**Restriction Fragment Length Polymorphism Mapping of Quantitative Trait Loci for Malaria Parasite Susceptibility in the Mosquito Aedes aegypti**

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**ABSTRACT**

Susceptibility of the mosquito *Aedes aegypti* to the malarial parasite *Plasmodium gallinaceum* was investigated as a quantitative trait using restriction fragment length polymorphisms (RFLP). Two F₂ populations of mosquitoes were independently prepared from pairwise matings between a highly susceptible and a refractory strain of *A. aegypti*. RFLP were tested for association with oocyst development on the mosquito midgut. Two putative quantitative trait loci (QTL) were identified that significantly affect susceptibility. One QTL, *pgs[F98]*, is located on chromosome 2 and accounted for 65 and 49% of the observed phenotypic variance in the two populations, respectively. A second QTL, *pgs[3,Mal]*, is located on chromosome 3 and accounted for 14 and 10% of the observed phenotypic variance in the two populations, respectively. Both QTL exhibit a partial dominance effect on susceptibility, wherein the dominance effect is derived from the refractory parent. No indication of epistasis between these QTL was detected. Evidence suggests that a tightly linked cluster of independent genes or a single locus affecting susceptibility to various mosquito-borne parasites and pathogens has evolved near the *LF98* locus; in addition to *P. gallinaceum* susceptibility, this general genome region has previously been implicated in susceptibility to the filarial nematode *Brugia malayi* and the yellow fever virus.

MALARIA maintenance and transmission among warm-blooded vertebrate animals are totally dependent upon the availability of competent mosquito intermediate hosts. Malarial parasites, *Plasmodium* spp., infect the mosquito after ingestion in a bloodmeal obtained from an infected vertebrate. *Plasmodium* must complete critical aspects of their life cycle, including gametogenesis and eventual development to vertebrate infective sporozoites, within the complex physiological milieu presented by the mosquito (*Warburg and Miller* 1991). Gamete maturation and fertilization to produce motile ookinetes occur within the mosquito midgut after gametocyte acquisition by female mosquitoes during blood-feeding. The ookinetes must migrate through the peritrophic matrix, penetrate the midgut epithelium and subsequently form oocysts. Oocysts undergo sporogony to produce sporozoites that must migrate to and invade the mosquito salivary glands. However, individual mosquito species, as well as individual mosquitoes within species, vary significantly in their competency to support development of the various *Plasmodium* spp. (*Boyd* 1949).

The conflicting literature regarding *Plasmodium* susceptibility in various mosquito species suggests a complex mode of inheritance. *Huff* (1929, 1931) reported that susceptibility of *Culex pipiens* to *P. cathemerium* was heritable as an apparently recessive trait, yet only 65% of the individuals were susceptible after three generations of selection. Conversely, *Dennhöfer* (1971) reported that *C. pipiens* susceptibility to *P. cathemerium* was due to a single gene with incomplete dominance. With *Aedes aegypti*, susceptibility to *P. gallinaceum* was associated with a single, dominant locus (*Frizzi et al.* 1975). With *A. gambiae*, although susceptibility to *P. berghei* was correlated with an incompletely dominant character, the influence of interacting polygenes was suggested (*Al-Mashhadani et al.* 1986). *Graves and Curtis* (1982) reported that refractoriness of *A. gambiae* to *P. yoelii nigeriensis* showed incomplete dominance, but their low susceptibility *A. gambiae* strains were highly susceptible to *P. falciparum*. *Ward* (1963) suggested that *A. aegypti* susceptibility to *P. gallinaceum* was primarily determined by a single locus with incomplete dominance but was unable to fix a strain for refractoriness despite 26 generations of selection. *Kilama and Craig* (1969) subsequently reported that *A. aegypti* susceptibility to *P. gallinaceum* was determined by a dominant gene, *pgs*, located on chromosome 2; however, to justify multifactorial inheritance they needed to invoke partial penetrance in the refractory phenotype. That is, individuals were considered refractory if they allowed development of 19 or less oocysts. We recently reported development of two *A. aegypti* strains that are highly refractory to and of intermediate susceptibility to *P. gallinaceum*, respectively (*Thathy et al.* 1994). Crosses involving these strains and highly susceptible strains suggested that susceptibility is largely determined by a...
major gene exhibiting at least partial dominance; how-
ever, these results also suggested that this locus either
exhibits allelic variability or that additional modifier
genes are involved.

Although A. aegypti is not a vector for human Plasmo-
dium spp., it is genetically the best characterized mos-
quito species and is the primary natural vector for the
avian malarial parasite, P. gallinaceaem. Since the primary
human malarial parasite, P. falciparum, shares a closer
phylogenetic relationship with P. gallinaceaem than with
other human, murine or primate malarias (WATERS et al.
1991), elucidation of the genetic basis for vector
competency in the A. aegypti / P. gallinaceaem system could
provide significant insight concerning the genetic rela-
tionship between P. falciparum and its Anopheles spp.

vectors. Our success in constructing a genetic linkage
map for A. aegypti, based on restriction fragment length
polymorphism (RFLP) markers, provides the tools to
partition genetic aspects of malaria parasite susceptibil-
ity into discrete Mendelian components (SEVERSON et al.
1993). In the present study, quantitative trait loci
(QTL) were sought for the genetic factors determining
P. gallinaceaem susceptibility (pgs) in A. aegypti using
RFLP markers. We have identified a QTL consistent
with the pbs locus on chromosome 2 and an additional
QTL affecting pgs on chromosome 3.

MATERIALS AND METHODS

Mosquito crosses and parasites: Genetic data are based on
F2 intercross progeny from pairwise matings between the
P. gallinaceaem-susceptible A. aegypti RED strain and a P. gallina-
ceaeem-refractory A. aegypti strain selected from the Moyo-In-
dry strain (MOYO-R). Selection of the MOYO-R strain and
related susceptibilities of the MOYO-R and RED strains are
described by THATHY et al. (1994). Two independently gener-
ated populations (T1 and T2) involving (RED X MOYO-R)F2
progeny were prepared using RED as the female. The origins
of the RED and MOYO-IN-DRY strains and RFLP-based esti-
mates of genetic diversity between them are described else-
where (SEVERSON et al. 1994a). Both strains are type form,
A. aegypti aegypti. The RED strain carried the red-eye (re)
locus on chromosome 1, the spot-abdomen (s) locus on chro-
mosome 2 and the black-tarsus (bt) locus on chromosome
3. Mosquitoes were reared as previously described (CHRIS-
tensen and SUTHERLAND 1984).

F2 female mosquitoes were allowed to engorge on re-
strained White Leghorn chicks that were naturally infected
with P. gallinaceaem essentially as described by KILAMA and
GIAIM (1969) with some modifications (THATHY et al. 1994).
Engorged females were dissected 6–7 days after blood-feeding
to determine whether they were permissive for P. gallinaceaem
development. The number of oocysts that had successfully
developed on the midgut of individual mosquitoes was
counted.

RFLP and statistical analysis: DNA extraction from individ-
ual mosquitoes, digestion with EcoRI, Southern blotting and
hybridizations were performed as previously described (SEV-
ERSON et al. 1993, 1994a). Sixteen informative RFLP probes
were selected to provide maximum coverage of the three A.
aegypti chromosomes at 10–20-cM intervals, based on known
chromosomal position (SEVERSON et al. 1993, 1994b and SEV-
ERSON, D. W., unpublished data).

RESULTS

Phenotypic variability in Plasmodium susceptibility: The oocyst frequency distribution was determined for
two populations of P. gallinaceaem-challenged F2 female
mosquitoes (Figure 1). These populations repre-
sented two independently prepared crosses between the
P. gallinaceaem-susceptible RED strain and a P. gallina-
ceaeem refractory MOYO-R strain. Mean oocyst counts and
within population SDs were 67.9 ± 103.4 (n = 154) and
45.7 ± 64.5 (n = 122) for populations T1 and T2, respectively.
Segregation of markers in the F2 from two populations evaluated for Plasmodium susceptibility

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Marker</th>
<th>Segregation ratio</th>
<th>T1</th>
<th>Segregation ratio</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LF178</td>
<td>45:98:11</td>
<td>26.8**</td>
<td>39:79:4</td>
<td>30.9**</td>
</tr>
<tr>
<td>2</td>
<td>LF248</td>
<td>29:85:40</td>
<td>3.4</td>
<td>24:81:17</td>
<td>14.5**</td>
</tr>
<tr>
<td></td>
<td>LF138</td>
<td>29:83:38</td>
<td>2.6</td>
<td>25:80:17</td>
<td>13.4**</td>
</tr>
<tr>
<td></td>
<td>s</td>
<td>30:124</td>
<td>6.9*</td>
<td>25:97</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>LF282</td>
<td>37:85:33</td>
<td>1.2</td>
<td>20:77:24</td>
<td>8.7*</td>
</tr>
<tr>
<td></td>
<td>LF98</td>
<td>36:85:29</td>
<td>3.6</td>
<td>22:79:21</td>
<td>11.2**</td>
</tr>
<tr>
<td></td>
<td>VCP</td>
<td>36:88:29</td>
<td>3.8</td>
<td>18:80:24</td>
<td>12.6**</td>
</tr>
<tr>
<td>3</td>
<td>blt</td>
<td>38:116</td>
<td>0.0</td>
<td>13:107</td>
<td>12.8**</td>
</tr>
<tr>
<td></td>
<td>LF218</td>
<td>37:89:24</td>
<td>7.8*</td>
<td>12:80:30</td>
<td>17.6**</td>
</tr>
<tr>
<td></td>
<td>LF261</td>
<td>41:87:26</td>
<td>5.7</td>
<td>6:84:32</td>
<td>28.9**</td>
</tr>
<tr>
<td></td>
<td>LF108</td>
<td>41:79:25</td>
<td>4.5</td>
<td>4:88:30</td>
<td>35.5**</td>
</tr>
<tr>
<td></td>
<td>LF168</td>
<td>47:79:27</td>
<td>5.4</td>
<td>3:95:24</td>
<td>45.8**</td>
</tr>
<tr>
<td></td>
<td>Mall</td>
<td>40:73:26</td>
<td>3.2</td>
<td>6:82:34</td>
<td>27.7**</td>
</tr>
<tr>
<td></td>
<td>LF347</td>
<td>43:75:33</td>
<td>1.3</td>
<td>17:68:37</td>
<td>27.2**</td>
</tr>
</tbody>
</table>

Marker loci are arranged in chromosomal order.

* Loci were scored for 1:3 ratio, all others 1:2:1.

** P < 0.01

Segregation of RFLP loci: Segregation ratios for each RFLP locus are shown in Table 1. Significant deviations from the expected 1:2:1 for F2 progeny are evident for all loci on chromosome 1. With T1, RFLP loci on chromosomes 2 and 3 generally fitted their expected 1:2:1 segregation ratios, with only a slight bias toward the heterozygote genotype. With T2, all loci on chromosomes 2 and 3 exhibited significant deviations from the expected 1:2:1 segregation ratios. An abundance of heterozygotes was observed for all loci on chromosome 2. All loci on chromosome 3 reflected a deficiency in the RED genotype. This deficiency increased progressively from the linkage group ends to the LF168 locus, suggesting the presence of a RED-strain-derived genetic lethal within the region of LF168.

RFLP analysis of Plasmodium susceptibility: QTL analyses for both populations identify associations between pgs and two independent QTL. QTL plots of LOD scores for chromosomes 2 and 3 provide a basis for estimating the individual QTL positions (Figure 2). Both populations identified a QTL at the interval defined by LF282 and LF98 on chromosome 2. This QTL, designated pgs[2,LF98], explained the largest fraction (single-locus model) of the observed phenotypic variance in each population (65 and 49% for T1 and T2, respectively). While both populations identified a QTL on chromosome 3, the interval placement varied. This QTL was defined by adjacent intervals, LF168 and Mall vs. Mall and LF347, for T1 and T2, respectively. The phenotypic variance (single-locus model) explained by this QTL, designated pgs[3,Mall], varied from 14 to 10% for T1 and T2, respectively.

Comparison of QTL genotype with pgs phenotype clearly illustrates their interrelationships (Table 2). A single individual accounts for the apparent increase in mean oocyst numbers observed for the S/R QTL genotype with T1. This individual exhibited the largest oocyst count (n = 520) observed during these studies. Because this individual also reflected a recombination event between Mall and LF347, it seems likely that it was actually heterozygous at the locus affecting susceptibility.

Because of the extensive segregation distortion observed in the T2 population (Table 1), some QTL genotype classes were represented by only a small number of individuals, including in some classes one or zero (Table 2). Therefore, the genetic effects of the individual QTL were assessed with T1 only.

Polynomial regression statistics indicating the significant effects of QTL associated with LF98 and LF347 on Plasmodium susceptibility in T1 are shown in Table 3. The linear and quadratic components both significantly reduce the residual variance with both RFLP loci. This suggests that each QTL exhibits a partial dominance effect on Plasmodium susceptibility. Further, this analysis indicates for both QTL that the dominance effect is derived from the refractory parent. Finally, the crossproduct term was not significant (P > 0.05), indi-
cating a lack of epistatic interaction between the two QTL.

**DISCUSSION**

Our results identify two independent QTL affecting pgs in *A. aegypti*. A major QTL on chromosome 2, \( pgs[2,LF98] \), accounts for the greatest fraction of the observed variance in pgs. With \( pgs[2,LF98] \), refractoriness exhibits partial dominance over susceptibility. The effect and map location of \( pgs[2,LF98] \) are consistent with the previously described \( pls \) locus (Kilama and Craig 1969; Thathy et al. 1984), even though susceptibility at \( pls \) was described as being dominant to refractoriness. Since \( pls \) was evaluated as a qualitative character in backcross progeny in these studies, the reported effect must be viewed with caution. The plus/minus scoring of a complex phenotype in a backcross is inadequate for determining mode of action. In this context, mode of action is best examined by using DNA-based markers to quantitatively evaluate \( F_2 \) intercross progeny (see Severson 1994).

The QTL on chromosome 3, \( pgs[3,MalI] \), while of lesser effect than \( pgs[2,LF98] \), does clearly have a significant yet apparently independent influence on pgs in *A. aegypti*. Also, with \( pgs[3,MalI] \), partial dominance effects are associated with refractoriness. The *A. aegypti* strain selected for an intermediate level of susceptibility to *P. gallinaceum* (MOYO IS) by Thathy et al. (1994) was likely fixed for refractoriness at \( pgs[2,LF98] \) and therefore reflects only the effect of \( pgs[3,MalI] \). In the present study, individuals homozygous for markers linked with refractoriness at \( pgs[2,LF98] \) and with susceptibility at \( pgs[3,MalI] \) carried a mean of 10.3 ± 15.7 oocysts. The MOYO IS line described by Thathy et al. (1994) carried a mean of 8.1 ± 9.2 oocysts.

Our results reflect some limits on the precision of estimating the location of individual QTL. For a QTL with a large effect, such as \( pgs[2,LF98] \), we identified a QTL corresponding to the same interval in two independent populations. However, for a QTL with lesser effects such as \( pgs[3,MalI] \), we were able to identify the QTL in both populations, but its location varied from the MalI-LF347 interval for T1 to the LF168-MalI interval for T2. This phenomenon is not surprising given our relatively small sample sizes and the lower

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**Figure 2.** LOD scores indicating QTL likelihood maps for pgs with chromosomes 2 (top) and 3 (bottom) in *A. aegypti* mosquitoes.
phenotypic variance (10–14%) attributable to this QTL. While a number of factors influence the accuracy of QTL identification and placement, population size and the magnitude of the genetic effect play highly significant roles in anticipated predictive power. Simulation studies suggest that our population sizes are barely sufficient for detecting QTL that explain 10% of the total phenotypic variance (Van Ooijen 1992).

The observed distortions in segregation ratios reflect at least two distinct phenomena. First, sex determination in A. aegypti is determined by a single autosomal gene on chromosome 1, with maleness being the dominant allele (Gilchrist and Haldane 1947). Therefore, the observed segregation ratios for female F2 progeny will reflect a bias toward the maternal RFLP genotype with all chromosomes 1 loci (Severson et al. 1993). Second, although the segregation distortion observed for chromosomes 2 and 3 could simply be due to sampling bias associated with relatively small populations, it likely reflects the effects of lethal loci on survival of F2 progeny homozygous for one or both parental types. Lethal genes have been reported to promote and maintain heterozygosity in mosquito strains (Matthews and Craig 1989; Münstermann 1994). Given the concordance in statistical analyses between T1 (chromosomes 1, 2, and 3 with only slight evidence for segregation distortion) and T2 (chromosomes 2 and 3 with highly distorted segregation ratios), it is likely that the distortion observed did not influence interpretation of the relationships between QTL and RFLP loci, although it may account for the observed variability in interval placement for pgs (Mall). The results of this study may provide some potential insights relative to the evolution of mosquito vector competence. We recently reported that, while susceptibility of A. aegypti to the filarial worm parasite Brugia malayi was primarily determined by a QTL on chromosome 1, ffl[1,LF178], the significant effect of a second QTL located near LF98 on chromosome 2, ffl[2,LF98], also was identified (Severson et al. 1994b). Additionally, significant correlations between yellow fever virus susceptibility and gene frequencies among mosquito populations at the Pgm isozyme locus have been reported (Tabachnick et al. 1985). The Pgm locus resides about four recombination units from the psl locus (Münstermann 1990); this suggests that a locus for yellow fever susceptibility also is linked with the LF98 locus. Therefore, independent studies involving several A. aegypti strains and their susceptibility to a protozoan and a metazoan parasite and an arbovirus all implicated the same genome region on chromosome 2. We conclude that either a tightly linked cluster of independent

TABLE 3
Regression statistics for RFLP markers defining QTL for Plasmodium susceptibility in the T1 population (R² = 0.85)

<table>
<thead>
<tr>
<th>Variable</th>
<th>d.f.</th>
<th>Regression coefficient</th>
<th>SE</th>
<th>t value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>6.05</td>
<td>0.25</td>
<td>25.80</td>
<td>0.0001</td>
</tr>
<tr>
<td>LF98</td>
<td>1</td>
<td>-3.13</td>
<td>0.44</td>
<td>-7.10</td>
<td>0.0001</td>
</tr>
<tr>
<td>Linear</td>
<td>1</td>
<td>0.46</td>
<td>0.21</td>
<td>2.21</td>
<td>0.0286</td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>-1.60</td>
<td>0.43</td>
<td>-3.69</td>
<td>0.0003</td>
</tr>
<tr>
<td>LF347</td>
<td>1</td>
<td>0.56</td>
<td>0.21</td>
<td>2.62</td>
<td>0.0098</td>
</tr>
</tbody>
</table>

Evaluated as the effect of adding alleles (0, 1 or 2) from the refractory parent at each locus.
genes or a single locus affecting susceptibility to various mosquito-borne parasites and pathogens has evolved in the genome region near the LF98 locus.

Plasmodium spp., lymphatic filarioids (Brugia spp. and Wuchereria bancrofti) and arboviruses must successfully cross the mosquito midgut to complete development. Midgut barriers to transmission or antagonistic hemolymph factors have been implicated for each of these disease agents (see MITCHELL 1983; CHRISTENSEN 1986; WARBURG and MILLER 1991). This apparent commonality in immune mechanisms could represent the phenotypic expression of the locus or loci linked with LF98. This hypothesis is strengthened by a QTL analysis of A. aegypti susceptibility to the filarial worm Dirofilaria immitis; in two populations, we have identified a major QTL for D. immitis susceptibility on chromosome 1 but have found no evidence for any QTL association with LF98 (D. W. SEVERSON, MORI, A. and CHRISTENSEN, B. W., unpublished data). This parasite migrates down the mosquito gut and into the lumen of the Malphigian tubules where it becomes intracellular and completes development; therefore, D. immitis does not traverse the midgut wall and is not exposed to hemolymph until it migrates to the probosis after development to the infective third-stage larva.

These studies need to be expanded with consideration for variance in pgs expression across different genetic backgrounds. That is, because pgs is a quantitative trait, it is probable that allelic variants exist for the two QTL identified in this study and that additional QTL remain to be identified. For example, the effect of fsb2[2,LF98] on B. malayi susceptibility is variable depending on the population under consideration (SEVERSON et al. 1994b). KILAMA (1973) examined pls expression among several geographic strains of A. aegypti and reported variability only among African strains. However, for this study susceptibility was evaluated as a single-locus, qualitative character. Our results demonstrate the necessity for evaluation of susceptibility as a quantitative trait.

Of further interest would be to evaluate the role of orthologous loci for the QTL identified for P. gallinaceum and A. aegypti with other mosquito and Plasmodium species. While some inconsistencies exist, our studies and the literature generally suggest that inheritance of Plasmodium susceptibility is determined primarily by a major gene, with additional effects attributable to undefined modifier genes (see Introduction). Therefore, if the general concept of whole arm homology for chromosome evolution in mosquitoes is correct (MATTHEWS and MUNSTERMANN 1994), it should be possible to rapidly evaluate the potential for homologies involving QTL linked with RFLP loci for A. aegypti. We provided some support for this hypothesis by demonstrating high levels of homology between our RFLP markers for A. aegypti and genomic DNA representing several mosquito species (SEVERSON et al. 1994a), and by the development of a comparative linkage map for A. albopictus using A. aegypti markers (SEVERSON et al. 1994c). Comparative linkage mapping has been used successfully to identify homologies for orthologous RFLP loci and QTL for seed weight in cowpea and mung bean, despite extensive genome rearrangements (FATOKUN et al. 1992).

Finally, the identification of RFLP loci linked with genes affecting Plasmodium susceptibility provides the physical landmarks to begin efforts to isolate these genes; map-based cloning is dependent on a series of very logical and independent steps, beginning with the mapping of target genes with DNA markers (WING et al. 1994).

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