

## Chromosomal mapping of two loci affecting filarial worm susceptibility in *Aedes aegypti*

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### Abstract

Two quantitative trait loci (QTL) affecting susceptibility of the mosquito *Aedes aegypti* to the filarial worm parasite *Brugia malayi* were identified using restriction fragment length polymorphism (RFLP) markers. The first locus, fsb[1,LF178], resides within a 10 cM interval on chromosome 1 and exhibits a recessive effect with respect to susceptibility. The second locus, fsb[2,LF98], resides within a 9 cM interval on chromosome 2 and exhibits an additive effect on susceptibility. Significant epistasis was detected between these loci, although the effect of fsb[2,LF98] was dependent on the genetic background of the mosquito strains. Suggestions for a standard QTL nomenclature are included.

**Keywords:** RFLP, genetic linkage, vector competency, quantitative trait loci, autosomal sex determination.

### Introduction

The lymphatic filarioid nematode parasites of humans, *Brugia malayi*, *B. timori* and *Wuchereria bancrofti*, are responsible for an estimated 90 million worldwide cases of lymphatic filariasis, with an additional 905 million people at risk (W.H.O., 1985). Efforts toward vaccine development against these parasites have been unsuccessful (Philipp *et al.*, 1988). Since their transmission to humans is totally dependent upon the availability of a competent mosquito vector, mosquito control remains the most successful strategy for preventing these diseases; however, the rapid development of genetic resistance to pesticides has diminished their effectiveness for mosquito control (Raymond *et al.*, 1991; Roush, 1993). An alternative control strategy would be to manipulate genetic aspects of vector competence within mosquito populations, thereby disrupting

parasite transmission (Crampton *et al.*, 1990; Eggleston, 1991; Cockburn & Seawright, 1990; Christensen & Severson, 1993).

*Aedes aegypti* is the best genetically characterized mosquito species. We recently described construction of a complete restriction fragment length polymorphism (RFLP) linkage map that presently includes seventy-five loci covering the *Ae. aegypti* genome at an average spacing of 1.8 cM (Severson *et al.*, 1993, and unpublished data). A classic genetic linkage map, based on seventy-seven morphological mutant and isozyme markers, also exists (Munstermann & Craig, 1979; Munstermann, 1990).

With *Ae. aegypti*, lymphatic filarial worm susceptibility is reportedly controlled primarily by a single sex-linked recessive gene designated  $f^m$ , although additional effects attributed to modifier or other genes have been suggested (Macdonald, 1962, 1963a; Macdonald & Ramachandran, 1965; Wattam & Christensen, 1992). RFLP markers provide the tools necessary to resolve a potential quantitative trait, such as filarial worm susceptibility, into discrete Mendelian components, commonly designated as quantitative trait loci or QTL (Paterson *et al.*, 1988; Lander & Botstein, 1989). Here we report the use of RFLP markers to examine the genetic basis for filarial worm susceptibility to *B. malayi* (fsb) in *Ae. aegypti*. Our results confirm the  $f^m$  locus on chromosome 1 and identify a second locus affecting fsb on chromosome 2.

### Results

Response to the parasite was determined in *B. malayi*-challenged  $F_2$  female mosquito progeny. These progeny reflected three independent crosses involving *B. malayi* susceptible (SUS) and refractory (REF) strains (Table 1). Use of (LIV  $\times$  RED) $F_2$  and (NAG  $\times$  MOY) $F_2$  populations allowed us to examine fsb in different genetic backgrounds. If fsb is determined solely by a single locus, autosomal sex-linked gene (Macdonald, 1962, 1983a, b; Macdonald & Sheppard, 1965), then the expected segregation ratio of SUS to REF is 1:1. It is important to note that sex determination in culicine mosquitoes is determined by a single autosomal gene (located on chromosome 1 in *Ae. aegypti*), with maleness being the dominant allele (Gilchrist & Haldane, 1947). Therefore the expected segregation ratios for single-sex  $F_2$  progeny will approach 1:1 instead of the

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Trial	Cross	No. refractory	No. susceptible	$\chi^2$ value	Maximum no. filarial worms <sup>a</sup>
T1	(LIV × RED)F <sub>2</sub>	148	39	63.5*	—
T2	(LIV × RED)F <sub>2</sub>	75	27	22.6*	10
T3	(NAG × MOY)F <sub>2</sub>	180	96	25.6*	23

<sup>a</sup> For T1, individuals were scored only as refractory or susceptible. For T2 and T3, the number of filarial worms that had successfully developed to the infective third-stage larva were counted for each individual.

\* $P < 0.001$ . Differences in the frequencies of refractory and susceptible were assessed for an expected 1:1 segregation ratio.

conventional 1:2:1 for this linkage group (Gilchrist & Haldane, 1947; Severson *et al.*, 1993). The observed segregation ratios deviated significantly from the expected 1:1 ratio for all three trials ( $P < 0.001$ , Table 1); fewer susceptible mosquitoes than expected were identified in each trial.

Segregation analyses for each of the three trials identify associations between *fsb* and two independent QTL. The composite RFLP genetic linkage map illustrates the intervals on chromosome 1 and chromosome 2 that exceed our LOD score threshold of 2.0 for declaration of a QTL (Fig. 1). We elected to describe individual QTL positions using the following format: *fsb*[*n*,*y*], where *fsb* = filarial worm susceptibility to *B. malayi*, *n* = the chromosome number and *y* = the RFLP flanking marker associated with the greatest LOD score. The putative QTL on chromosome 1 is located within an estimated 10 cM interval defined by the LF178 and LF198 loci, with the greatest LOD score associated with LF178 in two of the three trials (Table 2). This QTL, hereafter denoted as *fsb*[1,LF178], explained the largest fraction of the observed phenotypic variance for *fsb* with each trial. Results for the (LIV × RED)F<sub>2</sub> progeny (T1 and T2) were similar (22–28%). With the (NAG × MOY)F<sub>2</sub> progeny (T3), the apparent effect of *fsb*[1,LF178] was greater, accounting for a much larger proportion (43%) of the variance. The putative QTL on chromosome 2 is located within an estimated 9 cM interval defined by the LF282 and VCP loci, with the greatest LOD scores associated with LF98 in T1 and T2 (Table 2). The phenotypic variance explained by this QTL, hereafter denoted as *fsb*[2,LF98], was again similar for T1 and T2 (10–16%), but was clearly lower for T3 (3%). For T3, *fsb*[2,LF98] was

**Table 2.** Biometrical parameters of QTL significantly affecting *fsb* of female mosquitoes.

Trial	Chromosome	Interval	Maximum LOD	Phenotypic variance explained (%)
T1	1	LF178–LF198	6.05	21.6
	2	LF98–VCP	3.89	9.8
T2	1	LF178–LF198	5.76	27.6
	2	LF98–LF181	3.05	15.6
T3	1	LF178–LF235	21.86	43.2
	2	LF282–VCP	1.39	2.7

**Table 1.** Phenotypes of (SUS × REF)F<sub>2</sub> female mosquitoes.

identified, based on LOD values exceeding the threshold of 2.0, only when the analysis was calculated with *fsb*[1,LF178] fixed as a dominant effect relative to the filarial worm refractory phenotype (see Lincoln & Lander, 1990).

In addition to obvious segregation distortion related to autosomal sex determination, we also observed evidence for distortion due to a deficit in the number of mosquitoes homozygous for the susceptible RFLP parental types (Table 3). This is likely a consequence of increased mortality levels due to the detrimental effects of developing filarial worms on highly susceptible mosquitoes (Lavoipierre, 1958). In instances where we were able to dissect mosquitoes that died prior to the 12-day extrinsic developmental period, the infection rate was greatly elevated; for trials T2 and T3, 91.7% (11/12) and 65.1% (28/43) of these mosquitoes carried developing or fully developed filarial worm larvae compared with only 26.5% and 34.8% respectively (Table 1) for the entire populations.

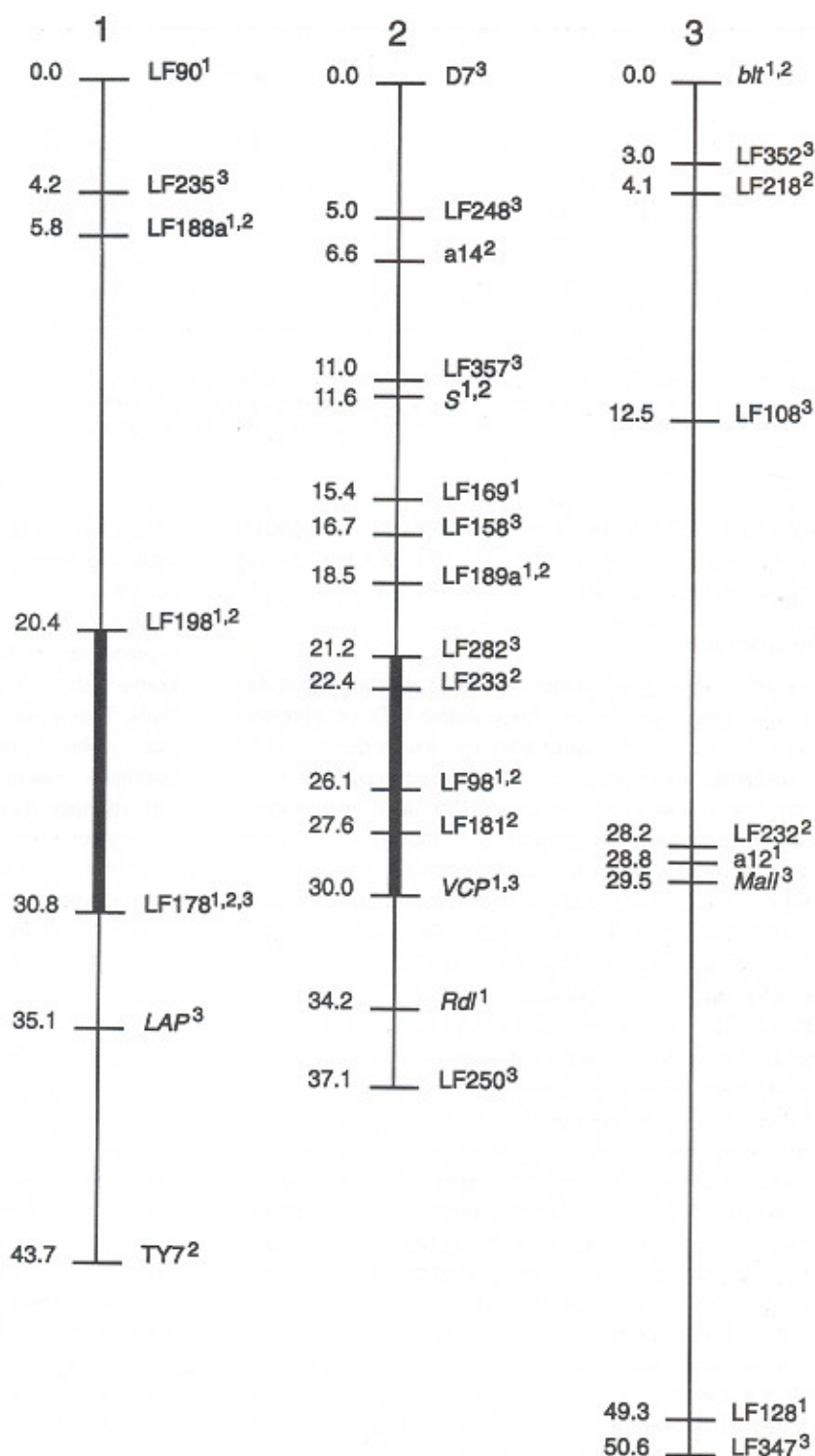
Examination of nonrecombinant progeny for flanking markers delimiting putative QTL clearly reflect their effects on *fsb* (Table 4). The analysis of variance for T2 and T3 indicated that the QTL genotypes had significant effects on *fsb* ( $P < 0.0001$ ,  $R^2 = 0.79$  and  $0.64$  for T2 and T3, respectively). With T2, significant effects of *fsb*[1,LF178] ( $P < 0.0001$ ), *fsb*[2,LF98] ( $P < 0.0001$ ), and the *fsb*[1,LF178] × *fsb*[2,LF98] interaction ( $P < 0.0001$ ) were demonstrated. The initial analysis with T3 indicated a significant

**Table 3.** Segregation of flanking markers for putative QTL.

Marker	Chromosome	Trial T1			Trial T2			Trial T3		
		S <sup>a</sup>	H	R	S	H	R	S	H	R
LF178	1	97	76	0*	35	46	1*	106	122	4*
LF198	1	76	87	10*	41	37	5*	—	—	—
LF235	1	—	—	—	—	—	—	82	113	33*
LF98	2	22	102	49*	18	46	17	—	—	—
VCP	2	20	100	53*	—	—	—	47	117	67
LF181	2	—	—	—	21	41	18	—	—	—
LF282	2	—	—	—	—	—	—	46	122	63

<sup>a</sup> Genotypic designations: S = filarial worm susceptible parental type; H = heterozygote; R = filarial worm refractory parental type.

\* $P < 0.001$ . Deviations from the 1:2:1 ratio expected for F<sub>2</sub> populations were assessed by  $\chi^2$  analysis.



**Figure 1.** Composite RFLP genetic linkage map indicating markers used for identification of QTL associated with *fsb* in three independent trials. The specific marker set for each trial is indicated by superscripts (1 = T1, 2 = T2 and 3 = T3). Intervals carrying putative QTL are identified by solid bars. Map distances are listed in Kosambi centiMorgans.

effect for *fsb*[1,LF178] ( $P < 0.0001$ ), but the effects of *fsb*[2,LF98] ( $P < 0.07$ ) and the *fsb*[1,LF178]  $\times$  *fsb*[2,LF98] interaction ( $P < 0.05$ ) were less obvious. However, the data for T3 were suggestive that individuals homozygous for the susceptible QTL genotypes (SS) were under-represented (segregation ratio 9:31:23). Additionally, the

SS individuals that did survive reflected a lower intensity of filarial worm infection when compared to SH individuals. This provided further evidence for our contention that the highly susceptible individuals are subject to increased parasite-induced mortality rates. The analysis of variance for the T3 data, with the SS category removed, indicated

QTL genotype		Filarial worm susceptibility phenotype							
		Trial T1		Trial T2			Trial T3		
QTL1 <sup>a</sup>	QTL2	Sus <sup>b</sup>	Ref	Sus	Ref	No. of worms	Sus	Ref	No. of worms
S <sup>c</sup>	S	6	5	7	0	7.1 ± 2.2	8	1	4.7 ± 3.3
S	H	12	20	11	4	2.1 ± 2.2	29	2	6.7 ± 5.5
S	R	0	20	0	7	0	17	4	3.1 ± 3.2
H	S	0	6	0	5	0	0	19	0
H	H	0	36	0	18	0	0	35	0
H	R	0	12	0	6	0	0	15	0

<sup>a</sup> QTL1 = fsb[1,LF178]; QTL2 = fsb[2,LF98].

<sup>b</sup> Phenotypic designations: Sus = filarial worm susceptible; Ref = filarial worm refractory.

<sup>c</sup> S = flanking markers both homozygous for the susceptible parental genotype; H = flanking markers both homozygous for the refractory parental genotype.

significant effects for fsb[1,LF178] ( $P < 0.0001$ ), fsb[2,LF98] ( $P < 0.03$ ), and the fsb[1,LF178] × fsb[2,LF98] interaction ( $P < 0.0004$ ).

### Discussion

Our data identify two independent QTL affecting fsb in *Ae. aegypti*. We conclude that the putative QTL on chromosome 1, fsb[1,LF178], carries the previously described  $f^m$  locus for fsb. Its effect is consistent for susceptibility being recessive (Macdonald, 1962; Macdonald & Ramachandran, 1965) and it also maps to the appropriate genome location (Macdonald, 1963b; Macdonald & Sheppard, 1965). We further suggest that the  $f^m$  locus may function to provide a permissive state for filarial worm development, but subsequent expression of the susceptible phenotype depends upon the genotype at the QTL on chromosome 2, fsb[2,LF98]. The significant fsb[1,LF178] × fsb[2,LF98] epistatic interactions observed with our trials T2 and T3 support this conclusion. Since the observed epistatic effect of fsb[2,LF98] was variable, depending on the  $F_2$  population being examined, our results also indicate the need for consideration of the variance between different genetic backgrounds when evaluating aspects of vector competency. Further, these results suggest that additional QTL affecting fsb may be identified as crosses involving other genetic backgrounds are examined.

We utilized a proposed standard methodology for QTL nomenclature. Individual QTL positions are described using the format abc[n,y], where abc = a lower case abbreviation for the phenotype associated with a putative QTL, n = the chromosome number, and y = the RFLP flanking marker exhibiting the greatest LOD score. This methodology is not only applicable to QTL defined by interval mapping techniques (Lander & Botstein, 1989), but also for QTL associated with individual markers as defined by analysis of variance (Jansen, 1992; Winkelman & Hodgetts, 1992) and multiple regression (Armstrong *et al.*, 1992; Moreno-Gonzalez, 1992) techniques. It is also

**Table 4.** Association of QTL genotype with fsb in (SUS × REF) $F_2$  female mosquitoes.

amenable to describing QTL using other marker systems including, for example, simple sequence repeats (Zheng *et al.*, 1993).

Autosomal sex determination in culicine mosquitoes represents a systematic segregation distorter on chromosome 1 that effectively limits the use of some statistical tests. That is, since males reflect heterozygotes at the sex locus, while females reflect the homozygous, recessive condition (Gilchrist & Haldane, 1947), gametic combinations such as males homozygous at the sex locus will never occur. This phenomena results in a significant sex-specific bias in the observed segregations of all loci on chromosome 1 (Severson *et al.*, 1993). The effect associated with individual markers (or intervals) on an observed phenotype is typically evaluated assuming that each marker is a treatment and that the three genetic states (maternal homozygotes, heterozygotes, paternal homozygotes) are levels within treatments (Edwards *et al.*, 1987). Linear comparisons measure additive effects at each locus, while quadratic deviations reflect dominant effects (Russell & Eberhart, 1970). Since QTL analyses for vector competency involve only female mosquitoes (male mosquitoes do not blood feed), statistical analyses involving loci on chromosome 1 reflect only two genetic states (maternal homozygotes and heterozygotes, Table 4). This, of course, limits the analysis to linear comparisons for markers on this linkage group as well as for their interactions with markers on other linkage groups.

Our results provide the first demonstration of the feasibility and power of using RFLP marker linkage studies to identify genetic loci involved in mosquito vector competence for transmission of human parasites and pathogens. We have established a framework for efforts to physically map and ultimately isolate genes associated with fsb in *Ae. aegypti*. These results need to be extended to other mosquito species, with consideration for the existence of conserved syntenies between mosquito genomes. We propose that some of the genetic mechanisms deter-

mining mosquito vector competency for a given pathogen will likely be highly conserved. That is, we hypothesize that the genetic capacity to transmit a given pathogen is unlikely to reflect completely independent evolutionary events between mosquito species. The high levels of interspecific hybridization observed with our RFLP markers (Severson *et al.*, 1994) should facilitate the rapid evaluation of this hypothesis.

## Experimental procedures

### Mosquito strains and parasite exposures

Four *Ae. aegypti* laboratory strains were used for these studies: Liverpool (LIV), Moyo-In-Dry (MOY), Nagasaki (NAG), and RED. The RED strain carries the red-eye (*re*) locus on linkage group 1, the spot-abdomen (*s*) locus on chromosome 2, and black-tarsus (*blt*) locus on chromosome 3. The origins of these strains and RFLP based estimates of genetic diversity between them are described elsewhere (Severson *et al.*, 1994). For each strain, the number of colonized generations is unknown. The LIV and NAG strains are susceptible (SUS) to *B. malayi*. The RED and MOY strains are refractory (REF) to *B. malayi*. Mosquitoes were reared as previously described (Christensen & Sutherland, 1984).

F<sub>1</sub> hybrids between SUS females and REF males were produced by pairwise matings. F<sub>2</sub> intercross populations were produced by pairwise mating of selected F<sub>1</sub> progeny. Three independent trials were included in this study. Two trials (T1 and T2) involved (LIV × RED)F<sub>2</sub> progeny, and the third trial (T3) involved (NAG × MOY)F<sub>2</sub> progeny. F<sub>2</sub> female mosquitoes were allowed to engorge on *B. malayi*-infected gerbils that were anaesthetized with a mixture of ketamine and Rompun (7:3) administered i.m. at a dosage of 0.1 ml/100 g of body weight. Microfilaraemia of gerbils used in these studies ranged from 200–296 microfilariae per 20 µl of blood. Engorged females were dissected 12–13 days after blood-feeding to determine whether they were permissive for *B. malayi* larval development. For T1, individuals were scored only as refractory (e.g. no larvae present) or susceptible (e.g. one or more larvae present). For T2 and T3, the number of filarial worms that had successfully developed to infective third-stage larvae were counted for each individual.

### DNA isolation, Southern blotting, and probe hybridization

Following dissections for determination of *fsb* phenotype, DNA extractions were performed on each mosquito carcass. They were homogenized in equal volumes of lysis buffer (0.5% NaDodSO<sub>4</sub>, 0.2 M NaCl, 25 mM EDTA, 10 mM Tris, pH 8.0) and phenol, followed by standard phenol/chloroform extraction procedures, including an incubation with RNase A (Sambrook *et al.*, 1989).

Individual mosquito genomic DNA preparations were digested with *EcoRI* 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 Gene Screen Plus (NEN) membranes were as previously described (Severson *et al.*, 1993).

Prior to their initial hybridization, membranes were incubated in a pre-blocking solution (2% NaDodSO<sub>4</sub>, 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). 50 ng of probe DNA was labelled with α<sup>32</sup>P-dCTP using random nonamer primers (Stratagene) to a specific activity of about 1.0–1.6 × 10<sup>9</sup> cpm/µg. Free dNTPs were separated by column chromatography through Biogel P60 (Bio-Rad) equilibrated in elution buffer (0.5% NaDodSO<sub>4</sub>, 1 mM EDTA, 5 µg/ml *Torula* yeast RNA, 20 mM Tris, pH 7.4). Kodak XAR-5 film was exposed to membranes for 5–7 days at –80°C with an intensifying screen. Membranes were regenerated for additional hybridizations by incubation in 0.4 N NaOH at 42°C for 30 min followed by neutralization (0.1 × SSC, 0.1% NaDodSO<sub>4</sub>, 0.2 M Tris, pH 7.4) at 42°C for 30 min.

### Probe selection and statistical analysis

For each trial, membranes were probed with a set of clones of known chromosomal position (Severson *et al.*, 1993, and unpublished data). Twenty-nine clones were selected to provide maximum coverage of the three *Ae. aegypti* chromosomes at 10–20 cM intervals. With trials T1, T2 and T3 we scored individual progeny for twelve, eleven and fourteen probes, respectively. Informative probes were identified by hybridization to membranes containing *EcoRI* digests of 10 µg of genomic DNA isolated from pooled samples of 100–200 mosquitoes representing the parent strains used in these studies.

Chi-square goodness-of-fit values for segregation and independent assortment ratios for all pairs of loci were calculated. Multi-point linkage analysis and QTL mapping were performed using the MAPMAKER/QTL computer package (Lander *et al.*, 1987; Lincoln & Lander, 1990). This package employs the method of interval mapping for QTL (Lander & Botstein, 1989). A LOD score of 2.0 was used as the threshold for declaring the presence of QTL. With the density of markers used in combination with a haploid genome consisting of only three chromosomes and a genetic length less than 1.5 Morgans, this represents a stringent threshold for our QTL analyses (Lander & Botstein, 1989).

A composite RFLP genetic linkage map was developed, based on the recombination frequencies between markers determined from this and previous studies (Severson *et al.*, 1993, unpublished data). This map was assembled using the JoinMap computer program (Stam, 1993) and reflects the best fit for all available RFLP mapping data. Recombination frequencies were converted into map distances (centiMorgans, cM) using the Kosambi function (Kosambi, 1944).

The effects of putative QTL identified for *fsb* with trials T2 and T3 were assessed using the general linear models (GLM) procedure of SAS (SAS Institute, 1990). Only non-recombinant mosquitoes were considered, wherein flanking markers for individual QTL shared the same parental RFLP genotypes (e.g. both markers homozygous for the maternal or paternal genotypes or both markers heterozygous).

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