Am. J. Trop. Med. Hyg., 69(1), 2003, pp. 98–104

UTILITY OF COMPARATIVE ANCHOR-TAGGED SEQUENCES AS PHYSICAL ANCHORS FOR COMPARATIVE GENOME ANALYSIS AMONG THE CULICIDAE

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Abstract. The development of comparative genetic maps in multiple species of mosquitoes could prove extremely useful in the search for those genes that contribute to mosquito vector competence or genes associated with other phenotypes of interest. To effectively compare these gene maps, markers must be developed that are based on chromosomal regions conserved throughout the Culicidae. We designed 35 polymerase chain reaction (PCR) primer pairs based upon orthlogous exons in *Aedes aegypti* and *Drosophila melanogaster* or *Anopheles gambiae*. Twenty-three of the primers yielded a single PCR product in at least one dipteran, in addition to *Ae. aegypti*, when screened with genomic DNA from seven dipterans, including five mosquito species. Eight of the primers amplified a single PCR product in only *Ae. aegypti*, while four primer pairs gave no PCR product in any species. The 23 successful comparative anchor-tagged sequence primer pairs give broad genome coverage in *Ae. aegypti*, and more importantly demonstrate an efficient strategy for developing comparative anchor marker loci for any species of Culicidae.

INTRODUCTION

The family Culicidae contains approximately 3,500 species of mosquitoes,[1] and historically most genetic research efforts have been directed towards the two principal disease vector organisms, *Aedes aegypti* and *Anopheles gambiae*. The first detailed genetic map for *Ae. aegypti* consisted of 77 morphologic, isozyme, and insecticide resistance markers.[2] Early efforts to produce genetic maps of the *Anopheles* spp. were less successful due to a dearth of morphologic and isozyme markers, along with the difficulty of rearing these mosquitoes in the laboratory. In addition, within both the Culicinae and Anophelinae, these markers were not applicable for detailed genome coverage in a single mapping population and were often non-neutral in nature. Despite the difficulty of working with such markers, genetic linkage maps were constructed for *Ae. togoi*, *Ochlerotatus triseriatus*, *Ae. aegypti*, *An. albimanus*, *An. quadrimaculatus*, and *An. gambiae.*[3,4] Furthermore, a limited comparison of isozyme loci suggested a high degree of synteny.[5] Synteny refers to loci that remain physically linked in different species.

The advent of DNA-based genetic markers including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), single-strand conformation polymorphisms (SSCPs), and microsatellites has allowed the construction of detailed linkage maps for a small number of mosquito species. The first such map, in *Ae. aegypti*, was generated using RFLP markers[6] and consisted of 50 DNA markers based mostly on random cDNAs that identified 53 loci across the three linkage groups. The most recent map for this species consists of 146 markers across 205 cM.[7] Two RAPD-SSCP linkage maps for *Ae. aegypti* were also constructed, the first consisted of 96 loci that covered 168 cM[8], while the second consisted of 94 SSCP markers based upon single nucleotide polymorphisms (SNPs) that covered 134 cM.[9] Within the anophelines, a DNA-marker-based genetic map of *An. gambiae* was constructed using 148 microsatellite markers covering 215 cM.[10] These genetic maps have proven useful within *Ae. aegypti* and *An. gambiae* in efforts to identify genomic regions or genes associated with mosquito competence to transmit a variety of pathogens.[11–16]

Of interest is the construction of detailed genetic linkage maps in a greater number of mosquito species. Comparative RFLP-based linkage maps for the mosquitoes *Culex. pipiens*,[17] *Ae. albopictus*,[18] *Oc. triseriatus* (formerly *Aedes*),[19] *Cx. tritaeniorhynchus,*[20] and *Armigeres subalbatus*[21] were previously constructed using *Ae. aegypti* cDNA clones as probes to Southern blots. This strategy was successful because many *Ae. aegypti* cDNA sequences are highly conserved across mosquito species.[22] These maps allowed for the direct comparison of these species because the markers are based upon expressed genes (Type I markers) as opposed to microsatellites (Type II markers); that is, although microsatellites have proven useful in developing gene maps in many organisms, they are less useful when making comparisons between organisms because they are usually not conserved between species.[23]

Despite the success in constructing comparative gene maps for several mosquito species using RFLP markers, there has been no attempt to develop polymerase chain reaction (PCR)–based DNA markers that can be used across species within the Culicidae. The utility of orthologous gene comparisons for development as tools for PCR-based identification of conserved syntenies has been demonstrated for both birds and mammals.[23–25] Markers developed in this manner have been referred to as comparative anchor-tagged sequences (CATS) and have been used to generate comparative genome maps in the human, dog, and horse.[26–28] The identification of such a set of markers within mosquitoes could prove to be extremely useful among those species that although not genetically well characterized, nonetheless are of great importance medically.

In this study, we investigated the utility of the CATS marker strategy for developing comparative genome maps among mosquito species. We have evaluated 35 putative CATS primer sets across six mosquito species and two more distant species within the Diptera. Twenty-three of these CATS amplified a single PCR product in at least one dipteran species in addition to *Ae. aegypti*. This simple strategy in which orthologous exons of two or more dipterans are identified, sequence alignments are made, and PCR primers are designed for highly conserved regions should facilitate the rapid identification of PCR-based genetic markers that provide physical anchor loci with broad application to genetically uncharacterized mosquito species and perhaps more distant dipteran species as well.

MATERIALS AND METHODS

Candidate selection for CATS. Identity searches were conducted on 71 previously mapped, partial cDNA clones or known genes from adult *Ae. aegypti* Liverpool females[7] by comparing nucleotide sequences against both the non-redundant as well as the non-human, non-mouse expressed sequence tag (EST) databases using the BLASTN computer program.[29] Thirty-five of the *Ae. aegypti* sequences showed significant nucleotide identities, based upon an expected value (E) of less than e^{-15} as our critical value for defining sequences as orthologs with sequences from *Drosophila melanogaster* and/or *An. gambiae*. Nucleotide sequences from the putative orthologs were aligned using the Multiple Alignment Program (MAP) on the Baylor College of Medicine (BCM) Search Launcher web site.[30]

Primer design. Primers were designed using the PRIMER program (Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA). We first attempted to design primers within regions of the sequences that exhibited complete nucleotide sequence identity between *Ae. aegypti*, *D. melanogaster*, and *An. gambiae*. In most instances, however, we designed primers to contain no more than 2–3 mismatches with complete sequence identity for the final 5–6 nucleotides at the 3′ end. When mismatches were unavoidable, we biased nucleotide selection to the *Ae. aegypti* sequence. We designed primers to be 18–22 nucleotides in length with a G + C content of approximately 50%. When possible, we placed a GC clamp at the 3′ end. The expected PCR product size was between 100 and 400 bases. Whenever possible, we avoided selecting primers with the 3′ nucleotide representing the third base position in a codon.

Isolation of DNA. Isolation of DNA from individuals representing *Ae. aegypti*, *Oc. triseriatus*, *Ae. togoi*, *An. gambiae*, *Ar. subalbatus*, and *D. melanogaster* were performed as previously described.[31] Genomic DNA representing individuals from *Cx. pipiens* was kindly provided by Dr. Akio Mori and that from *Rhagoletis pomonella* was kindly provided by Dr. Jeff Feder (both at the University of Notre Dame, Notre Dame, IN).

Primer optimization. The PCRs were conducted in 96-well plates using a Hybaid (Ashford, Middlesex, United Kingdom) Omnigene thermal cycler. Each 25-μL PCR mixture contained 1× *Taq* buffer (10 mM KCl, 2 mM Tris, pH 9.0, 0.02% Triton X) 1.5 or 3.0 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP, and dTTP, 5–10 pmoles of each primer, 1 unit of *Taq* DNA polymerase, and 5 ng of genomic DNA. Six primer sets were screened with eight dipteran species per plate. We screened each primer set at three anneal temperatures, 50°C, 54°C, and 58°C. The standard PCR conditions were 94°C for five minutes, followed by 30 cycles at 94°C for one minute, an annealing step at one of the three previously mentioned temperatures for one minute, 72°C for two minutes, and a final extension at 72°C for 10 minutes. Primer sets with an expected PCR product size less than 200 basepairs were subjected to an alternate set of PCR conditions: 94°C for 10 minutes, followed by 40 cycles at 94°C for 20 seconds, annealing at one of the three temperatures for 20 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The PCR products were size fractionated by electrophoresis in 3% agarose gels, stained with ethidium bromide, and visualized under UV light.

Sequencing. Amplified PCR products were spin-column purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The purified PCR products were then quantified visually after electrophoresis on 3.0% agarose gels. The PCR product templates were subjected to cycle sequencing using the ABI Prism Big Dye Terminator kit according to the suppliers recommendations (Applied Biosystems, Inc., Foster City, CA) and an ABI Prism 310 Genetic Analyzer. Single-pass sequence information was obtained from each strand of each PCR product. Sequence data were then subjected to BLASTN and BLASTX searches of the GenBank non-redundant and EST databases using default settings,[29] as well as pairwise sequence alignment to the original *Ae. aegypti* clone sequence using the Blast2 program.[32]

Linkage map. The linkage map was drawn using the DrawMap computer program.[33] Map distances are reported in Kosambi centiMorgans.

RESULTS

From an initial screening of 71 *Ae. aegypti* cDNAs, we designed PCR primers for 35 putative CATS. Of the 36 cDNAs for which no primers were designed, 15 showed insufficient nucleotide identity with *D. melanogaster* or *An. gambiae*, based on our primer design criteria, to allow amplification of a minimum fragment size of at least 100 basepairs, while the remaining 21 *Ae. aegypti* cDNAs showed no nucleotide identity with either *D. melonogaster* or *An. gambiae* when screened against either the non-redundant or non-human non-mouse EST databases using the BLASTN program.

The 35 CATS primer sets were used to screen genomic DNA from six mosquito species including *Ae. aegypti*, *Oc. triseriatus*, *Ae. togoi*, *An. gambiae, C. pipiens*, and *Ar. subalbatus*, as well as two more distant species within the dipterans, *D. melanogaster* and *R. pomonella.* Twenty-three primer pairs (Table 1) yielded a single PCR product with at least one species in addition to *Ae. aegypti*, while 8 primer pairs produced a single PCR product only in *Ae. aegypti*. Four of the primer sets failed to produce a PCR product in all of the eight dipterans screened, including *Ae. aegypti.* Two typical CATS primer screening results are seen in Figure 1. The genetic locations in *Ae. aegypti*[7] of all CATS for which we successfully obtained a PCR product in at least one non-*Ae. aegypti* species are shown in Figure 2.

We observed varying rates of success for amplification of individual CATS within each of the eight species tested (Table 2). Amplification of a single PCR product varied among species relative to *Ae. aegypti* with a low of 13% seen in *R. pomonella* and a high of 60% seen in *Oc. triseriatus*. Thirteen of the twenty-three CATS primer pairs (56%) amplified with four or more of the species, while the remaining 10 CATS primer pairs (44%) amplified between two to three different species.

We selected three CATS markers, LF178, LF158, and LF103, for sequence analysis and identity verification. They were selected as random representatives of each of the three *Ae. aegypti* linkage groups. Also, these CATS amplified a single PCR product in at least five of the eight species screened. We sequenced the PCR products from these CATS following amplification of genomic DNA from *Ae. aegypti*, *A. subalbatus*, and/or *D. melanogaster*.

TABLE 1
Comparative anchor-tagged sequence (CATS) primers and PCR conditions*

CATS no.	Accession no.	PCR conditions Annealing temperature, °C/mM Mg+	Predicted product size (bp)	Primer sequences 5′-forward/reverse-3′
LF90	T58320	50/1.5	110	GAAGAGGTGCAGGTCTCGCT/ACAGATCCGTGACATGGACG
LF92	BM005493	55/1.5	277	CCCAGATCAGCAGCGTTTG/TTACTTCAGCTTCTTCTTCGGG
LF96	BM005491	50/1.5	164	ATCAAGCTCGCCAAGGTC/GGGTTGTAGACGGCGTTG
LF99	BM005477	54/1.5	356	AAGCGCAAGCACAAGAAG/TTGATCGACTTGTTCAGCT
LF103	BM005488	54/1.5	181	ATGCGCGATCTTCGGATC/AGTGGACGGCAATCTTTTCG
LF106	BM005490	54/1.5	175	CAAAGCAGCCGCAAAAGA/GGGTGATCAGATTGTAGG
LF108	T58322	50/1.5	265	AAGTCCGGCAAGCTGAAGG/GCTTGATCTGTTCCAGGGC
LF124	BM005518	54/1.5	103	GAGGAGAAGCGCAAGCAC/TTGGGCATGGCTGAACAC
LF129	BM005504	54/3.0	300	GCACCAGGAAAGTCGGAATC/AGTACACCCAGGCACCGC
LF138	T58332	54/1.5	204	CGTGTTCAACTACGACAT/TCCTTCAATGTACGATGAG
LF158	BM005485	50/3.0	239	GGAAGGGTCTGAAGATCACC/AGCACTTCCTTGCAGGTACC
LF168	R47184	54/1.5	113	GGAGGACGCTGCAAGCAC/ACGATGTTCCGGATCACG
LF178	T58309	50/1.5	207	GCCGTGGTGTCCCAGATC/GCAGACGCATACGGATGTG
LF179	BM005479	50/3.0	234	AACAAAGTGACCCAACTGACTG/ACACCTTGACATTGGCCTTG
LF188	BM005472	54/1.5	208	CTTTGACGACTTCAAGCGC/ACGGAAGCGAAGGCC
LF218	BM005487	50/1.5	176	AAGGGCCCAGTTCGCATG/CCGGGTTCGATGTTGATCGA
LF231	BM005478	50/1.5	100	TTGCTGAAGGTGTCCAAGGA/TGTTGGACAGTTCCTCACGC
LF250	T58311	54/1.5	152	GTGGAGACTGGCCGCG/TCAGGTGCAGGTGGTTCAC
LF253	T58331	58/1.5	214	TGAAGACTCTCCGCCAGG/CGGATGATATCGGAATCT
LF272	BM005484	54/3.0	147	GTGCGTGGTATCTCCATCAA/GATGTTGTTGAAGTCCAGCTG
LF291	BM005482	54/1.5	250	GAACACCTTGGCCGAGTG/GTGCACAGGGAACCATCG
LF334	BM005506	54/1.5	200	GTTCATGAAGTACGCCAAGG/CTTGCCGATGCATTCTCC
LF417	BM005499	54/1.5	228	GAAGACCAGGAAGCTACGTGG/GACCAGCGACCACAGCTT

* PCR = polymerase chain reaction; bp = basepairs.

For LF178, we sequenced the PCR product obtained from one individual each from *Ae. aegypti*, *Ar. subalbatus*, and *D. melanogaster*. The CAT PCR products from *Ar. subalbatus* and *D. melanogaster* exhibited 92% and 83% nucleotide identity, respectively, when subjected to a pairwise alignment with the original *Ae. aegypti* LF178 cDNA sequence using the Blast2 program.[32] A representative nucleotide and amino acid sequence alignment for all CAT PCR products based upon LF178, LF158, and LF103 is shown in Figure 3.

For CAT PCR products designed from A*e. aegypti* LF158, we sequenced one individual each from *Ae. aegypti* and *D. melanogaster*. The *D. melanogaster* sequence was interesting in that it was 394 nucleotides in length while the *Ae. aegypti* PCR product was only 241 nucleotides in length. BLAST analysis of the *D. melanogaster* PCR product showed 100% nucleic acid identity and 100% amino acid identity to the *Drosophila* ribosomal protein L12 gene, GenBank accession number AAF47152, which contains four introns. The *D. melanogaster* CAT sequence spanned the fourth intron. The first exon in the *D. melanogaster* CAT PCR product showed 82% nucleotide identity with the original *Ae. aegypti* LF158 clone (nucleotides 1-135) and the second exon showed 74%

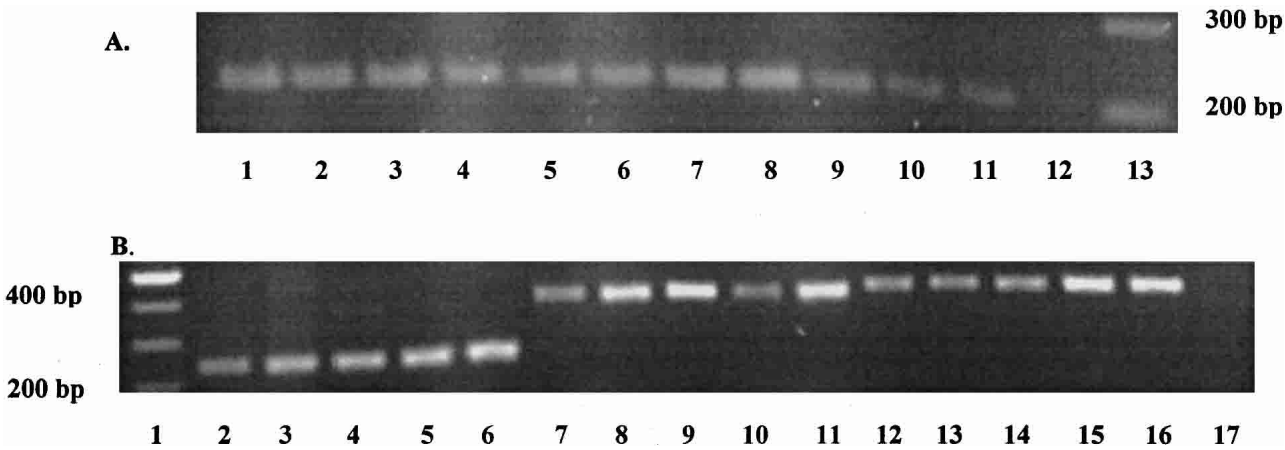

FIGURE 1. Representative agarose gels of single individual dipterans after amplification with comparative anchor-tagged sequences (CATS) polymerase chain reaction (PCR) primer pairs. **A**, Amplification products with the CATS LF178 primer pair. Lanes 1–3, products from single *Aedes aegypti* individuals; lanes 4–6, single *Anopheles gambiae* individuals; lanes 7 and 8, single *Drosophila melanogaster* individuals; lanes 9–11, single *Culex pipiens* individuals; lane 12, negative control lane; lane 13, 100-basepair molecular weight marker. **B**, Amplification products with the CATS LF158 primer pair. Lane 1, 100-basepair molecular weight marker; lanes 2–6, single *Ae. aegypti* individuals; lanes 7–11, single *An. gambiae* individuals; lanes 12–16, single *D. melanogaster* individuals; lane 17, negative control. The CAT LF158 PCR products from the *An. gambiae* and *D. melanogaster* individuals each contain an intron, while the product from the *Ae. aegypti* individuals does not. bp = basepairs.

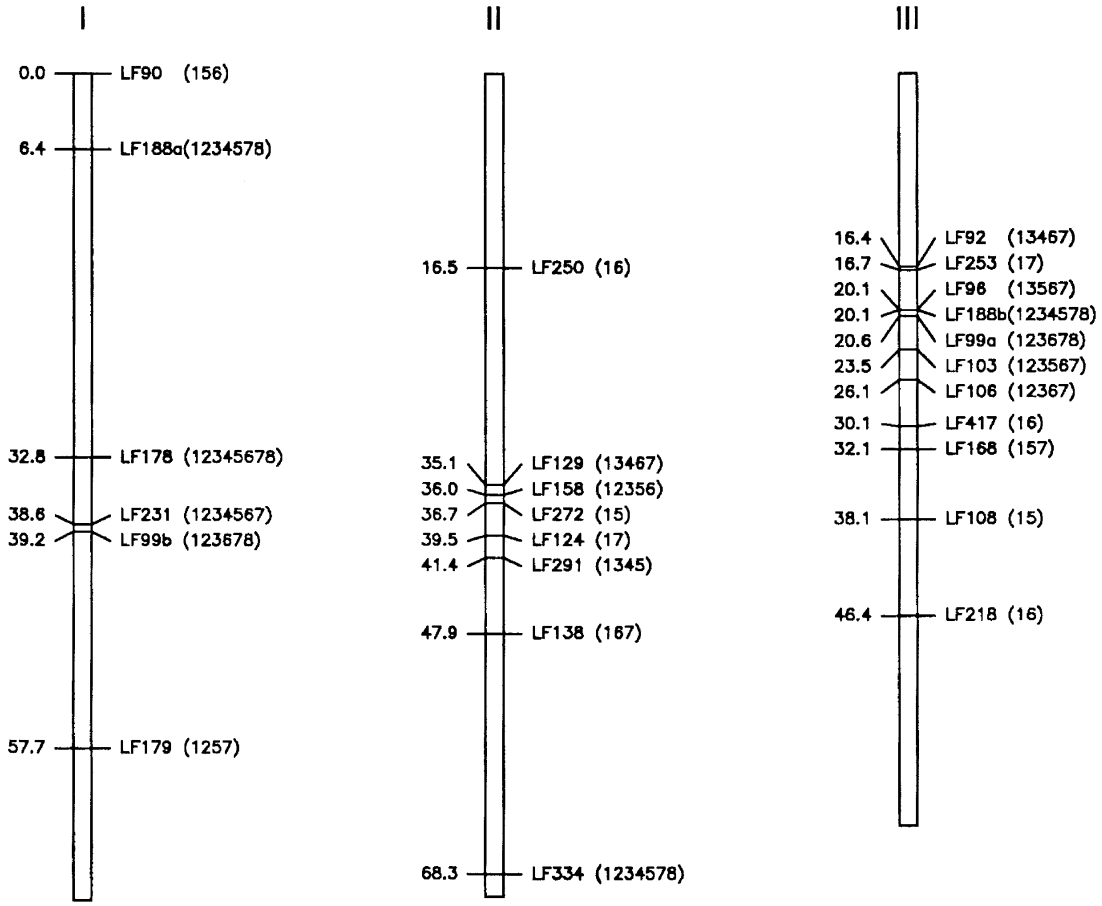

FIGURE 2. Relative distribution of comparative anchor-tagged sequence markers based on restriction fragment length polymorphism loci on the *Aedes aegypti* genetic map. The numbers in parentheses indicate the species amplified by that marker. 1 = *Ae. aegypti*; 2 = *Drosophila melanogaster*; 3 = *Anopheles gambiae*; 4 = *Ae. togoi*; 5 = *Armigeres subalbatus*; 6 = *Culex pipiens*; 7 = *Ochlerotatus triseriatus*; 8 = *Rhagoletis pomonella*. I, II, and III refer to chromosome numbers. Map distances are listed in Kosambi centiMorgans.

nucleotide identity with the same clone sequence (nucleotides 289-394). The CAT PCR product obtained from *An. gambiae* also seemed to contain an intron (Figure 1B).

Finally, for LF103 we sequenced PCR products obtained from one individual each of *Ae. aegypti* and *Ar. subalbatus*. The *Ar. subalbatus* CAT sequence showed 88% nucleotide identity with the original *Ae. aegypti* LF103 cDNA when the two sequences were subjected to a pairwise sequence alignment using the Blast2 computer program.[32]

TABLE 2
Comparative anchor-tagged sequence amplification success rate in Diptera

Genus and species	Family	% single product*
Aedes aegypti	Culicidae	100
Ochlerotatus triseriatus	Culicidae	61
Armigeres subalbatus	Culicidae	52
Anopheles gambiae	Culicidae	48
Culex pipiens	Culicidae	48
Drosophila melanogaster	Drosophilidae	35
Aedes togoi	Culicidae	26
Rhagoletis pomonella	Trypetidae	13

* The percentage of the 23 primers listed in Table 1 that produced a single polymerase chain reaction product.

DISCUSSION

Mosquitoes, members of the insect order Diptera and family Culicidae, are divided into three subfamilies: Culicinae, Anophelinae, and Toxoryhnchitinae.[1] A limited number of species within each of the former two subfamilies serve as obligate intermediate vectors for a variety of diseases such as malaria, dengue fever, yellow fever, and lymphatic filariasis. Malaria alone accounts for 2.7 million deaths annually, lymphatic filariasis afflicts and disables upwards of 120 million people each year[34] and dengue strikes 50 million individuals each year with an ever increasing number of accompanying cases of dengue hemorrhagic fever.[34]

The development of CATS markers with utility across a wide range of mosquito species could prove to be very useful for identifying species-specific as well as common genes involved in vector competence for malaria, filariasis, and arboviruses. That is, a comparative genomics approach facilitated by such markers would allow us to rapidly determine if the same genomic regions play a role in vector competence. Positional cloning efforts to isolate and characterize the individual genes from these regions could then be better directed toward mosquito species that are more fully characterized genetically and more amenable to laboratory manipulations because many of the mosquito species responsible for disease

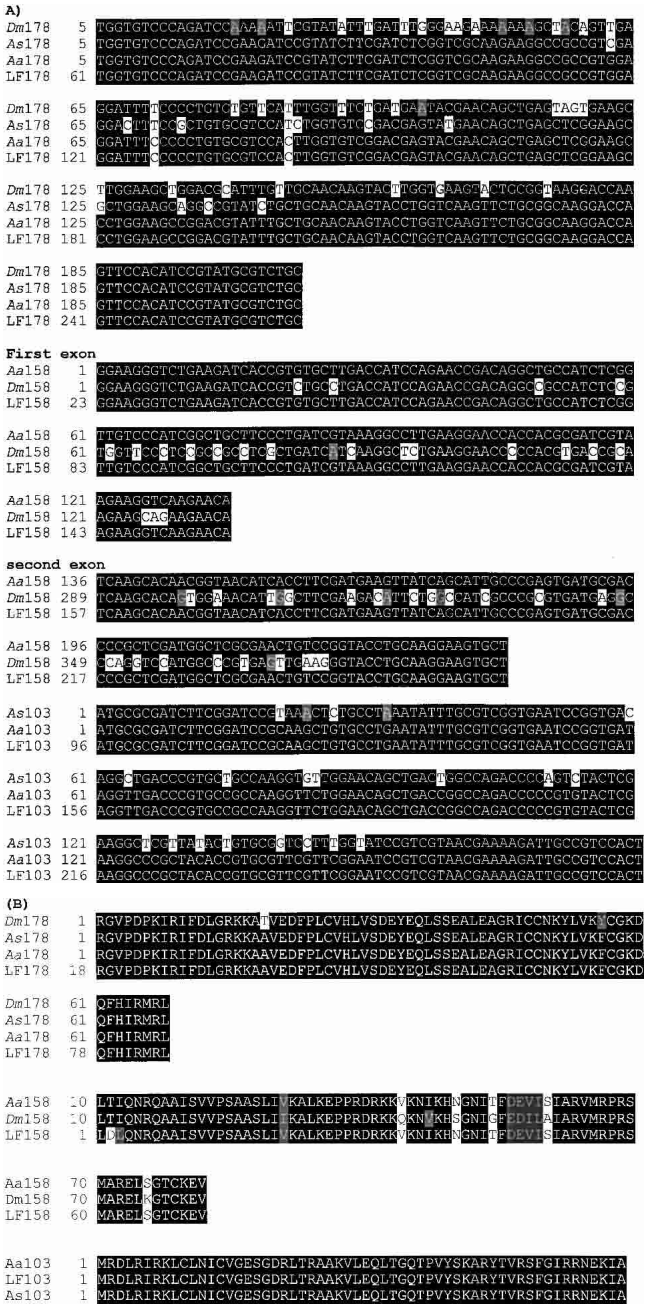

FIGURE 3. Nucleotide (**A**) and deduced amino acid (**B**) sequences from *Aedes aegypti* (*Aa*), *Armigeres subalbatus* (*As*), and *Drosophila melanogaster* (*Dm*), and LF178, 158, and 103 sequences from *Ae. aegypti*. Black shading signifies complete nucleotide or amino acid identity and similar amino acids are shaded in gray.

transmission to humans are extremely difficult to colonize in the laboratory or have never been successfully colonized. Indeed, genome regions containing quantitative trait loci (QTL) have previously been identified that are associated with vector competence of *Aedes aegypti* to *Brugia malayi*, a filariod nematode,[11,13] to *Plasmodium* gallinaceum,[12] and to the dengue-2 virus.[16] In *An. gambiae,* QTL have been associated with encapsulation of *P. cynomolgi B* and *P. berghei.*[14,15]

We have developed a PCR-based strategy for producing genetic markers that can be used as a comparative core set for rapid genetic mapping of multiple species within the Culicidae. We developed these markers by using a comparative genomics approach based upon CATS. Using available sequence data from *Ae. aegypti* ESTs and genes, we were able to develop 23 CATS primer pairs that generated a single-copy PCR product in *Ae. aegypti* and at least one of seven additional mosquito or more distantly related dipteran species. These markers exhibit broad genome coverage within *Ae. aegypti* and thus broad potential coverage within other dipterans and clearly demonstrate the utility of a CATS-based strategy for marker development within the diptera.

Failure of the CATS primers to amplify a PCR product from genomic DNA was likely due to sufficient nucleotide divergence from the *Ae. aegypti* sequence, leading to the selection of primers within those regions of nucleotide divergence or to the presence of an intron. Interference by introns could include intron/exon boundaries residing within the selected primer sequence or due to instances in which an intron was too large to allow for amplification.

Our CATS markers can be easily applied to genetic studies in previously uncharacterized mosquito species. For example, the CAT PCR products can be rapidly screened for polymorphism and subsequent mapping using SSCP screening with subsequent silver staining.[9,35] Single nucleotide polymorphisms can be identified within CATS and genotyping performed through the use of various techniques including melting curve analysis of single nucleotide polymorphisms.[36]

The development and subsequent mapping of CATS within the Diptera should also be useful in identifying syntenic relationships among species. Comparisons of linkage relationships of biochemical and morphologic markers suggested that linkage group conservation existed among the higher flies, *Ceratitis capitata*, *D. melanogaster*, and *Musca domestica,* but the relationship of mosquito linkage groups to these species was not clear.[37] Recent reports within the *Drosophila* indicates that while syntenic regions are common, there has been extensive rearrangement of gene orders such that the estimated length of conserved chromosomal fragments between any two species of *Drosophila* is expected to be no greater than approximately 20–600 kilobases.[38,39] A comparative genomic analysis of *D. melanogaster* and *An. gambiae* uncovered microsytenic blocks of 2–3 genes between these two species that were estimated to correspond to approximately 50–80-kilobase genomic segments.[40] A further analysis of the full *D. melanogaster* and *An. gambiae* genomes revealed the presence of 948 microsyntenic blocks, with a microsytenic block being defined as having at least two orthologous groups within the region.[41] The total fraction of *Anopheles* orthologs assigned to these microsyntenic blocks is 34% and constitutes a significant level of local conservation.[41] Previously, six microsyntenic clusters composed of 17 orthologues between *An. gambiae* and *D. melanogaster* were identified within the *Pen1* region of *An. gambiae*, thus demonstrating that gene arrangements in their last common ancestor persist in these two species to some degree.[42] When 157 *An. funestus* cDNAs were physically mapped to polytene chromosomes of this species as well as mapped *in silico* (electronically, i.e., by sequence comparisons with gene databases) to *An. gambiae*, perfect preservation of synteny was observed but substantial shuffling of gene order was also seen.[43] In contrast to this, comparisons within the *Culicinae* show conservation of gene order.[19]

The development of high-density CAT maps could provide an excellent means for eventually delineating the boundaries

of syntenic regions[23] and identifying regions of additional microsynteny, which may exist between dipterans in general and particularly among the great majority of mosquito species who are members of the sub-family *Culicinae*.

Additionally, CATS could prove valuable for phylogenetic studies. Using CATS, sequence comparisons can be made between a wide variety of mosquito species, which would contribute toward a greater understanding of systematic and taxonomic relationships between mosquitoes. It is believed that the lower diptera (mosquitoes, midges, gnats) diverged from the higher flies (*Drosophila* and house flies) about 250 million years ago.[44] Traditional evolutionary systematics in mosquitoes was based on morphologic characters[44] that placed the Anophelinae at the basal position, with Toxoryhnchitinae intermediate and Culicinae the most recently derived.[44] Recent molecular data has supported this assertion, as shown by the use of the *white* gene by Besansky and Fahey[45] to evaluate taxonomic relationships among the Culicidae. It is interesting to note that our success in amplification with CATS primer pairs was highly correlated with the reported evolutionary relationship among the various species tested. Those species that had diverged more recently from *Ae. aegypti*, e.g., *Oc. triseriatus* and *C. pipiens*, exhibited greater success in amplification as CATS, while those that diverged earlier, e.g., *An. gambiae*, *D. melanogaster*, and *R. pomonella*, were less successful in amplifying with the CATS primer pairs.

Given that the total genome sequence is available for *D. melanogaster,*[46] and just recently for *An. gambiae*,[47] and an *Ae. aegypti* genome project has recently been initiated (Severson DW, unpublished data), the application of comparative genomics to other Culicidae as well as to the Diptera in general is likely to grow rapidly.[48] Our demonstration of CATS utility for inter-specific comparisons should facilitate that process.

Received December 27, 2002. Accepted for publication April 3, 2003.

Financial support: This work supported by National Institute of Health grants AI-33127 and AI-34337.

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