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An initial linkage map of the West Nile Virus vector *Culex tarsalis*

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Abstract

We have constructed the first genetic linkage map for the North American arboviral vector *Culex tarsalis*. 120 F_2 offspring from a cross between two colonies were genotyped using 25 microsatellites and six inter-simple sequence repeat (ISSR) markers. We resolved four linkage groups which likely correspond to two full-length chromosomes and two arms of the final chromosome. The longest linkage group contains the sex locus and corresponds to chromosome 3. Recombination rates around the sex locus were dramatically higher in females compared to males. The majority of microsatellite loci share sequence identity with regions of the *Culex quinquefasciatus* genome, whose assembly should aid in anchoring linkage groups to physical chromosomes. This map will aid in identification of loci involved with variable phenotypes in *C. tarsalis* including WNV susceptibility.

Keywords: *Culex tarsalis*, linkage map, West Nile virus, QTL.

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Introduction

The mosquito *Culex tarsalis* is a major vector of West Nile Virus (WNV), Western Equine Encephalitis Virus (WEEV) and St. Louis Encephalitis Virus (SLEV) in North America. The species distribution of *C. tarsalis* spans from the west coast to the Mississippi River and extends into portions of Canada and Mexico (Darsie & Ward, 1981). It is one of the most efficient vectors of WNV known (Goddard *et al*., 2002, 2003), reaches very large population sizes (Reisen & Reeves, 1987; Reisen & Lothrop, 1995) and serves as both an amplifying vector in bird reservoirs (Reisen *et al*., 2004) and a bridge vector to human hosts in rural areas (California DHS, 2008).

Decades of extensive field and laboratory research on *C. tarsalis* have revealed a great deal of variation in its physiology, vector competence and vectorial capacity. Autogeny (the ability to generate eggs without a bloodmeal) is commonly observed in both laboratory and field-caught females, and is known to vary both temporally (Spadoni *et al*., 1974) and spatially (Hardy & Reeves, 1973; Reisen, 1995). The ability to diapause varies within populations (Reisen & Reeves, 1987) while body size and early season survival has been shown to vary among populations when reared under controlled conditions (Reisen, 1995). Perhaps most importantly, *C. tarsalis* exhibits high intra-species variation in its ability to transmit arboviruses. Early studies on transmission of WEE indicated that populations from different parts of California varied greatly in ID_{50} (viral dose at which 50% of mosquitoes were infected 14 days post-inoculation) and infection rate by oral challenge (Hardy *et al*., 1976; Hardy & Reeves, 1987). More recent work on WNV has shown that susceptibility, oral and vertical transmission of West Nile virus varies significantly among populations (Goddard *et al*., 2003).

While phenotypic variation in this mosquito has been well-described, the dearth of molecular tools for *C. tarsalis* has made it difficult to investigate the relationship between phenotypic and genotypic variation in laboratory colonies or wild populations. Until recently, genetic studies in *C. tarsalis* were limited to crude linkage associations of a limited number of morphological mutations (Asman *et al*., 1987)

Table 1. Microsatellite markers used in this cross

Locus	Accession no.	Forward Primer	Reverse Primer	Allele size range (bp)		Sequence identity to Cx. pip genome
Linkage group 1 (Chromosome 3)						
CUTC105	DQ296487	5′-GCCGGTTGTTGTTGTTGTAC-′3	5′-TCCTCGTCAATTTCATCGAC-′3	209	230	
CUTB210*	DQ682690	5′-ACCCACTGTTTGCGTATGAA-′3	5′-ACACTCACACCACCTTGTGC-′3	262	280	X
CUTB218*	DQ682694	5′-TGCTGAGGCCGTTTTACC-′3	5′-CCCTGGAAAAGCATCAAACT-′3	150	214	X
CUTB228*	DQ682697	5′-CATCACCATCAATCGTTTCC-′3	5′-GAAAACTTCCGGCACACAC-′3	145	195	X
CUTB224*	–	5′-CGAAGAGCAACAACATTCCA-′3	5′-CTCTGAAATCGATACACCAAGC-′3	204	232	
CUTB101*	DQ682680	5′-GGGGTTCTTCGTGAGTTC-′3	5′-AGCAAGCGATTTCCCTAC-′3	208	254	
CUTA220*	DQ682674	5′-TGAGCACGGGTGAGTTACAC-′3	5′-CCAATCGACGGGAAATTACA-′3	148	168	X
CUTC203*	DQ682700	5′-AGGCCATGCAACATCCTTAC-′3	5′-CGACTTTATCTAGGCGCTCTC-′3	192	225	X
CUTB112	DQ682681	5′-AACCCCAGATTCTTAATGGC-′3	5′-GGAATTGGCTCAAACAACC-′3	154	184	
Linkage group 2						
CUTB203*	DQ682687	5′-ACGAACGCGAAAGAAGAGAG-′3	5′-CACACCCGATTGTAGAGTGC-′3	230	256	X
CUTA6	DQ682664	5′-ACTCACACCCGATTGTAGAG-′3	5′-AGCCAGTCAGTCAGTCAGTG-′3	259	307	X
CUTC12	DQ296486	5′-GTGGAGAACCCGTATTCAAC-′3	5′-TACAATCACGACTCGCACATA-′3	184	208	X
CUTB223*	DQ682696	5′-CGATATTTTGCTCCCACTTTG-′3	5′-AACTCCTTCGGGCTACACTG-′3	145	177	X
CUTC102*	DQ682698	5′-GGAACCACAATCATCATAACC-′3	5′-GCAACAAACGAATCTTAGAAAC-′3	255	282	X
CUTD211*	DQ682706	5′-TTCTGTTGTTGGGATTGCTG-′3	5′-GTCCGCACCCTGAATTGTA-′3	251	278	X
CUTB212*	DQ682691	5′-TGTCGAGGTGAAACAACCAG-′3	5′-CCGAACGAAAAGCAAAAGTC-′3	148	176	X
CUTD114	DQ296492	5′AGGAAGAGTGGTTCGTTTTC′3	5′GGGTAAGTTTCAGGGCTATC-′3	180	198	X
CUTD102*	DQ682703	5′-CAGTTCCAGCAGCAGTCA-′3	5′-CAGGTGATGGGGGTGTAG-′3	117	142	X
CUTD113	DQ296491	5′-ATCATACCACTGCCCATAGTC-′3	5′-AACCAGCAGGGACAAGTC-′3	159	185	X
CUTA109.7*	DQ682667	5′-CCATCACATTGAACATCACTT-′3	5′-CGAGTTGCCGATAGAAGAT-′3	236	288	X
Linkage group 3						
CUTB1	DQ296484	5′-GAAAAAAAGGCGCAACAT-T-′3	5′-GAAGGTGCCAGCCTACTTG-′3	104	138	X
CUTB214*	DQ682693	5′-GCAGTAGCTGGAACGTGCT-′3	5′-GCGCATAAAATACACAGCAAA-′3	156	190	
CUTA105.7*	DQ682666	5′-TCGCCTTACTTCCCACAT-′3	5′-AGGACCCAACAACAGCAC-′3	244	282	
Linkage group 4						
CUTD120	DQ296493	5′-TACCCTCGCAAACAAAACAA-′3	5′-GTCGGCTTCCATTCCACTAC-′3	159	183	X
CUTD203*	DQ682708	5′-TATCCGGCAGCAGAACTTG-′3	5′-ACAAGCACCACAGCAAACTG-′3	214	241	X

*Allele size range includes 26 bp 5′ M13 tag.

and a single population study using allozymes in California and Nevada (Gimnig *et al*., 1999). The characterization of genetic variation in this mosquito has recently been facilitated by the development and validation of a large panel of microsatellite markers (Rasgon *et al*., 2006; Venkatesan *et al*., 2007a). A preliminary analysis of microsatellite variation across populations in five states showed significant genetic differentiation in southern California, New Mexico and Nebraska (Venkatesan *et al*., 2007b). These results are consistent with the possibility that genetic variation may condition phenotypic variation among individuals and/or populations. The presence of genetic structure along with phenotypic differences in WNV susceptibility and transmission in *C. tarsalis* suggest that genetic polymorphism at particular loci may condition WNV infection phenotypes, and that genetically disparate populations of *C. tarsalis* may also be phenotypically distinct with respect to vector competence for WNV.

Quantitative trail loci (QTL) associated with pathogen susceptibility and transmission have been identified in other mosquitoes and arboviral systems. Linkage mapping has been used extensively to identify dengue virus QTL in *Aedes aegypti* involved in barriers to both midgut infection and dissemination (Bosio *et al*., 2000; Gomez-Machorro *et al*., 2004; Bennett *et al*., 2005). QTL have also been identified for transovarial (Graham *et al*., 2003) and oral (Anderson *et al*., 2005) transmission of La Crosse virus in *Ochlerotatus triseriatus*. However, virus-related QTL have not yet been identified for vectors of WNV or for any members of the genus *Culex*, which includes several significant, globally distributed arboviral vector species.

In order to lay the foundation for QTL mapping of phenotypic traits such as virus susceptibility, autogeny and diapause in *C. tarsalis*, we have generated the first molecular genetic linkage map for this species. Linkage groups were derived from an F_2 intercross between two laboratory colonies using microsatellite markers (Table 1) and inter-simple sequence repeats (ISSRs; Table 2). We anticipate that this map will facilitate genetic characterization of traits of medical and biological interest in this important arboviral vector. Additionally, we discuss the possibility of physically anchoring multiple loci mapped in our cross based on orthologous regions in the recently sequenced *Culex quinquefasciatus* genome.

Results

F_2 cross

High mortality in 4th instar F_2 larvae led us to select multiple F_2 families for the mapping population. A total of 120 F_2

Table 2. ISSR primers and loci used in this cross

Primer	Scorable loci	Segregating loci	Mapped loci	Label	Location
$(AG)_8$CG	4	2	1	$(AG)_8$CG_B	Linkage group 1 (Chromosome 3)
$(AC)_8$TGA	9	2	1	$(AC)_8$TGA_B	Linkage group 1 (Chromosome 3)
$(AG)_8$TGA	8	6	2	$(AG)_8$TGA_A	Linkage group 4
				$(AG)_8$TGA_E	Linkage group 3
$(AC)_8$TCT	3	2	2	$(AC)_8$TCT_A	Linkage group 4
				$(AC)_8$TCT_B	Linkage group 2

offspring (41 females, 79 males) from the five largest families were successfully reared to adulthood and used for linkage analysis. Family 3 contained 22 individuals (five females, 17 males), Family 5 contained 12 (five females, seven males), Family 6 contained 18 (eight females, 10 males), Family 12 contained 23 (12 females, 11 males) and Family 22 contained 45 (11 females, 34 males).

Microsatellites

Parental F_0 individuals were successfully genotyped at 53 of the 57 available microsatellite loci. 28 markers were identical in the two F_0 individuals and were thus uninformative for linkage analysis. Of the remaining 25 microsatellites, 13 loci segregated fully between the two F_0 parentals and 12 segregated partially, with one allele varying between F_0's. Twenty of the 25 markers were informative for all five families with a total of 22 microsatellites in Family 5, 25 in Families 5 and 6, and 24 in both Families 12 and 24.

In general, microsatellite loci conformed to expected Mendelian inheritance ratios (Table 3). After correcting for multiple tests, seven in 123 comparisons demonstrated statistically significant deviations. No family-specific deviation pattern was observed and none of the loci exhibited significant deviations in more than a single family. The newly described locus CUTB224 appears to segregate in accordance with expected Mendelian inheritance ratios (Table 3) but has not yet been tested for deviation from Hardy-Weinberg allele frequencies in natural populations.

ISSR markers

Each of the 12 ISSR primers produced between three and nine scorable bands. Two to six bands per primer segregated in the F_0 parentals (Table 2), producing 12 informative loci. Six of the 12 loci were mapped (Fig. 1) while the other six were excluded from the map because they did not exhibit linkage to any other markers or linkage groups.

Mendelian inheritance ratio patterns were similar to those observed in microsatellites. All six ISSR loci deviated from expected ratios in at least one of the five families (Table 3; Table S1), but no family-specific patterns were observed. Eight of 30 total comparisons exhibited deviation from expected Mendelian inheritance ratios after a correction for multiple tests.

Linkage Map

Genotypic information from 32 loci (25 microsatellites + 6 ISSRs + sex locus) in 120 F_2 progeny from five families was used to construct a genetic map. Family-specific linkages along with a composite multi-family linkage analysis integrating recombination information from each family were constructed. The composite map resolved four linkage groups (Figs 2 and 3). The total map length was 510 cM, with an average recombination distance between markers of 18.22 ± 2.12 cM (s.e.). Family-specific maps showed similar marker order to each other and to the composite map, although there was significant variation in calculated recombination rates and map lengths between families, likely due to sample size effects (Fig. 2).

Across most of the genome, there was no significant difference in recombination rates between sexes, and male and female-specific maps resolved identical linkage groups and marker order, except in the area directly flanking the sex locus. In this region only, the recombination rate was dramatically higher in females compared to males, resulting in the region containing the sex locus being unlinked from the rest of the linkage group in females (Fig. 4).

As it contains the sex locus, the first linkage group likely corresponds to chromosome 3 (McDonald *et al.*, 1978). Nine microsatellites and two ISSRs mapped to linkage group 1 at an average genetic distance of 20.38 cM ± 4.03 cM and a total length of 224 cM. The sex locus mapped to 1.4 cM from CUTB218, representing the shortest distance between markers on linkage group 1. The greatest distance occurred between microsatellite loci CUTB218 and CUTB228.

The second largest linkage group, spanning 131.6 cM, group consisted of 11 microsatellites and one ISSR with an average distance of 11.96 cM ± 2.44 cM between markers. Initially, an additional microsatellite CUTA6R.7, was mapped to this linkage group in the exact same position as CUTA11. Further examination revealed that the primer sets for CUTA6 (Accession no. DQ682664) and CUTA11 (Accession no.

Table 3. Chi-squared values of Mendelian inheritance ratios for microsatellite and ISSR markers

		Chi-squared values				
		Family 3	Family 5	Family 6	Family 12	Family 22
Locus	No. individuals	22	12	18	23	45
CUTC105	Linkage group 1 (Chromosome 3)	3.45	2.00	10.89	0.80	5.95
CUTB210		6.55	0.82	0.00	0.47	11.52
CUTB218		**35.10***	1.00	3.00	5.68	3.42
CUTB228		7.36	6.00	2.00	3.38	2.33
CUTB224		0.55	4.00	6.00	2.00	6.91
CUTB101		8.52	0.20	3.00	0.50	10.22
CUTA220		0.18	5.18	1.89	1.00	14.58
$(AG)_8$CG_B		10.24	8.33	**10.89**	0.53	**23.27**
CUTC203		2.91	5.33	2.00	1.31	**26.27**
$(AC)_8$TGA_B		0.06	4.00	2.00	0.00	**12.30**
CUTB112		3.57	3.18	**24.18**	0.16	7.10
$(AC)_8$TCT_B	Linkage group 2	**16.40**	0.09	0.06	4.00	3.76
CUTB203		–	4.45	0.06	4.00	0.21
CUTA6R.7		–	5.33	0.06	–	0.03
CUTC12		5.18	5.33	8.22	2.41	2.12
CUTB223		3.60	4.67	14.44	0.23	3.82
CUTC102		5.18	8.00	9.11	1.00	2.76
CUTD211		–	7.25	0.89	8.07	0.68
CUTB212		8.91	7.36	0.89	9.00	1.14
CUTD114		1.64	4.45	6.00	**27.60**	2.00
CUTD102		0.59	3.00	0.89	8.00	1.52
CUTD113		0.00	0.33	0.89	11.84	1.19
CUTA109.7		4.55	3.60	5.44	0.40	1.19
CUTB1	Linkage group 3	–	0.50	3.67	0.30	0.24
CUTB214		**19.48**	5.67	5.35	4.14	2.60
$(AG)_8$TGA_E		0.18	0.33	**21.41**	2.58	0.56
CUTA105.7		2.91	0.00	0.22	0.47	–
$(AG)_8$TGA_A	Linkage group 4	**14.73**	1.78	0.07	6.37	**11.76**
CUTD120		12.55	12.82	7.71	**17.00**	2.00
CUTD203		3.45	1.00	1.80	15.13	**21.21**
$(AC)_8$TCT_A		0.05	0.81	0.02	**30.42**	0.20

*Chi-squared values that deviate significantly from expected Mendelian inheritance ratios are shown in bold.

DQ296483) amplify the same microsatellite locus. CUTA11 was subsequently removed from the analysis and a note was made to GenBank to correct this error.

The third and fourth linkage groups were the smallest, spanning 82.5 cM and 71.8 cM, respectively. Linkage group 3 consisted of three microsatellites and one ISSR with an average distance of 27.5 cM ± 1.77 cM). Linkage group 4 encompassed two microsatellites and two ISSRs spaced with an average distance of 24 cM ± 1.11 cM.

Synteny with C. quinquefasciatus

Microsatellite-containing sequences from *C. tarsalis*, obtained during initial marker development described in Rasgon *et al*. (2006), were compared to the *C. quinquefasciatus* genome (www.vectorbase.org) using BLASTN. Flanking sequences of 19 of the 25 mapped microsatellites exhibited high similarity to regions of the *C. quinquefasciatus* genome (Fig. 3; Table 4). Aligned regions showed 70%–96% sequence similarity in ~100–600 bp segments. The actual microsatellite repeat motif was present in 17 of the *C. quinquefasciatus* sequences. CUTA109.7 showed similar sequence identity to portions of two separate supercontigs (Table 4).

Discussion

We present in this study the first genetic linkage map of *C. tarsalis* using modern molecular markers. We resolved four linkage groups using a total of 32 loci. Based on estimates of the genome sizes of *C. quinquefasciatus* at 580 megabases (Mb) (CpipJ1.2 genome assembly; http://cpipiens.vectorbase.org/SequenceData/Genome/), mapped loci in the *C. tarsalis* genome occur at an approximate frequency of one marker per every 18 Mb assuming that the genome sizes of *C. quinquefasciatus* and *C. tarsalis* are similar.

Our map at 510 cM is larger than those constructed for other mosquitoes. To confirm that this result was not due to problems with the mapping algorithms implemented by R/qtl, we validated the maps by separate analyses with the CRI-MAP software package, which is widely used in human genetics (Green *et al*. 1990). Analysis with CRI-MAP required the omission of a small portion of the genotypes at

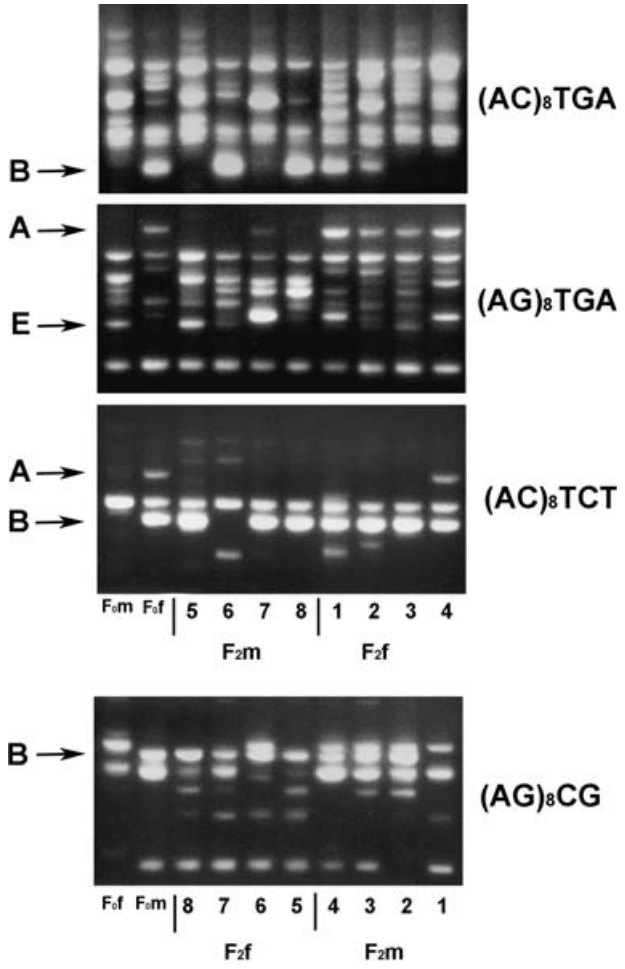

Figure 1. ISSR loci used in this cross. A total of 6 bands from 4 primers were mapped. Mapped band positions are indicated by arrows. Genotypes of the F_0 male and F_0 female are shown along with 4 F_2 males and 4 F_2 females (1–8). m = male, f = female.

dominant markers, as the software was constructed for use with co-dominant markers only. Nevertheless, the maps constructed with CRI-MAP exactly matched those constructed with R/qtl to within 0.1 cM except for a single 30 cM interval on linkage group 3, for which the distance estimates differed by 2 cM (data not shown).

Variation among family-specific maps due to sample size effects could in part account for the relatively large composite map distances observed in this study. However, at approximately 500 cM, the *C. tarsalis* map is not unreasonably larger than those generated for some other mosquitoes. For instance, both the *Anopheles funestus* map and a map generated in a hybrid cross between *Ochlerotatus trisariatus/hendersoni* were almost 400 cM (Anderson *et al*. 2005; Wondji *et al*. 2005). Future mapping experiments will serve to clarify the issue of map size for *C. tarsalis*.

An early study of sex linkage in *C. tarsalis* showed that the sex locus is located on the longest chromosome (McDonald *et al*., 1978), corresponding to chromosome 3. Convention holds that mosquito chromosomes are labeled as 1, 2, and 3 in order by increasing size, chromosome 1 being the shortest and chromosome 3 being the longest (Rai, 1963). Here we term our longest linkage group, which also contains the sex locus, as linkage group 1/chromosome 3. In contrast, the sex locus in *C. pipiens*, *Ae. aegypti* and most culicines is located on chromosome 1, the shortest chromosome (McDonald & Rai, 1970; Jost & Laven, 1971). Interestingly, in some populations of *Culex tritaeniorhynchus*, the sex locus is found on chromosome 1 (Baker *et al*., 1971; Selinger, 1972) while in others it is found on chromosome 3 (Baker & Sakai, 1976; Baker *et al*., 1977; Mori *et al*., 2001).

Variation among linkage groups in marker number and length suggests that apart from linkage group 1/chromosome 3, another chromosome likely corresponding to linkage group 2 has been well-resolved. We suspect that the smaller linkage groups 3 and 4 correspond to two arms of the remaining chromosome. This phenomenon is not uncommon in lower-resolution linkage analyses of other mosquitoes where chromosomal arms appear to assort independently or are separated by large genetic distances (*An. funestus*; Wondji *et al*., 2005; *Ochlerotatus spp*.; Anderson *et al*., 2006), particularly if centromere-spanning markers are lacking. Full resolution of the remaining chromosome may require higher marker density and/or a larger mapping population. Alternatively, since microsatellite homologues from each of the two smallest linkage groups exist in the *C. quinquefasciatus* genome, we may gain insight into the identity of these linkage groups in *C. tarsalis* when the *C. quinquefasciatus* genome is fully assembled.

Sex-specific recombination rates are known to vary in insects including *Bombyx mori* (Rasmussen, 1977) and *Drosophila* species (Clements, 1992). Many studies have found that *C. tritaeniorhynchus* females do not undergo recombination (e.g. Baker & Rabbani, 1970; Baker & Sakai, 1973a,b; Mori *et al*., 2001), though recombination occurs in both sexes in *Culex pipiens* (D. Severson, pers. comm.; Rasgon & Scott, 2004). We did not observe evidence of sex-specific differences in recombination for the majority of markers in *C. tarsalis*. However, recombination does appear to vary significantly between males and females in the region directly adjacent to the sex locus, where females exhibited dramatically higher rates of recombination. This phenomenon warrants further investigation.

ISSR markers are commonly used in plant linkage mapping (Irzykowska *et al*., 2002; Hashuzime *et al*., 2003; Irzykowska & Wolko, 2004) and have been tested for population variation in invertebrates (Abbot, 2001). In our hands, 12 ISSR bands segregated clearly and reliably in F_0's, F_1's and F_2's, suggesting that ISSR markers are variable and informative for mapping purposes. However,

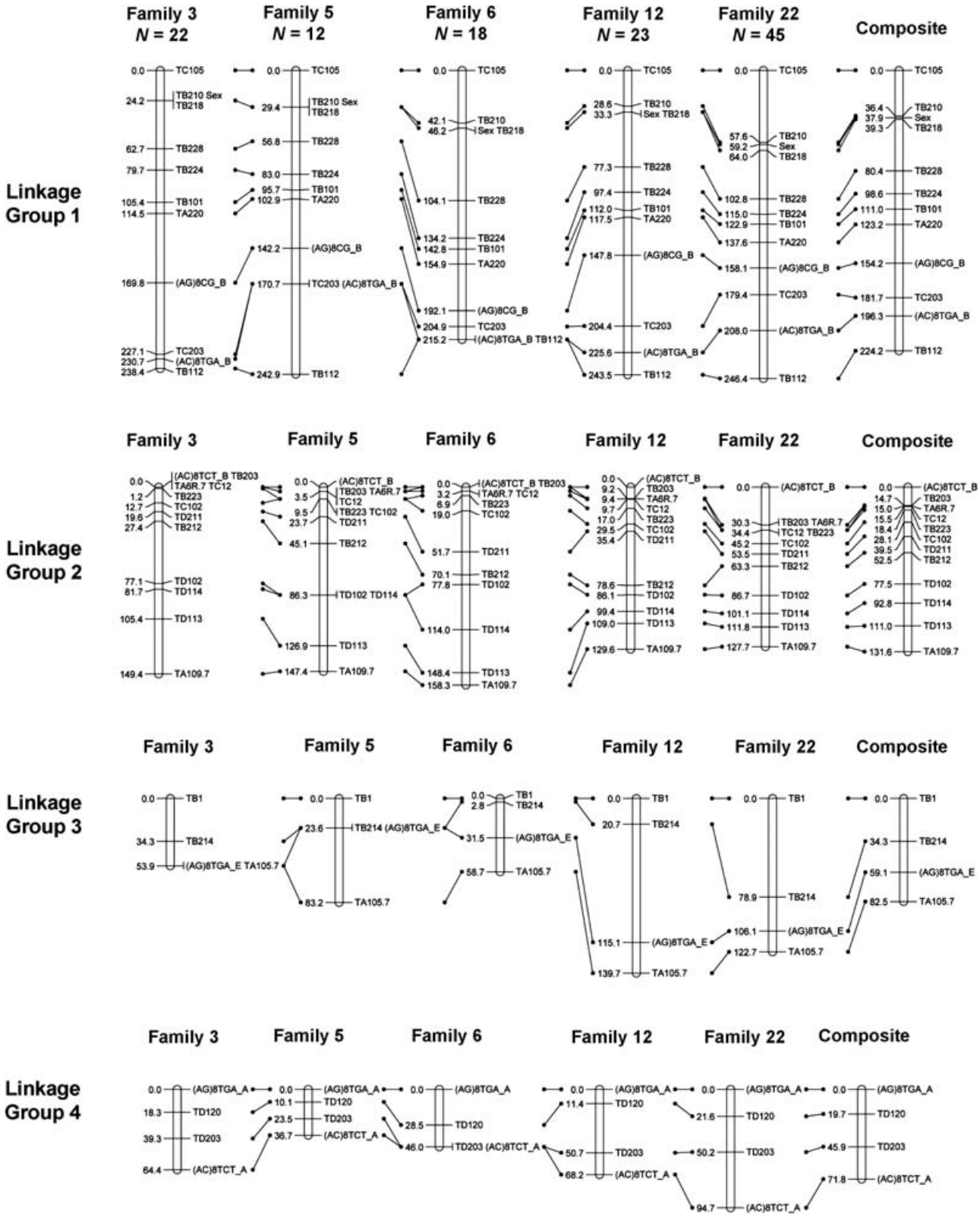

Figure 2. Family-specific and composite linkage maps of *Culex tarsalis* based on recombination frequencies observed in an F_2 cross. Maps were derived from recombination estimates using the Kosambi mapping function. Map distances are listed in cM. Those few markers that were uninformative in family-specific maps (Table 3) were placed by linear interpolation for comparative purposes.

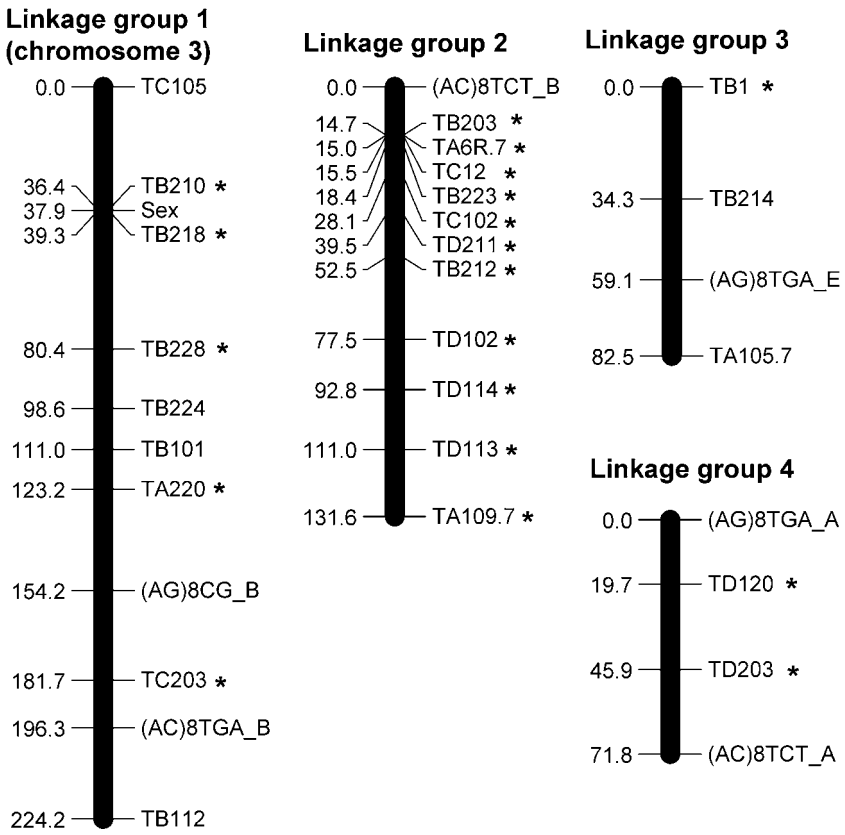

Figure 3. Composite F_2 linkage map of *Culex tarsalis* showing loci mapped by homology to the sequenced *C. quinquefasciatus* genome (indicated by asterisks). Map distances are listed in cM. Map was constructed using the Kosambi function.

Table 4. Mapped microsatellite loci in *Culex tarsalis* with orthologs to flanking regions in the *Culex quinquefasciatus* genome

Locus	Location	Alignment length (no. of bp)	Corresponding regions in *C. quinquefasciatus* genome			Sequence identity
			Supercontig	From (bp)	To (bp)	
CUTB210	Linkage group 1 (Chromosome 3)	257	3.22	1047680	1047937	0.91
CUTB218*		92	3.10	749087	749179	0.89
CUTB228		481	3.355	192600	193081	0.72
CUTA220		435	3.16	1042459	1042894	0.86
CUTC203		214	3.198	92748	92962	0.71
CUTB203	Linkage group 2	459	3.185	332456	332915	0.81
CUTA6R.7		476	3.185	332456	332932	0.81
CUTC12		375	3.369	252315	252690	0.80
CUTB223		315	3.561	223790	224105	0.74
CUTC102**		469	3.384	128435	128904	0.78
CUTD211		240	3.50	892111	892351	0.84
CUTB212		400	3.297	90515	90915	0.70
CUTD114		299	3.805	61858	62157	0.88
CUTD102		628	3.258	59592	60220	0.91
CUTD113		251	3.1	2851920	2852171	0.96
CUTA109.7***		268	3.1233	65213	65481	0.69
		355	3.1424	55752	56107	0.70
CUTB1**	Linkage group 3	151	3.32	76478	76629	0.78
CUTD120	Linkage group 4	317	3.49	490661	490978	0.79
CUTD203		407	3.1235	59619	60026	0.71

All sequence positions in *C. quinquefasciatus* refer to genome version CpipJ1, released in March 2007.
*Corresponding supercontig in *C. quinquefasciatus* begins at position 371 of *C. tarsalis* microsatellite-containing sequence.
**Microsatellite repeat region absent in corresponding *C. quinquefasciatus* sequence.
***CUTA109 shares similarity to two regions in the *C. quinquefasciatus* genome.

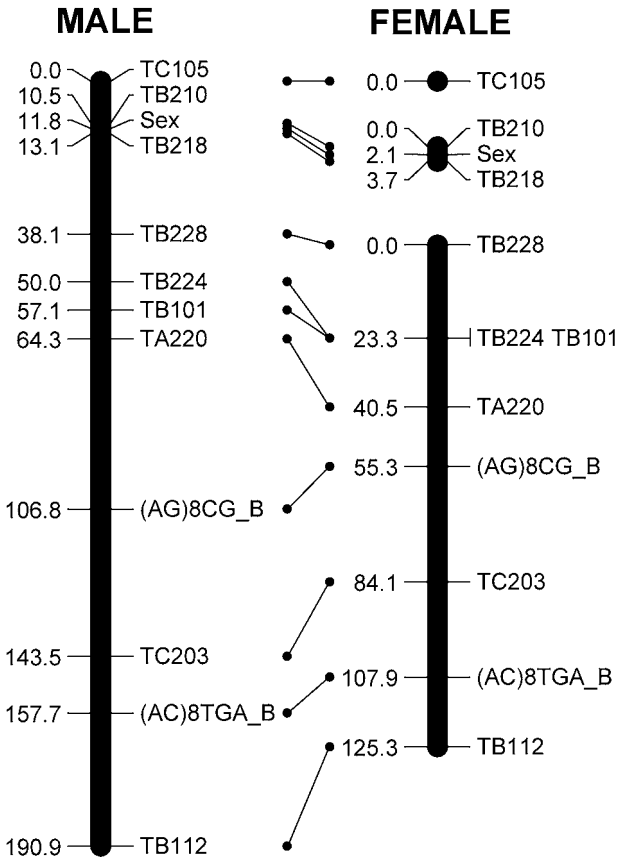

Figure 4. Comparison of sex-specific composite maps of linkage group 1/chromosome 3, highlighting the dramatically increased recombination rate around the sex locus in females. Recombination rates across the remainder of the genome did not vary significantly between sexes.

only six of the 12 mapped to the four defined linkage groups. The reasons for such poor mapping performance are unclear, but this phenomenon does not necessarily appear to be specific to the cross presented here or to insect mapping in general. Mapping studies of various plant species, where ISSRs have been used extensively, show a high percentage (up to 80%) of non-mapping ISSR markers (Kojima *et al*. 1998; Duran *et al*. 2004; Yu *et al*. 2006; Gupta *et al*. 2007). We recommend that ISSRs be used in future insect mapping studies only if a large number of segregating bands can be identified, since a significant proportion may not map to any linkage group.

Of the 53 reliably amplifying microsatellite primers, nearly half were informative in the cross. The mapping population arose from a cross between two California colonies which were established from sites 240 miles apart and reared independently for at least four years. Recent evidence suggests that there is moderately differentiation between populations in the two sites (Venkatesan *et al*., 2007b); yet the majority of microsatellite loci did not segregate between the F_0 individuals. If possible, future studies should attempt to cross colonies established from populations at an even greater genetic distance from each other to increase the number of informative loci from the available panel of markers.

The total number of mapped loci, at 32, lies well within the range of marker number for QTL analysis in other mosquito species such as *An. funestus*, at 49 or 56 loci (Wondji *et al*., 2005, 2007), *Ochlerotatus sp*., at 25 loci (Anderson *et al*., 2005, 2006) *C. tritaeniorhynchus*, at 14 loci (Mori *et al*., 2001) and *C. pipiens*, at 9–13 loci (Mori *et al*., 2007). While the availability and density of mapping markers may increase in future efforts, these studies suggest that the existing set of 60 + available loci and linkage map of 32 loci should be sufficient to identify QTL in *C. tarsalis*.

Several of the flanking sequences of mapped microsatellites in *C. tarsalis* exhibit high similarity to regions of the recently released *C. quinquefasciatus* genome (Fig. 3; Table 4). Seventeen of the 19 repeat motifs are present in *C. quinquefasciatus*, suggesting that microsatellite loci are well-conserved between the two species. Additionally, others have used microsatellite loci developed for *C. tarsalis* to investigate population genetic questions in the *C. pipiens* species complex (Kent *et al*., 2007). Future assignment of *C. quinquefasciatus* supercontigs to chromosomes will assist in validating marker placement in our linkage map and contribute to an improved physical positioning of microsatellites in *Cx. tarsalis*. Additionally, genome assembly should allow us to make estimates of physical distance among markers for comparison with linkage distances, explore synteny between the two species and aid in cloning and characterization of genes of interest.

The linkage map presented in this report is an important tool for genetic studies of *C. tarsalis* and comparative analysis of related *Culex* species. We anticipate that it will be useful as a basis for QTL studies of variable phenotypes such as autogeny, insecticide resistance and susceptibility to arboviruses in this important vector mosquito.

Experimental procedures

Mosquito strains and rearing conditions

The two *Culex tarsalis* strains used in the cross were KNWR, colonized from the Kern National Wildlife Refuge in Kern, CA and CTC from Coachella, CA in 2003 by W. Reisen (UC Davis). Colonies were acquired by our group at the Johns Hopkins School of Public Health in 2005 (KNWR) and 2007 (CTC) and maintained independently. Colonies were reared at 27 °C and 90% relative humidity on a 16:8 light-dark cycle. Larvae were fed a 1:2:2 mixture of ground rabbit pellets, liver powder and fish flakes at 7.5 mg/individual. Adults were provided with 10% sucrose solution *ad libitum*. KNWR females were maintained autogenously while CTC and F_1 cross females were allowed to bloodfeed on anesthetized mice according to JHU Animal Welfare Assurance protocol A3272-01.

Crosses

Harem crosses were conducted in pint cages with six to eight virgin F_0 CTC females (2–3 days old) and a single F_0 KNWR male per cage. Due to an insectary lighting malfunction, mating in the attempted reciprocal cross was unsuccessful. Females were bloodfed after five days and placed in individual cages to oviposit. Each F_1 egg raft was reared independently and offspring were sibling-harem-mated as described above. Mated F_1 females were bloodfed up to three times to produce the F_2 generation. F_2 families were reared to adulthood and preserved for subsequent DNA extraction along with the original F_0 parents and productive F_1 mosquitoes.

DNA preparation and microsatellite genotyping

The five largest F_2 families were selected for genotyping. DNA was extracted by salt extraction/ethanol precipitation as previously described (Black & DuTeau, 1997) and suspended in nuclease-free water at a concentration of 30 ng/uL. We successfully genotyped F_0 mosquitoes at 53 of 57 available microsatellite loci (Rasgon *et al*., 2006; Venkatesan *et al*., 2007a) using previously described methods (Boutin-Ganache *et al*., 2001; Venkatesan *et al*. 2007a). PCR products were resolved on an ABI Prism Genetic Analyzer 3100 Avant (Applied Biosystems, Foster City, CA). Allele sizes were automatically determined with an internal ROX-500 size standard (Applied Biosystems) using GeneScan v. 3.1 and Genotyper software (Applied Biosystems). Loci informative in the F_0's were genotyped in F_1's and 120 F_2's. Microsatellite markers used in the cross are shown in Table 1 and include a previously unpublished microsatellite, the (AG) repeat CUTB224. 5′ M13-labelled amplification of CUTB224 was carried out as described in Venkatesan *et al*., 2007a. This locus appears to segregate in accordance with expected Mendelian inheritance ratios (Table 3) but has not yet been tested for deviation from Hardy-Weinberg allele frequencies in natural populations.

ISSR genotyping and scoring

The segregating population was genotyped at 12 inter-simple sequence repeat (ISSR) loci derived from four 3′ anchored repeat primers shown in Table 2. The 25 uL PCR mixture contained 2.5 μl of 10X reaction buffer (New England Biolabs, Ipswich, MA, USA), 30–40 ng of DNA template, 0.8 μM of a single ISSR primer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP and 1 μl (5 units) of *Taq* DNA Polymerase. PCR products were amplified using a DNA Engine thermal cycler (Biorad, Hercules, CA, USA), under the following conditions adapted from Abbot (2001): an initial denaturation step at 94 ° for 2 min, 13 cycles of 94 ° for 30 s, 68 ° for 30 s with a 0.7 ° reduction per cycle and 72 ° for 1 min, followed by 36 cycles of 94 ° for 30 s, 55 ° for 30 s and 72 ° for 1 min, finishing with a 10 min final extension at 72 °. 20 μl of each PCR product was loaded into 2% agarose gels buffered with 1X TBE. Gels were run at 60 V for 300 minutes. Marker sizes were estimated using a 100-bp DNA ladder. All PCRs and gels were run twice to ensure reproducibility of the markers. Reproducible bands were scored as present or absent in each individual (Fig. 1). Parental (F_0) PCR products for each primer were run in each set of gels for band size comparison. Amplicons of the same size were assumed to be the same locus regardless of band intensity.

Statistical analysis and map construction

Deviation from expected Mendelian inheritance ratios for all markers within each family was determined using Chi-square analysis. Mapping calculations were performed with R/qtl (Broman *et al*., 2003), an add-on package to the R statistical software (Ihaka and Gentleman, 1996). The Kosambi mapping function was used for all maps. We focused on microsatellite markers for initial map construction. For each pair of markers, the recombination fraction between them, r, was estimated and a LOD score was calculated for the test of $r = 0.5$. Initial linkage groups were formed on the basis of the pairwise marker linkage information, where two markers were placed in the same linkage groups if LOD > 3. For each linkage group, a rough initial marker order was established by a greedy algorithm: markers were added, one at a time, in the position giving the maximum likelihood, until all markers had been placed. An improved marker order was identified by considering all possible shuffles of a sliding window of eight markers, and choosing the order with the minimal number of obligate crossovers. The final marker order was chosen by maximum multipoint likelihood, considering all possible shuffles of a sliding window of four markers. Multipoint calculations were performed assuming a genotyping error rate of 1%. Once the genetic map for the microsatellite markers was constructed, the placement of the dominant ISSR markers was considered. All possible F_1 genotypes, and all possible positions for each marker, were considered. The dominant markers were placed, one at a time, in the position giving the highest likelihood, provided that there was good evidence for linkage between the marker and a linkage group. Linkage maps were graphically depicted using MapChart v. 2.2 (Voorrips, 2002).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Observed and expected Mendelian inheritance ratios for microsatellites and ISSRs

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