Targeted identification of markers linked to malaria and filarioid nematode parasite resistance genes in the mosquito *Aedes aegypti*

DAVID W. SEVERSON*, DAVID ZAITLIN† AND VICTORIA A. KASSNER

Department of Animal Health and Biomedical Sciences, 1655 Linden Drive, University of Wisconsin, Madison, WI 53706, USA

(*Received 31 August 1998 and in revised form 17 December 1998*)

Summary

Quantitative trait loci (QTL) have been identified for competence of the mosquito *Aedes aegypti* to transmit the avian malaria parasite *Plasmodium gallinaceum* and the human filarial parasite *Brugia malayi*. Efforts towards the map-based cloning of the associated genes are limited by the availability of genetic markers for fine-scale mapping of the QTL positions. Two F2 mosquito populations were subjected to bulked segregant analysis to identify random amplified polymorphic DNA (RAPD)-PCR fragments linked with the major QTL determining susceptibility to both parasites. Individual mosquitoes for the bulks were selected on the basis of their genotypes at restriction fragment length polymorphism (RFLP) loci tightly linked with the QTL. Pool-positive RAPD fragments were cloned and evaluated as RFLP markers. Of the 62 RAPD/RFLP fragments examined, 10 represented low-copy number sequences. Five of these clones were linked with the major QTL for *P. gallinaceum* susceptibility (*pgs1*), of which one clone mapped within the flanking markers that define the QTL interval. The remaining five clones were linked with the major QTL for *B. malayi* susceptibility (*fsb1*), and again one clone mapped within the flanking markers that define the QTL interval. In addition, nine RAPD/RFLP fragments were isolated that seem to be of non-mosquito origin.

1. Introduction

Malaria and lymphatic filariasis are both parasitic diseases with an increasing prevalence worldwide, particularly in the developing tropics (World Health Organization, 1997). Malaria remains a major cause of morbidity and mortality in 90 countries or territories, resulting in 1·5–2·7 million deaths per year, mainly among children less than 5 years of age. Lymphatic filariasis affects 120 million people and remains a leading cause of long-term and permanent disabilities in 73 endemic countries. Current efforts to limit or prevent transmission of these diseases are increasingly hampered by a number of factors. First, efforts to develop effective vaccines against these parasites remain remarkably disappointing, and are unlikely to achieve success in the foreseeable future. Secondly, the emergence of antimalarial drug resistance among *Plasmodium*, the causal organism for malaria, has reduced the effectiveness of drugs as prophylactic and treatment agents. Thirdly, deteriorating socioeconomic conditions in many endemic areas have resulted in the collapse of once-effective disease monitoring and control programs. Fourthly, mosquito control efforts are increasingly reduced through the loss of established mosquito control programs and the rapid development of insecticide resistance in mosquito populations. However, because the transmission to humans of both parasites is totally dependent upon the availability of competent mosquito vectors, mosquito control remains the most successful mechanism for disease prevention.

An alternative strategy for controlling mosquito-borne diseases would involve the genetic manipulation of mosquito populations to reduce their effectiveness as disease vectors (Eggleston, 1991; Collins & Besansky, 1994). The development of contemporary

* Corresponding author. Present address: Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556-5645, USA.
† Present address: AviGene Services Inc., 565 Science Drive, Madison, WI 53711, USA.

molecular markers for mosquitoes provides the tools to partition complex genetic traits, such as vector competence, into discrete Mendelian components (Severson, 1994). The mosquito *Aedes aegypti*, while the primary vector for yellow fever and dengue fever viruses, also provides an excellent system for studying the genetic basis for the transmission of the avian malaria parasite *Plasmodium gallinaceum* (Kilama & Craig, 1969) and the human filarial parasite *Brugia malayi* (Macdonald, 1962*a*, 1963). A linkage map based on restriction fragment length polymorphisms (RFLP) has been developed for *A. aegypti* (Severson et al., 1993), and these markers have been used to identify quantitative trait loci (QTL) that determine vector competence for both parasites. Two QTL determining *P. gallinaceum* susceptibility have been identified (Severson et al., 1995). One QTL, *pgs*[*2*,*LF98*] or *pgs1*, has a major effect, accounting for 49–65% of the observed phenotypic variance in susceptibility. A second QTL, *pgs*[*3*,*MalI*] or *pgs2*, has a minor effect, accounting for 10–14% of the observed phenotypic variance. Two QTL determining *B. malayi* susceptibility have also been identified (Severson et al., 1994*a*). One QTL, *fsb*[*1*,*LF178*] or *fsb1*, has a major effect, accounting for 22–43% of the observed phenotypic variance. A second QTL, *fsb*[*2*,*LF98*] or *fsb2*, has a minor effect, accounting for 3–16% of the observed phenotypic variance. These QTL could, therefore, represent potential candidate genes as targets for genetic manipulation of mosquito populations.

Identification of map positions for genes determining parasite susceptibility provides an initial framework for efforts to isolate the associated genes using established map-based cloning procedures (Rommens et al., 1989; Martin et al., 1993). The next step is to saturate the genome regions defining QTL with additional markers in order to narrow the physical distance that must be resolved. Bulked segregant analysis provides a rapid and efficient method for identifying markers linked with a target phenotype (Michelmore et al., 1991) or genotype (Giovannoni et al., 1991). Briefly, DNA samples from individual segregants, previously characterized for a trait of interest, are used to prepare bulked pools representing both states of the trait. The two pools will reflect DNA polymorphisms associated with the genome region around the trait, but will be monomorphic at all other areas of the genome. Markers reflecting pool-specific polymorphisms are, therefore, linked with the trait and can be used for fine-scale QTL mapping.

The present study was conducted to determine the utility of bulked segregant analysis for targeted marker development within the major QTL determining *P. gallinaceum* (*pgs1*) and *B. malayi* (*fsb1*) susceptibility in *Aedes aegypti*. DNA pools based on RFLP genotypes of markers defining the QTL were screened using random amplified polymorphic DNA (RAPD)-PCR analysis (Williams et al., 1990). Because of the dominant inheritance of most RAPD markers and the use of largely outcrossing mosquito populations for genetic studies, pool-specific RAPD fragments were cloned and evaluated as RFLP markers.

2. Materials and methods

(i) *Mosquitoes*

Segregating F2 intercross progeny for bulked segregant analysis were produced by pairwise matings between *Aedes aegypti* LIV[SD] strain females and MOYO strain males. The LIV[SD] strain had been selected for high susceptibility to *Dirofilaria immitis* from the filarial worm susceptible Liverpool strain originally selected at the London School of Hygiene and Tropical Medicine (Macdonald, 1962*b*). The MOYO strain originated from the Mombasa district in Kenya. Both strains had been laboratory-reared for an indefinite number of generations. Two independently generated populations were studied. Each population consisted of a mixture of full and half-sib families. Additional mapping populations included Hamburg (HAM) strain by MOYO strain F2 intercross progeny and formosus (FOR) strain by RED strain F2 backcross progeny. Other strains used in screens for polymorphisms included: Liverpool (LIV), *D. immitis* refractory Liverpool (LIVRD), *B. malayi* high susceptibility Liverpool (LIV[SB]), *B. malayi* refractory Liverpool (LIV[RB]), bronze (BZ), BLPCOFZMIN (MIN) and DLSI (SIL). The origins of these strains are described elsewhere (Miller & Mitchell, 1991; Severson et al., 1994*b*; Beernsten et al., 1995). Mosquitoes were reared as previously described (Christensen & Sutherland, 1984). Also included in screens for polymorphisms were the mosquitoes *Anopheles gambiae* (Arr strain), *Culex pipiens pipiens* (Iowa), *C. p. pipiens* (Nile Delta), *C. p. molestus* as well as *Drosophila melanogaster*.

(ii) *Selective genotyping using RFLP analysis*

DNA extraction from individual mosquitoes, digestion with *Eco*RI, Southern blotting and hybridizations were performed as previously described (Severson, 1997). The two bulked segregant analysis populations, SDAM1 and SDAM2, consisted of 99 and 191 individuals, respectively. All individuals of the SDAM1 population were initially genotyped at the LF178 and LF198 loci, that flank the *fsb1* QTL (Severson et al., 1995) and at the LF98 and LF282 loci that flank the *pgs1* QTL (Severson et al., 1994*a*).
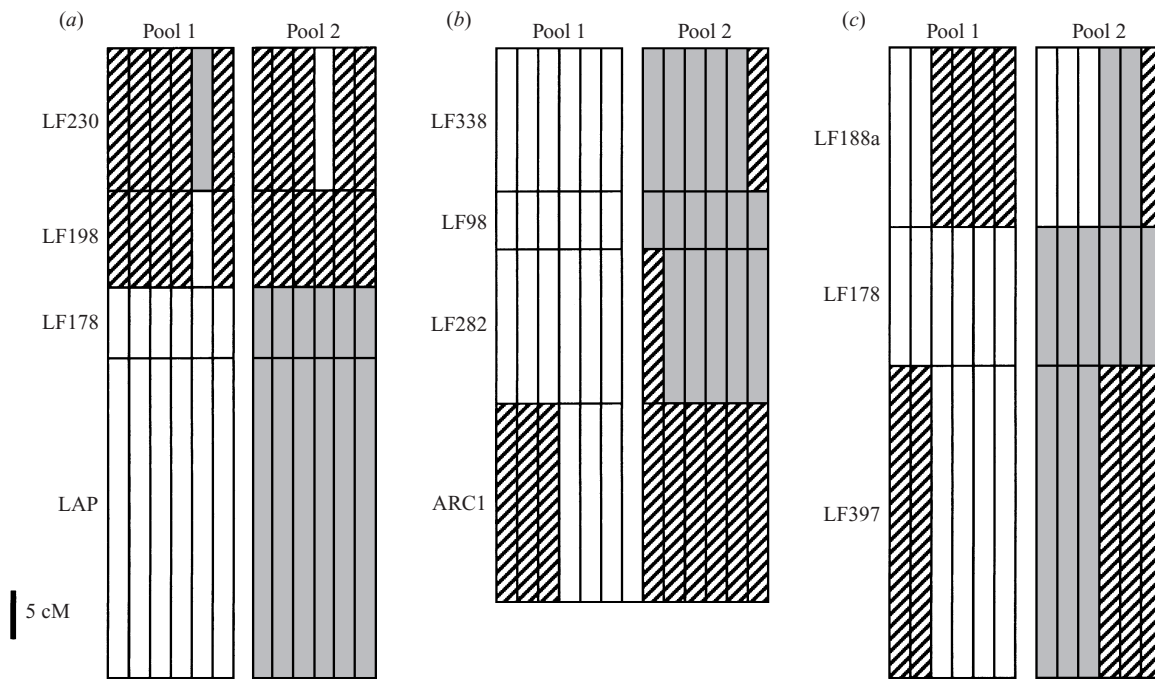
Fig. 1. Graphical genotypes for individual mosquitoes from F2 segregating populations used to prepare bulked DNA pools. Progeny were scored as LVP[SD] strain parental type (open bars), MOYO strain parental type (black bars) or heterozygotes (hatched bars) at selected RFLP loci. (*a*) SDAM1 chromosome 1, (*b*) SDAM1 chromosome 2, (*c*) SDAM2 chromosome 1. Individual markers reflect the half-recombinational distance between adjacent markers or between an adjacent marker and the end of the linkage group.

Following some preliminary analyses, the SDAM1 individuals were also genotyped at RFLP loci outside the QTL regions including LF230 and *LAP* loci on chromosome 1 and the ARC1 and LF338 loci on chromosome 2. For the SDAM1 bulks, four DNA pools were prepared each representing six individuals determined to be homozygous for the maternal and paternal genotypes respectively at the LF178 locus for *fsb1* and the LF98 locus for *pgs1*; in addition, individuals were selected so that at least some of them also reflected recombination events with markers linked to but outside the QTL regions (LF230 and *LAP* for *fsb1*; ARC1 and LF338 for *pgs1*). All individuals of the SDAM2 population were genotyped at the LF188a, LF178 and LF397 loci on chromosome 1. The SDAM2 bulks consisted of two DNA pools each representing six individuals determined to be homozygous for the maternal or paternal genotypes respectively at the LF178 locus for *fsb1* and also reflecting some recombination events with the LF188a and LF397 loci. Graphical genotypes for individual mosquitoes used to prepare bulked DNA samples were constructed as previously described (Severson & Kassner, 1995) and are shown in Fig. 1. Briefly, the RFLP genotypes are graphically represented to indicate the allelic configurations at selected chromosomal segments. This provides a method for rapidly examining genome structure within our segregating populations and identifying individuals suitable for preparing DNA bulks using a simple visual analysis.

(iii) *Bulked segregant analysis using RAPD-PCR*

PCR amplification of DNA fragments from individual pools was performed using commercially prepared random ten-mer oligonucleotides (Operon Technologies, Alameda, California). A total of 94 primers selected from the OA through OE kits were examined, although each of the bulk groups (SDAM1, SDAM2) were not screened with each primer. Individual PCR reaction mixtures (15 μl total volume) contained $1 \times$ reaction buffer (Promega), 2 mM-MgCl$_2$, 0·2 mM each dNTP, 0·8 μg/μl BSA, 25 ng primer, 5 ng template DNA and 1 unit of *Taq* DNA polymerase (Promega). PCR was performed using a Hybaid Tempcycler as follows: one cycle of denaturation at 94 °C for 4 min followed by 45 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. PCR products were size-fractionated in 1·2% Trevigel 500 agarose gels and visualized by UV light following ethidium bromide straining.

(iv) *PCR product cloning and analysis*

PCR products that appeared specific to an individual pool within one of the bulked groups were excised as plugs from the gel, placed in 100 μl H$_2$O and incubated at 65 °C for 5 min, followed by incubation at 37 °C overnight to facilitate diffusion of the RAPD fragment into the H$_2$O. One microlitre of this preparation provided the template for a second PCR amplification, using the same reaction mixture concentrations as the

initial PCR except that it contained 50 ng primer and 2 units of *Taq* DNA polymerase in 30 μl total volume. PCR was performed using Touchdown PCR (Don *et al.*, 1991) as follows: 1 cycle at 94 °C for 10 min; 2 cycles of 94 °C for 20 s, 50 °C for 60 s and 72 °C for 30 s; 28 cycles as previously except that the annealing temperature was sequentially changed to 2 cycles each at 48 °C, 46 °C and 45 °C, and the final 22 cycles at 44 °C.

The pool-specific PCR products were then purified through low-melting-temperature agarose. Some PCR products were cloned using commercial kits including TA Cloning (Invitrogen) and pGEM-T (Promega) as directed by the supplier. Most PCR products were cloned as blunt-end fragments in a ligation reaction mixture that contained a 3:1 molar ratio of PCR product to *Hinc*II- or *Sma*I-digested pGEM3Z plasmid vector, $1 \times$ T4 DNA ligase buffer, 0·3 mM each dNTP, 1 unit T4 DNA polymerase, 1·5 units T4 kinase and 0·5 units T4 DNA ligase (GibcoBRL). Recombinant plasmids were transformed into JM109 (Promega) or ElectroMAX DH10B (GibcoBRL) bacterial cells according to the supplier's recommendations. Inserts were isolated directly from recombinant bacterial colonies as PCR products and labelled with ^{32}P as previously described (Severson, 1997). They were then used to probe Southern blots carrying *Eco*RI-digested DNA obtained from pooled samples of adult mosquitoes, each representative of single *Aedes aegypti* strains, *Anopheles gambiae*, *Culex pipiens* spp. and *Drosophila melanogaster*. Clones that identified strain-specific RFLP were then used to probe Southern blots of individual mosquitoes representing segregating populations. Linkage analyses were performed using the MAPMAKER (Lander *et al.*, 1987) and JoinMap (Stam, 1993) computer programs.

(v) *Sequence analysis*

Plasmids containing RAPD inserts were purified using the alkaline lysis technique (Sambrook *et al.*, 1989). Plasmid templates were subjected to cycle sequencing using the ABI Prism BigDye Terminator kit according to the supplier's recommendations (PE Applied Biosystems) and sequenced using the ABI Prism 310 Genetic Analyzer. Single-pass sequence information was obtained for about 300–500 nucleotides at the T7 end of each clone. Homology searches of the partial DNA sequences were conducted by comparing the six-frame conceptual amino acid translations against protein sequence databases using the BLAST program (Altschul *et al.*, 1990).

3. Results

(i) *RAPD and bulked segregant analysis*

The SDAM1 *fsb1* and *pgs1* bulked samples were screened with 83 RAPD primers while the SDAM2

fsb1 bulked samples were screened with 94 RAPD primers. A typical positive screening result is shown in Fig. 2. A total of 62 RAPD fragments were cloned and subsequently screened as potential RFLP markers (Table 1). The two parental sources were equally represented. The isolated inserts were used as probes for hybridization to Southern blots containing *Eco*RI-digested genomic DNA from pooled samples of mosquitoes representing a number of strains and species. These analyses revealed that most (43 of 62) of the cloned RAPD fragments represented high-copy number sequences within the *Aedes aegypti* genome. A total of 10 RAPD/RFLP fragments seemed to represent low-copy number sequences. In addition, nine RAPD/RFLP fragments seemed to be of non-mosquito origin as they hybridized only to identical restriction fragments in the *Aedes aegypti* LVP^{SB}, LVPRD, LVP^{SD} and HAM strains and to *Anopheles gambiae*, but not to any other *Aedes aegypti* strain or other mosquito species (Fig. 3). Further, each of these sequences was not always present in each of the *Aedes aegypti* LVP^{SB}, LVPRD, LVP^{SD} and HAM strains, but was always present in the *Anopheles gambiae* strain. Interestingly, each of these fragments was isolated from the SDAM 2 bulked segregant analysis population and was specific to the pool representing the paternal (MOYO) parent.

(ii) *Characterization of selected RAPD/RFLP clones*

Eight of the nine clones that appeared to be of non-mosquito origin were subjected to partial sequence analysis (one clone was lost prior to sequence analysis). Database comparisons revealed putative protein identifications for three of these clones (Table 2). Interestingly, high amino acid homologies were observed only to genes isolated from prokaryotes. These results, therefore, seem to corroborate a non-mosquito origin for these cloned sequences.

(iii) *Chromosomal location and genetic linkage of RAPD/RFLP clones*

The 10 RAPD/RFLP clones, identified as low-copy number in preliminary Southern screenings, were subjected to linkage analysis. Chromosomal map positions of all clones were consistent with their targeted QTL bulk pools. Five clones were determined to be linked with each QTL region (Fig. 4). Of this group, one clone (B8L260) mapped between flanking markers defining the *fsb1* QTL, and one clone (A13L975) mapped between flanking markers defining the *pgs1* QTL.

4. Discussion

Five RAPD/RFLP clones were linked with flanking markers for the *fsb1* QTL on chromosome 1 (Severson
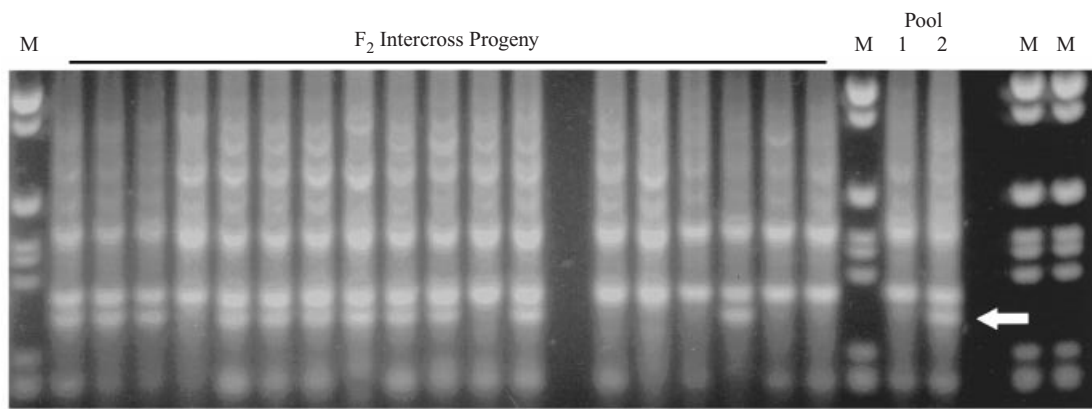
Fig. 2. RAPD-PCR products obtained from total genomic DNA in bulked (Pool 1 and 2) and individual mosquito samples using the OPA20 primer. Lanes M, *Hae*III-digested bacteriophage PM2 marker DNA. The arrow identifies a pool-specific product.

Table 1. *Characteristics of RAPD/RFLP fragments that were isolated using bulked segregant analysis on pools based on flanking marker RFLP genotypes at the* fsb1 *and* pgs1 *QTL*

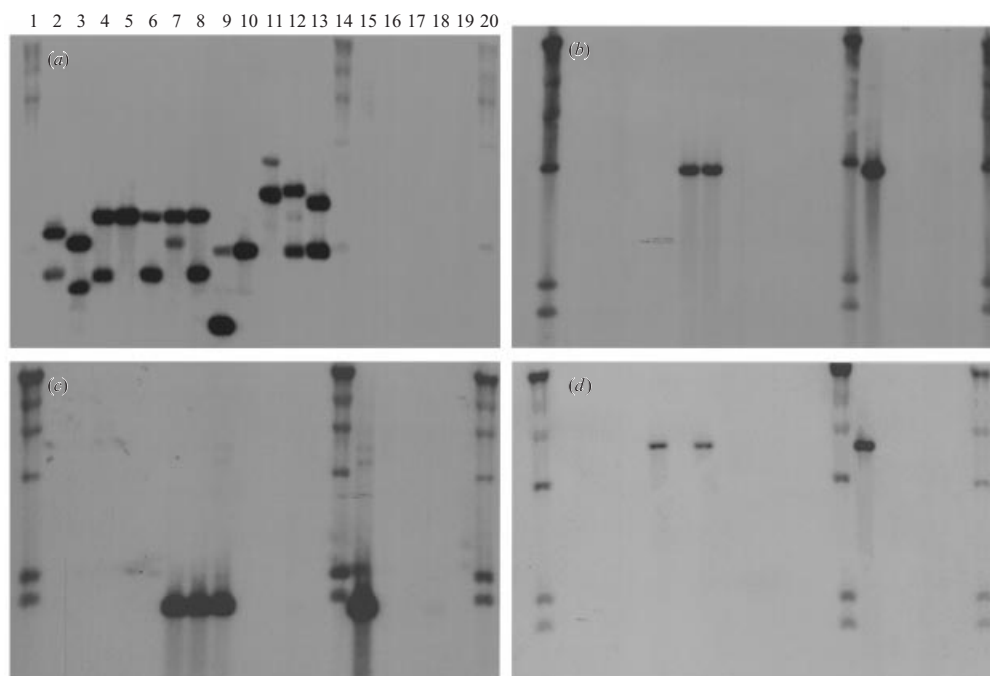|	Targeted QTL		Bulk pool		
	fsb1	*pgs1*	LIV^{SD}-specific	MOYO-specific	Total
Number of cloned fragments	43	19	31	31	62
Number of mapped clones	5	5	6	4	10
Number of putative non-mosquito clones	9	0	0	9	9
Number of high-copy/non-mappable clones	29	14	25	18	43

Fig. 3. RFLP observed following hybridization with one RAPD/RFLP clone of mosquito origin (*a*) and three RAPD/RFLP clones of apparent non-mosquito origin (*b–d*). Each clone was isolated using bulked segregant analysis on *Aedes aegypti* DNA. Lanes 2–13, *Aedes aegypti* strains, bronze, formosus, Liverpool, Liverpool refractory *Brugia*, Liverpool susceptible *Brugia*, Liverpool refractory *Dirofilaria*, Liverpool susceptible *Dirofilaria*, Hamburg, BLPCOFZMIN, Moyo-In-Dry, RED, Silver; lane 15, *Anopheles gambiae*; lanes 16–18, *Culex pipiens* subspecies; lane 19, *Drosophila melanogaster*; lanes 1, 14, 20, *Hind*III-digested bacteriophage λ marker DNA.

Table 2. *Putative identification of RAPD/RFLP fragments isolated using bulked segregant analysis that appear to be of non-mosquito origin*

Clone	Putative identification	ID[a] (%)	Similar[a] (%)	Overlap[a]	Score[b]	Organism
B10M410	LfgE, gi, U51896	46	60	43	101	*Vibrio parahaemolyticus*
C13M750	Hypothetical 60·2 kDa protein, sp, P54608	32	53	58	88	*Bacillus subtilis*
C14M590	Formate dehydrogenase, PIR, JS0628	98	99	131	729	*Escherichia coli*

[a] The number of identical (ID) or similar (Similar) amino acids in a contiguous region of overlap (Overlap).
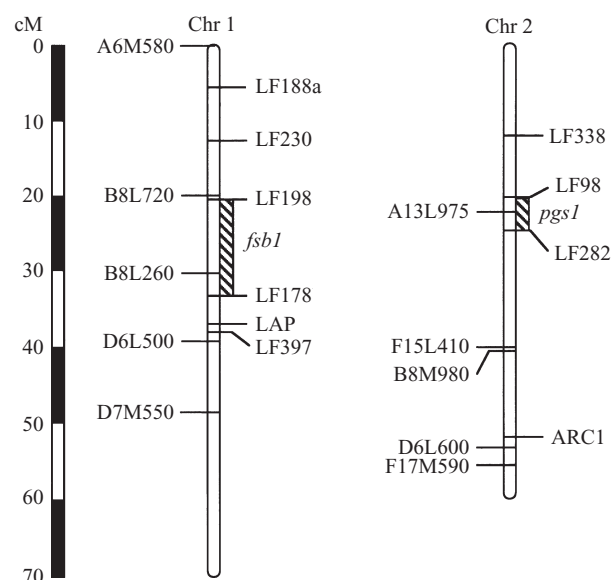[b] BLASTX PAM120 score.

Fig. 4. Composite RFLP linkage map for *Aedes aegypti* indicating the map positions of RAPD/RFLP markers (designations to the left) isolated using bulked segregant analysis relative to RFLP markers (designations to the right) defining intervals for QTL (hatched bars) determining susceptibility to *Brugia malayi* (*fsb1*) and *Plasmodium gallinaceum* (*pgs1*) and RFLP markers used to select progeny for bulked pools.

et al., 1994*a*). One of these clones, B8L260, mapped within the QTL interval itself. Fine-scale QTL mappings of *B. malayi* susceptibility with this locus included has indicated that the associated gene resides between the B8L260 and LF178 loci (D. W. Severson & B. M. Christensen, unpublished data). Bulked segregant analysis therefore provided a mechanism to reduce the *fsb1* QTL interval from 10·4 cM (flanking markers LF198 and LF178) (Severson *et al.*, 1994*a*) to 2·9 cM (flanking markers B8L260 and LF178). However, comparisons of the physical and genetic maps for *Aedes aegypti* (S. E. Brown, D. W. Severson & D. L. Knudson, unpublished data) indicate that the physical size of a 2·9 cM interval in this genome remains large. That is, the distal 0·41 physical portion

of the short arm of chromosome 1 (containing *fsb1*) represents *c*. 52·8 % of the genetic map for that chromosome. Using an average haploid genome size of 0·82 pg derived from independent estimates (Rao & Rai, 1987; Warren & Crampton, 1991) and the conversion factor 965×10^6 bp/pg (see Arumuganathan & Earle, 1991), the 2·9 cM interval represents about 7 Mb.

Five RAPD/RFLP clones were linked with flanking markers for the *pgs1* QTL on chromosome 2 (Severson *et al.*, 1995). Again, one of these clones, A13L975, mapped within the QTL interval itself. Although the A13L975 locus has yet to be included in fine-scale QTL mapping studies with *P. gallinaceum*, its identification will reduce the size of the interval from 4·3 cM (flanking markers LF282 and LF98) (Severson *et al.*, 1995) to 2·1 cM (flanking markers LF282 and A13L975) or 2·2 cM (flanking markers A13L975 and LF98). Again, comparisons of the physical and genetic maps for *Aedes aegypti* (S. E. Brown, D. W. Severson & D. L. Knudson, unpublished data) indicate that the physical size of a 2·2 cM interval in this genome region remains relatively large. The distal 0·34 physical portion of the short arm of chromosome 2 (containing *pgs1*) represents *c*. 47·4 % of the genetic map for that chromosome. Using the aforementioned calculations, the interval represents about 7·6 Mb.

Although RAPD-PCR was performed on DNA pools representing *Aedes aegypti* bulked segregants, nine RAPD/RFLP fragments were isolated that seem to be of non-mosquito origin. The apparent serendipitous isolation of these fragments is difficult to explain. However, several of their characteristics suggest that they are likely to be of prokaryotic origin. Southern blot analyses indicated that these sequences were only present in particular mosquito DNA preparations: the *Aedes aegypti* LVP[SB], LVP[RD], LVP[SD] and HAM strains and a strain of *Anopheles gambiae*. The sequences were not present in any of the other *Aedes aegypti* strains tested or in three *Culex pipiens* subspecies or *Drosophila melanogaster*. Also, with each fragment the restriction fragment patterns

observed were identical in the *Aedes aegypti* strains and in *Anopheles gambiae*. Finally, partial sequence analysis of the nine cloned fragments identified significant homologies for three of them with genes isolated from three different bacterial species. While bacterial endosymbionts such as *Wolbachia pipientis* have been isolated from several insect species, including the mosquitoes *Culex pipiens* and *Aedes albopictus* (see O'Neill *et al.*, 1992), the source identification and characterization of these RAPD/RFLP fragments are beyond the scope of the present studies and remain, therefore, an enigma.

Although segregating individuals were selected for the bulk pools based on RFLP markers tightly linked with each QTL, and also at markers some distance from the QTL (Fig. 1), four of five RAPD/RFLP loci targeted to each QTL mapped outside the desired interval (Fig. 4). This strategy has been suggested for increasing the likelihood that pool-specific products will reflect sequences internal to the target interval (Giovannoni *et al.*, 1991). We initially selected segregating individuals for the SDAM1 population based solely on flanking RFLP markers at the QTL. Subsequently, additional more distant markers were examined. These results indicated that with some of the preliminary bulked pools, none of the selected individuals exhibited recombination between markers defining the QTL region and markers outside the targeted interval (data not presented). That is, with some individuals all markers examined were homozygous for one or the other parental genotype across the targeted chromosome. Under this condition, the bulked segregant approach selected for RAPD polymorphism across the entire chromosome rather than the intended QTL region. It seems imperative, therefore, to include additional markers outside the target region when preparing bulked samples. In addition, the likelihood of obtaining pool-specific products that reflect sequences internal to the target interval can probably be increased significantly by selecting segregating individuals homozygous at the flanking markers, and also heterozygous at markers a short genetic distance from them.

This study demonstrates that bulked segregant analysis can be used effectively to develop genetic markers within a targeted QTL interval. However, the estimated physical size of even the newly defined intervals still represents a daunting challenge for our efforts towards map-based cloning of genes determining parasite susceptibility. The observed levels of recombination in *Aedes aegypti* seem extremely low for the reported genome size. Our map-based cloning efforts are presently directed towards the development of genetic markers from cosmid and BAC clones that have been and continue to be physically mapped to these genome regions (Brown *et al.*, 1995; S. E. Brown & D. L. Knudson, unpublished data).

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