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Chromosomal Evolution among Six Mosquito Species (Diptera: Culicidae) Based on Shared Restriction Fragment Length Polymorphisms

The approximately 3500 species in the mosquito family Culicidae have been traditionally divided into three subfamilies: Anophelinae, Toxorhynchitinae, and Culicinae (Knight and Stone, 1977). The chromosome complement among nearly all mosquito species is fixed at $2N = 6$, with the one known exception being the anopheline *Chagasia bathana* (Dyar) ($2N = 8$) (White, 1980). Despite this conservation, genome size varies widely both across species (Black and Rai, 1988; Rao and Rai, 1987a) and within species (Kumar and Rai, 1990).

Until recently, genetic linkage analyses of various mosquito species were based principally on morphological mutant markers or isozyme markers. Because these markers are limited in number and utility across species, little comparative genetics was possible. Nonetheless, Matthews and Munstermann (1994) suggested that karyotype evolution in culicines occurs through chromosomal rearrangement while linkage block synteny is maintained. These syntenies were also apparent when culicines were compared to *Drosophila melanogaster* (Matthews and Munstermann, 1994). Giemsa C-banding analysis also provided some insight into karyotypic evolution (Rao and Rai, 1987b). Rai and Black (1999) provide a recent review of mosquito genomic organization and evolution.

The introduction of DNA markers has allowed for the saturation of linkage maps with large numbers of markers and for interspecific comparative maps based on conservation of these markers. Severson *et al.* (1993) produced a linkage map of the yellow fever mosquito, *Aedes aegypti* (L.), using 50 restriction fragment length polymorphism (RFLP) markers. This map now has >130 RFLP markers across the three linkage groups (D. W. Severson, unpublished data). Interspecific hybridization of these markers ranges from about 50% with *Anopheles gambiae* Giles to 100% with *Aedes albopictus* (Skuse) (Severson *et al.*, 1994). Subsequently, comparative maps have been developed for *A. albopictus* (Severson *et al.*, 1995), *Armigeres subalbatus* (Coquillett) (Ferdig *et al.*, 1998), *Culex pipiens* L. (Mori *et al.*, 1999), and *Culex tritaeniorhynchus* Giles (Mori *et al.*, 2001).

We here report the construction of a comparative map for the North American eastern treehole mosquito, *Aedes triseriatus* (Say), the natural vector of La Crosse virus (LACV; Bunyaviridae, *Bunyavirus*, California serogroup), using *A. aegypti* RFLP markers. We next examine evolutionary relationships by comparing karyotypic rearrangements among the six culicine mosquito species for which these markers have been used to construct linkage maps. These relationships

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are then compared to published morphological and molecular culicine phylogenies.

Three *A. triseriatus* strains were used in this study. The WALTON strain from northern Indiana has been in colony since 1969. The VERO strain was colonized from eggs collected in Florida in 1977, and the SALADO strain originated from Texas in 1972. Bulk DNA Southern analysis showed extensive similarity between the VERO and the WALTON strains, but reflected considerable polymorphism compared to the SALADO strain. Because *A. triseriatus* does not readily mate in small cages, all crosses were done by induced copulation (Horsfall and Taylor, 1967; Gerberg *et al.*, 1994).

DNA extractions from bulked and individual mosquitoes, restriction digestion, Southern blotting, and hybridization were performed as previously described (Severson, 1997). All markers used in this study are *A. aegypti* cDNA or in one instance (TY7) a genomic clone containing a coding sequence. Markers were initially hybridized to bulk DNA digests of all three *A. triseriatus* strains to confirm hybridization and to identify informative clones. Mapping blots were washed twice in 2× SSC, 0.1% SDS as described (Severson, 1997) and then checked for background signal with a handheld Geiger–Muller counter. Blots were washed as necessary in one or two washes of 0.2× SSC, 0.1% SDS.

Analysis of bulk DNA extractions of the three *A. triseriatus* strains showed that 48 of the 83 (58%) markers tested were polymorphic and informative between at least two strains. Generally, the VERO and WALTON strains shared at least one restriction fragment. Another 16 (19%) markers hybridized with restriction fragments shared across the three strains; the remaining 19 (23%) did not hybridize. Twenty-one markers were hybridized to VERO X SALADO F_1 intercross mapping populations and scored as homozygous for either parental type or as heterozygous. In 14 of 20 (70.0%) of the loci examined, significant deviations from the expected 1:2:1 ratio were observed (Table 1). Similar deviations have been reported for *A. aegypti* (Severson *et al.*, 1994), *A. albopictus* (Severson *et al.*, 1995), *A. subalbatus* (Ferdig *et al.*, 1998), and *C. pipiens* (Mori *et al.*, 1999). Linkage group I markers showed a lack of the VERO homozygote, most likely maintained by homozygous lethal loci in the parental genotypes (Matthews and Craig, 1989; Munstermann, 1994). LGIII loci were also characterized by fewer than expected VERO homozygotes. Loci on LGII were not significantly different from the expected ratio, with the exception of LF264a, which was deficient in heterozygotes.

Twenty-one markers were used to construct a linkage map containing 22 loci and spanning 270.7 cM (Fig. 1). Linkage group I covered 44.6 cM with 6 markers and contained the sex-determining locus. Sex in culicine mosquitoes is determined by a single locus, with maleness being dominant (Gilchrist and Haldane, 1947). One marker (LF90) was not linked to any marker on LGI, nor to any marker on the other linkage groups. Linkage group II contains 6 markers and spans 57.2 cM, whereas LGIII has 8 markers and covers 168.9 cM (Fig. 1). As expected, there appear to be whole-arm rearrangements between these linkage groups compared to *A. aegypti*, which also reflect several inversions. A region bracketed by markers LF103 and LF168 has been transposed from *A. aegypti* LGIII to *A. triseriatus* LGII. One additional marker (LF377) remains unlinked in *A. triseriatus;* there is also a duplication of the LF264 locus in that species. We cannot explain why LF90 and LF377 remain unlinked; however, others have reported a similar phenomenon. For example, two small linkage groups were unlinked in the Solanaceae when tomato markers were used to construct a linkage map of the pepper *Capsicum*, though this may be a result of the interspecific cross used in that study (Livingstone *et al.*, 1999).

We performed comparative karyotype analyses on six mosquito species for which *A. aegypti* RFLP markers have been used to construct linkage maps (Table 2).

TABLE 1

Segregation of *Aedes aegypti* RFLP Markers in F_1 Intercross Populations from *Aedes triseriatus* Crosses[a]

	No. of individuals[b]			
	P1	H	P2	χ^2
Chromosome 1				
LF235	53	67	0	48.45**
AeW	15	76	29	11.80**
LAP	17	77	26	10.98**
LF179	17	74	29	9.93**
TY7	17	70	33	7.60*
LF159	19	57	43	9.89**
Chromosome 2				
LF115	23	61	26	1.47
LF264a	32	38	33	7.10*
LF272	28	65	17	5.84
LF103	26	60	21	2.05
LF168	28	62	20	2.95
Chromosome 3				
LF128	23	46	40	7.96*
LF227	21	51	34	3.34
LF352	17	33	58	47.46**
LF315	17	32	58	59.73**
LF264b	23	37	39	11.49**
LF248	7	58	44	25.57**
LF335	3	61	43	31.91**
LF334	5	62	43	28.04**

[a] Linkage group I (LGI) was believed to be conserved between *A. aegypti* and *A. triseriatus*, whereas rearrangements were predicted between linkage group II (LGII) and linkage group III (LGIII) (Matthews and Munstermann, 1994). Therefore, one F_1 intercross mapping population was used for LGI markers, and a second population was used to map LGII and LGIII markers.

[b] P1, VERO strain; H, heterozygote; P2, SALADO strain.

* $P < 0.05$; ** $P < 0.01$ (loci tested for expected 1:2:1 ratio).

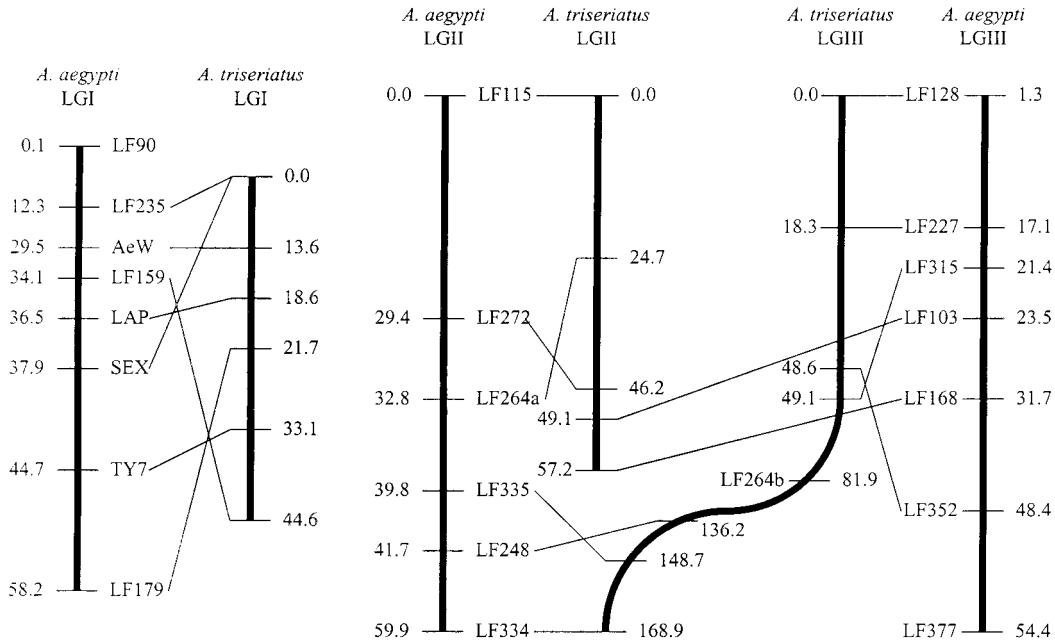

FIG. 1. Comparative map between *Aedes triseriatus* and *Aedes aegypti*. χ^2 goodness-of-fit values were calculated for segregation and independent assortment of alleles for all loci. Multipoint linkage analyses were performed with the Mapmaker computer program (Lander *et al.*, 1987), with a minimum LOD threshold of 3.0 used to identify linkage between markers. Recombination frequencies were converted to Kosambi centiMorgans (cM; Kosambi, 1944). LG, linkage group.

Sixteen chromosomal rearrangements were identified and scored as character states across all six species. For comparisons in which a different subset of closely linked markers was used, markers were assumed to retain synteny and linear order with markers to which they are linked in *A. aegypti*. These data were then input in the PAUP* 4.0b4a computer program as standard data for phylogenetic analysis (Swofford, 2000). Phylogenies were constructed by exhaustive searches with distance and parsimony methods. Bootstrapping (Felsenstein, 1985) was performed on all analyses with the heuristic search option, 5000 replicates with 10 random additions of taxa each, and tree bisection–reconnection branch swapping. Ross (1951) considered *Armigeres* to be ancestral to *Culex* and *Aedes;* we therefore rooted the tree with *Armigeres*.

TABLE 2

Minimum Evolutionary Distance for Pairwise Comparisons Using the Number of Rearrangement Steps between Species (above Diagonal) and Total Character Differences as Given by PAUP* (below Diagonal)

	1	2	3	4	5	6
1. *A. subalbatus*[a]	—	4	4	10	8	9
2. *A. aegypti*[b]	2	—	0	9	8	10
3. *A. albopictus*[c]	2	0	—	9	8	10
4. *A. triseriatus*	3	3	3	—	11	13
5. *C. pipiens*[d]	7	7	7	5	—	2
6. *C. tritaeniorhynchus*[e]	6	8	8	6	1	—

Note. Karyotypic comparisons are given in the references listed.
[a] Ferdig *et al.*, 1998.
[b] Severson *et al.*, 1993.
[c] Severson *et al.*, 1995.
[d] Mori *et al.*, 1999.
[e] Mori *et al.*, 2001.

The number of rearrangements between pairwise species comparisons yielded a minimum evolutionary distance between those species. Eight characters were uninformative for parsimony and were excluded. *A. subalbatus* was used as the outgroup taxon. A single most parsimonious tree was generated (Fig. 2). The genus *Aedes* was paraphyletic, with the *Stegomyia* forming one clade and *A. triseriatus* clustering with the two *Culex* species. A heuristic bootstrap search using 5000 replicates with random addition sequence and tree bisection–reconnection branch swapping indicated that there was strong support for *Culex*, but only moderate support for *Stegomyia* and for the clade containing *Culex* and *A. triseriatus*. Use of the mean character difference as a measure of distance also produced a single best tree, which was identical to that produced by parsimony. Bootstrapping, with the same settings as those used for parsimony, gave strong support for *Culex* and for *Stegomyia*, with moderate support for the *A. triseriatus/Culex* clade.

Reciprocal translocations are evident in the lineage leading to the genus *Culex* and in the *A. triseriatus* lineage. These rearrangements differ at their breakpoints and the segments with which they recombine.

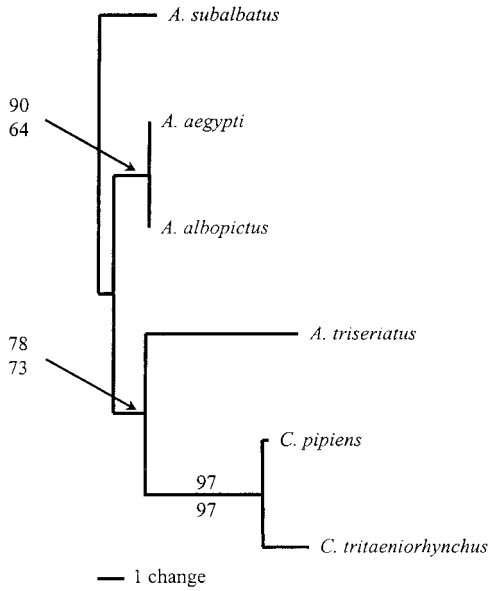

FIG. 2. Phylogenetic tree based on chromosomal rearrangements scored as individual characters among six culicine mosquito species. Numbers above and below branches indicate bootstrap values >50% for distance and parsimony analyses, respectively.

With the exception of a few minor inversions and transpositions, *A. subalbatus*, *A. aegypti*, and *A. albopictus* chromosomes show nearly identical linkage associations of RFLP markers. In the evolution of the culicine mosquitoes, inversions appear to have occurred very frequently, whereas transpositions occurred only rarely. In determining the smallest number of rearrangements in one species necessary to reproduce the linear order of another species, we have given each step (inversion, transposition, or translocation) equal weight. However, studies of bacterial and mitochondrial evolution suggest that transpositions should be given slightly more than twice the weight of inversions (Blanchette *et al.*, 1996). This would then increase the number of inversions by two in any pairwise comparison in which a transposition has occurred on the same chromosome (Sankoff *et al.*, 1992). Weighting transpositions more heavily would have virtually no effect on markers that had transposed between chromosomes.

Within the family Culicidae, morphological comparisons place the Anophelinae in a basal position, the Culicinae as most derived, and the Toxorhynchitinae as intermediate (Ross, 1951). Molecular data appear to support these placements. Rao and Rai used C-banding and comparative karyotyping (1987b) and haploid nuclear DNA amounts (1990) to reach the same conclusions. Besansky and Fahey (1997) used the *white* gene to examine evolutionary relationships among the Culicidae, including several species examined here. Use of all informative characters placed the Anophelinae in the most derived position, with the Culicinae basal. Exclusion of the third codon position, however, recovered the placement of these taxa as described by Ross (1951). Examination of 18S and 5.8S rDNA sequences also recovered these relationships (Miller *et al.*, 1996). However, a recent phylogenetic study that examined morphological characters in 38 genera (Harbach and Kitching, 1998) differed significantly from the accepted phylogeny. Anophelinae remained basal; however, the Aedini and Mansoniini formed a sister clade to the clade of Culicini and Sabethini. In addition, there was no support for the subfamily status of Toxorhynchitinae; the authors proposed lowering this taxon to tribe status.

The linkage maps for *A. aegypti* and *A. albopictus* are entirely conserved (Severson *et al.*, 1995). This is not unexpected as both belong to the subgenus *Stegomyia*, albeit in different species groups (Bates, 1949). Similarly, *C. pipiens* and *C. tritaeniorhynchus* were also highly conserved, differing by only one inversion and one transposition. These species are both placed in the subgenus *Culex* (Knight and Stone, 1977), again in different species groups (Miller *et al.*, 1996). Comparisons between species in different subgenera give larger numbers of rearrangements (e.g., nine steps between *A. (Stegomyia) aegypti* and *A. (Protomacleaya) triseriatus).*

A. aegypti and *A. albopictus* share an inversion, corresponding to the region bracketed approximately by markers LF203 and LF264, that is not present in the remaining four species examined. This suggests that the order present in *A. subalbatus*, *Culex* sp., and *A. triseriatus* is ancestral in the Culicinae and that the inversion arose in the lineage leading to the subgenus *Stegomyia*. We assume that the ancestral order is the same in these species and has not arisen from different inversions giving the same genotype. This assumption may not be valid, however. Caccone *et al.* (1998) showed that inversions that appeared identical in polytene chromosomes in the *A. gambiae* complex in fact arose from separate inversion events.

The lineages leading to *Culex* sp. and to *A. triseriatus* clearly exhibit a greater frequency of rearrangements than the *Stegomyia* lineage. Nine steps separate *A. triseriatus* and a minimum of eight steps separate the *Culex* clade from *Stegomyia*. *A. triseriatus* is the only species mapped to date that exhibits major rearrangements on LGI. The rates of synteny disruption in various mammalian lineages have been shown to vary by approximately 25-fold (Erlich *et al.*, 1997). The genus *Aedes* in our reconstruction was paraphyletic. The *Stegomyia* formed a basal clade, with *A. triseriatus* forming a sister taxon to the genus *Culex* (Fig. 2). Besansky and Fahey (1997), using the *white* gene, also showed that *Aedes* was paraphyletic. In their phylogeny, *A. triseriatus* also clustered with a member of a different genus, *Haemagogus equinus* Theobald, another New World species. Wesson and co-workers (1992) also separated *Stegomyia* from *H. equinus* and *A. triseriatus* on the basis of rDNA ITS2 sequence data. Most recently, Reinert (2000) proposed dividing the

genus *Aedes* into two genera: *Aedes* and *Ochlerotatus*. His genus *Aedes* contains the *Stegomyia*, whereas the genus *Ochlerotatus* contains the *Protomacleaya*.

The use of common markers across many mosquito species may provide additional insight into the evolution of the family Culicidae. To resolve more clearly the evolutionary relationships, many more species need to be mapped. Most mapping to date has focused on major vectors of disease agents, principally *A. aegypti* and *A. gambiae*. We have discussed herein six linkage maps in only three genera. A representative species from each subgenus may be sufficient to determine the chromosomal arrangement of that subgenus, since both the *Aedes (Stegomyia)* and the *Culex (Culex)* groups appear to share much of the linear order of markers. Our analyses, along with those of Besansky and Fahey (1997), Wesson *et al*. (1992), and Harbach and Kitching (1998), appear to support Reinert's (2000) recent proposal to split the large genus *Aedes* into two genera.

A wide range of pathogens is vectored by the Culicidae, including the malarial parasites, filarial worms, and many arboviruses such as yellow fever virus and the dengue viruses. Mapping of a gene of interest in one species will predict the location of the gene in another species. This will prove especially useful for those species that are difficult to colonize and/or maintain in the laboratory. Comparative mapping will also begin to allow elucidation of differences between vector species and nonvector species. For example, *A. triseriatus* is the natural vector of LACV, whereas the closely related *Aedes hendersoni* is incapable of transmitting the virus due to a salivary gland escape barrier (Paulson *et al.*, 1989). Quantitative trait mapping of oral transmission in *A. triseriatus* would identify a corresponding region in *A. hendersoni* that is presumably responsible for the salivary gland barrier. Identification of the genes responsible for such traits and interspecific comparisons of their associated functions could identify novel genetic approaches to limiting, or even preventing, mosquito-borne disease transmission.

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Ddc and *amd* Sequences Resolve Phylogenetic Relationships of *Drosophila*

With about 3000 species, the family Drosophilidae is large and diverse even by Dipteran standards. This diversity provides biologists with distinctive opportunities to investigate evolutionary patterns, but also poses taxonomic and other challenges. Thus, the traditional classification (e.g., Wheeler, 1981) is inconsistent with phylogenetic relationships based on morphology (Throckmorton, 1975; Grimaldi, 1990) or molecular data (review in Powell, 1997). The two comprehensive phylogenetic hypotheses of Throckmorton (1975) and Grimaldi (1990) have been tested against recent molecular studies, which have resolved some important discrepancies between them (see Remsen and DeSalle, 1998; Tatarenkov *et al.*, 1999a; Kwiatowski and Ayala, 1999; Katoh *et al.*, 2000). Yet, many phylogenetic relationships remain unsolved, such as those among *Hirtodrosophila*, *Zaprionus*, *Dorsilopha*, and s.g. *Drosophila* (e.g., Tatarenkov *et al.*, 1999a; Kwiatowski and Ayala, 1999). Some genera, such as *Liodrosophila* and *Samoaia*, have received scarce attention (Pélandakis and Solignac, 1993; Tamura *et al.*, 1995; Tatarenkov *et al.*, 1999a) and their phylogenetic placement remains largely unknown. One problem with the molecular phylogenies is the incompleteness of taxa sampling. Although representatives of some *Drosophila* groups have been included together in some studies, often different studies include different groups, which prevents construction of a reliable higher-level phylogenetic framework.

We seek to define a robust framework of relationships in the Drosophilidae at the species-group and higher taxonomic levels. We have investigated 29 species (Table 1) from several drosophilid genera and subgenera and from representative species groups for two nuclear genes, dopa decarboxylase (*Ddc*) and α-methyl dopa (*amd*). These are closely linked paralogous genes, arisen by an ancient gene duplication (Eveleth and Marsh, 1986; Tatarenkov *et al.*, 1999b). We earlier used *Ddc* to address some issues of *Drosophila* systematics (Tatarenkov *et al.*, 1999a). We now extend our previous investigation by including additional taxa for longer sequences of *Ddc* and a new gene, *amd*, which previously had been sequenced only in *D. melanogaster* (Marsh *et al.*, 1986), *D. simulans*, and *Scaptodrosophila lebanonensis* (Tatarenkov *et al.*, 1999b).

DNA preparation and sequencing were as described by Tatarenkov *et al.* (1999a; method b). The 963- to 966-bp-long sequences of *Ddc* previously reported are now extended to 1131–1134 bp. *Ddc* sequences of five more species, *D. pseudoobscura*, *D. robusta*, *D. phalerata*, *D. funebris*, and *D. gymnobasis*, are added. Amplification and sequencing of *amd* was as for *Ddc*, except that the annealing temperature was 60°C and the extension time was 3 min. The *amd* amplifying primers were 5′-MAYATGCAYGSCTAYTAYCCCAC-CAG-3′ (Amd-un2, forward primer) and 5′-ACCA-TRTAGATYTTYTTNCGNTCCAT-3′ (Amd-bw, reverse primer). The amplified region of *amd* encompasses an intron. The amplified fragment varied in length from 1269 bp in *D. hydei* to nearly 2600 bp in *D. tripunctata*, depending on the length of the intron. Only the coding regions, 1032-bp-long, were used in this study (66 bp