Reviews

Applications of Molecular Marker Analysis to Mosquito Vector Competence

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A rapidly expanding cadre of molecular biology techniques is being developed for human and plant genetics, including development of the technology to identify large numbers of genetic markers and to evaluate these markers relative to phenotypic observations. In this review, David Severson discusses applications of these techniques for the analysis of mosquito vector competence.

Mosquito vectors serve as obligate intermediate hosts for numerous diseases that collectively represent a major source of human morbidity and mortality worldwide. These disease groups include malaria (*Plasmodium*), lymphatic filariasis (*Wuchereria* and *Brugia*) and many arboviruses (most notably yellow fever and dengue fever). The most successful efforts towards controlling these diseases have been accomplished through strategies designed to control the mosquito host. This approach is limited by the rapidly increasing emergence of multiple pesticide-resistant vector populations[1,2].

An alternative strategy for controlling these diseases would be the genetic manipulation of mosquito populations to reduce their inherent competence as disease vectors. Evidence for genetic components in the determination of vector competence has been extensively reviewed[3–6]. The prospects for identifying specific genes involved in vector competence and developing genetic control strategies have been limited because of the small number and minimal utility of genetic markers available for mosquitoes. Traditionally, a large number of genetic crosses were necessary to develop a linkage map because a small number of mutant marker and isozyme marker loci segregated in each cross. New classes of genetic markers, based on DNA sequence polymorphisms, permit the rapid development of detailed genetic maps from a limited number of crosses.

Development of molecular markers

Virtually any genomic DNA-derived sequence has potential as a molecular marker for linkage analysis. Several general criteria are fundamental in determining the type of sequence that can efficiently function as a genetic marker. Foremost, they must represent single copy, low copy number, or tandemly repeated sequences, and they must be inherited in a mendelian fashion. These sequences must be distributed randomly throughout the genome and must exhibit distinct sequence polymorphisms between genetic lines. In addition, these sequences should exhibit a co-dominant segregation pattern; that is, an in-depth genetic analysis of a complex phenotype requires the ability to identify not only individuals that are homozygous for both parental genotypes, but also those that are heterozygotes. This criterion limits the utility of one class of molecular markers, random amplified polymorphic DNA (RAPD), since the observed polymorphisms generally segregate as dominant characters[7]. Finally, to be useful as genetic markers, DNA sequences must be identifiable as easily scorable polymorphisms and also must be compatible for the simultaneous tracking of a number of markers for each individual in a population.

Restriction fragment length polymorphisms

The most widely used approach to molecular marker development is based on identifying specific regions of a genome using restriction fragment length polymorphisms (RFLP)[8]. Genomic DNA from individuals is cleaved with DNA sequence-specific restriction endonucleases, size fractionated with agarose gel electrophoresis, and transferred to nylon membranes. The nylon membranes are hybridized with labeled, low copy number genomic DNA clones or cDNA clones. RFLP specific to each individual are identified by autoradiography.

An RFLP marker genetic linkage map has been developed for the yellow fever mosquito, *Aedes aegypti*[9]. This map now consists of 81 RFLP loci that cover 153 cM across the *Ae. aegypti* genome. Determination of linkage associations between RFLP markers and several mutant marker loci allowed for their partial integration into an existing classical genetic linkage map for *Ae. aegypti*. The classical genetic linkage map consists of various mutant markers and allozyme markers[10]. Most of the RFLP markers consist of random cDNA clones obtained from an *Ae. aegypti* Liverpool strain cDNA library. However, several represent clones of known genes, including those for *Ae. aegypti* salivary gland proteins (*MalI* and *D7*)[11,12], vitellogenic carboxypeptidase (*VCP*)[13], lysosomal aspartic protease (*LAP*)[14] and a gamma-aminobutyric acid receptor subtype A receptor (*Rdl*)[15].

There are advantages and disadvantages in using RFLP markers for examining mosquito vector competence. Southern blotting techniques and probe hybridizations can be difficult, and day-to-day inconsistency in blot performance is common. The small amount of genomic DNA per mosquito essentially limits an RFLP analysis to a single restriction-endonuclease

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digest and a single Southern blotting effort with each individual. However, we have been able to regenerate Southern blots for up to 24 rounds of hybridization and routinely achieve 12–15 rounds[9]. Coupled with the ability to examine two or three different probes with each hybridization[9], we can easily examine 20–35 RFLP loci for each mosquito.

Probably the most significant attribute of RFLP markers developed from cDNA clones is their long-term potential for examining genome structure in other mosquito species. The hybridization, under high stringency conditions, of several *Ae. aegypti* RFLP markers to genomic DNA digests of other mosquito species including, *Ae. albopictus*, *Ae. togoi*, *Armigeres subalbatus*, *Culex pipiens* and *Anopheles gambiae* has been demonstrated[16]. This suggests that these markers could be used for rapidly examining linkage associations in other mosquito species. Obviously, some level of chromosome rearrangement would be expected, including translocations, inversions, duplications and deletions. While the linear order or linkage group for some markers is likely to vary between species, the basic architecture of the mosquito genome may be largely conserved[17]. We have successfully used *Ae. aegypti* RFLP markers to document the existence of extensive syntenies between the *Ae. aegypti* and *Ae. albopictus* genomes (D.W. Severson *et al.*, unpublished).

Simple sequence repeats

Another popular approach to molecular marker development is based on the existence of highly abundant dinucleotide repeats distributed throughout many eukaryotic genomes[18]. These sequences are commonly referred to as microsatellite sequences or simple sequence repeats (SSR). For example, the most common SSR in humans and mice is $(CA)_n$ and occurs about once every 30 kb (Ref. 19).

The basic strategy for SSR marker development involves the initial selection of cloned genomic DNA fragments containing SSR via screening with oligonucleotide probes. Positive clones are then sequenced to define polymerase chain reaction (PCR) primer sequences that flank the SSR. Polymorphisms between genetic lines are characterized as differences in the number of repeats within individual SSR. The PCR-amplified sequences from individuals are discriminated as alleles on polyacrylamide sequencing gels.

Zheng and co-workers[20] are developing an SSR marker genetic linkage map for the principal human malaria vector, *An. gambiae*. They identified SSR clones in genomic libraries by screening with $(GT)_{15}$, $(CA)_{15}$ or $(GA)_{15}$ oligonucleotide probes. By determining recombination frequencies between SSR markers in a backcross population, they were able to develop a genetic linkage map for the X chromosome of *An. gambiae*. This map consists of 31 SSR markers that are resolvable at 23 loci and cover a map distance of about 44 cM. In addition, linkage associations between SSR markers and a white eye mutation (*w*) were determined. They were able to determine physical locations for many of these markers relative to their origin either from microdissected chromosome libraries[21] or by *in situ* hybridization to polytene chromosomes[20]. Linkage maps for the remaining two *An. gambiae* chromosomes are forthcoming.

SSR markers also have advantages and disadvantages for examining mosquito vector competence. The initial clone isolations and sequencing efforts are relatively laborious and time-consuming. However, once primer sequences are defined, the PCR protocols used for SSR identification provide fairly simple and rapid assays of allele frequencies. Publication of primer sequences provides ready marker access to any laboratory equipped to conduct PCR assays. SSR markers are particularly attractive, given the limited amount of genomic DNA per mosquito; they could be used to characterize upwards of 200 loci per individual as only 10 ng of genomic DNA template is used per PCR reaction[20]. A long-term limitation of SSR markers could be their utility for interspecific comparisons. That is, because dinucleotide repeats are highly unstable[22–24], it is unlikely that SSR loci are conserved between mosquito species.

Vector competence as a quantitative trait

Most phenotypic characters of interest are likely to exhibit continuous or quantitative variation. Quantitative variation results from the segregation of multiple mendelian genetic factors coupled with modification by the environment (Box 1).

The susceptibility of *Ae. aegypti* populations to yellow fever, dengue fever and several other flaviviruses has been correlated with several isozyme loci on chromosome 2, suggestive of a major single-gene effect[25–27]. However, these studies and others[28,29] also indicated

Box 1. Quantitative Variation of Phenotypic Characters

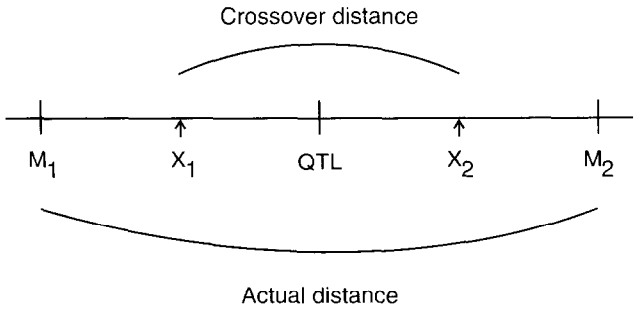
Quantitative variation represents differences in the degree of expression rather than the simple presence or absence of a particular character. Measurements of the variation observed between individuals for a quantitative trait typically represent the summation of both genetic and environmental effects, and (occasionally) interactions between genotype and environment. Powerful statistical procedures have been developed for the resolution of a quantitative trait into its discrete components[67]. These procedures provide for a partitioning of the observed phenotypic variance (V) into variance components as follows:

$$V_P = V_A + V_D + V_I + V_E + V_{GE}$$

where V_P = variance in phenotype within a population, V_A = variance due to additive genetic effects, V_D = variance due to dominance genetic effects, V_I = variance due to epistatic interactions between individual genetic effects, V_E = variance due to environmental influence, V_{GE} = variance due to interactions between genotype and environmental factors. The contributions of each of these components can be estimated by examining progeny from appropriate genetic crosses. V_P can most simply be addressed in terms of V_G (total genetic variance) and V_E. These values can be calculated based on the general premise that individuals of identical genotype will reflect only V_E, while individuals exposed to a uniform environment will reflect only V_G.

Box 2. Quantitative Trait Loci (QTL) Mapping

Various statistical approaches to QTL identification have been developed. QTL can be defined by their association with individual molecular markers using analysis of variance[47,68] and multiple regression techniques[69,70]. In addition, QTL can be defined by identifying two linked markers that flank an interval containing the QTL[71]. The ability to detect QTL using molecular markers is dependent on the magnitude of the individual QTL effects, the size of the population under investigation, and the recombination frequency between QTL and individual markers[41]. For example, the relationship between a gene contributing to a quantitative trait (QTL) and two molecular markers (M_1 and M_2) can be evaluated in terms of crossover events (X_1 and X_2) (see Fig.). We assume that the two parental lines were homozygous for opposite alleles at each locus. By examining progeny from segregating populations, map distances between markers can be calculated from the observed crossover frequencies. How closely the map distance will correlate with the physical distance (eg. number of base pairs between markers) depends on genome organization and chromosomal location of individual QTL.



that at least one additional gene influences the observed phenotype.

In a series of classic studies, Macdonald and coworkers[30–33] demonstrated that, with *Ae. aegypti*, lymphatic filarial worm susceptibility is controlled primarily by a single, sex-linked recessive gene, designated as f^m. They also suggested that effects of additional modifier genes were involved. Linkage analyses involving the red-eye (*re*) locus and the sex-determination locus identified their linear order on chromosome 1 as f^m-SEX-*re*. Multiple gene influences have been indirectly supported by comparisons of *in vivo* polypeptide synthesis between susceptible and refractory mosquito strains; six polypeptide differences, unique to refractory strains, were consistently identified following bloodfeeding[34,35].

A strain of *An. gambiae* has been selected that exhibits varying degrees of resistance to different *Plasmodium* species[36]. The phenotypic response to the parasite involves its encapsulation in melanotic materials[36,37] and is determined by at least two loci, one of which exhibits linkage with an esterase locus[38,39]. In addition, strains of *Ae. aegypti* have been selected that are highly permissive, intermediate in susceptibility and completely refractory for *P. gallinaceum* transmission[40]. Evaluation of crosses between these strains indicates that a major gene exhibiting partial dominance is primarily responsible for susceptibility. However, these results also suggest a more-complex mode of inheritance for susceptibility.

Efforts to identify systematically quantitative trait loci (QTL) associated with vector competence have been limited by the inability to evaluate crosses with genetic markers that delineate the entire mosquito genome. The successful development of high-density molecular marker linkage maps for mosquitoes provides a mechanism for the resolution of these complex phenotypic traits into discrete genetic components. Individuals from a segregating population (such as an F_2 intercross or backcross) are evaluated for a character of interest and for their genotypes at molecular marker loci spanning regular intervals (10–20 cM) throughout the genome[41]. Linkage associations between the segregating molecular markers and the observed phenotype can then be determined (Box 2). Molecular marker assisted QTL analyses of medically and agronomically important phenotypes have become relatively commonplace[42–50].

Case study

A QTL analysis of vector competence is dependent on identifying mosquito populations that represent the phenotypic extremes. Selection of strains of *Ae. aegypti* that are highly susceptible or refractory to *Brugia malayi* is easily accomplished following about three generations of selective inbreeding[34]. This phenomena seems to apply for most, if not all, mosquito-borne pathogens[27,36,40,51] and suggests that vector competence is determined primarily by a small number of genes, probably two or three.

The relatively small genome size in mosquitoes (~150–200 cM) provides for a complete genome evaluation with only 12–15 appropriately located genetic markers. We used RFLP markers to conduct QTL analyses for filarial worm susceptibility to *B. malayi* (fsb) in *Ae. aegypti*[52]. These analyses identified and described the combined effects of two independent QTL affecting fsb (Fig. 1). Both QTL were identified in each of three independent intercrosses in which individual mosquitoes were scored for 11, 12 or 14 RFLP markers. One QTL, fsb[1,LF178], is located within an estimated 10 cM interval on chromosome 1, with susceptibility reflecting a recessive character. It is likely that this QTL carries the f^m locus, since it maps to the appropriate genome location[33,53] and exhibits the same genetic effect[30,32]. The second QTL, fsb[2,LF98], is located within an estimated 9 cM interval on chromosome 2 and contributes to susceptibility in an additive manner. Epistatic interactions observed between the 2 QTL 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 fsb[2,LF98] locus. The observed effect of fsb[2,LF98] was variable depending on the F_2 population being examined. Therefore, the potential for variance between different genetic backgrounds must be considered when evaluating aspects of vector competence. These studies also suggest that additional QTL affecting fsb may be identified as crosses involving other genetic backgrounds are examined.

Projections for map-based cloning

The rigorous genetic evaluation of vector competence is ultimately limited, not by the total number of markers available, but by the design of the experimental crosses and the total number of individuals examined. For an

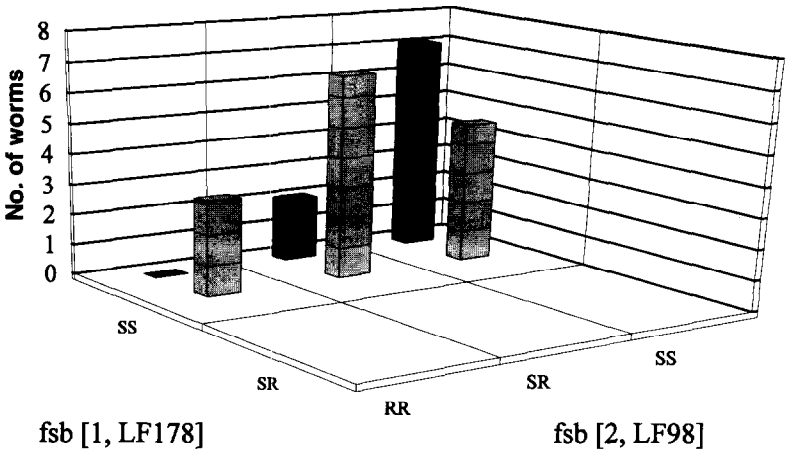


Fig. 1. Histogram showing the association of the Aedes aegypti *genotype and the observed filarial worm susceptibility to* Brugia malayi *(fsb) phenotype with quantitative trait loci (QTL) analysis. Note that autosomal sex determination influences segregation ratios for all loci on chromosome 1 in culicine mosquitoes[9]; this affects QTL analyses since male mosquitoes do not bloodfeed and the male genotype (RR in this instance) is observed only in females following recombination. Individual mosquitoes were evaluated for the number of infective third-stage filarial worm larvae (L3) they carried. SS, flanking markers both homozygous for the susceptible parental genotype; SR, flanking markers both heterozygous; RR, flanking markers both homozygous for the refractory parental type; cross 1, shaded bars; cross 2, solid bars. (Adapted from Ref. 52.)*

initial QTL analysis, F_2 intercross progeny are preferred. That is, since molecular markers such as RFLP can be completely classified, an examination of an F_2 intercross population will provide two times the average amount of genetic information per individual as that provided by an F_2 backcross population. Consequently, the intercross design generally requires the evaluation of about half the number of individuals than the backcross design for equivalent statistical power[54,55]. This has the greatest importance for evaluating the mode of action for individual loci (Box 3). Once QTL are identified and their mode of action characterized, marker development and analysis can be targeted to genome regions carrying QTL. This fine resolution is accomplished most efficiently using the appropriate backcross progeny[54,55].

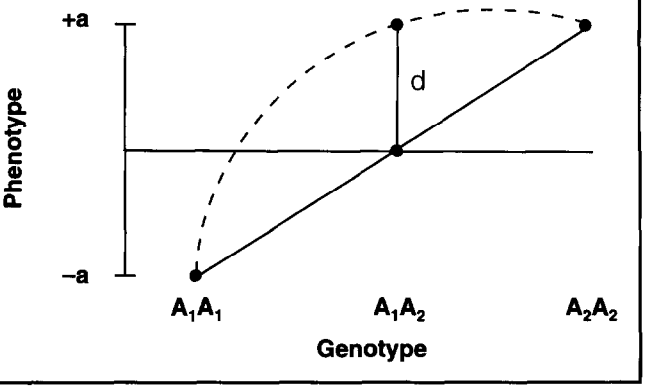
In physical distance terms, a QTL defined by flanking molecular markers may represent several megabases (Mb) of genomic DNA to resolve. The approach used to isolate specific genes from QTL will probably vary depending on the mosquito species. For mosquitoes with well-defined polytene chromosomes, such as *An. gambiae*, chromosomes have been microdissected, PCR amplified, and cloned to generate pools for direct physical mapping[21]. Different approaches for physical mapping must be used for mosquitoes with poorly defined polytene chromosomes, such as *Ae. aegypti*. For example, the development of yeast artificial chromosome (YAC) cloning vectors provides a means for the rapid screening of megabase-sized regions of a genome, because stable insert sizes approaching 1000 kb can be obtained[56,57]. Overlapping regions between YAC clones (which provide the basis for chromosome walking) can be identified by subcloning the termini of YAC inserts to generate end-specific probes[58]. Another approach uses fluorescent *in situ* hybridization (FISH) to identify the physical position of probes on metaphase chromosomes with roughly 1 Mb resolution[59]. Application of FISH mapping to interphase nuclei can increase the resolution to 50–100 kb or less[60,61]. A physical map of *Ae. aegypti* is being prepared using FISH techniques (D.L. Knudson *et al.*, unpublished).

Irrespective of the approach, map-based cloning of genes associated with vector competence will require segregating populations comprising large numbers of individuals. A good estimate for prospective sample sizes is likely offered by the successful map-based cloning of a disease resistance gene, *Pto*, in the domestic tomato (genome size 950 Mb)[62]. An RFLP analysis of populations totaling 1300 individuals was necessary to confirm that a 400 kb YAC-clone encompassed the *Pto* locus.

Once candidate clones are isolated, reliable techniques for verifying their influence on vector competence are essential. Genetic complementation assays for gene activity are presently limited in mosquitoes by the lack of stable germ-line transformation systems[63–65]. However, this limitation may be circumvented by the

Box 3. QTL Mode of Action

The phenotypic effect associated with individual markers or with flanking markers defining an interval is typically evaluated assuming that markers are treatments and that the three genetic states (maternal homozygotes, heterozygotes and paternal homozygotes) are levels within treatments[72]. Consider a marker exhibiting no recombination with a QTL (eg. complete linkage disequilibrium) with two alleles A_1 and A_2, where the effect of genotypes A_1A_1, A_1A_2 and A_2A_2 are a, d and –a, respectively (see Fig.). Additive genetic effects will approximate a linear function, while dominance genetic effects will approximate a quadratic function[73]. That is, for an additive effect d=0 and the heterozygote will exhibit a phenotypic value near the midpoint of the two parental types, while for a dominance effect d=a and the heterozygote will exhibit a phenotypic value identical to one of the parental types. For instances involving recombination between the marker and the QTL, frequency-adjusted mean expression calculations for QTL genetic effects have been developed[72].



recent development of an alphavirus expression system for the *in vivo* expression and functional analysis of specific genes in mosquitoes[66].

Conclusions

Mosquito-transmitted diseases have been controlled most successfully by control programs directed toward the mosquito. The development of molecular marker systems for mosquitoes provides the necessary tools to begin realistic efforts to isolate genes responsible for vector competence. These efforts could ultimately lead to disease control strategies involving the genetic manipulation of mosquito populations.

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