liverpool (L1)

DNA isolation and RFLP markers

To determine RFLP allele frequencies, DNA was extracted from individual adults, *Eco*RI-digested and Southern blotted to nylon membranes, and the membranes were probed with radiolabelled RFLP markers as previously described (Severson, 1997). A total of six RFLP markers were selected for analysis. A tightly linked pair of loci was chosen from each of the three chromosomes (Severson *et al*., 1993, 2002). These markers and their map position included: chromosome 1, B8L720 (19.4 cM) and LF198 (20.0 cM); chromosome 2, LF98 (27.2 cM) and LF409 (28.9 cM); and chromosome 3, LF168 (32.1 cM) and a12 (29.2 cM). Molecular weights of the individual RFLP fragments were estimated by comparison to λ-*Hind*III size markers included on each blot.

Relatedness measures and sibling discrimination

Pairwise relatedness coefficients were calculated using the Queller & Goodnight (1989) statistic, r_{xy}, which is an estimate of true relatedness that adjusts for sampling biases. The r_{xy} equation is:

$$r_{xy} = \frac{\sum_x \sum_k \sum_l (P_y - P^*)}{\sum_x \sum_k \sum_l (P_x - P^*)},$$

where x indexes individuals in the data set, k indexes loci, and l indexes allelic position. The variables in the ratio are:

P_x = the frequency within the current x individual of the allele found at x's locus k and allelic position l. This value in a diploid must be 0.5 or 1.0;
P_y = the frequency of that same allele in the individuals to which x's relatedness is being measured;
P^* = the frequency of the allele in the population at large, with all putative relatives of x excluded (bias correction option).

Additional bias correction can be achieved by excluding any putative relatives from the allele frequency calculations for the pair of individuals whose relatedness is being calculated. Population simulations and pairwise relatedness calculations were made using the KINSHIP and RELATEDNESS computer software (Goodnight & Queller, 1999). These simulations create virtual randomly mating populations, with individual genotypes constructed by choosing alleles from the observed allele frequency distribution. The simulated individuals are then used to generate distributions of expected pairwise relatedness values. For the simulated unrelated pairwise relatedness calculations, 1000 pairs of individuals were created with random parent genotypes (each individual is created from randomly chosen, unrelated, parents). Full-sibling pairwise relatedness calculations were made for 1000 pairs of individuals that share common parent genotypes. The distribution mean of the simulated unrelated and full-sib pairwise r_{xy} coefficients was calculated and the midpoint (mp) between both means was then used as the discriminating value for estimating sibling families (Blouin *et al*., 1996). This calculation was performed separately for each unique population of mosquitoes using their respective allele frequencies.

The laboratory mosquito families, including full-siblings and half-siblings, were grouped together to model a collection of individuals with unknown relatedness. Calculations of r_{xy} were performed and an unweighted pair-group method arithmetic average (UPGMA) cluster analysis of the pairwise r_{xy} matrix was used to create a dendrogram. Clusters of individuals with r_{xy} values greater than the discriminating value were grouped as sibling families.

To better simulate a field oviposition site and test the accuracy of the technique, 20 random samples of 20 individuals each were created from the original laboratory population. Calculations of r_{xy} were performed and the calculated mp value was then used to estimate the number of full-sibling families in each sample. Estimations were then compared to the actual number of families and the misclassification rate was determined. These randomized samples were also used to calculate a critical r_{xy} value for prediction of true full-sibling pairs. The probability of unrelated pairs clustering above the critical r_{xy} value was calculated by solving for α at the critical r_{xy} value with a one-tailed significance test using the unrelated pairwise r_{xy} distribution (Sokal & Rohlf, 1981).

Field collections in Trinidad

To estimate sibling families at field oviposition sites, *Ae. aegypti* larvae were collected from rural settlements on the island of Trinidad, West Indies. Mosquito larvae were collected from multiple containers at sites in Felicity, Flanigan Town and Malick (Fig. 1). The most common containers for oviposition were large water storage drums placed in close proximity to individual households. The observed water volumes were highly variable. A representative sample, consisting of second, third and fourth instar larvae, was collected from each container. Samples were collected during the dry season in February 1998 and March 1999. The collection sites shared similar environments for oviposition and larval development. Larvae were collected in vials, stored on ice and transported to the laboratory in St. Joseph, Trinidad where they were identified using standard taxonomic keys. All *Ae. aegypti* larvae were then preserved in 100% ethanol for transport to Notre Dame for genetic analysis.

The ethanol-preserved larvae were allowed to completely dry on filter paper and thereafter were subjected to DNA extraction as previously described. Individual larvae were genotyped at the same six RFLP loci used for the laboratory population analysis. Independent simulations, using RFLP allele frequencies for each unique population, were then used to calculate mp. Calculations of pairwise r_{xy} and subsequent UPGMA cluster analysis were used to estimate the number of sibling families at the field oviposition sites.

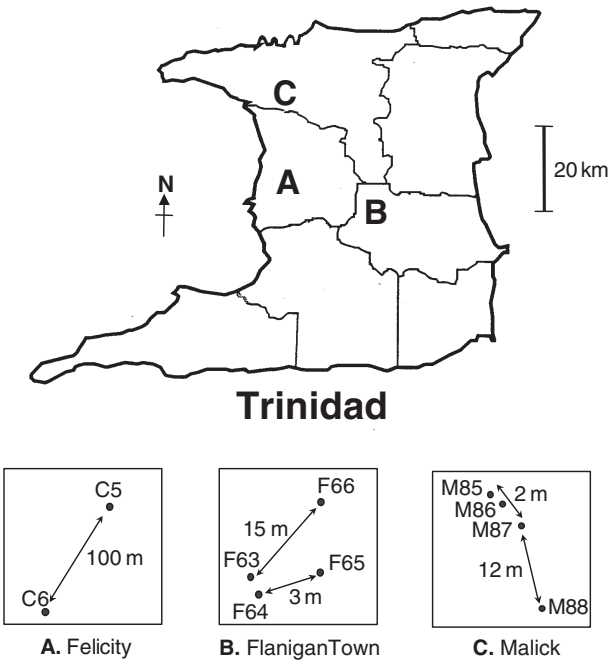

Fig. 1. Outline map of the Trinidad island showing the relative locations of mosquito collection sites: Felicity (A), Flanigan Town (B) and Malick (C). Details of individual collection sites show location of water containers and their relative distances in metres.

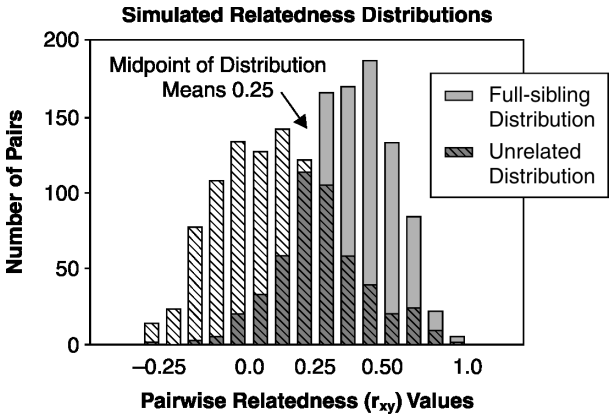

Fig. 2. Pairwise relatedness distribution of simulated full-sibling and unrelated populations generated using observed laboratory RFLP genotype data. The midpoint between the two distribution means is used as the discriminating value for sibling estimation.

Results

Laboratory populations

RFLP allele frequencies were calculated for eight full-sibling families including two sets of paternal half-sibling families. The number of alleles per locus ranged from six to 13 with an average of eight alleles across all loci (Table 2). For the simulated relatedness distributions, the mean r_{xy} values were 0 and 0.502 for unrelated and full-sib pairs, respectively (Fig. 2). The midpoint between the distribution means, 0.25, was established as the discrimination value for sibling family estimations. These distribution means, and the midpoint between the means, are as expected for average r_{xy} in random mating populations. The actual r_{xy} was calculated for all possible pairwise comparisons across families and a UPGMA dendrogram created. All calculations were made using the basic Queller & Goodnight (1989) algorithm that uses a bias-corrected value for the population allele frequencies. We also examined the effects of bias correction wherein putative relatives are excluded from allele frequency calculations, but found it had little effect on our estimations of sibling families. The actual average r_{xy} for the eight laboratory full-sibling families was 0.533, and the average r_{xy} for all unrelated individuals from the eight families was 0.055.

Sibling analysis was first applied to the four independent full-sibling families (e.g. excluding the half-sibling families), for a total sample size of 48 individuals. Individual members of all four families clearly grouped as discrete clusters, and three out of four families were correctly classified as full sibs

Table 2. Number of individuals tested (*n*), number of alleles per locus (*a*) and observed heterozygosity (*h*).

	Laboratory test families		Felicity			Flanigan			Malick		
RFLP markers	*n*	*a*	*n*	*a*	*h*	*n*	*a*	*h*	*n*	*a*	*h*
Chromosome 1											
B8L720	48	9	29	5	0.68	46	5	0.77	47	4	0.82
LF198	48	6	29	6	0.86	46	6	0.70	47	4	0.66
Chromosome 2											
LF98	48	7	29	5	0.65	46	5	0.82	47	4	0.77
LF409	48	6	29	3	0.20	46	1	na	47	2	0.33
Chromosome 3											
a12	48	7	29	6	0.79	46	5	0.80	47	4	0.72
LF168	48	13	29	8	0.86	46	6	0.53	47	4	0.54
Average		8		5.5	0.67		4.6	0.724		3.6	0.64

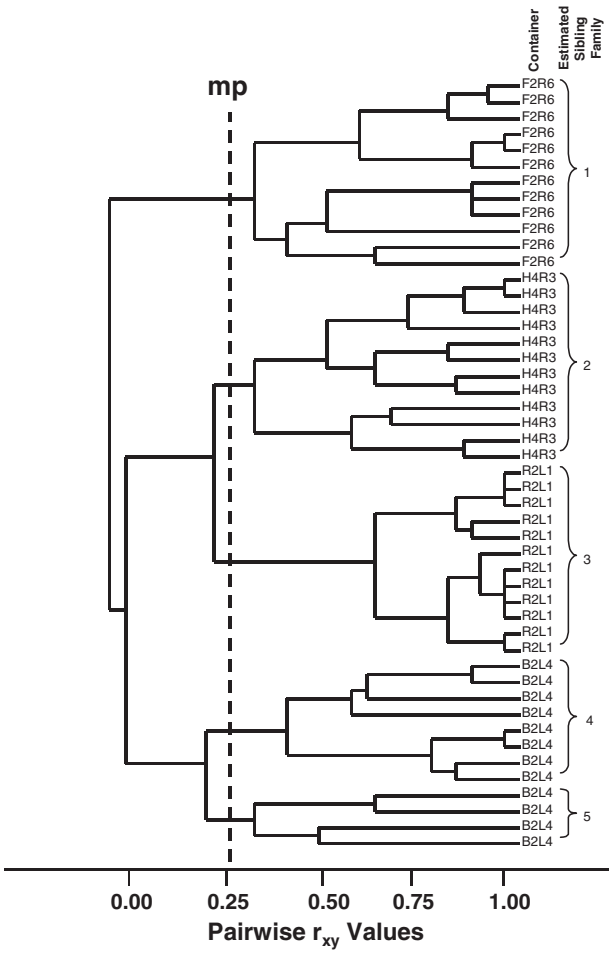

Fig. 3. Dendrogram of an UPGMA cluster analysis of 48 individuals included in the four independent full-sibling laboratory families. The discriminating value (mp) of 0.25 represents the cutoff for grouping clusters of individuals as sibling families.

using the calculated 0.25 mp value (Fig. 3). The fourth family (B2L4) was misclassified as consisting of two independent family groups. Misclassification was likely a consequence of the highly polymorphic nature of the parent individuals at the RFLP loci and the distribution of actual progeny genotyped. For example, the two parents were heterozygous for completely different alleles at two of the six loci. Most importantly, none of the individuals were falsely classified as siblings across families despite three families sharing a common genetic background (the RED strain provided one parent in each of these families).

Results from the combined analysis of the four independent full-sibling families and the two paternal half-sibling families (total families = 8), indicated that the method has difficulty discriminating among some such closely related groups (data not presented). Using the calculated mp value, a total of only six families were predicted, wherein three families were misclassified as a single family. Interestingly, the misclassification did not involve misclassification of either of the half-sibling family pairs as a single family, as they correctly clustered as four distinct groups. The analysis was, however, unable to discriminate among one of the half-sibling families (H1R2) and two of the full-sibling families (H4R3 and R2L1). Again, this likely relates to the common genetic background of the parents that produced these families. Still, the individual progeny for these three families did cluster as distinct groups, but at relatedness values greater than the calculated mp value. That is, inclusion of the half-sibling families resulted in inflated r_{xy} calculations for all individuals.

To simulate field collections, 20 samples of 20 individual mosquitoes were randomly selected from the full laboratory population and r_{xy} calculations were performed on each sample. Cluster analyses estimated an average of 6.3 families per sample, slightly less than the actual average of 7.4 families (Table 3). The actual number of individuals per family varied from one to nine. Only two samples overestimated the number of families, and 14 samples underestimated the actual number by one or two families. The percentage misclassified was calculated as the number of individual mosquitoes assigned to the wrong family among each population of 20 individuals. Misclassification percentages ranged from 10 to 30% with an average of 20.25. It appears that, although the mp value is suitable for estimations of sibling families at oviposition sites, it is not appropriate for establishing true full-sibling pairs. These 20 random populations were also used to establish confidence limits for a pairwise r_{xy} value that could be used to predict true sibling pairs. Examination of pairwise clustering showed that with an r_{xy} value of 0.67, only one pair of individuals clustered incorrectly (at 0.80 in family 11). Solving for α using a one-tailed test using the laboratory unrelated pairwise simulation distribution, at a critical value of 0.67, $t = 3.742$ and $\alpha = 0.0000962$. Therefore, greater than 99.99% of simulated unrelated pairs cluster at r_{xy} values less than 0.67. Moreover, inspection of the pairwise r_{xy} clustering results from the laboratory full/half-sibling family analysis ($n = 96$) revealed that there were no half-sibling pairs that clustered together above a 0.67 r_{xy} value, further supporting true sibling designation for pairs that cluster above 0.67 r_{xy}.

Trinidad field populations

For field populations, the average number of alleles per locus across all six RFLP loci was 4.6, ranging from one to eight alleles per locus (Table 2). The results from pairwise relatedness simulations and cluster analysis are shown in Table 4. For the Felicity site, 29 individuals from two oviposition containers separated by 100 m were analysed (Fig. 1A). Cluster analysis estimated a total of 11 families: nine families in C5 and seven families in C6 (Fig. 4). At the Flanigan Town site, 46 individuals from four oviposition containers within 15 m of each other were analysed (Fig. 1B). Cluster analysis estimated 12 families: one family in F63, 11 families in F64, three families in F65 and seven

Table 3. Twenty groups of 20 individuals selected at random from full/half-sibling families.

Group (n = 20)	Estimated families	Actual families	Actual family size			Percentage misclassified
			Average	Minimum	Maximum	
1	7	8	2.5	1	5	20
2	8	8	2.5	1	4	15
3	6	7	2.8	1	5	10
4	6	8	2.5	1	4	30
5	6	6	3.3	2	7	10
6	5	8	2.5	1	4	20
7	6	8	2.5	1	4	25
8	6	8	2.5	1	4	25
9	6	7	2.8	1	5	25
10	7	6	3.3	2	5	30
11	6	7	2.8	1	4	25
12	5	7	2.8	1	5	10
13	7	8	2.5	1	3	30
14	5	8	2.5	1	4	25
15	6	7	2.8	1	4	25
16	7	7	2.8	1	4	20
17	6	8	2.5	1	4	15
18	8	7	2.8	1	5	10
19	6	7	2.8	2	3	20
20	8	8	2.5	1	5	15
Average	6.35	7.4	2.7	1.15	4.4	20.25

families in F66 (Fig. 5). At the Malick site, 47 individuals from four oviposition containers within 14 m of each other were analysed (Fig. 1C). Cluster analysis estimated 12 families: seven families in M85, four families in M86, four families in M87 and nine families in M88 (Fig. 6). Additionally, of the 35 estimated families at the three sites, cluster analysis indicated that 19 families were distributed across multiple containers. Descriptions of the size and the volume of water in each container are shown in Table 5.

Noteworthy are pairs of individuals that cluster across containers and measure above $0.67\,r_{xy}$: individuals from families 1, 5, 6 and 7 at the Flanigan town site and individuals from families 3 and 8 at the Malick site (Figs 5 and 6). These pairs are likely true full-siblings and provide strong molecular evidence for skip-oviposition behaviour at a typical *Ae. aegypti* egg-laying site. At the collection site in Felicity (Fig. 4), family 7 included individuals from both containers C5 and C6 (100 m apart) which clustered at $0.64\,r_{xy}$, suggesting they are also true full-siblings.

Table 4. Trinidad mosquito population analysis. Number of individuals genotyped (*n*), number of containers (*ct*), unrelated distribution mean r_{xy} (um), full-sib distribution mean r_{xy} (*fsm*), midpoint of the means (*mp*), number of estimated families (*families*).

Population	*n*	*ct*	*um*	*fsm*	*mp*	*families*
Felicity	29	2	−0.013	0.49	0.26	11
Flanigan	46	4	0.002	0.49	0.25	12
Malick	47	4	0.006	0.48	0.24	12

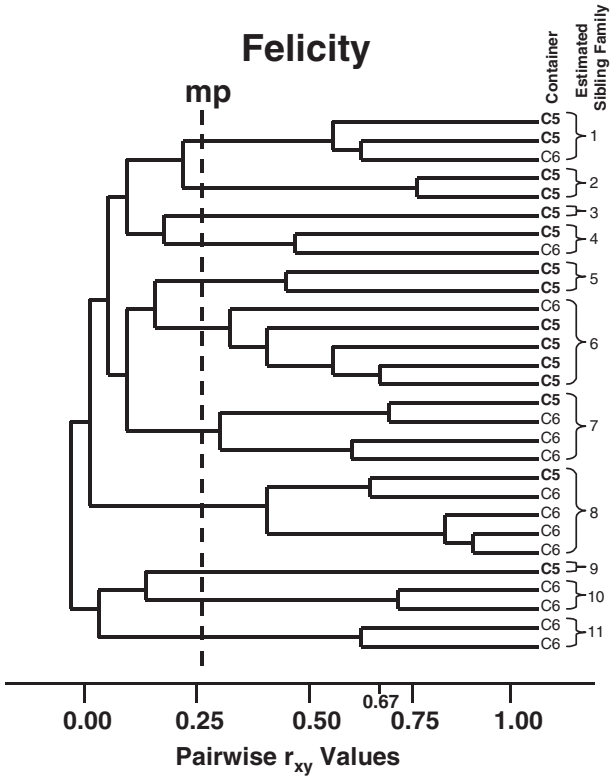

Fig. 4. Dendrogram of an UPGMA analysis of pairwise r_{xy} values from Felicity, Trinidad. Twenty-nine individuals from containers C5 and C6 were genotyped.

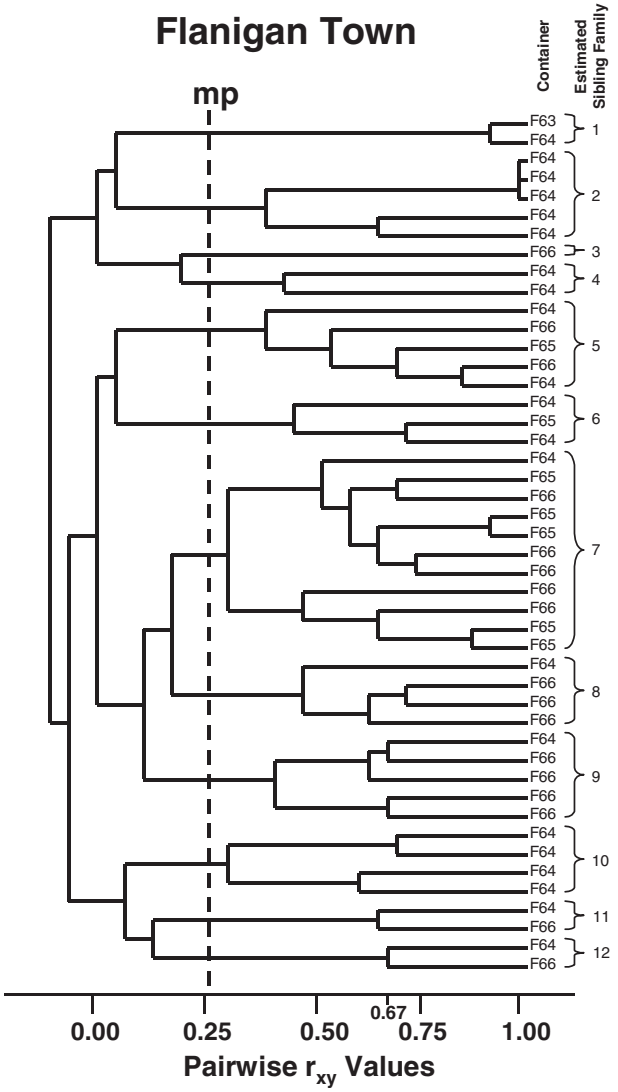

Fig. 5. Dendrogram of an UPGMA analysis of pairwise r_{xy} values from Flanigan Town, Trinidad. Forty-six individuals from containers F63, F64, F65 and F66 were genotyped.

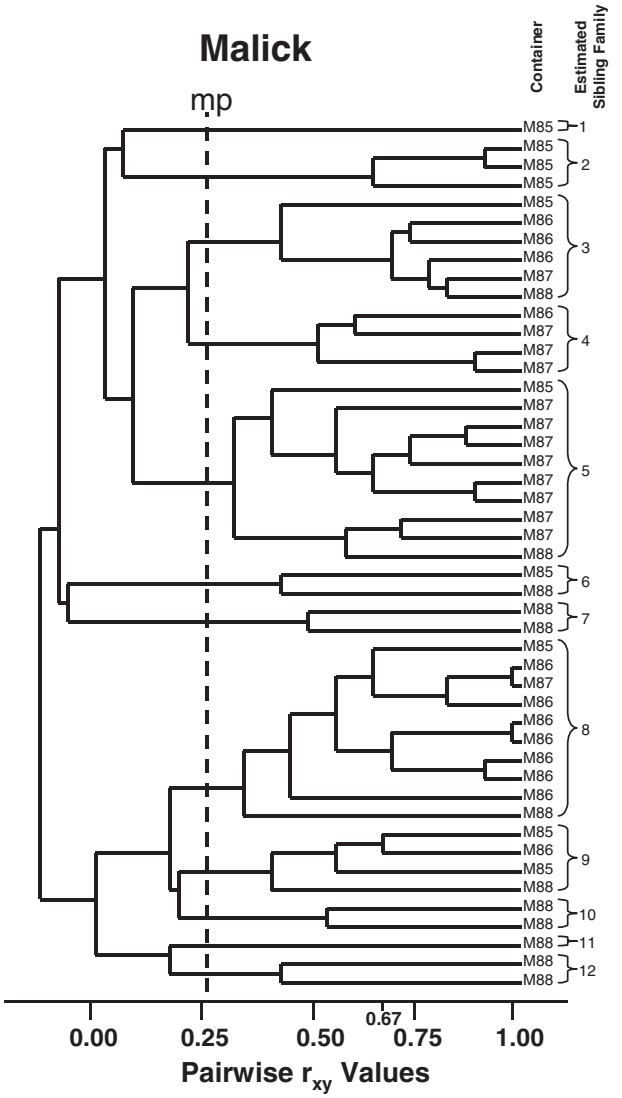

Fig. 6. Dendrogram of an UPGMA analysis of pairwise r_{xy} values from Malick, Trinidad. Forty-seven individuals from containers M85, M86, M87 and M88 were genotyped.

Discussion

This study describes a predictive method that estimated the number of sibling families at *Ae. aegypti* oviposition sites. Gravid females were found to deposit eggs across several oviposition sites, confirming earlier laboratory and field observations that a single oviposition container may contain a mixture of several families (Corbet & Chadee, 1993). The midpoint between the means of simulated pairwise relatedness distributions for unrelated and full-sibling

Table 5. Size and approximate water volume of the Trinidad oviposition containers from which larvae were collected prior to genotyping and cluster analysis. Description includes: volume of water (size and type of container) (see Fig. 1).

Felicity		Flanigan Town		Malick	
Container	Description	Container	Description	Container	Description
C5	220 L (900 L tank)	F63	180 L (180 L drum)	M85	100 L (1800 L tank)
C6	75 L (250 L drum)	F64	250 L (250 L drum)	M86	2 L (14 L bucket)
		F65	250 L (250 L drum)	M87	400 mL (4.5 L bucket)
		F66	250 L (250 L drum)	M88	50 L (250 L drum)

families was assigned as a discriminating value for resolving independent family groups (Blouin *et al*., 1996). In a related skip-oviposition study, Apostol *et al*. (1993), using alleles from 46 RAPD–PCR loci, estimated multiple *Ae. aegypti* sibling families in single oviposition containers. Lynch & Milligan (1994) suggested that the utility of multilocus markers for relatedness estimations, such as RAPD–PCR loci, is rather limited. Because of the dominant nature of RAPD–PCR, it is difficult to estimate allele frequencies with the accuracy necessary for relatedness calculations (Yan *et al*., 1999). Lynch & Milligan (1994) also showed that more loci and larger samples were necessary for RAPD loci to achieve the same confidence as RFLP or isozyme loci. The present results were derived from a set of highly polymorphic RFLP loci, which provide a more accurate estimate of allele frequencies and genotypic similarities among individuals within and across oviposition containers.

Using a set of only six RFLP loci, laboratory full-sibling mosquito families were clustered from non-sibling individuals (Fig. 3). This is quite remarkable given the initial degree of relatedness among the individual parents used to create test families (Table 1). Splitting of the B2L4 family into two clusters is attributable to the relatively high polymorphism at loci within this sibling family. The addition of paternal half-sibling families further complicated the background allele sharing, but true full-siblings continued to be clustered from their half-siblings. However, cluster analysis using the calculated mp value did create groups of distantly related individuals that were not true full-siblings. This is likely due to the historical relatedness of the laboratory strains and the small number of RFLP loci used in the study. One of the disadvantages of working with RFLPs is the limited amount of genotyping that can be performed on individual mosquitoes. In this study, these limitations were combined with the challenge of extracting and Southern blotting DNA from mosquito larvae. Sibling groups could be resolved with even greater fidelity by using an increased number of highly polymorphic markers. A future solution to these challenges would be the use of PCR-based codominant genetic markers, such as single-strand conformational polymorphisms (SSCP) (Fulton *et al*., 2001) or single nucleotide polymorphisms (SNPs) (Brookes, 1999), which would allow for more extensive genotyping and greater efficiency.

Cluster analysis was also performed on small, randomized, samples meant to simulate collections made from natural oviposition sites. For 20 samples the average estimated number of families was 6.3, whereas the true average was 7.4 families. Most of the randomized samples underestimated the number of sibling families and resulted in an average individual misclassification rate of 20.5% (measured as the ratio of individual misclassifications to the total population, Table 3). Apostol *et al*. (1993), using RAPD–PCR alleles from Puerto Rican *Ae. aegypti* populations, showed a misclassification rate of 14.4% (0–38.7%), with most misclassifications from overestimation of actual family numbers. A similar study using 20 microsatellite loci to cluster sibling families of mice had a 15–17% misclassification rate in discriminating half-sibs and full-sibs from unrelated individuals (Blouin *et al*., 1996). Although the current technique provides robust estimation of the number of sibling families, the relatively small number of highly polymorphic RFLP loci used limits our ability to establish true relatedness. However, further inspection of the randomized samples revealed that pairs of individuals that clustered at or above $0.67\,r_{xy}$ were consistently true full-siblings. For the distribution of the simulated pairwise r_{xy} comparisons, over 99.99% of the unrelated pairs showed values less than 0.67 ($t = 3.742$, $\alpha = 0.000096$). Therefore, pairs of individuals that cluster at or above $0.67\,r_{xy}$ are most likely true full-siblings.

This study provides genetic evidence for females laying eggs in multiple containers at Trinidad oviposition sites. Previous studies in Trinidad have shown that small egg batches are common at oviposition sites (Chadee, 1992). Artificial ovitraps were changed at weekly intervals for a year and the average number of eggs was 32 (range 1–337). These small family sizes may be explained by egg dispersal over a number of sites. Of the 35 estimated families at field collection sites in the present study, 19 were clusters of individuals from multiple containers. The relatively large container sizes utilized by females in our study indicate that skip-oviposition is not a function of small container size. The strongest evidence for skip-oviposition behaviour is provided by individuals that cluster across containers with r_{xy} values greater than 0.67. Using this stringent criterion, skip-oviposition was clearly evident at the two collection sites where containers for oviposition were relatively close (*c*. 2–15 m) and was likely at the site where the containers were separated by about 100 m. A distance of 100 m is not beyond the expected flight range of *Ae. aegypti* females, especially considering sample collections were made during the dry season when water containers are scarce. Reiter *et al*. (1995) found, by recovering rubidium marked eggs, that female *Ae. aegypti* deposited their eggs over a period of several days and distances up to 432 m (average of 181 m).

A recent study of *Ae. aegypti* egg laying behaviour in Thailand compared the number of developing eggs vs. mature eggs among resting indoor caught adult females (Harrington & Edman, 2001). If skip-oviposition does occur, one would expect to find gravid females that had laid some but not all of their mature eggs, which would cause a decrease in the average number of mature eggs compared with the average number of developing eggs. The samples from Thailand showed no significant differences between the mean immature and mature eggs per female, suggesting that skip-oviposition does not occur at sites in Thailand. However, several factors could have biased these results. The exclusive sampling of indoor resting females may have missed females that were in-between outdoor egg laying sites. The availability of outdoor oviposition sites may cause different egg laying behaviour than the oviposition-site-rich environment found indoors (Edman *et al*., 1998). The study was also unable to rule

out the possibility of single females ovipositing in multiple containers before returning to the indoor resting areas where the collections were made.

Finally, this technique also provides the potential for tracking unique genotypes through a population. Earlier studies (Bond *et al*., 1970; Hausermann *et al*., 1971) released laboratory strains of *Ae. aegypti*, with several phenotypic markers, in suburban Mississippi. They used phenotypic markers to observe dispersal of eggs laid by gravid females. They also measured the extent of laboratory strain males mating with wild females by collecting eggs up to nine weeks after release and examining the resulting adults for the presence of the phenotypic markers. Positive mating of laboratory reared males and females with native field *Ae. aegypti* was shown. After post-release egg collections from 160 ovitraps, Hausermann *et al*. (1971) reported 1.5% of the resulting adults were offspring of the previously released mosquitoes. The Mississippi studies suggest that a very well-characterized genotype, with unique alleles, could be introduced into a foreign population and genetically traced over time and space (genome-release-tracking). Codominant molecular markers could be used to analyse dispersal and introgression of a specific gene after the release of transgenic mosquitoes. This method should be valuable for predicting the success of releasing transgenic mosquitoes engineered to be refractory to pathogen transmission, and to test the efficiency of mechanisms that may be used to drive genes into populations of disease vectors (Curtis, 1994).

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