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Aedes aegypti genomics

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Abstract

The mosquito, *Aedes aegypti*, is the primary, worldwide arthropod vector for the yellow fever and dengue viruses. As it is also one of the most tractable mosquito species for laboratory studies, it has been and remains one of the most intensively studied arthropod species. This has resulted in the development of detailed genetic and physical maps for *Ae. aegypti* and considerable insight into its genome organization. The research community is well-advanced in developing important molecular tools that will facilitate a whole genome sequencing effort. This includes generation of BAC clone end sequences, physical mapping of selected BAC clones and generation of EST sequences. Whole genome sequence information for *Ae. aegypti* will provide important insight into mosquito chromosome evolution and allow for the identification of genes and gene function. These functions may be common to all mosquitoes or perhaps unique to individual species, possibly specific to host-seeking and blood-feeding behaviors, as well as the innate immune response to pathogens encountered during blood-feeding. This information will be invaluable to the global effort to develop novel strategies for preventing arthropod-borne disease transmission.
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1. Introduction

The mosquito, *Aedes aegypti* is the primary, worldwide arthropod vector for the yellow fever and dengue viruses. It has a cosmotropical distribution between 30°N and 20°S (Christophers, 1960; Knight and Stone, 1977), and exhibits a distinct preference for human habitats, including artificial oviposition sites, e.g., tires, flower vases, water storage containers (Tabachnick, 1991). The dengue viruses are a threat to >2.5 billion people, with an annual incidence in the tens of millions and ~24,000 deaths per year (WHO, 2002). Overall, mosquito-borne diseases have emerged or re-emerged as significant human heath problems due to a number of factors including lack of progress in vaccine development, emergence of drug resistance in pathogens and insecticide resistance in mosquitoes, and the decline in socioeconomic conditions in many disease endemic countries that limits disease monitoring and mosquito control efforts (Gubler, 1998). With few exceptions, mosquito control remains the only viable strategy for preventing dengue and other mosquito-borne diseases.

Ae. aegypti is considered the most tractable mosquito species for laboratory culture, and has been used for detailed laboratory investigations of mosquito biology including, morphology, physiology, genetics, and vector competence (Clements, 1992), and recently, molecular evolution applications (Severson et al., 2001). Determining the complete genome sequence of *Ae. aegypti* is a logical complement to the recently completed effort for the African malaria disease vector, *Anopheles gambiae* (Holt et al., 2002). These two species represent the best characterized and most significant members of the two medically important mosquito subfamilies, Culicinae and Anophelinae. Transmission of arboviruses and lymphatic filariasis is largely associated with the Culicinae, while the

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Anophelinae contain the primary vectors for malaria transmission. These subfamilies differ significantly in many biological characteristics and in their genomic structure (Knudson et al., 2002; Rai and Black, 1999).

2. *Ae. aegypti* provides broad utility for investigating mosquito/pathogen interactions

Successful mosquito-borne transmission of a pathogen 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, the pathogen must avoid an arsenal of internal active defense mechanisms dedicated to the recognition and subsequent destruction of nonself (foreign) entities (Barillas-Mury et al., 2000; Lowenberger, 2001). Vector competence varies significantly both within and among mosquito species and also relative to the specific pathogen. It is well-documented that (1) this variability is heavily influenced by genetic factors and (2) that genetic control of vector competence is due to the joint action of two or more individual genes (Severson et al., 2001; Black et al., 2002). Traits with variable phenotypic outcome determined by the combined effects of several genes and their interactions with the environment, such as vector competence, are generally referred to as multigene 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 chromosome location or their gene product. Development of DNA-based marker loci has, however, provided the tools that allow us to resolve complex traits into their individual genetic components (Lander and Botstein, 1989).

2.1. *Genetic basis for dengue vector competence*

Oral infection of *Ae. aegypti* with dengue virus has repeatedly been shown to vary both within and among geographic strains (Gubler et al., 1979; Tardieux et al., 1990; Sumanochitrapon et al., 1998; Bennett et al., 2002). Several environmental factors have been shown to affect the extrinsic incubation period (EIP) of arboviruses in mosquitoes, including temperature, humidity, and titer of the virus in the human (Black et al., 2002). Selection experiments confirmed a genetic component in flavivirus vector competence, but also showed considerable variability in success at selecting for highly refractory and highly susceptible *Ae. aegypti* strains (Wallis et al., 1985; Miller and Mitchell, 1991). The EIP takes ca. 10–14 days, but has been shown in some *Ae. aegypti* populations to be interrupted at the stage where the virus infects the midgut epithelium or prior to dissemination from the midgut epithelium. These are commonly referred to as a midgut infection barrier (MIB) and midgut escape barrier (MEB), respectively (Black et al., 2002). Laboratory studies have shown that dengue vector competence in *Ae. aegypti* is a quantitative trait and have documented the existence of genes that determine both a MIB and a MEB (Bosio et al., 1998). QTL mapping studies confirmed a multigene mode of dengue vector competence and defined genome regions containing three independent QTL that are involved in MIB and MEB (Bosio et al., 2000). Two QTL were associated with a MIB on chromosomes 2 and 3 that accounted for 44% and 56% of the total phenotypic variance, respectively. A minor effect QTL for MEB was identified on chromosome 3.

2.2. *Model for genetic basis of vector competence to malaria parasites*

Nearly 500 million clinical cases of malaria caused by infection with *Plasmodium* parasites occur each year, resulting in ca. 3 million deaths, mainly among children in subSaharan Africa (WHO, 1998). *Ae. aegypti* is not a vector for the human malarial parasites, including *Plasmodium falciparum*, but is highly susceptible to the avian parasite *Plasmodium gallinaceum*. Most strains of *Ae. aegypti* are highly susceptible to *P. gallinaceum* (Kilama and Craig, 1969). However, a highly refractory strain (MOYO-R) has been selected (Thathy et al., 1994). Genetic studies indicated that *P. gallinaceum* susceptibility was determined in part by the effects of a single gene (*pls*) on chromosome 2 (Kilama and Craig, 1969). QTL analyses identified a major effect QTL (*pgs1*) on chromosome 2 that likely corresponds to the *pls* locus and a minor effect QTL (*pgs2*) on chromosome 3 (Severson et al., 1995). Recent studies suggest that up to four additional linked QTL on chromosome 2 also influence *P. gallinaceum* susceptibility (Meece, 2002).

2.3. *Model for genetic basis of vector competence to filarial worms*

Lymphatic filariasis is caused by parasitic nematodes and is the second leading cause of permanent and long-term disability worldwide, with 120 million people annually presenting clinical morbidity (WHO, 2000). *Ae. aegypti* is not a natural vector for lymphatic filariasis and most laboratory strains and field populations are highly refractory to filarial worm development. However, a strain (Liverpool) was selected that is highly susceptible to the parasites that infect humans, *Brugia malayi* and *Wuchereria bancrofti* (Macdonald, 1962a,b). Classical studies indicated that susceptibility in *Ae. aegypti* is determined primarily by a single, recessive gene (f^{m}) on chromosome 1 (Macdonald, 1963; Macdonald and Ramachandran, 1965). However,

susceptibility has since been shown to be determined by at least two QTL, one on chromosome 1 (*fsb1*) that likely carries the f^m locus and one on chromosome 2 (*fsb2*) (Severson et al., 1994). The effect of the individual QTL, including significant epistatic interactions, varies among *Ae. aegypti* populations. An additional QTL (*idb1*) has been shown to influence both the number of microfilariae ingested while blood-feeding and the number that successfully penetrate the midgut epithelium (Beernsten et al., 1995).

2.4. Comparative genomics

Ae. aegypti and *An. gambiae*, as representatives of the Culicinae and Anophelinae, reflect the subfamily-specific extremes in mosquito chromosome evolution. The Anophelinae have smaller genome sizes: the 278 Mb *An. gambiae* genome is only ca. 1.6× larger than the 179 Mb *D. melanogaster* genome (Holt et al., 2002). Genome sizes in the Culicinae are typically larger (Black and Rai, 1988), with *Ae. aegypti* at 813 Mb (Warren and Crampton, 1991) or ca. 2.9× and 4.8× the *An. gambiae* and *D. melanogaster* genomes, respectively. Because the mosquito/*Drosophila* lineages diverged about 250 Myr and the Culicinae/Anophelinae lineages diverged ca. 95 Myr (Krzywinski et al., 2001), limited local synteny conservation is evident. However, comparisons of both *Ae. aegypti* and *An. gambiae* to *D. melanogaster* indicate that some ancestral chromosome elements remain conserved among chromosome arms of these species (Bolshakov et al., 2002; Severson et al., 2004; Zdobnov et al., 2002).

Comparative studies of chromosome locations for highly conserved orthologs indicate unequivocally that *Ae. aegypti* chromosome regions share extensive homology to the five *An. gambiae* chromosome arms (Severson et al., 2004). Whole arm or near whole arm homology was only contradicted with two genes among 75 *Ae. aegypti* genes for which orthologs to *An. gambiae* were identified. When compared, the two genomes reflect large evolutionarily conserved chromosome segments that generally correspond to break/fusion events and a reciprocal translocation with extensive paracentric inversions evident within, indicating only very tightly linked genes are likely to retain conserved linear orders within chromosome segments. However, local synteny conservation may be evident within the Culicinae (Anderson et al., 2001; Severson et al., 2001). Conversely, both *Drosophila* spp. and *Anopheles* spp. show extensive inter- and intra-specific reshuffling of gene order within chromosome arms (Ranz et al., 2001; Sharakhov et al., 2002).

Recent studies (Jaillon et al., 2003; McCue et al., 2002; Thomas et al., 2003) clearly indicate that comparative genome sequence analyses will significantly enhance our understanding of fundamental evolutionary and genetic mechanisms that define genome organization. The *An. gambiae* gene sequence already has demonstrated the tremendous benefits to be gained from comparative genome analyses and indicates that *Ae. aegypti*/*An. gambiae*/*Drosophila* genome comparisons (as well as other arthropods) will facilitate the discovery of conserved functional elements unique to the Culicidae. This should provide important insight into mosquito chromosome evolution and allow for the identification of genes and gene function, either common to mosquitoes or perhaps unique to individual mosquito species that are specific to host-seeking and blood-feeding behaviors, as well as the innate immune response to pathogens encountered during blood-feeding. Comparisons of *An. gambiae* with *D. melanogaster* have shown that while about half the proteome is highly conserved between them, there are significant numbers of proteins that appear to be unique to each species (Zdobnov et al., 2002). Also, some protein families show considerable expansion in *An. gambiae*, and include several families that likely evolved with hematophagy (Zdobnov et al., 2002) as well as those implicated in the innate immune response (Christophides et al., 2002).

3. The preferred laboratory model

Ae. aegypti has been and will remain the preferred mosquito species for laboratory investigations of virtually all aspects of mosquito biology. Much of this relates directly to its superior tractability in the laboratory that facilitates basic research, and indeed the mosquito research community recognized early the broad utility of *Ae. aegypti* as one of the most tractable mosquito species for laboratory studies (Christophers, 1960).

3.1. Life history traits and suitability for experimentation

The evolution in *Ae. aegypti* of an egg quiescence period following oviposition is an extremely favorable basic biological attribute. That is, the life cycle for most mosquito species is continuous, wherein eggs are laid individually or in rafts, typically on the water surface and hatch soon thereafter. Such species, that includes *Anopheles* and *Culex*, can be difficult to adapt to laboratory culture, and even when successfully adapted require constant maintenance. This physically severely limits the number of laboratory colonies that can be maintained. In contrast, *Ae. aegypti* preferentially oviposit away from the water surface and their eggs show considerable tolerance to desiccation and can be stored on dried oviposition substrates (such as paper towels) for several months. The eggs can be

induced to hatch by simply placing them in deoxygenated water; they rapidly develop to the adult stage within ca. 7 days. *Ae. aegypti* collected from the field also readily adapt to laboratory rearing conditions, including single-pair matings in small containers that greatly facilitates genetic studies. As such, individual laboratories are able to maintain a number of *Ae. aegypti* strains with a reasonable level of effort. While not comparable to the ease of maintaining hundreds or thousands of *Drosophila* cultures, individual laboratories can likely maintain >100 *Ae. aegypti* strains with a moderate level of effort.

3.2. Demand for genome sequence

Many laboratories that study mosquito biology include *Ae. aegypti* in their research program, either as their primary organism or as a critical complement to their target mosquito species. This is clearly evidenced by the PubMed citation data listed in Table 1. When compared with two other commonly studied mosquitoes, *An. gambiae* and *Culex pipiens*, research on *Ae. aegypti* has resulted in a greater number of total publications, and of publications in nearly every biological category we examined. Availability of the complete genome sequence will clearly enhance interest in *Ae. aegypti* research within the mosquito research community and will undoubtedly attract interest from investigators outside medical entomology. A general assessment of the *An. gambiae* literature supports this scenario, and the availability of genome sequence data for both *An. gambiae* and *Ae. aegypti* will accelerate that interest for both species.

3.3. Genetic mapping

Ae. aegypti has been the subject of numerous genetic studies conducted in laboratories throughout the world. A relatively large number of morphological mutant stocks have been identified, and with isozymes, provided the tools for development of the first detailed genetic linkage map for any mosquito species (Munstermann and Craig, 1979). Many of the morphological mutant stocks are still available in individual laboratories around the world, and are valuable tools for investigations coupled with other molecular markers and technologies. Detailed DNA-based genetic maps have been constructed using RFLP (Severson et al., 1993), RAPD (Antolin et al., 1996), and SSCP (Fulton et al., 2001) marker loci. Of note, microsatellite markers have not proven useful or abundant in *Ae. aegypti*, likely due to its genome organization (Fagerberg et al., 2001). That is, microsatellites tend to be underrepresented in the *Ae. aegypti* genome and those that are present are often imbedded in repetitive elements that prevent their utilization as single copy markers. Nonetheless, a 205 cM composite linkage map that includes 141 RFLP, SSCP and SNP marker loci was recently described (Severson et al., 2002). SNPs seem to be abundant in the *Ae. aegypti* genome (Morlais and Severson, 2003) and, due to the potential for high-throughput analysis, will likely become the marker of choice for large-scale mapping and genotyping.

3.4. Physical mapping

As with nearly all mosquito species, the *Ae. aegypti* genome is organized in three chromosomes. Of interest, no sex chromosome dimorphism is evident in *Ae. aegypti* or among other culicine mosquitoes: sex determination appears to be a function of a single autosomal gene locus (Gilchrist and Haldane, 1947; Anderson et al., 2001). As we expect the total number of genes in *Ae. aegypti* to be similar to that for *D. melanogaster* and *An. gambiae* (~13–15,000), the large genome size differences between culicine and anopheline mosquitoes have been attributed to the amount and pattern of repetitive sequences (Black and Rai, 1988). Culicines exhibit a short period interspersion pattern wherein repetitive sequences of ~100–300 bp are interspersed with ~1–2 kb single copy sequences (Warren and Crampton, 1991), which is typical of most higher eukaryote genomes including humans. Physical mapping in *Ae. aegypti* is based on in situ hybridization to metaphase chromosomes, as its genome organization (likely due to the repetitive nature) is not conducive to producing useable polytene chromosome preparations. Both cosmid and BAC genomic libraries are available and have been used for developing a physical map using FISH technology (Brown et al., 1995, 1997). In addition, the linkage and physical maps have been integrated by FISH mapping clones contain-

Table 1
PubMed entries (as of 9/12/2003)

	Total	Physiology	Development	Immunity	Genetics	Insecticide	Parasite	Pathogen
Ae. aegypti	2843	1723	676	253	530	340	151	144
An. gambiae	1179	786	238	156	450	183	173	34
C. pipiens[a]	1121	598	195	94	218	234	38	51

[a] Search query included '*pipiens* or *quinquefasciatus*'.

ing sequences for markers on the linkage map (Brown et al., 2001).

4. Facilitating the whole genome sequencing effort

We already have initiated an effort to develop the critical preliminary molecular tools to support a whole genome shotgun (WGS) sequencing project for *Ae. aegypti* that is presently funded and well-advanced (NIH-NIAID, UO1-AI50936). Considerable progress has been achieved in generating the appropriate background information to justify a WGS sequencing effort for *Ae. aegypti* with a high probability for success. This includes bacterial artificial chromosome (BAC) end sequencing, physical mapping of select BAC clones, and extensive expressed sequence tag (EST) production from normalized and enriched full-length cDNA libraries. We also suggest that the WGS sequencing effort should entail a minimum of ~8-fold coverage as this will greatly facilitate broad-scale scaffold assembly for most of the genome and will allow for systematic comparative genomic analyses of *Ae. aegypti* with *An. gambiae*, *D. melanogaster*, and other arthropods. High coverage of the *Ae. aegypti* genome also will facilitate WGS sequencing of other culicine species at low to moderate coverage levels, because assembly into large scaffolds should benefit from synteny conservation with *Ae. aegypti* (Anderson et al., 2001).

4.1. BAC end sequencing and physical mapping

Paired BAC end sequence data are extremely important as sequence-tagged-connectors to assist in assembling scaffolds for WGS sequencing projects. These data also will provide valuable preliminary information on genome structure including, for example, repetitive element type, frequency and variability as well as putative open reading frame frequency. We have completed end sequencing of clones from three independent BAC libraries, prepared from two *Ae. aegypti* strains (Liverpool and Rexville) and the ATC-10 cell line. A total of 117,937 end sequences were obtained: these assemble into 103,085 contigs that represent ca. 8.2% of the whole genome. All BAC end sequences have been deposited in the GSS database at NCBI.

We also have conducted a small-scale project to gain some insight into the general feasibility for a WGS sequencing effort. We performed shotgun sequencing on four whole BAC clones containing genetic marker sequences that map within major euchromatin regions in *Ae. aegypti* (Brown et al., 2001). About 3000 paired sequence reads (ca. 12× coverage) were obtained for each BAC clone from random 3–4 kb plasmid subclone libraries. We were able to assemble each of them into large scaffolds without difficulty suggesting that repetitive sequences within clones are sufficiently diverged to minimize their impact on assembly. Assemblies for each clone are available at the TIGR *Ae. aegypti* website (http://www.tigr.org/tdb/e2k1/aabe/). We also combined enough sequence reads from each of these BACs to represent ca. 6× coverage and performed an assembly to determine if they would assemble individually or if repetitive sequences would interfere with proper assembly: the pooled assembly mirrored the individual assemblies. In summary, although preliminary, these results are supportive of a WGS sequencing approach for *Ae. aegypti*.

Another aim of our current project is to place an additional 1000 BAC clones to metaphase chromosomes using FISH as these data will greatly assist with orienting scaffold assemblies generated from WGS sequencing. Although most of these clones will be randomly selected, a core set of clones containing 106 genetic marker sequences have been identified (Jiménez et al., 2004) as well as clones identified as containing open reading frames, based on BAC end sequence analysis: these clones have been or will be given priority for FISH mapping.

4.2. Gene discovery

Generation of large-scale EST data is important for several reasons. First, they represent an opportunity to identify genes expressed collectively among various developmental stages and tissues including, for example, midgut, ovaries and salivary glands, and thus are generally reflective of the entire transcriptome. Second, the examination of tissues from naïve, blood fed or pathogen-infected mosquitoes will provide the opportunity to clone genes that are expressed in response to infection as well as those associated with blood-feeding. Comparison of genes from these sources with their homologues in *Drosophila* and *An. gambiae* also may shed light on those evolutionary adaptations that have been necessary for obtaining, digesting and utilizing blood. Third, these libraries will represent a valuable immediate community resource, which will permit both subsequent full-length sequencing as desired and can be used for the production of sequence-verified, unique gene microarrays. Fourth, they will be important for training gene-finding software and subsequent annotation of the full genome. Finally, they will likely be useful in helping to determine whether *Drosophila* ORFs with no known homologues are in fact functional genes and for comparative functional genomics with *An. gambiae*.

We and others are targeting two general areas for the discovery of *Ae. aegypti* genes: those genes for which transcription levels are likely life-stage or tissue specific; and those genes whose transcription levels are likely influenced by normal and pathogen-infected

blood meals, including dengue virus, the malarial parasite *P. gallinaceum*, and the filarial worm parasite *B. malayi*. Several investigators (see GenBank) have developed ESTs from targeted tissues including, Malpighian tubules, midgets from naïve and blood fed females, salivary glands and hemocytes, and efforts to develop ESTs from a variety of sources are underway or planned. We are focusing on the production and end sequencing of directionally cloned cDNA libraries constructed with an emphasis on producing a high percentage of full-length clones and normalized to minimize repeated sequencing of abundant mRNA species (Bonaldo et al., 1996). We already have completed some of these cDNA libraries and sequencing efforts are currently ongoing. Other cDNA libraries are in various phases of construction. Of immediate utility to the research community, all sequences are available at TIGR (http://www.tigr.org/tdb/e2k1/aabe/) and we have recently announced the *Ae. aegypti* Gene Index (http://www.tigr.org/tdb/tgi/aegi/) that presents partially annotated gene information for these data and also incorporates all *Ae. aegypti* gene sequence data previously submitted to GenBank by the entire research community.

5. Conclusions

Ae. aegypti has been and will remain one of the most intensively studied mosquito species. Considerable genome information has been obtained for this mosquito, yet much remains unknown. Efforts to generate important molecular tools to support a WGS sequencing effort have been successful and, therefore, *Ae. aegypti* seems well poised to proceed with whole genome sequence determination. The results of this effort will undoubtedly have profound effects on research to better understand, and thereby develop novel methods to prevent, pathogen transmission to humans.

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