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Analysis of mosquito genome structure using graphical genotyping

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Abstract

Restriction fragment length polymorphism (RFLP) markers provide effective tools for establishing the genotype at many loci for individual mosquitoes. RFLP genotypes can be organized to provide information concerning parental origin or the allelic configuration of various chromosome segments. Whole genome genotypic information for individuals can be represented in a simple graphic format, termed graphical genotyping. In this study, RFLP markers were used to demonstrate the utility of using graphical genotyping to easily and quickly evaluate the entire genome of the mosquito *Aedes aegypti*. Two substrains selected for susceptibility and refractoriness, from a strain highly susceptible to the filarial worm parasite *Brugia malayi*, were compared. Graphical genotyping provided a mechanism to rapidly evaluate genome structure both within and between the two substrains with a simple visual analysis. This information was used in related studies to identify a new locus influencing *B. malayi* susceptibility in *A. aegypti*. Graphical genotyping also provided perspectives on some features of genome structure in *A. aegypti*. It suggested the presence and location of a genetic lethal on chromosome 3 and possible inversion polymorphisms associated with each of the three *A. aegypti* chromosomes.

Keywords: RFLP, genetic similarity, QTL, *Aedes aegypti*.

Introduction

A growing body of evidence suggests that mosquito vector competence to transmit various parasites and pathogens to their vertebrate hosts is not only under genetic control, but also is the consequence of the combined activities of several genes (Vernick *et al.*, 1989; Severson *et al.*, 1994a, 1995). The phenotypic expression of vector competence seems to reflect continuous or quantitative variation (see Severson, 1994). The development of DNA-based genetic marker technology has provided the tools necessary to partition quantitative traits into discrete genetic components, commonly designated as quantitative trait loci or QTL (Paterson *et al.*, 1988; Lander & Botstein, 1989). However, some difficulties remain in the statistical interpretation of QTL mapping data, most importantly in separating the effects of linked QTL and in determining an appropriate significance threshold for declaring a QTL. Although elegant statistical procedures have recently been developed to address these issues (Jansen, 1993; Jansen & Stam, 1994; Churchill & Doerge, 1994; Zeng, 1993, 1994), their efficacy for empirical data analysis has not been thoroughly demonstrated.

The relatively small genome size and haploid complement of three chromosomes, characteristic of most mosquito species (see Kumar & Rai, 1993), may represent a difficult challenge for QTL analysis. That is, these characteristics increase the probability that two or more QTL affecting a trait of interest will reside on a single chromosome. The uncertainty for the resolution of linked QTL can result in erroneous placement of QTL genome positions (Knott & Haley, 1992; Martinez & Curnow, 1992). This obviously can impede map-based cloning efforts in mosquitoes.

We previously described construction of a genetic linkage map for the mosquito *Aedes aegypti*, based on restriction fragment length polymorphism (RFLP) markers (Severson *et al.*, 1993). These RFLP markers were subsequently used to identify QTL affecting susceptibility of *A. aegypti* to the filarial worm parasite *Brugia malayi* (Severson *et al.*, 1994a) and the malarial parasite *Plasmodium gallinaceum* (Severson *et al.*, 1995).

RFLP markers also provide a mechanism to compare genetic variation within and among mosquito populations (Severson *et al.*, 1994b). RFLP markers can be used to determine the genotypes of individual mosquitoes at many loci distributed along the entire genome. The RFLP genotypes can be organized to provide, for example, information concerning parental origin or the allelic configuration of various chromosome segments. These data are most amenable to representation in a simple graphic format, termed graphical genotyping (Young & Tanksley,

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Table 1. Average heterozygosity of G_{14} LVP^{sbm} and LVP^{rbm} substrain *Aedes aegypti* females.

	No. of RFLP loci examined	LVP^{sbm}	LVP^{rbm}
Chromosome 1	7	0.27	0.20
Chromosome 2	9	0.03	0.30
Chromosome 3	7	0.29	0.33
Total	23	0.18	0.28

1989). That is, whole genome genotypic information for individual mosquitoes can be easily visualized graphically.

The concept of graphical genotyping should be applicable to comparisons of genome structure between individuals from populations reflecting contrasting phenotypes (i.e. refractory versus susceptible to a particular parasite). It would provide a rapid method to identify chromosome regions reflecting population-specific allelic configurations. That is, if we obtain genome-wide genotypic information for individual mosquitoes in populations for which we have *a priori* information concerning an expected phenotype, we may be able to use this information to: (1) provide indirect evidence supporting putative QTL locations; and (2) identify other genome regions for further study that also may have an influence on the phenotype. In this paper we demonstrate the use of graphical genotyping to examine genome structure in *A. aegypti* and to gain additional insight into the genetic basis for *A. aegypti* susceptibility to *B. malayi*.

Results

We determined genotypes at twenty-three RFLP loci for fifty LVP^{sbm} females and forty-eight LVP^{rbm} females. A total of forty-nine different alleles were identified for the twenty-three loci. However, only twenty-two alleles (46%) were common to both substrains; fifteen alleles were unique to the LVP^{rbm} substrain and twelve alleles were unique to the LVP^{sbm} substrain. The average heterozygosity was lower in the LVP^{sbm} substrain, apparently due to fixation of alleles at most loci on chromosome 2 (Table 1). Most loci met Hardy-Weinberg expectations with each substrain. However, two genome regions reflected significant segregation distortion in the LVP^{rbm} substrain: (1) one end of chromosome 2 defined by the LF115 locus, and (2) a region on chromosome 3 defined by the LF168, a12 and LF106 loci (Table 2).

Graphical genotypes for all individuals examined in both substrains are shown in Fig. 1. This simple method illustrates the complete genome for each mosquito and provides easily interpretable information for comparing the two substrains. Of particular interest to our understanding of vector competence, the substrains are essentially fixed for identical alleles at only two loci across the entire genome: (1) at the LF178 locus on chromosome 1 and (2) at the

Table 2. Allele frequencies among G14 LVP^{sbm} and LVP^{rbm} substrain *Aedes aegypti* females (marker loci are arranged in chromosomal order).

	LVP^{sbm}		LVP^{rbm}		
Marker	Pa_1	Pa_2	Pa_1	Pa_2	Pa_3
Chromosome 1					
LF235	0.71	0.29	0.37	0.64	—
LF188a	0.84	0.16	—	0.40	0.61
LF230	0.69	0.31	—	0.40	0.61
LF198	0.71	0.29	—	1.00	—
LF178	1.00	—	0.96	—	0.02
LF204	0.92	0.08	0.02	0.98	—
TY7	0.80	0.20	0.01	0.99	—
Chromosome 2					
ARC1	0.14	0.86	0.61	—	0.40
LF138	1.00	—	—	—	1.00
LF357	1.00	—	0.52	—	0.48
LF180	1.00	—	—	—	1.00
LF282	1.00	—	0.21	0.12	0.68
LF98	1.00	—	1.00	—	—
VCP	1.00	—	—	—	1.00
LF250	1.00	—	—	—	1.00
LF115	1.00	—	0.50*	—	0.50
Chromosome 3					
LF352	0.48	0.52	—	1.00	—
LF261	0.56	0.44	—	1.00	—
LF168	1.00	—	0.55*	—	0.45
a12	1.00	—	0.55*	—	0.45
LF106	1.00	—	0.58*	—	0.42
LF227	0.45	0.55	—	1.00	—
LF347	0.63	0.37	—	1.00	—

*$P<0.01$. Allele frequencies at each locus were assessed for expected Hardy-Weinberg equilibria.

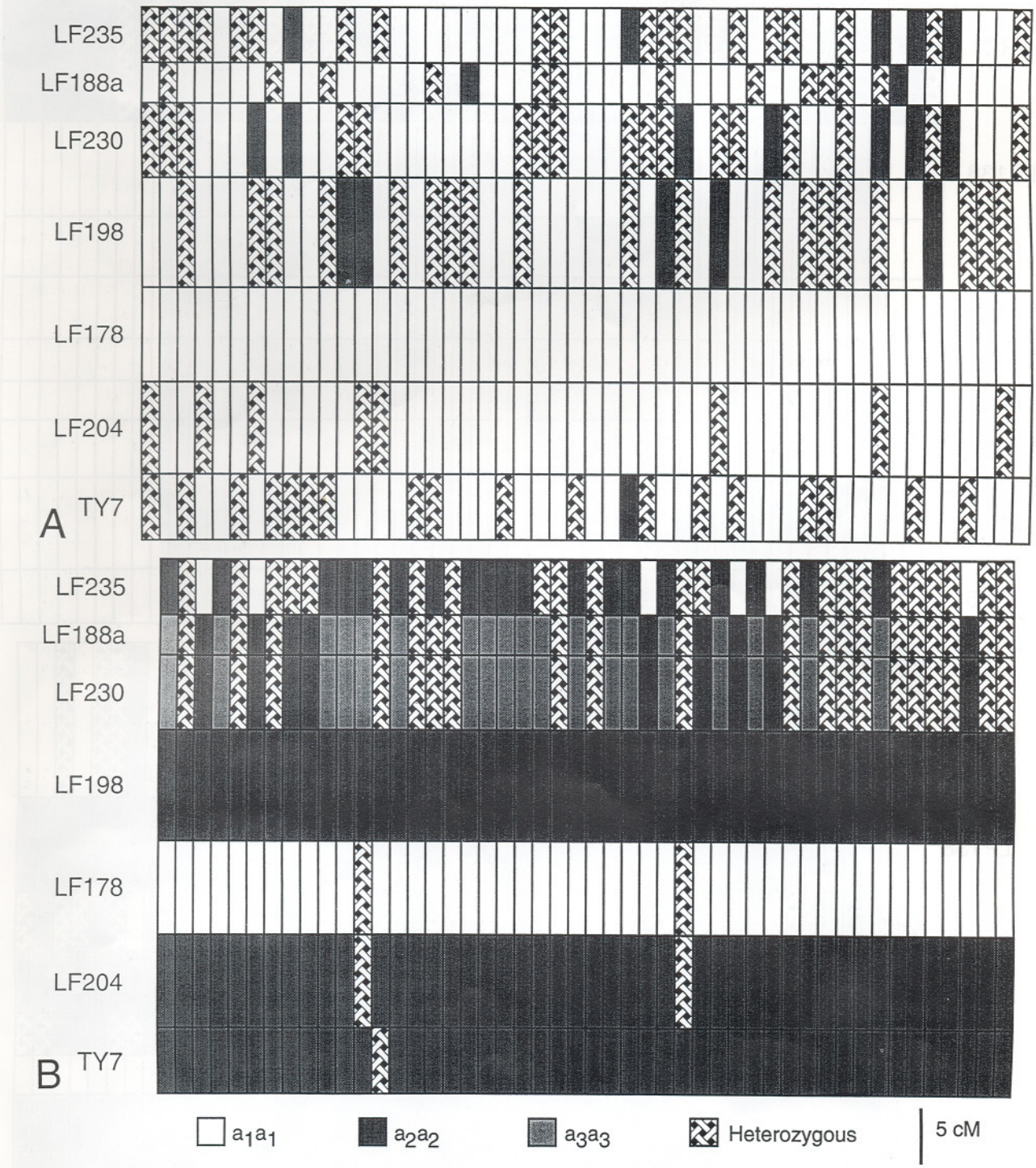

Figure 1. Graphical genotypes for individual mosquitoes representing the LVP^{sbm} and LVP^{rbm} substrains of *Aedes aegypti*. (A) chromosome 1 for LVP^{sbm}; (B) chromosome 1 for LVP^{rbm}; (C) chromosome 2 for LVP^{sbm}; (D) chromosome 2 for LVP^{rbm}; (E) chromosome 3 for LVP^{sbm}; and (F) chromosome 3 for LVP^{rbm}. 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.

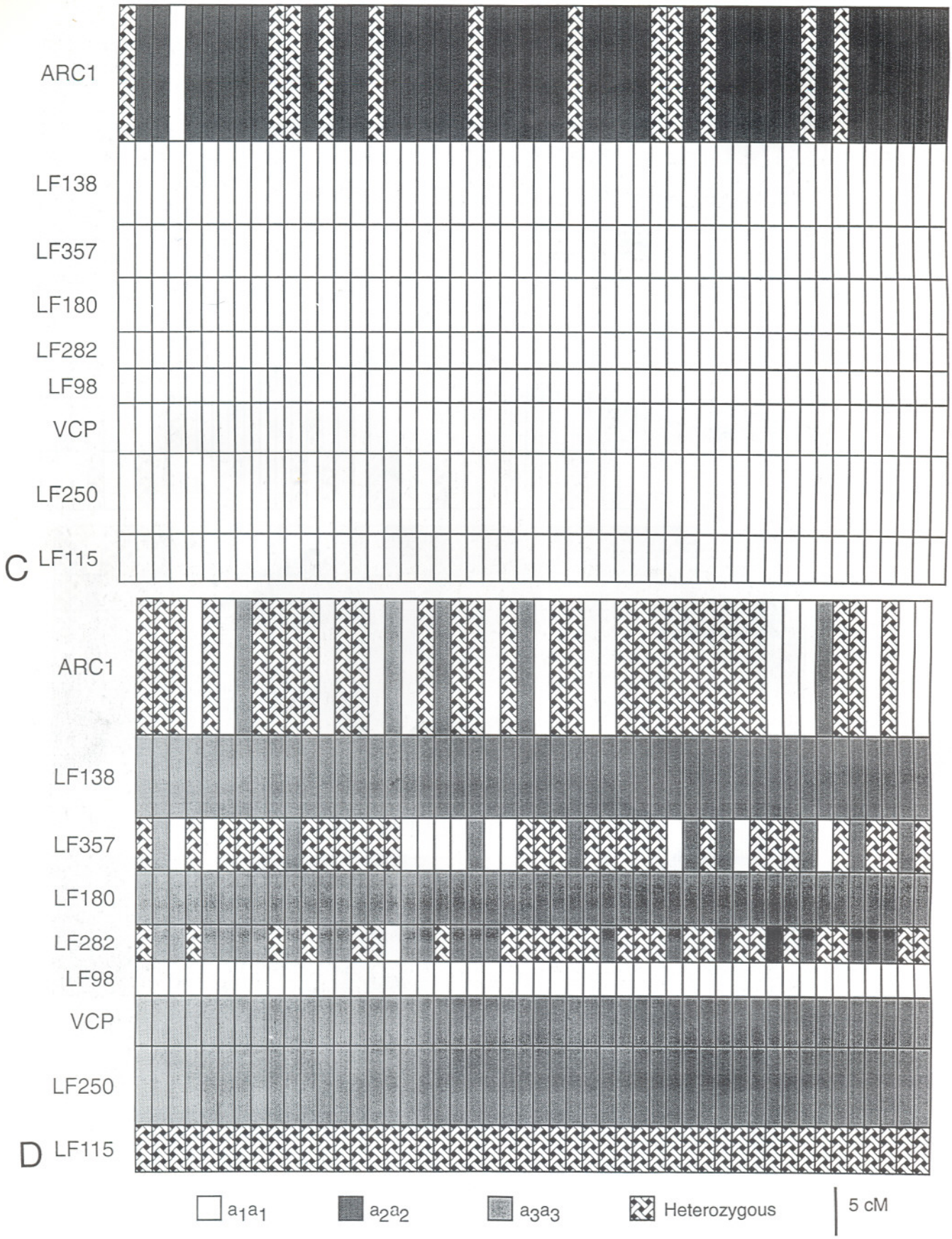

Figure 1 (*continued*).

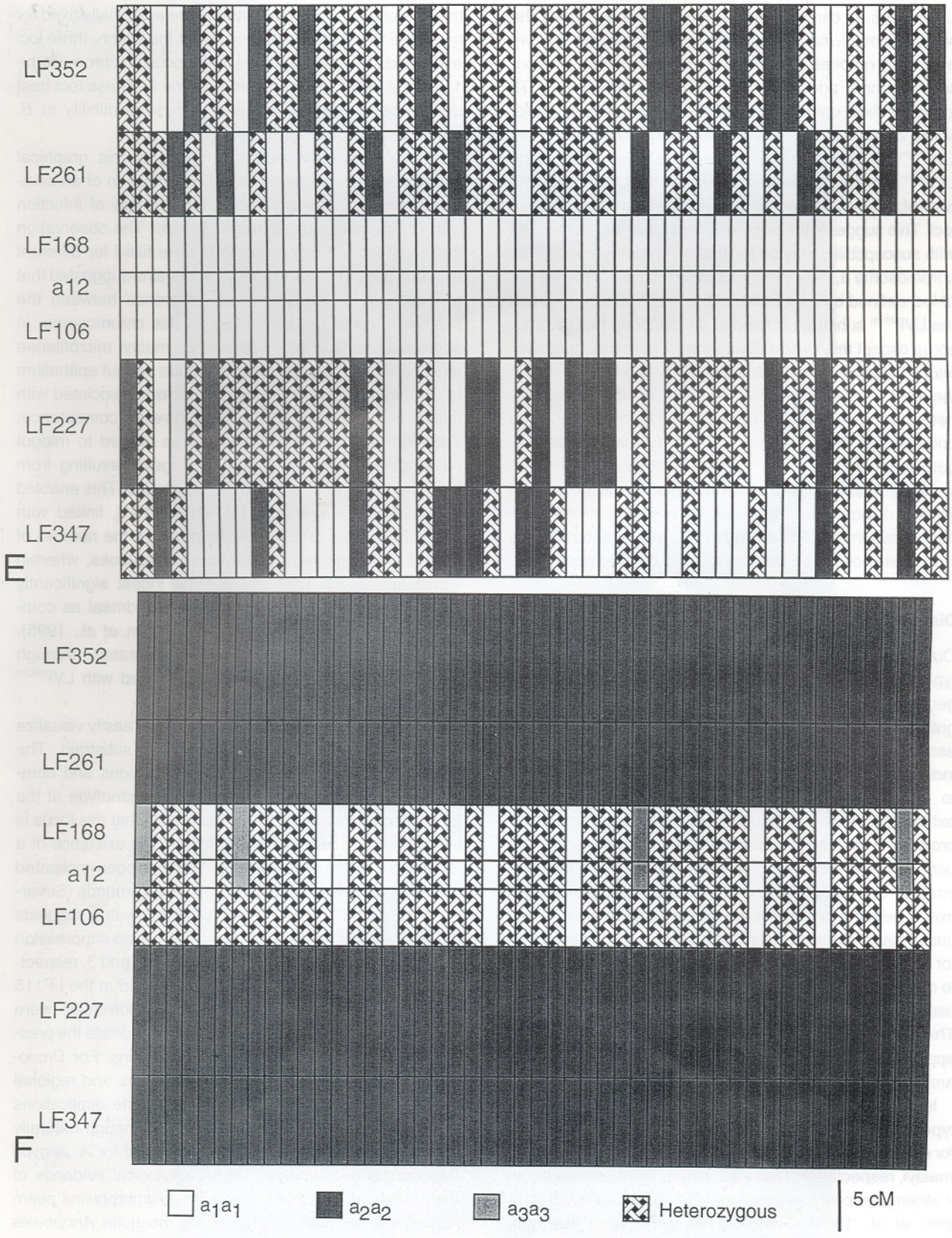

Figure 1 (*continued*).

LF98 locus on chromosome 2. These loci best define the two QTL for *B. malayi* susceptibility in *A. aegypti* that we previously reported (Severson *et al.*, 1994a). With chromosome 1, three additional loci contiguous with the LF178 locus are fixed only in the LVP^{rbm} substrain. With chromosome 2, eight of the nine loci examined are fixed in the LVP^{sbm} substrain and five of these nine loci are fixed in the LVP^{rbm} substrain; however, outside of the LF98 locus, the two substrains are fixed for different alleles at each of these loci. This suggests the possibility that other loci associated with susceptibility may be located on chromosome 2. With chromosome 3, the LVP^{sbm} substrain is fixed at the cluster of loci defined by the LF168, a12 and LF106 loci, whereas the LVP^{rbm} substrain is fixed at all loci along this chromosome except this cluster. Evidence for nonrandom associations between some alleles and loci was observed only in the LVP^{rbm} substrain. For example, all individuals are heterozygous at the LF115 locus on chromosome 2 and only heterozygotes and one of the two expected homozygote genotypes are evident at the LF106 locus on chromosome 3. In addition, two genome regions reflect complete linkage disequilibrium because no recombination is evident between the LF188a and LF230 loci on chromosome 1 and between the LF168 and a12 loci on chromosome 3.

Discussion

Our results demonstrate the utility of using graphical genotyping to easily and quickly evaluate the entire *A. aegypti* genome. Young & Tanksley (1989) demonstrated that graphical genotyping allowed for rapid and concise assessment of data for large numbers of markers for individuals in a population. We have expanded this concept to include whole genome comparisons of populations exhibiting contrasting phenotypes. Graphical genotyping provides a method for formulating hypotheses about the genetic basis for vector competence in mosquitoes. It enables one to rapidly identify putative associations between genome segments and vector competence. Obviously, these associations alone are not sufficient evidence for genetic relationships. However, these data can be used to corroborate results from QTL mapping studies or can be used to assist in establishing QTL mapping populations. This strategy may prove particularly useful for designing appropriate crosses to resolve instances where linked QTL are suspected.

In this study we were able to compare graphical genotypes of mosquitoes representing two substrains selected for refractoriness (LVP^{rbm}) and susceptibility (LVP^{sbm}) to *B. malayi*, respectively. These substrains were selected from a strain previously selected for high susceptibility (Beerntsen *et al.*, 1995). Therefore we anticipated that both substrains would be fixed for alleles conferring susceptibility at one or more loci. Our present results seem to support this contention; the two substrains are essentially fixed for the same RFLP alleles at only two of the twenty-three loci examined. This includes the LF178 locus on chromosome 1 and the LF98 locus on chromosome 2. These loci best define the two QTL associated with susceptibility to *B. malayi* (Severson *et al.*, 1994a).

We have already used the results of this graphical genotyping effort to assist in the identification of an additional genetic factor influencing the intensity of infection with *B. malayi* in susceptible *A. aegypti*. The observation that most loci on chromosome 2 were fixed for different alleles in the LVP^{sbm} and LVP^{rbm} substrains suggested that at least part of the phenotypic difference between the substrains could be due to loci on this chromosome, in addition to fsb[2,LF98]. Because *B. malayi* microfilariae must penetrate and cross the mosquito midgut epithelium to complete development, genetic factors associated with midgut penetration may play a role in vector competence. Therefore we examined some factors related to midgut penetration among F_2 intercross progeny resulting from crossing the LVP^{sbm} and LVP^{rbm} substrains. This enabled us to identify a second QTL, idb[2,LF181], linked with fsb[2,LF98]. This QTL seems to influence the number of microfilariae ingested by individual mosquitoes, wherein individuals of the LVP^{rbm} genotype ingest significantly fewer microfilariae while obtaining a bloodmeal as compared to the LVP^{sbm} genotype (Beerntsen *et al.*, 1995). Additionally, fewer microfilariae penetrated through midguts from LVP^{rbm} individuals compared with LVP^{sbm} individuals.

Graphical genotyping also allowed us to easily visualize some genetic anomalies in the LVP^{rbm} substrain. The departure from Hardy-Weinberg expectations and complete lack of one expected homozygote genotype at the LF106 locus on chromosome 3 suggests that this locus is linked with a recessive genetic lethal. The existence of a lethal locus in this genome region also has been implicated with crosses involving other genetic backgrounds (Severson *et al.*, 1995). Further, graphical genotyping suggests the existence of some form of recombination suppression in two genome regions on chromosomes 1 and 3, respectively. Also, a duplication event is suggested at the LF115 locus on chromosome 2, because all individuals were heterozygous. These observations could indicate the presence of inversions in these genome regions. For *Drosophila*, the relationships between inversions and regional recombination suppression as well as gene duplications are well known (see Burnham, 1980). Although naturally occurring inversions have been suggested for *A. aegypti* (Macdonald & Sheppard, 1965), cytological evidence of their presence is lacking. Inversion polymorphisms seem responsible for maintenance of the mosquito *Anopheles gambiae* as a complex of six sibling species (Coluzzi *et al.*, 1985).

Graphical genotypes also are amenable to expression as actual physical segments of DNA (Young & Tanksley, 1989). This could be particularly useful for mosquito species, such as *A. aegypti*, for which polytene chromosome preparations are consistently of poor quality. An active effort to develop a physical map for *A. aegypti*, using fluorescence *in situ* hybridization (FISH) mapping techniques (Brown *et al.*, 1995), should provide the basis for the integration of physical and genetic linkage data.

Experimental procedures

Mosquito strains

Two *A. aegypti* substrains derived from the black-eyed Liverpool (LVP) strain were compared in this study. Details for the selection of these substrains were described previously (Beerntsen *et al.*, 1995). Briefly, the LVP strain was initially selected for high susceptibility to *B. malayi* and used in seminal studies to demonstrate a genetic basis for parasite susceptibility in mosquitoes (Macdonald, 1962, 1963a, b). The substrains were selected for contrasting phenotypes in *B. malayi* susceptibility from this LVP strain with three generations of selective inbreeding. That is, one substrain, LVP^{sbm}, was selected for very high susceptibility (e.g. increased prevalence and intensity of infection) and the second substrain, LVP^{rbm}, was selected for refractoriness (e.g. very low prevalence of infection). Following selection, the substrains were each maintained as random mating laboratory colonies. In this study we examined generation 14 (G_{14}) following the initial substrain selections from the LVP strain.

DNA isolation, Southern blotting, and probe hybridization

DNA was extracted from newly eclosed female mosquitoes representing G_{14} for both the LVP^{sbm} and LVP^{rbm} substrains. Individual mosquitoes were homogenized in a lysis buffer (0.5% $NaDodSO_4$, 0.2 M NaCl, 25 mM EDTA, 10 mM Tris, pH 8.0) containing 125 ng/μl RNase A and incubated at 37°C for 60 min. The homogenate was then digested with Proteinase K at 0.5 μg/μl at 50°C for 60 min, followed by a single extraction with an equal volume of phenol:chloroform (1:1). The supernate was precipitated in ethanol and the purified DNA resuspended in TE, pH 8.0. The individual mosquito DNA preparations were digested with *Eco*RI essentially as recommended by the supplier (Promega), except that 4 mM spermidine was added. Restriction fragments were size-fractionated on 0.9% agarose gels at 1.8–2.0 V/cm. Subsequent gel preparations and transfer to Hybond N^+ nylon membranes (Amersham) were as previously described (Severson *et al.*, 1993).

Prior to the initial hybridization, membranes were incubated in a pre-blocking solution (2% $NaDodSO_4$, 0.5% BSA, 1 mM EDTA, 1 mM orthophenanthroline) for 3–5 h at room temperature with gentle shaking. Prehybridization, hybridization and membrane washes were conducted at 65°C in glass bottles in a rotating oven (Hybaid) as previously described (Severson *et al.*, 1993). Inserts for probe synthesis were generated from transformed bacterial cultures using SP6 and T7 primers in a polymerase chain reaction (PCR) as previously described (Severson *et al.*, 1993). Probe DNA was radiolabelled using PCR to a specific activity of about 1.0–1.6 $\times 10^9$ cpm/μg. Each PCR mixture (total volume of 50 μl) contained reaction buffer (Promega), 1.5 mM $MgCl_2$, 200 μM dATP, dGTP, dTTP, 50 μCi α^{32}P-dCTP (Dupont), 0.12 μM each of SP6 and T7 primers, 1 unit of Taq DNA polymerase (Promega), and 50 ng of PCR-derived insert DNA. The reaction mixtures were subjected to one cycle of PCR with 2 min at 94°C, 3 min at 50°C, and 5 min at 72°C. Free dNTPs were separated by column chromatography through Biogel P60 (Bio-Rad) equilibrated in elution buffer (0.5% $NaDodSO_4$, 1 mM EDTA, 5 μg/ml Torula yeast RNA, 20 mM Tris, pH 7.4). Kodak XAR-5 film was exposed to membranes at −80°C with an intensifying screen. Membranes were regenerated for additional hybridizations by incubation in 0.5 N NaOH at 42°C for 30 min followed by neutralization (0.1 × SSC, 0.1% $NaDodSO_4$, 0.2 M Tris, pH 7.4) at 42°C for 30 min.

Probe selection and data analysis

Membranes carrying genomic DNA from individual mosquitoes were probed with a set of clones of known chromosomal position (Severson *et al.*, 1993, 1994a, 1995, and unpublished data). Twenty-three clones were selected for analysis that provide coverage of the entire *A. aegypti* genome, with an average resolution of 7.2 cM. All fragments detected by Southern analysis had previously been shown to represent unique alleles for each probe. The average heterozygosity within substrains and among chromosomes within substrains was calculated as described by Weir (1990) as the average of the observed heterozygote frequencies across the loci examined. Allele frequencies were calculated for each locus within each substrain and evaluated for Hardy-Weinberg equilibrium (Weir, 1990). Graphical genotypes for each individual in both substrains were prepared essentially as described by Young & Tanksley (1989) using CorelDraw.

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