GENETIC AND PHYSICAL MAPPING IN MOSQUITOES: Molecular Approaches

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Key Words *Aedes*, *Anopheles*, positional cloning, malaria, filariasis

■ **Abstract** The genetic background of individual mosquito species and populations within those species influences the transmission of mosquito-borne pathogens to humans. Technical advances in contemporary genomics are contributing significantly to the detailed genetic analysis of this mosquito-pathogen interaction as well as all other aspects of mosquito biology, ecology, and evolution. A variety of DNA-based marker types are being used to develop genetic maps for a number of mosquito species. Complex phenotypic traits such as vector competence are being dissected into their discrete genetic components, with the intention of eventually using this information to develop new methods to prevent disease transmission. Both genetic- and physical-mapping techniques are being used to define and compare genome architecture among and within mosquito species. The integration of genetic- and physical-map information is providing a sound framework for map-based positional cloning of target genes of interest. This review focuses on advances in genome-based analysis and their specific applications to mosquitoes.

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PERSPECTIVES AND OVERVIEW

Mosquito-borne diseases continue to cause significant human health problems, largely in the subtropics and tropics, and their incidence has increased significantly within the last 2 decades. Estimates from the World Health Organization indicate that three mosquito-borne diseases are among the leading causes of morbidity and mortality in developing countries around the world. Nearly 500 million clinical cases of malaria caused by infection with *Plasmodium* parasites occur each year, resulting in 2.7 million deaths, mainly in children. Lymphatic filariasis is caused by parasitic nematodes and is the second leading cause of permanent and long-term disability worldwide, with 120 million people presenting clinical morbidity. Dengue fever virus, particularly its hemorrhagic form, is a threat to >2.5 billion people, with an annual incidence in the tens of millions and ~24,000 deaths per year.

Current efforts to limit or prevent transmission of these and other mosquito-borne diseases are increasingly hampered by a number of factors, as reviewed by Gubler (88). First, efforts to develop effective vaccines against these pathogens remain remarkably disappointing, and these efforts are unlikely to achieve success in the foreseeable future. Second, the emergence of antimalarial-drug resistance among *Plasmodium* parasites has reduced the effectiveness of drugs as prophylactic and treatment agents. Third, deteriorating socioeconomic conditions in many endemic areas have resulted in the collapse of once effective disease-monitoring and-control programs. Fourth, the effectiveness of mosquito control efforts has been increasingly reduced due to the loss of established mosquito control programs and the rapid development of insecticide resistance in mosquito populations. Nonetheless, because the transmission to humans of these pathogens is totally dependent on the availability of competent mosquito vectors, mosquito control remains the most successful mechanism for disease prevention (11).

Only a small number of mosquito species are genetically competent to transmit pathogens to a vertebrate host and competence varies within populations of a given species (49, 67, 90). An alternate strategy for controlling mosquito-borne diseases is the genetic manipulation of natural mosquito populations to reduce their effectiveness as disease vectors (14, 46, 53, 54, 64, 65, 74). Clearly, this strategy necessitates efforts directed toward an in-depth understanding of mosquito genetics, particularly as it relates to the genetic basis for vector competence and subsequent interference with that competence. In this review, we focus on advances made during the last 10 years in understanding mosquito genetics and vector competence at the molecular level. This research has been fueled largely

by the renaissance in quantitative and population genetics resulting from contemporary advances in DNA-based marker development that can readily be applied to arthropods (128, 190).

CONTEMPORARY GENETIC MAPPING

Genetic markers provide powerful tools for tracking groups of genes or genome segments and evaluating their influence on a phenotype of interest. Construction of and access to detailed linkage maps permits identification and localization of genes that control both simple and complex traits. Historically, mosquito genomics has been the subject of considerable research interest, yet advances were eventually limited by the prevailing technology (110). Available genetic marker systems included morphological mutants, isozymes, and some insecticide resistance loci. Although clearly instrumental to our present knowledge of mosquito genomics, these markers could not be used to provide dense marker coverage with single mapping populations. In addition, some of these markers, especially morphological mutants, had significant deleterious effects on particular genotype classes (e.g. were nonneutral markers) and, therefore, directly influenced the outcome of genetic crosses.

The foundation for contemporary mosquito genomics is in the demonstration that recombinant-DNA technology provides a mechanism for developing genetic markers that detect polymorphism at the level of the individual nucleotide (40). Such markers are selectively neutral, often codominant in inheritance, and seemingly unlimited in number. The result has been a proliferation of densely populated genetic maps for an abundance of organisms, including mosquitoes.

All mosquitoes are members of the family Culicidae, which is divided into three subfamilies—Culicinae, Anophelinae, and Toxorhynchitinae (109). Toxorhynchitinae spp. do not feed on blood and therefore are not involved in transmitting diseases to vertebrates. Consequently, contemporary molecular-mapping strategies have not been applied to these species. Both the Culicinae and Anophelinae contain species recognized globally as principal vectors for mosquito-borne diseases. The Culicinae are commonly associated with arbovirus transmission, including dengue fever and yellow fever, and also transmission of lymphatic filariasis. The Anophelinae are the primary vectors for malaria transmission. Considerable research over the past decade has been directed toward development of molecular approaches for genetic analyses of selected species within both subfamilies. Because the two subfamilies vary significantly in their fundamental genome architecture (110, 172), the specific approaches used for genetic analyses at the molecular level reflect, at least in part, some of these differences.

Culicinae

***Restriction Fragment Length Polymorphism Markers and* Aedes aegypti**   Historically, from a genetic viewpoint, the most widely studied mosquito is the yellow

fever mosquito *Aedes aegypti*. A classical genetic map for *Ae. aegypti*, which consists of 77 morphological, isozyme, and insecticide resistance markers that cover 171 recombinational map units, was the first extensive genetic map for a mosquito species (63, 152). Development of this map provided a springboard for partial map development in several other mosquito species and, more recently, creation of a standard for interspecific comparisons concerning chromosome evolution in mosquitoes (137).

Ae. aegypti has maintained its presence at the forefront of studies of mosquito genetics. The first DNA-based, complete mosquito genetic map was constructed for *Ae. aegypti* (195). This map was developed using molecular techniques that identify specific regions of a genome using restriction fragment length polymorphisms (RFLPs) (191). Briefly, 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. RFLPs specific to each individual are identified by autoradiography. The initial RFLP map for *Ae. aegypti* consisted of 50 DNA markers that covered 134 recombinational units across the genome and included several morphological marker loci (195). This allowed partial integration of the RFLP map with the classical genetic map. At present, this map consists of 120 RFLP loci that cover 187 Kosambi centiMorgan units (cM) across the *Ae. aegypti* genome and includes 6 morphological marker loci (DW Severson, unpublished data).

RAPD-Single-Strand Conformation Polymorphism Markers   Antolin et al (5) also developed a genetic map for *Ae. aegypti*, using a different DNA-based marker strategy. Two molecular strategies were combined to identify DNA polymorphisms. First, the basic marker strategy used the polymerase chain reaction (PCR) to amplify arbitrary genome regions using a single 10-base primer (236), commonly referred to as RAPD-PCR. This technique permits amplification and identification of relatively short genome regions in which the primer sequence exists as an inverted repeat. Using single-strand conformation polymorphism (SSCP) analysis of the products increased the efficiency of RAPD-PCR assays. SSCP analysis identifies point mutation differences in single-stranded DNA fragments that result in their altered electrophoretic mobilities through nondenaturing acrylamide gels (93, 163), and this method has been adapted for mosquitoes (33).

The RAPD-SSCP map developed for *Ae. aegypti* consisted of 96 loci that covered 168 cM (5). The map size is therefore similar to that reported for the map created using RFLP markers (195; DW Severson, unpublished data). Although useful for genetic studies in mosquitoes, a significant drawback to RAPD markers is that individuals are usually scored only for the presence or absence of a particular amplified fragment, and, therefore, the observed polymorphisms segregate as dominant characters. This reduces their informativeness for genetic studies because homozygotes and heterozygotes carrying the amplifiable allele cannot be distinguished. Antolin et al (5) did report, however, that 11% of their RAPD-SSCP

loci segregated as codominant loci in *Ae. aegypti*. Some additional cautions to be observed with RAPD markers are that loci mapped in one segregating population may not be evident in analyses of additional populations and amplified fragments across populations may represent fragment size convergence (homoplasy) rather than allelic identity by descent. In practice, a new genetic map must be prepared for each segregating population investigated. Finally, the RAPD-PCR technique is inherently subject to reproducibility problems, both within and between laboratories, which significantly reduces its utility as a genetic marker (32). Mutebi et al (153) used RAPD-SSCP marker analysis to construct a genetic map for the Asian tiger mosquito, *Aedes albopictus*. This map consisted of 77 loci that covered 225 cM, with an average of 12.5 loci per RAPD primer examined. In contrast to observations with *Ae. aegypti*, none of the RAPD-SSCP loci identified for *Ae. albopictus* segregated as codominant markers. Mutebi et al (153) suggest that this may reflect the use of selected laboratory strains for preparing mapping populations in *Ae. albopictus*, whereas one of the parental strains used in the *Ae. aegypti* mapping efforts arose from an unselected field strain.

Microsatellites   Another very popular DNA-based genetic marker system is based on simple sequence repeat (SSR) or microsatellite DNA. Microsatellites are tandemly repeated sequences composed of simple repeat units of 1 to 5 base pairs, and they seem to be ubiquitous and randomly distributed throughout most eukaryote genomes (102, 208). These repetitive elements tend to be hypervariable among populations, because slippage events during DNA replication can increase or decrease the number of simple repeat units within individual elements (207). Of particular interest in genetic studies, size polymorphisms between individuals can be identified using primers specific to unique DNA sequences flanking the repeat element in a simple PCR amplification, with subsequent allele discrimination using gel electrophoresis (232). Indeed, microsatellites have become a preferred genetic marker system. The literature reflects several excellent examples among arthropods of abundant microsatellite identification and development as genetic markers using standard small-fragment, partial-genome libraries and enrichment strategies (76, 99, 177, 213, 240–242).

It is curious that, using standard procedures, microsatellites have proved to be under-represented in some arthropod species, including some culicine mosquitoes. All 10 repeat motifs tested for abundance among two mite species, *Tetranychus urticae* and *Amblyseius fallacis*, reflected severalfold under-representation compared with typical vertebrates (157). Furthermore, the usually common dinucleotide repeats $(CT)_n$ and $(GT)_n$, when present, were composed of a very low number of repeats; generally, there were no more than four repeat units. The common microsatellite motifs were also in relatively low abundance among four phlebotomine sandfly species (69). It is interesting that the most abundant microsatellite in sandflies is the trinucleotide repeat $(AAT)_n$. Both dinucleotide and trinucleotide repeat motifs appear to be under-represented in the mosquitoes *Culex pipiens quinquefasciatus* (80) and *Ae. aegypti* (98; WC Black, unpublished data; SE Brown,

unpublished data; JK Meece & DW Severson, unpublished data). At present, no explanation exists for this phenomenon, although it probably is related to some unique aspects of genome organization in these organisms. Recent results with *C. pipiens* indicate that microsatellite repeats are adequately represented to develop a primary linkage map (L Zheng, unpublished data).

Anophelinae

Restriction Fragment Length Polymorphism Markers   The lack of suitable polymorphic markers and difficulties associated with the laboratory maintenance of *Anopheles* spp. have, until recently, limited the development of detailed genetic maps for individual species. Before the development of DNA-based marker systems, composite maps, which consisted of modest numbers of morphological, isozyme, and insecticide resistance markers, were available for a handful of species (141, 166, 186, 188, 211). Even today, nearly all efforts at developing DNA-based genetic markers for anophelines have focused on the principal malaria vector, *Anopheles gambiae*.

The relatively small amount of DNA per mosquito and technical difficulties associated with the technique also have limited the development and utility of RFLP markers for anophelines. Romans et al (184) reported independent assortment between RFLP for a diphenoloxidase gene (*Dox-A2*) located on chromosome 3 and an esterase locus that correlated with refractoriness of *An. gambiae* to the malaria parasite *Plasmodium cynomolgi*, and more recently, provided direct evidence of linkage between the *Dox-A2* RFLP locus and the *Plasmodium*-refractory phenotype (183). Five cDNA clones were mapped as RFLP loci in a study that investigated the quantitative genetics of CM-Sephadex bead melanization after their injection into individuals of a segregating population of *An. gambiae* (86).

RAPD Markers   This marker type has received limited application to anopheline genetics. Favia et al (77) identified ∼60 RAPD fragments that were differentially amplified among strains of *An. gambiae* and *Anopheles arabiensis*. Subsequently, 15 RAPD fragments were integrated into an existing genetic map for *An. gambiae* (72). Sequence analyses indicated that 4 of the 15 fragments contained microsatellites, and they were therefore mapped genetically as microsatellite markers rather than as RAPD markers.

Microsatellites   Unlike the culicine mosquitoes, microsatellites are both abundant and highly polymorphic within anopheline genomes. A detailed genetic map has been constructed for *An. gambiae* that consists of 148 microsatellite markers that cover ∼215 cM (72, 227, 242). About 89% of readily amplifiable $(GT)_n$ or $(GA)_n$ microsatellite repeats proved to be polymorphic among the *An. gambiae* strains tested (241). Although most of the polymorphic microsatellites contained perfect repeat arrays of 6–40 units, ∼23% were interrupted with variant sequences (241, 242). Interrupted sequence arrays were about twice as common (47%) among

the nonpolymorphic microsatellite repeat group (241). This supports suggestions that interruptions within the core repeat tend to stabilize the entire repeat array, making interrupted sequence arrays less variable (168). Procedures have been described for semiautomated high-throughput microsatellite analysis in *An. gambiae* using fluorescent primers and automated DNA-sequencing technology (227).

Comparative Genomics

Advances in insect genomics have been limited by the general lack of genetic information for individual species, caused in part by the lack of suitable cytogenetic or genetic tools with broad applicability across species (95). A feasible strategy for determining the extent of chromosome conservation and rearrangement across species is to compare the map positions of homologous genes. Homologous genes can be identified directly by DNA-DNA cross-hybridization and by sequence analysis. Development of DNA-based genetic markers provides a suitable basis for comparative genome mapping, as evidenced by a rapidly expanding body of literature in this area dealing with both animal and plant species. For example, conserved syntenies are evident across mammal species (75) and across cereal grasses (3, 169, 220). Comparative linkage maps can be used effectively to predict the location of a particular gene in one species based on knowledge of its position in another species (154). These maps could prove extremely effective for application to mosquito genetics, given the biological differences between species. That is, a comparative mapping approach could be used initially to evaluate gene functions in mosquito species that are more tractable to laboratory culture and controlled matings, and thereafter these gene functions could be efficiently evaluated in more recalcitrant, yet medically important, species.

A synthesis by Matthews & Munstermann (137) of available isozyme linkage data representing mosquito species across all three subfamilies provided considerable insight into chromosome evolution in mosquitoes. Linkage conservation, even across diverse genera, was sufficient to permit identification of six syntenic arrangements of enzyme loci, which is suggestive of ancestral linkage groups. Matthews & Munstermann suggested that these six syntenic groups probably represented whole chromosome arms. They further suggested that karyotype evolution in mosquitoes may, therefore, have largely involved gross structural changes, including Robertsonian translocations and paracentric inversions.

An important feature of the *Ae. aegypti* RFLP map is that most of the markers are random cDNA clones or cDNA clones of known *Ae. aegypti* genes (195). Therefore, these sequences probably represent single loci that will be conserved across species. Severson et al (197) demonstrated that *Ae. aegypti* RFLP markers derived from cDNA clones would be extremely useful for comparative gene mapping across mosquito species. They showed that homology of these loci could be examined through hybridization with genomic DNA from several culicine mosquitoes including *Ae. albopictus, Ae. togoi, Armigeres subalbatus*, and *C. pipiens* and also

with an anopheline, *An. gambiae*. Homology was guaranteed by performing all hybridizations under highly stringent conditions.

The RFLP markers developed for *Ae. aegypti* have subsequently been used to develop comparative linkage maps for several culicine species. The first comparative RFLP linkage map was developed for *Ae. albopictus* (194). This map included 18 RFLP markers that provided broad coverage of the *Ae. aegypti* genome. The linkage group associations and linear orders of these markers appeared to be identical between the two species. The loci mapped in *Ae. albopictus* covered 98.4 cM, whereas the same loci covered 129.1 cM in *Ae. aegypti*. The difference between the two species largely reflected reduced recombination levels for chromosome 1 in *Ae. albopictus*.

A comparative map developed for *Ar. subalbatus* also showed considerable conservation with *Ae. aegypti* (79). For 25 of the 26 RFLP markers examined, the linkage groups for *Ar. subalbatus* were consistent with *Ae. aegypti*, although the chromosome 2 marker order reflected an inversion that involved several markers in the centromere region. A significant difference between *Ae. aegypti* and *Ar. subalbatus* is that the sex determination locus in *Ar. subalbatus* maps to chromosome 3, whereas in *Ae. aegypti* and most other culicines, it maps to chromosome 1. It is interesting that no evidence was observed for the translocation of RFLP markers that are tightly linked with sex determination. Although this could reflect a difference in sex determination mechanisms between the two species, cytogenetic evidence revealed species-specific chromosomal differences in the presence of a female-determining heterochromatic band (172), which suggests that a common mechanism does exist. That is, the heterochromatic band is located on different chromosomes in *Ae. aegypti* and *Ar. subalbatus*.

The comparative linkage map constructed for *C. pipiens* reflects extensive chromosomal rearrangements when compared with *Ae. aegypti, Ae. albopictus, or Ar. subalbatus* (142). The *C. pipiens* comparative map consists of 21 RFLP markers that provide broad coverage of the *Ae. aegypti* genome. These markers identify 22 loci covering 165.8 cM. Chromosome 1 markers seem to be conserved across all four species examined to date, although, as observed with *Ae. albopictus* (194), the recombination frequencies for these markers are remarkably reduced in *C. pipiens*. However, as predicted by Matthews & Munstersmann (137), *C. pipiens* chromosomes 2 and 3 largely reflect apparent whole-arm translocations when compared with *Ae. aegypti* chromosomes 2 and 3. In addition, several inversion polymorphisms are evident.

From a broader comparative-mapping perspective, partial sequence analyses of several *Ae. aegypti* cDNA-based RFLP loci (200) suggested that comparative map analysis with *Drosophila melanogaster* is feasible. Recent success in sequencing the *Drosophila* genome will facilitate this analysis (1). From a whole-genome perspective, considerable genome divergence is evident between the two species (233). However, a potential for conserved localized syntenies (microsynteny) was previously noted by Matthews & Munstermann (137), because some isozyme linkage homologies seemed to be conserved between mosquitoes and *D. melanogaster*. Conserved syntenies are segments of homology composed of two or more pairs of

homologous genes located on the same chromosome regardless of order, whereas conserved linkages are segments within which synteny and gene order are retained (154). At present, we have identified 49 homologous genes in *Ae. aegypti* and *D. melanogaster* (DW Severson, unpublished data). Of particular interest are conserved genome regions carrying genes that determine vector competence in *Ae. aegypti*. This strategy seems to be working well for genetic diseases in humans and their mouse homologs (48, 68, 94, 121, 154, 156, 162).

QUANTITATIVE GENETICS

The joint action of two or more individual genes, as well as a balance between the individual gene effects and their interactions with each other and with the environment, determines many important phenotypic characteristics. The phenotypic outcome usually reflects continuous variation among individuals within populations. These traits are generally referred to as multigenic or quantitative traits, and the individual gene locations as quantitative trait loci (QTLs). For most quantitative traits, little is known about the number of genes involved, their chromosomal location, or their gene product.

The development of complete (saturated) genetic linkage maps that consist of DNA-based marker loci provides a useful mechanism for the resolution of these complex phenotypic traits into their individual genetic components (118). Briefly, individuals from genetic crosses (such as backcross or F_2 or recombinant inbred lines) between strains with contrasting phenotypes are evaluated for the trait of interest and for their genotypes at DNA-based marker loci at regular 10- to 20-cM intervals across the genome. Statistical differences between the mean phenotype and marker genotype classes are then used to determine the genome positions and the effects of QTLs that contribute to the phenotype (190).

Successful mosquito-borne transmission of a parasite to its vertebrate host reflects the culmination of a complex series of events within the mosquito. After ingestion by a female mosquito in a blood meal obtained from an infected vertebrate, a parasite must avoid an arsenal of internal active defense mechanisms dedicated to the recognition and subsequent destruction of nonself (foreign) entities (50). Successful metazoan parasites are able to avoid triggering an active immune response by the mosquito during their migration from the blood meal to a tissue-specific site, where they complete development intracellularly. In some instances, however, the parasite is subject to active cellular and humoral immune responses by the mosquito or reaches the appropriate target site for development but fails to initiate or complete development and subsequently dies. The basis for incompatibility between parasite and mosquito host appears to be, at least in part, determined by genetic factors (49).

Filarial Worm Transmission

Macdonald (129, 130) selected a strain of *Ae. aegypti* (Liverpool strain) that was highly susceptible to a lymphatic filarioid nematode parasite of humans, *Brugia*

malayi, and subsequently demonstrated that susceptibility was determined primarily by a single, recessive, sex-linked gene designated f^m (131, 133). *B. malayi* migrates to and completes development within the thoracic musculature of the mosquito. Three-point linkage estimates indicated a linear order of *re* (*red-eye*)-sex determination-f^m, with an observed recombination fraction of 3.4%–7.8% between sex determination and f^m. The f^m gene also determined susceptibility to other filarial worm species that developed within the thoracic musculature, including *Brugia pahangi* and *Wuchereria bancrofti* (132). Nonetheless, these studies and others (231) also suggested that additional genes were involved because f^m did not entirely control susceptibility.

Severson et al (196) provided the first demonstration of the feasibility and power of using DNA-based marker linkage studies to identify genetic loci involved in mosquito vector competence to transmit parasites to humans. Severson et al essentially repeated the classical experiments of Macdonald and coworkers with *Ae. aegypti* and *B. malayi*, but they used RFLP markers, which allowed them to conduct whole-genome scans for loci that determined susceptibility. Two QTLs that determined *B. malayi* susceptibility were identified. 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. The *fsb1* QTL is probably the f^m locus, because it exhibits a recessive effect on susceptibility (129, 132) and it maps to the same genome region on chromosome 1 (131, 133).

Although no gene products have been identified, Severson et al (196) also suggested that *fsb1* seems to permit filarial worm development, but subsequent expression of the susceptible phenotype depends on the genotype at *fsb2*, as reflected by the significant epistatic interaction between the two loci. Furthermore, as has been indicated by QTL analyses in other organisms (12, 125), the effect of the individual QTL that determine filarial worm susceptibility and interactions between loci varied considerably, depending on the genetic background of the mosquito strains that contributed to the individual segregating populations (196).

Several studies have shown that environmental effects and the genetic background of the mosquito midgut significantly influence the number of microfilariae (mf) ingested and subsequently their penetration into the hemocoel (15, 83, 160, 204). Substrains of *Ae. aegypti*, highly susceptible and highly refractory to *B. malayi* infection, were selected from the susceptible Liverpool strain (15); whole-genome RFLP analyses of individuals representing both substrains indicated that they differed in allelic configuration at a number of loci genome wide (193). Most of the loci examined on chromosome 2 were fixed for different alleles in the two substrains, suggesting that at least part of the phenotypic difference between them could be caused by genes located on this chromosome. Genetic studies involving these strains confirmed that a QTL on chromosome 2, *idb*[*2,LF181*] or *idb1*, significantly influences the number of mf ingested (15). Additionally, *idb1* also seems to influence midgut penetration by mf, because in vitro assays indicated that

genetically susceptible mosquitoes not only ingested more mf, but the percentage of ingested mf that actually penetrated the midgut was always significantly greater than observed with genetically refractory mosquitoes (15).

Identification of QTLs determining *B. malayi* (or other parasite) susceptibility in mosquitoes provides an initial framework for efforts to isolate the associated genes using established map-based or positional-cloning procedures (92, 116, 159, 179, 206, 237). It is then necessary to saturate the genome regions defining QTLs with additional markers to narrow the physical distance that must be examined. Bulked segregation analysis has been used as an effective tool to identify novel markers linked with a target phenotype or genotype. 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 other areas of the genome. Severson et al (199) used this approach to identify RAPD-PCR fragments that are linked with markers flanking *fsb1*. The fragments were cloned and mapped as RFLP loci. Of the five RAPD-RFLP loci linked with *fsb1*, one mapped directly within the *fsb1* region, which reduced the *fsb1* interval from 10.4 cM to 2.9 cM (196).

Malaria Transmission

The majority of *Plasmodium* and mosquito combinations are incompatible, because most mosquito species are totally incapable of supporting the growth and development of a particular *Plasmodium* species. A primary factor limiting the ability of some *Plasmodium* spp. to successfully complete development in several mosquito species, including *An. gambiae*, seems to be the inability of the ookinete to complete development to the oocyst. Not surprisingly, the midgut-specific, blood meal-activated genes of mosquitoes, particularly *Anopheles* spp., have been intensively studied as potential vehicles for driving the expression of anti-ookinete factors (8, 9, 10, 73, 150, 151, 203). This parasite/vector incompatibility seems to have strong, yet complex, genetic components, including apparent variation in the observed phenotype associated with refractoriness. Nonetheless, there presently is no gene or gene product that has been shown, at the molecular level, to directly affect phenotype with respect to genetic refractoriness, although molecules in the kynurenine pathway have recently been shown to affect microgametocyte exflagellation (30, 82).

Anophelinae   Collins et al (57) selected for a refractory strain of *An. gambiae* that encapsulates most *Plasmodium* spp. in a layer of melanin at the ookinete or early oocyst stage. It is interesting that this strain is nearly completely refractory to a wide variety of simian, rodent, and avian *Plasmodium* spp., but exhibits a variable response to human *Plasmodium* spp. The strain seems to be highly refractory to *Plasmodium vivax, Plasmodium ovale*, and neotropical and Southeast Asian isolates of *P. falciparum*. However, it reflects limited refractoriness to African isolates

of *P. falciparum* and to *Plasmodium malariae*, suggesting that the refractoriness phenotype is determined by interactions involving both the mosquito and the parasite genomes. It also is important that the refractory phenotype is manifested solely by an encapsulation response and reflects no obvious influence relative to the observed intensity of infection in individual mosquitoes.

Initial genetic analyses indicated that the melanotic-encapsulation response to malaria parasites involved the effects of at least two unlinked genes, *Pif-B* and *Pif-C*, one of which (*Pif-B*) has a partially dominant effect and is associated with the 2La region of chromosome 2 (57, 66, 222, 223).

Zheng et al (243) used microsatellite markers to identify QTLs associated with the melanotic-encapsulation response among segregating progeny of five backcross families. The major QTL, *Pen1*, is located within the 2R region of chromosome 2 and accounts for ∼54% of the phenotypic variability in the phenotype. A second QTL, *Pen2*, is located within the 3L region of chromosome 3 and accounts for ∼13% of the variability. The third QTL, *Pen3*, is linked with *Pen1* and contributes a very minor effect on the phenotype. Additionally, genetic analyses of the ability of the same *An. gambiae* strains to melanize CM-Sephadex beads (167) identified a single, major locus, *mep1*, that accounted for 44% of the observed phenotypic variance (85, 86). Interestingly, the *mep1* locus colocalizes with the major locus associated with melanotic encapsulation of the parasite on chromosome 2, *Pen1* (243), thus supporting earlier evidence that bead and parasite encapsulations share some common genetic mechanisms (84). Fine-scale mapping efforts are targeting *Pen1* (56).

It is curious that neither of these QTL mapping studies identified a locus within the 2La region as initially indicated (57, 66, 222, 223). Zheng et al (243) suggested that the reported association with the 2La region may be an artifact of the previously available strains or that a locus within this region is required for the expression of *Pen1, Pen2,* and *Pen3* but is not directly involved in encapsulation. Moreover, a recent study suggests that a locus on chromosome 3 that is independent of *Pen2* also influences refractoriness (183). Again, however, such variable and seemingly conflicting results are not unprecedented with analyses of complex traits (12, 125).

A nonmelanizing mechanism of *Plasmodium gallinaceum* refractoriness in *An. gambiae* also has been described, wherein oocysts fail to develop as a result of ookinete lysis after successful invasion of midgut epithelial cells by ookinetes (224). This intracellular ookinete-killing mechanism could be active in natural *An. gambiae* populations because many *P. falciparum* ookinetes in a blood meal never become oocysts (221). The intracellular ookinete-killing mechanism seems to be genetically determined by a single dominant locus on chromosome 3L in *An. gambiae* (KD Vernick, unpublished data). It remains unclear, however, whether the lytic and melanotic encapsulation phenotypes are influenced by the same gene(s). Failure of oocysts to develop also has recently been investigated at the genetic level with *P. falciparum* and *Anopheles stephensi* (78). These studies found that refractoriness is autosomal and semidominant to susceptibility.

Culicinae   Although ookinete death has not been examined at the ultrastructural level in refractory *Ae. aegypti*, it is possible that the mechanism is identical to that observed in *An. gambiae*. That is, melanotic encapsulation of oocysts is rarely observed in refractory *Ae. aegypti*, but, instead, oocysts fail to develop on the midgut and are presumably lysed (107, 198, 212). Kilama & Craig (107) demonstrated that *Ae. aegypti* susceptibility to *P. gallinaceum* was primarily determined by a single gene, *pls*, located on chromosome 2.

Severson et al (198) used RFLP markers to examine *P. gallinaceum* susceptibility in *Ae. aegypti* as a complex trait, and identified two QTLs with significant effects on the observed phenotype. One QTL on chromosome 2, designated *pgs*[*2,LF98*] or *pgs1*, accounted for the largest fraction of the observed phenotypic variance in two independently prepared populations (49% and 65%, respectively) and exhibited a partial dominance effect on susceptibility. The *pgs1* QTL likely represents the previously described *pls* locus, because its effect is consistent with that of the *pls* locus, in which susceptibility is a partially dominant characteristic (107, 212), and, most importantly, it also maps to the genome region previously reported to contain *pls* (107). A second QTL on chromosome 3, designated *pgs*[*3,MalI*] or *pgs2*, accounted for a smaller fraction of the observed phenotypic variance in the two populations (10% and 14%, respectively) and exhibited an additive effect on susceptibility. Evaluations of pairwise interactions between the two genes indicated a lack of epistasis between them. This indicates that the effects of the two genes are independent of each other. It also means that, for each copy of a susceptible allele that an individual mosquito carries at each of these loci, the greater the number of oocysts that will develop. The bulked segregant procedure also has been used to identify new markers linked with or internal to the *pgs1* region (199).

Flavivirus Transmission

Susceptibility of *Ae. aegypti* to oral infections with flaviviruses also reflects multigenic inheritance (38, 39, 89, 140, 205, 226). Bosio et al (38) performed a classical quantitative genetic analysis of dengue-2 virus development within two *Ae. aegypti* strains differing in their oral susceptibility to the virus, and found that both midgut epithelial cell infection and subsequent dissemination, as measured by head titers, were highly heritable traits. With an *Ae. aegypti aegypti* strain from Puerto Rico, ∼50% of the phenotypic variation in midgut infection and ∼39% of the phenotypic variation in dissemination were attributable to a combination of additive and dominant genetic effects. With an *Ae. aegypti formosus* strain from Nigeria, the total genetic effects accounted for ∼39% and 68% of the phenotypic variation in midgut infection and dissemination, respectively. Also, two sets of genes likely influenced vector competence, with one set controlling a midgut infection barrier and another controlling a midgut escape and dissemination barrier. Recent QTL analyses with a segregating population derived from these same mosquito strains have identified two QTL-controlling a midgut infection barrier on chromosomes

2 and 3, respectively, and a single QTL on chromosome 3 controlling a midgut escape and dissemination barrier (39).

PHYSICAL MAPPING

Efforts to construct physical maps of genomes are founded in the science of cytogenetics, and these initial cytogenetic physical maps of mosquito chromosomes were used to study genetic relationships among and between mosquitoes (110). The inherent banding patterns in anopheline polytene chromosomes were useful, resulting in polytene chromosome maps for anophelines (60, 61, 108) and in culicines, primarily *C. pipiens* (210). Polytene chromosome physical maps for most culicines have been difficult to construct. Examination of mitotic characters and differentially stained or treated metaphase chromosomes has been used for most culicine cytogenetic studies (145, 171, 174).

The future for physical mapping in mosquitoes is clear. While cytogenetic physical mapping is considered low resolution, these maps will progress in their degree of resolution until the primary sequences of the mosquito genomes are determined. Recently, an ∼120-Mbase (Mb) sequence of *D. melanogaster* euchromatic genome was determined and annotated (1). *D. melanogaster* is not only the model dipteran, but also a model eukaryote. While *D. melanogaster* research embarks on the era of functional genomics, mosquito physical mapping is in an early stage of discovery of its genome organization. Mosquito genomics will clearly benefit from the *D. melanogaster* paradigm, but physical mapping in mosquitoes has been motivated toward the identification of those gene(s) that influence mosquitoes to function as a pest species to humans and other animals. Mosquito physical mapping is a work in progress, and it is focused primarily in map-based positional cloning strategies directed toward identifying important pest-related genes.

Genome Size and Organization

The direct manipulation of chromosomal DNA is prohibited because of its size. With the advent of recombinant-DNA technologies, this physical-size dilemma was circumvented by the production of libraries that represent the genomic complement as subgenomic clones. These important reagent libraries are needed in map construction and map-based gene identification. Genome size variation among and between organisms also reflects the ratio of unique or single-copy sequences relative to sequences that are found repetitively in the genome.

In the Culicidae examined, the genome complement is three paired chromosomes ($2n = 6$), with one exception, an anopheline *Chagasia bathana* ($2n = 8$) (111). The haploid genome consists of three metacentric chromosomes (95, 235). The chromosome arrangement consists of a smaller set of metacentric sex-determining chromosomes and two larger metacentric or submetacentric autosomal chromosomes. In culicines, the chromosome-1 pair is similar in size and it contains an autosomal sex locus. Anophelines have sex chromosomes akin to

those of *D. melanogaster*, where the Y chromosome is smaller than the X chromosome. In addition, the anopheline Y chromosome is not found as a polytene in the salivary glands. The male and female karyotypes are dimorphic, and the mechanics for sex determination are similar to those of mammals (95).

The genome sizes for mosquitoes from the Anophelinae, Culicinae, and Toxorhynchitinae have been estimated both by Feulgen cytophotometry and by DNA reassociation kinetics as reviewed elsewhere (21, 110). The anopheline genomes are the smallest and possibly the most primitive members of the family because they exhibit the least variation in size (176). The genome size estimate for *An. gambiae* is 0.27 pg (265 Mb, where 1 pg = ~980 Mb) (24), and six additional anopheline species range from 0.23 to 0.29 pg (225–284 Mb) (104). Culicinae have the largest genomes and exhibit the most interspecies and intraspecies variation in size (176). While the genome size range for *Ae. aegypti* is 0.78–0.83 pg (764–813 Mb) (175, 229), the size range for *Aedes* species is 0.59–1.9 pg (578–1862 Mb) (31, 104, 114, 175). The genome size variation is likely explained by the complexity of repetitive elements (31, 52, 113, 138).

In mosquitoes, 18%–84% of the genome is repetitive (31). In eukaryotes, the organization of repetitive elements in the genome has been described as having either a short-period or long-period interspersion. Long-period interspersion patterns are characteristic of smaller genomes like that of *D. melanogaster* (135) and anophelines (31, 117). Most of the 18% repetitive DNA found in anopheline species is either interspersed in <100 blocks or not interspersed. Short-period interspersion patterns are typical for larger eukaryotic organisms, such as the human genome, and, oddly, aedines (229). *C. quinquefasciatus*, which has an intermediate genome size, has been described as having intermediate- to short- or long-period interspersion (52). However, the genus *Culex* has been classified as having short-period interspersion based on results from DNA reassociation kinetics (31).

Repetitive sequences fall generally into two classes; moderately repeated sequences usually occur in 10–300 copies in the genome, and highly repeated sequences might range from 5000 to 15,000 copies. In mosquitoes, the repetitive elements that have been described are microsatellites, minisatellites, various transposable elements, and ribosomal DNA (rDNA; 20, 110, 244).

GenBank (release 117) holds 1835 culicine and 24,714 anopheline sequences, representing 1.5 Mb and 17.8 Mb, respectively. Most of the anopheline sequences result from the large sequence-tagged connector program that yielded end-sequence data on the *An. gambiae* strain PEST bacterial artificial chromosome (BAC) library (58). When the repetitive elements in these mosquito sequences are tabulated by subfamily and compared with known *Drosophila* elements using RepeatMasker software (Table 1), similarities and differences are seen between the anopheline and culicine genomes. Even though there is a 10-fold difference in the amount of sequence data available for analysis, both subfamilies exhibit a 45.9% GC content (Table 1), and 2% of the sequence data in each subfamily was identified as repetitive. The distribution of the retroelements between the two subfamilies is also similar at ~0.2%. Differences are seen in the simple repeats and low-complexity

TABLE 1 Repetitive elements in anopheline and culicine sequences from GenBank (release 117) relative to *Drosophila* spp.

Repetitive elements	Anophelinae Subfamily[a]			Culicinae Subfamily[b]		
	Number of elements[c]	Length occupied (bp)	Percentage of sequence	Number of elements[c]	Length occupied (bp)	Percentage of sequence
Retroelements	289	34,798	0.2	17	3552	0.24
LINEs:	168	13,959	0.08	15	3388	0.23
SINEs:	0	0	0	0	0	0
LTR elements:	121	20,839	0.12	2	164	0.01
Gypsy-type	21	8621	0.05	0	0	0
Copia-type	4	328	0	0	0	0
PAO-type	96	11,890	0.07	2	164	0.01
DNA elements:	9	467	0	1	447	0.03
Tcl-type	9	467	0	0	0	0
Unclassified:	0	0	0	0	0	0
Total interspersed repeats:		35,265	0.2		3999	0.27
Small RNA:	12	1505	0.01	0	0	0
Satellites:	1	121	0	0	0	0
Simple repeats:	3955	167,033	0.94	170	8071	0.54
Low complexity:	3107	160,084	0.9	281	21,119	1.42

[a]The Subfamily Anophelinae comprised 24.715 GenBank sequences with a total length of 17,780,773 bp. The GC level was 45.93% and 363,772 base pairs (2.05%) were masked.

[b]The Subfamily Culicinae comprised 1835 GenBank sequences with a total length of 1,483,919 bp. The GC level was 45.90% and 33,123 base pairs (2.23%) were masked.

[c]Most repeats fragmented by insertions or deletions have been counted as one element. The sequence(s) were assumed to be of *Drosophila* origin. RepeatMasker version 04/04/2000 default settings, ProcessRepeats version 04/04/2000, Repbase version 03/31/2000 (AFA Smit & P Green, RepeatMasker, unpublished results at http://repeatmasker.genome.washington.edu/cgibin/RM2_req.pl). The RepeatMasker drosophila.lib file was the repeat library screened, and there are no SINEs annotated in this library.

sequence elements, with 0.9% and 0.9% seen in anophelines and 0.5% and 1.4% seen in culicines, respectively. The culicine sequences exhibit 0.23% long interspersed elements and 0.01% *Drosophila* long-terminal repeat (LTR) elements as compared with 0.08% and 0.12%, respectively, seen in anophelines. Whether these differences are representative of the two subfamilies or are the result of inadequate culicine sample size remains to be demonstrated. Clearly, the list of mosquito repetitive elements has grown as a result of recent sequencing efforts in Culicidae (Table 2).

It has been estimated that the *An. gambiae* genome has ∼10,000 SSRs of the $(dG-dT)_n$ motif in the haploid genome, whereas the dG-dT motif is infrequent in the genome of *Ae. aegypti* (110, 244). Simple interpolation of the simple repeat data resulting from RepeatMasker analysis (Table 1) suggests that there may be >58,000 SSR elements in the anopheline genome. The numbers of loci and their polymorphic nature lend to their use in the study of population genetics and gene flow (98, 105, 119, 120, 185). SSRs distributed along the genome have also been

TABLE 2 Repetitive elements of anophelines and culicines[a]

Repetitive elements	*Drosophila* homolog	Anophelines	Culicines	Reference(s)
Retroelements				
LINEs or nLTR				
		LINE-like		62
		Q	*Q*-like	19, 217
		RT1		23
		RT2		23
		T1		18
	Bilbo		*Bilbo*-like	RepeatMasker
	DMCR1A	*DMCR1A*-like		RepeatMasker
	G		*G*-like	RepeatMasker
	Jockey	*JUAN*-like	*JUAN*	RepeatMasker; 2, 17, 146, 147
	LINEJ1	*LINEJ1*-like	*LINEJ1*-like	RepeatMasker
	R2B	*R2B*-like	*R2B*-like	RepeatMasker
	TART_B1	*TART_B1*-like		RepeatMasker
			Lian	218
	I	*MosquI*-like	*MosquI*	RepeatMasker; 217
		I-like		RepeatMasker
SINEs		*Feilai*-like	*Feilai*	RepeatMasker; 215
LTR elements				
	Gypsy	*Accord*-like		RepeatMasker
		Bloodi-like		RepeatMasker
		DM412-like		RepeatMasker
		HMSBeagle-like		RepeatMasker
		Mdg1-like	*Mdg1*-like	RepeatMasker; cited in 170
		Mdg3-like		RepeatMasker
		Quasimodo-like		RepeatMasker
		Tabor-like		RepeatMasker
		Tirant-like		RepeatMasker
		Ty3-like		62
		Ulyss-like		RepeatMasker
		Zam-like		RepeatMasker
	Copia	*DM1731*-like		RepeatMasker
		Moose		29
		Ty1-like		62
		ZebeedeeI-like	*ZebeedeeI*	RepeatMasker; 230
		ZebeedeeII-like	*ZebeedeeII*	RepeatMasker; 230
			MosqCopia	217

(*Continued*)

TABLE 2   (*Continued*)

Repetitive elements	*Drosophila* homolog	Anophelines	Culicines	Reference(s)
	Pao	*Batumi*-like		RepeatMasker
		Bel-like	*Bel*-like	RepeatMasker; 217
		Diver-like		RepeatMasker
		Ninja-like		RepeatMasker; 62
		Pao-like		
		Roo-like	*Roo*-like	RepeatMasker
DNA elements		*Odysseus*		136
	Het-A		*CM-gag*	16
	mariner	*mariner*		180, 181
	Tc1	*Pegasus*		22
		FB4-like		RepeatMasker
		Ikirara		182
		Paris-like		RepeatMasker
		Quetzal		106
		Uhu-like		RepeatMasker
MITEs			*Dufu*	Cited in 170
			Kaizoku	215
			Pony	216
			Wujin	214
			Wukong	214
			Wuneng	214
			Youzi	215
Satellites	*HSATII*	*HSATII*-like		RepeatMasker

[a]RepeatMasker analysis was done using the GenBank release 117 sequences for anopheline and culicine described in Table 1 against the *Drosophila* repeat library. In addition, the anopheline sequences were also used with RepeatMasker using a culicine repeat library (DL Knudson, unpublished data). If the repeat name is noted as *repeatname*-like, this indicates a higher percentage of divergence from the core repeat element. LINE, long interspersed element; LTR, long terminal repeatname; MITE, miniature inverted-repeat transposable element; SINE, short interspersed element.

used to help in the integration of *An. gambiae* genetic-linkage maps and physical maps (58, 241, 242, 244).

Minisatellites are complex regions dispersed throughout the genome that contain variable numbers of tandem repeats. When the core variable numbers of tandem repeat unit is used as a probe of endonuclease-restricted genomic DNA, DNA fingerprint patterns are generated, and these patterns can be used to detect polymorphisms. For example, a house fly minisatellite yielded a DNA fingerprint for *Ae. aegypti* (36). As a group, mosquito minisatellites have not been examined in depth.

Transposable elements are moderately repetitive DNA sequences that are interspersed in the genome, and these elements move about the genome maintaining a copy at the original locus. There are two types of transposable

elements—transposons and retrotransposons. Transposons transpose from DNA to DNA, and retrotransposons transpose via RNA intermediates (37). Most of the knowledge available on dipteran transposable elements comes from *D. melanogaster*. Transposable elements are responsible for mutations and chromosomal rearrangements. The search for mosquito transposable elements or elements that will transpose in mosquitoes has intensified because of their potential use as transformation vectors that orchestrate the integration of recombinant genes into the mosquito genome (Table 2).

Retrotransposons can be divided into two groups; the LTR elements or the non-LTR retrotransposons (Table 1 and Table 2; 239). Non-LTR retrotransposons are classified as either random elements or siteposons based on their preferred integration site. Random elements are distributed across the genome, whereas the siteposons insert at specific locations (4).

The mosquito rDNA cistron is an arrangement of tandem repeats of between 100 and 1000 copies, and it has been found in one or more chromosomes. In *An. gambiae* and *An. arabienses*, the rDNA is found on the X chromosome, and it is found on chromosome 1 in *Ae. aegypti* and *C. quinquefasciatus*. X and Y chromosomes contain the rDNA cistron in *Anopheles quadriannulatus, Anopheles melas, Anopheles merus*, and *Anopheles quadrimaculatus*. Chromosomes I and III contain the rDNA in *Aedes triseriatus* (51). The variability found in the internal transcribed spacers and the intergenic spacers of the cistron has become an important target that is used for distinguishing species and complexes in Culicidae (13, 34, 55, 81, 112, 148, 164, 189, 209, 238).

Clustered, highly repetitive satellite DNA, found in thousands to millions of copies, has been studied in the mouse and *D. melanogaster* (41, 158), but there is little information on these elements in mosquitoes. The function of these nontranscribed repeats may be physical maintenance, replication, and transmission of chromosomes (103). The centromere plays a role in the distribution of chromosomes to daughter cells, and chromosome integrity is maintained by telomeric sequences (35, 149). Whereas centromeric (96, 234) and telomeric (25, 28, 122, 143, 161) sequences have been described for a number of organisms, the mosquito centromere sequences have not been delineated. A BAC clone has been isolated that shares sequence similarity with the centromeres of all three *Ae. aegypti* chromosomes, and it is being characterized (SE Brown & DL Knudson, unpublished data). The 2L chromosome telomeric region of a transgenic and wild-type strain of *An. gambiae* has been described (26, 27), and its structure is similar to the organization of the 2L chromosome telomeric region of *D. melanogaster*. Telomeric sequences have not been described for other mosquito species.

Cytogenetics and the Impact of Polytene and Metaphase Chromosomes on Low-Resolution Physical Mapping

Polytene chromosomes provide a distinct advantage in generating and integrating genetic and physical maps. Microdissection strategies have been used to generate a low-resolution genome map for *An. gambiae* (241, 245). Methods for in situ

hybridization to anopheline polytenes are well developed (187), and they have been used to physically map microsatellites, cDNAs, polymorphic RAPD clones, RAPD-derived sequence-tagged sites (STSs), and cosmids and BACs that contain genomic DNA (70, 72, 225, 241). The ability to map cDNAs or other small probes directly to polytenes by using standard in situ techniques highlights the real advantage of mapping with polytenes. This approach works because the chromosomal DNA has been amplified 2^n, where n may be as large as 10, that is, 1024 times, resulting in sufficient target sequence to yield a probe signal that is strong enough to visualize.

In Culicinae, useful polytene chromosomes have been isolated from *C. pipiens* (210). Although a preliminary physical map of polytene chromosomes for *Ae. aegypti* was reported (201), the difficulty in preparing consistent, reliable polytene chromosomes renders them unusable for further mapping studies (66, 144). The primary problem in their preparation is getting the polytene chromosomes to spread, and this is likely caused by ectopic pairing resulting from regions of highly repetitive DNA.

Integration of Genetic and Physical Maps

The recombinant mosquito libraries containing either large genomic fragments or cDNAs have been the essential reagents needed for the integration of genetic and physical maps. Cosmid and BAC libraries have been constructed from two different strains of *Ae. aegypti* (43, 44; DW Severson, SE Brown, & DL Knudson, unpublished data) and from *An. gambiae* (58, 70, 225). A number of mosquito cDNA libraries have also been constructed from mosquitoes at different life stages and from mosquitoes that are refractory and susceptible to filarial infection (7, 71, 197, 200; SS Gill, GenBank submissions).

The *An. gambiae* genetic and physical maps were correlated using microsatellite genetic markers and in situ hybridization. Low-resolution physical maps were constructed for *An. gambiae* by using microdissection and in situ hybridization to polytenes in 1991. Polytene chromosome divisions were microdissected into 54 groups (245). The groups were enzyme restricted and PCR amplified into pools. The divisional pools had a high complexity and were division specific by in situ hybridization, except near the centromere. A detailed genetic map for the X chromosome with 24 sex-linked microsatellite markers at a resolution of 2 cM (242) was anchored to the *white* eye mutation, which was then placed into the physical map by hybridization. Some of the microsatellites were division specific, and the other microsatellites were mapped to polytenes by in situ hybridization. In 1996, more integrated maps using microsatellites and RAPDs were generated. In situ hybridization was used to physically map 85 random cosmid clones, cDNA clones, or cloned RAPD to the five chromosome arms (70). The first genetic map from markers that covered all three chromosomes, with an average distance between markers of 1.6 cM, was reported (241). Of these markers, 47 were located on the polytenes by in situ hybridization or by division-specific probe isolation. An

additional 31 RAPD-derived STSs were integrated into the genetic and physical map of *An. gambiae* (72). In addition to the genetically mapped markers, 300 random cDNAs and genomic DNA clones have been put into the *An. gambiae* physical map (59). The maps and data are available from the *Anopheles* database (99a).

In contrast to the anopheline approach, in situ hybridization methods have been used primarily to map genes and recombinant clones to culicine metaphase chromosomes (110). Early in situ hybridization probes used on metaphase chromosomes were repetitive probes that were labeled isotopically (113, 115). Due to the technological advances resulting from the human genome project, fluorescent in situ hybridization (FISH) combined with digital-imaging microscopy opened the door for low- and high-resolution FISH physical mapping in *Ae. aegypti* (43, 44, 110). The hybridization target, that is, chromosomes, interphase nuclei, or DNA fibers, used in FISH determines the level of resolution—the ability to resolve two probes—that may be achieved (97, 139, 228). FISH mapping with metaphase chromosomes has a resolution of ∼1 Mb. If signals colocalize on metaphase chromosomes, the interphase nuclei may be used at a resolution of 50 to 100 kb. The relationship between kb and distance measured between signals in interphase nuclei is linear up to 2 Mb (219). FISH probes to decondensed DNA or extended chromatin fibers have a resolution as low as 100 bp (91, 165). The ability to resolve multiple probes at these different resolutions explains, in part, why FISH has become such an important tool in physical mapping. Briefly, FISH is used to assign a probe to a specific chromosome and regional location or map coordinate. If the probe sequence contains repetitive elements, then suppression hybridization is used to dampen the repeat signal, allowing the unique sequence signal to be visualized (124). When the unique FISH signal is detected, its map coordinates are expressed as a fractional length from the smaller arm or *p*-arm terminus (*p*ter) relative to the total length of the chromosome [%FL*p*ter (123)].

In 1995, a preliminary FISH physical map for *Ae. aegypti* using 37 different recombinant cosmid probes was reported (44). An unambiguous system for marking and orienting the three *Ae. aegypti* chromosomes was clearly needed. Three repetitive sequences were identified and were combined in one plasmid, p2392, and this plasmid provided FISH landmarks that uniquely labeled and oriented the three chromosomes (43). The *Ae. aegypti* FISH landmark probe has been used to locate 349 random cosmids or BACs to the *Ae. aegypti* FISH physical map (42; SE Brown & DL Knudson, unpublished data). This landmark probe was also tested against other culicine genera, but unfortunately it was species specific (SE Brown & DL Knudson, unpublished data). The *Ae. aegypti* FISH physical maps and data are and will be available at the Mosquito Genomics WWW Server (59a).

As has been the case with anophelines, FISH methods have also been used to correlate the genetic with the FISH physical map (SE Brown, DW Severson, & DL Knudson, unpublished data). Briefly, STSs were produced from the cDNA-based RFLP genetic markers. Specific PCR primers were designed from these STSs, and they were used to screen cosmid libraries. A larger cosmid probe was

needed to correlate the maps by FISH because cosmid-sized probes would yield a more reliable signal that could be visualized by using standard FISH techniques. Probes in standard FISH reactions need to be >5 kb for single-copy sequences, and more consistent results are seen with DNA probes of >10 kb. The PCR screening procedure yielded eight cosmids that contained genetic RFLP marker STSs. Later, cDNA probes were mapped directly by using an ultrasensitive FISH method, wherein the faint cDNA signal is amplified with a specific reporter molecule resulting in a detectable signal. Probes as small as 400 bp have been successfully mapped by this approach, and 21 cDNAs from the genetic map were placed into the physical map. The chromosome numbers for the genetic linkage map and the physical map were identical, but the genetic linkage map did not completely represent the physical map (SE Brown, DW Severson, & DL Knudson, unpublished data). Although chromosome 2 maps were complete, most of the genetic map markers for chromosome 1 were found in the small arm. The genetic map of chromosome 3 was found in 85% of the physical map. The genetic map for *Ae. aegypti* does not represent the entire physical map, and additional markers are needed to cover to the genome.

Map-Based Positional Cloning of Genes

A target gene is found because it has been located on a genetic linkage map, which in turn has a physical-map correlate. Recombinant libraries are screened for large insert clones that are found in the region, and the clones are ordered into a contiguous series of overlapping fragments or contigs so that the region is completely covered. A transcript map of the region is created to identify coding sequences, and likely candidate exons are identified. These exons are then characterized for functional nature by the examination of the different phenotypes, by directed mutation and correlation with changes in the appropriate phenotype, or by "knock-out" through a number of strategies.

Map-based cloning for vector susceptibility in *An. gambiae* has shown great promise (58, 59). Using an *An. gambiae* strain that is able to encapsulate and destroy *Plasmodium* ookinetes that reach the area between the midgut epithelial cells and the basal lamina, high-resolution genetic mapping has identified three genes, *Pen1, Pen2*, and *Pen3*, that influence this phenotype. *Pen1* maps to chromosome 2R within 1.5 cM of the H175 marker, *Pen2* maps to chromosome 3L within 8 cM of the H758 marker, and *Pen3* maps to chromosome 2R within 4 cM of H135 marker (243). The *Pen2* and *Pen3* intervals are large and need to be narrowed. If the regions containing the genes were microdissected, and the pool was used as a probe to identify recombinant BAC clones from the regions, then these new BACs could be screened for new microsatellite markers that would refine the region (243). Progress on the fine-scale mapping of the *Pen1* region has been reported (58). A BAC library has been screened with the H175 marker and with primers from flanking genetic markers to isolate BACs near the *Pen1* region. These clones were physically mapped to chromosome 2R division mid 8C to distal

8D, using in situ hybridization. A total of 146 BAC clones have been identified from this region by using microdissection techniques, random clone mapping or walking from mapped clones. Since these clones provide a sevenfold coverage of the region, they will be fingerprinted by restriction endonuclease digestion and assembled into contigs, and the minimal tiled path will be sequenced. In addition, the ends of the 12,288 clones from the *An. gambiae* strain PEST, BAC library have been sequenced, providing a sequence-tagged connector resource (134) that will be essential in future sequencing efforts (58).

Map-based positional cloning in *Ae. aegypti* has focused on the two QTL regions, *fsb1* and *pgs1* (also near *fsb2*), involved in filarial and malarial worm transmission (199). *fsb1* is located in a 2.9-cM interval between markers *LF178* and *B8L280* located at 26.9% FL*p*ter on chromosome 1. *pgs1* is a 2.1-cM interval between markers *LF98* and *A13L975* at 12.6% FL*p*ter. These two intervals are estimated to be 7–8 Mb in size (199; SE Brown, DW Severson, & DL Knudson, unpublished data). A chromosomal walking strategy of STS-based contig building (87) is being used to build contigs across the two intervals. While bringing the genetic and physical maps together, STSs were identified from *LF98* and *LF178*, PCR primers were designed, and the genomic libraries were PCR screened. One cosmid LSBC29.1 was identified that contained *LF178*, and its ends were sequenced and new, end-specific *LSBC29.1* STSs were identified. The libraries were screened again with primers representing these new *LSBC29.1* STS. One additional clone from the *fsb1* interval was found from one end STS, and the other end STS was likely to be in an undefined repetitive element because the PCR reactions were positive in a number of pools. No clones were identified from the *pgs2* interval in these initial screens. An exon-trapping technique (45) was used with *LSBC29.1* and resulted in an internal STS that has added at least three new clones to the contig (SE Brown & DL Knudson, unpublished data). The STS-based contig building has been hampered by the presence of undefined repetitive elements in the *Ae. aegypti* genome. Fortunately, random BAC and cosmid clones were FISH mapped to the two QTL regions, providing 15 cosmids and 4 BACs mapped to the *fsb1* region and 18 cosmids and 10 BACs mapped to the *pgs1* region (SE Brown & DL Knudson, unpublished data) These cosmids and BACs have been fingerprinted; end-sequence STS have been identified, which will be used in linkage-mapping studies and as new initiation points for chromosomal walks (SE Brown & DL Knudson, unpublished data).

Additional targets for map-based positional cloning in mosquitoes are genes that might be used to control the mosquito or to alter its ability to vector pathogens. Candidates include genes that encode antipathogen products such as cecropins and defensins, genes that are critical to melanization pathways such as proteases and phenoloxidases, genes that effect resistance to insecticides, and genes that are tissue specific in their expression. To this end, a number of genes have been mapped. Cecropin and a BAC containing the gene have been located to *An. gambiae* chromosome X division 1C by in situ hybridization (225). The gene encoding prophenoloxidase, which is involved in the melanization process, was

located to *An. gambiae* chromosome 2, division 13B. The gene encoding dopa decarboxylase, which is involved in melanin production, has been linked to the *Dox-A2* locus, which has been located in chromosome 3R division 33B (183). Class I glutathione S-transferase enzymes are likely involved in the resistance of *An. gambiae* to DDT, and the genes encoding these enzymes were characterized and located sequentially to *An. gambiae* chromosome 2R division 18B by in situ hybridization (173). The *H2A, H3*, and *H4* histone genes, which were isolated from ovarian tissue after a blood meal, have been mapped to *An. gambiae* chromosome 2L division 20 (178). Histone genes from *An. albimanus* have been located to chromosome 3 division 34A by in situ hybridization (155). A number of genes have been isolated from salivary gland tissue, but their map location has not been reported (6).

Other genes that might be important in *Aedes* control or affect its vector competence have been identified, and some have been mapped. The majority of these candidate genes were mapped previously to the genetic-linkage map and then mapped physically (SE Brown, DW Severson, & DL Knudson, unpublished data). The gene encoding a secreted protein in the salivary glands, *D7*, has been isolated, characterized (100), and located on chromosome 2. Salivary gland-specific genes for maltase-like I (MalI) and apyrase (47, 101) both have been located to chromosome 3. The *Rdl* γ-aminobutyric acid receptor gene is the cyclodiene-resistance locus and maps to chromosome 2; the acetylcholinesterase gene *Ace*, which is associated with resistance to organophosphorus and carbamate insecticides, maps to chromosome 1 (192). Two genes coding for defensins in *Ae. aegypti* have been genetically mapped to chromosome 3 (127), and a gene coding for cecropin has been characterized and not mapped (126). The *DHFR* gene from methotrexate resistant *Ae. albopictus* cells has been characterized and not mapped (202).

Genomics

The reagents and methodologies are now in place for fine-scale mapping in Culicidae, and map-based positional cloning in *Ae. aegypti* and *An. gambiae* has come of age. The near future will bring an explosive growth in functional gene information from the dipteran paradigm *Drosophila*, of which much will be directly transferable to mosquitoes, particularly *An. gambiae*. Foresighted efforts are underway to initiate an *An. gambiae* genomics project in which expressed sequence tags (ESTs) will be identified. It seems a relatively safe prediction to suggest that the genomes of one or more mosquitoes will be sequenced in toto and highly likely that technology transfer from other genomics projects will make important contributions to our understanding of vector competence and mosquito control. The next few years should be exciting for mosquito genomics, with great strides being made in the study of refractoriness and susceptibility in these vectors of disease. Web sites available to track the progress in this exciting area are the Mosquito Genomics WWW Server (http://klab.agsci.colostate.edu) and the AnoDB (http://konops.imbb.forth.gr/AnoDB).

ACKNOWLEDGMENTS

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