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Identification of quantitative trait loci for larval morphological traits in interspecific hybrids of *Ochlerotatus triseriatus* and *Ochlerotatus hendersoni* (Diptera: Culicidae)

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Abbreviation: API – anal papillae index; CL – confidence limits; D/V – dorsal-to-ventral gill ratio; IM – interval mapping; LG – linkage group; LR – likelihood ratio; OR – odds ratio; QTL – quantitative trait loci; SNP – single nucleotide polymorphism

Abstract

Ochlerotatus triseriatus is the natural vector of La Crosse virus, a common cause of pediatric encephalitis in the United States; the closely related *Ochlerotatus hendersoni* transmits this virus at low frequency. Adults of these mosquito species are difficult to distinguish morphologically; however, the larval stages show species-specific differences in several characters. We identified genomic regions contributing to the differences between the larvae of these species through interspecific hybridizations. Quantitative trait loci (QTL) were identified by standard interval mapping techniques and by univariate marker association analyses. We examined 159 F_2 progeny from an *Oc. hendersoni* female by *Oc. triseriatus* male interspecific cross for variation in the number of saddle and siphon hair branches, attachment of the acus, and morphology of the anal papillae. At least one putative QTL was identified for each of the phenotypes examined. QTL most commonly mapped to linkage group (LG) III, although QTL were identified on LGI and LGII for three phenotypes each. Several of these QTL, and particularly those on LGIII, also map to genome regions controlling adult female body size and ability to orally transmit La Crosse virus. Further studies are required to elucidate the relationships among these traits and the impact they may have had on the ecological specialization and speciation of these mosquitoes.

Introduction

Ochlerotatus triseriatus (Say), the Eastern treehole mosquito, and the closely related *Ochlerotatus hendersoni* Cockerell are distributed largely sympatrically throughout the eastern United States (Zavortink, 1972). Morphologically, adult mosquitoes of the two species are difficult to differentiate. When the specimens are missing a large proportion of their thoracic scales, they are virtually indistinguishable. Nonetheless, Zavortink (1972) described differences between the two. *Ochlerotatus hendersoni* are characterized by the lateral patches of white or light scales extending mesad of the more numerous mesoscutal setae, whereas in *Oc. triseriatus* these white patches do

not extend past the setae, and the mesoscutum appears much darker (Zavortink, 1972).

Ochlerotatus triseriatus is medically important as the natural vector of La Crosse virus (LACV; family *Bunyaviridae*, genus *Bunyavirus*, species *California encephalitis virus*), a primary cause of pediatric encephalitis in North America (Watts et al., 1972). Approximately 70 cases of La Crosse encephalitis are reported annually (Centers for Disease Control and Prevention, 2005); affected individuals often suffer long-lasting neurological sequelae (McJunkin et al., 2001). Both *Oc. triseriatus* and *Oc. hendersoni* readily support replication, dissemination, and transovarial transmission (vertical transmission of virus to offspring) of LACV at similar rates and to similar viral titers (Paulson & Grimstad, 1989). However, *Oc. hendersoni* transmits the virus only at low rates (Grimstad, Paulson & Craig, 1985; Paulson, Grimstad & Craig, 1989). Recently, genomic regions influencing the ability of *Oc. triseriatus* to transmit LACV transovarially (Graham et al., 2003) and of interspecific hybrids to orally transmit LACV (Anderson et al., 2005) were identified.

Despite the extensive sympatry between these species, interspecific hybridization has been documented only rarely in field studies. Truman and Craig (1968) found only a single larval individual out of 205 exhibiting characteristics of both species. In addition, only two of 34 field-collected populations appeared to have hybrids among the larvae (Grimstad, Garry & DeFoliart, 1974). Interspecific hybrids generated from laboratory crosses reflected morphologies characteristic of both species (Truman & Craig, 1968; Grimstad, Garry & DeFoliart, 1974). The lack of hybridization may be due in part to vertical stratification; *Oc. hendersoni* prefers to feed and oviposit in the forest canopy, whereas *Oc. triseriatus* resides nearer the forest floor (Copeland & Craig, 1990).

The preferred larval habitats of *Oc. hendersoni* and *Oc. triseriatus* differ in type, location, and potentially chemical makeup. Copeland and Craig (1990) (see also Kitching, 1971) described natural larval habitat preferences in northern Indiana; they distinguished habitats among pans and rotholes, and included elevation in their analyses. Pans are defined as shallow natural depressions associated with exposed tree roots and buttresses. Rotholes are decaying cavities in tree trunks that can result following the death of individual branches. Larvae of *Oc. triseriatus* could be isolated from all larval habitat types, although very few individuals were found in deep rotholes (Copeland & Craig, 1990). Most *Oc. triseriatus* were found in pans irrespective of elevation. However, *Oc. hendersoni* was almost never found in pans; 97% of individuals collected came from elevated rotholes (Copeland & Craig, 1990). In laboratory experiments testing for oviposition preference in relation to water types, *Oc. hendersoni* preferentially oviposited in water from deep rotholes over pans, but *Oc. triseriatus* did not exhibit an oviposition preference (Copeland & Craig, 1992a).

In contrast to the relative lack of morphological differences between adults, larvae of these species exhibit a number of diagnostic traits. These may relate to the evolution of reproductive isolation mechanisms that facilitated *Oc. triseriatus* and *Oc. hendersoni* speciation. That is, the vertical stratification in habitat preferences likely included larval adaptation to the ground level versus canopy level oviposition sites. In order to further characterize the genetic differences between *Oc. triseriatus* and *Oc. hendersoni*, we elucidated the genetic bases for four of these distinguishing traits. To that end, interspecific crosses were produced, and the F_1 intercross larvae were examined for segregation of genes influencing the acus, siphon and saddle hairs, and anal papillae morphology. We identified quantitative trait loci (QTL) for each trait using interval mapping (IM) techniques as well as univariate marker association.

Materials and methods

Mosquito rearing and mating

The *Oc. triseriatus* strain used in these experiments was originally derived from eggs collected in 1969 in northern Indiana; the generation used is unknown, but is likely over F_{100}. *Ochlerotatus hendersoni* eggs were collected in Peoria, Illinois, by Jack Swanson of the Illinois Department of Public Health. The colony was maintained by forced copulation (McDaniel & Horsfall, 1957), and the F_3 generation was used here. All colonies were maintained in an insectary at 21°C and ~80% relative humidity. Larvae were reared in enamel pans and fed a 60 mg/ml bovine liver powder suspension (ICN Biochemicals, Aurora, OH)

ad libidum (Munstermann & Wasmuth, 1985). Pupae were transferred to small paper cups to emerge. Adults were held in either 60 cm ×60 cm ×60 cm (*Oc. triseriatus*) or 18.9-l cages (*Oc. hendersoni*); honey-soaked cotton was given as a sugar source. Mice anesthetized with 0.25–0.3 ml rodent cocktail (nine parts Ketamine (100 mg/ml), nine parts xylazine (20 mg/ml), three parts Acepromazine (10 mg/ ml) and 79 parts saline for a total volume of 100 ml) were offered as a blood source. All procedures involving vertebrate animals were approved by the Institutional Animal Care and Use Committee of the University of Notre Dame. Oviposition substrates were either white paper towels placed in plastic cups (*Oc. triseriatus*) or balsa wood strips in black-painted beverage cans (*Oc. hendersoni*).

These species do not readily mate in small cages; therefore, individual male *Oc. triseriatus* were force-mated to female *Oc. hendersoni* 1–2 days after being offered a bloodmeal (McDaniel & Horsfall, 1957). Females were anesthetized in a nitrogen atmosphere; males were decapitated and the last pair of legs was removed. The genitalia were brought into proximity, and clasping generally occurred immediately. Following mating, females were held at 21°C and ~80% humidity. Larvae were reared on a bovine liver powder suspension at 21°C and 80% relative humidity as described above. Sexes were separated at the pupal stage. F_1 progeny were intercrossed, again by force-mating, to produce an F_2 segregating population.

Larval characteristics

The morphological traits characterized in this study include the siphon acus, siphon hair (seta 1-S), lateral saddle hair (seta 1-X), and anal papillae morphology (Truman & Craig, 1968; Grimstad, Garry & DeFoliart, 1974). The acus is a sclerotized protrusion at the proximal end of the siphon. In *Oc. triseriatus*, the acus is attached to the siphon, or lightly detached, whereas in *Oc. hendersoni* it is broadly detached. The siphon hair has two branches in *Oc. triseriatus*, but three in *Oc. hendersoni*. The hair on the lateral aspect of the saddle has only two to three branches in *Oc. hendersoni*, but four or more in *Oc. triseriatus*. Lastly, the anal papillae (or anal gills) of *Oc. triseriatus* are rather short (approximately 1.5- to 2-fold longer than the saddle), taper to slightly pointed ends (i.e. 'lanceolate'; Hopkins, 1952), and are unequal in length (dorsal gills 1.5–2 times as long as the ventral). The anal gills of *Oc. hendersoni*, in contrast, are long (2.5- to 3-fold longer than the saddle), round on the ends, and equal (or slightly subequal) in length (Grimstad, Garry & DeFoliart, 1974; Taylor, 1982).

Individuals of the F_2 generation were allowed to pupate, and the shed 4th instar larval skins were transferred to individually labeled microcentrifuge tubes containing 95% ethanol until they were scored. Larval skins were examined under a stereo microscope in a drop of distilled water for four traits following the methods of Taylor (1982). The acus was scored as either detached or attached. For the siphon and saddle hairs, the number of branches on either side was scored if present, and the larger number of branches was recorded. We assumed that each side would have an equal number of hairs, and that any differences were due to loss of some hairs. The length of the anal papillae was measured from the base of segment X to the tip of the longest gill using an ocular micrometer; both the dorsal and ventral gills were measured and recorded separately. In addition, the morphology of the gill tips was noted – either rounded or pointed. Finally, the length of the saddle was used for comparison with the gills. Gill lengths were transformed in two ways. First, the length of the dorsal gill divided by the saddle length yielded the anal papillae index (API) described by Taylor (1982). Second, the ratio of the dorsal gill to the ventral gill was calculated to account for the difference in relative length between the two species.

DNA extraction, genetic mapping, and data analysis

Genomic DNA was extracted from individual mosquitoes following our standard protocols (Severson, 1997). Genetic markers consisted of species-specific single nucleotide polymorphisms (SNPs) identified in direct-sequenced PCR amplicons of comparative anchor tagged sequences (Chambers, Lovin & Severson, 2003), *Aedes aegypti*-specific primers, *Oc. triseriatus* Genbank sequences, or *Oc. triseriatus* cDNA sequences (Graham et al., 2003). SNPs were visualized either by restriction digestion or polyacrylamide gel electrophoresis (Fulton et al., 2001; Meece, 2002) following standard protocols.

The 159 F_2 progeny from a single cross were genotyped at 23 loci. Linkage associations for 24 loci and the sex locus mapped in 502 individual F_2 progeny, from this experiment and other works in preparation, are shown in Figure 1; each of these markers was scored in the present study, with the exception of CAT158 on linkage group (LG) III. In order to ameliorate potential confounding effects of the large map distance (52.5 cM) separating ATE001 and LF334 on LGIII, data for this linkage group were evaluated as two independent linkage groups (LGIIIa and LGIIIb; see Figure 1).

QTL were identified for binary or categorical phenotypes using the QTL-by-SAS program (Xu & Xu, 2003) using a step size of 1 cM; significance thresholds for declaring a QTL were estimated using the method of Piepho (2001).

For continuous traits, QTL were identified using QTL Cartographer (Basten, Weir & Zeng, 2001). Markers with significant partial regression coefficients were identified with the SRmapqtl function by forward–backward stepwise regression. Quantitative trait loci affecting each trait were identified by composite interval mapping

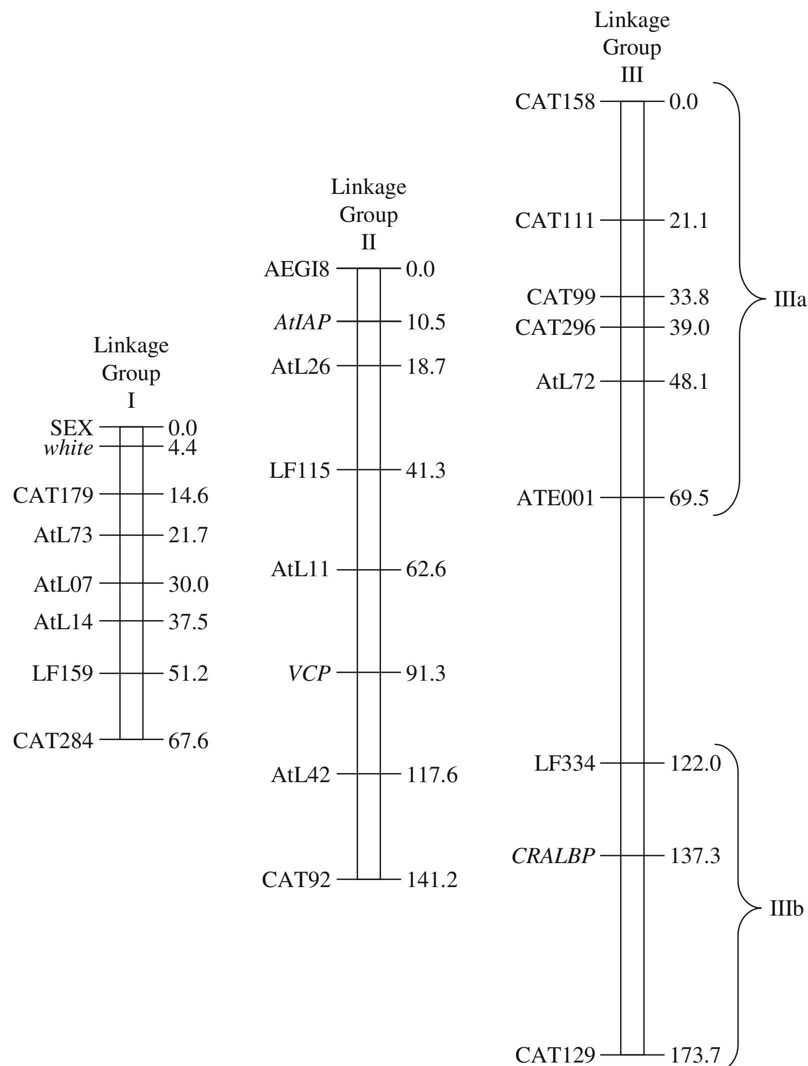

Figure 1. Linkage map for 502 progeny of four *Ochlerotatus hendersoni* female by *Ochlerotatus triseriatus* male crosses. Genetic markers are given to the left of the chromosome; Kosambi (1944) centiMorgan distances are to the right. Braces indicate the two linkage groups (designated IIIa and IIIb) into which LGIII was divided for interval mapping analyses. All markers, with the exception of CAT158, were used to identify QTL regions for the larval morphological traits examined.

(CIM; Zeng 1993, 1994) using the Zmapqtl function with model 6. An experiment-wise likelihood ratio (LR) threshold for declaring a QTL was determined by permutation test (Churchill & Doerge, 1994). The data were permuted 1000 times, and a critical LR value was determined for the $\alpha = 0.05$ significance level.

Additionally, we performed univariate Fisher's exact test (for binary/categorical traits) or linear regression (for continuous traits) to identify markers associated with each phenotype. These procedures, while inadequate for accurately defining QTL positions, are rigorous for identifying significant marker associations (Doerge, 2002). We included these analyses as (1) interval mapping has low power across large intervals and (2) the experimentwise thresholds for interval mapping are conservative and likely to exclude valid QTL (Churchill & Doerge, 1994). All statistical analyses were performed using the SAS computer package (SAS Institute, Cary, NC). The effect of replacing *Oc. hendersoni* alleles with *Oc. triseriatus* alleles was manually calculated from the beta coefficients given by proc GENMOD in SAS. Markers at $p < 0.05$ were considered to have a significant association with the phenotype, while markers with $p < 0.10$ were considered as suggestive of an association. Adjustments for multiple tests (e.g., Bonferroni corrections) were not performed for two reasons. First, linkage data, and thus QTL analyses, are likely not appropriate for these adjustments as markers on individual chromosomes are not independent, and multiple loci affecting a phenotype may not be independent even across chromosomes. Second, because of the relatively small numbers of unique marker tests involved in our study ($n = 23$), at the 5% significance level we expect only a single false positive per trait examined.

Results

An interspecific cross between a single *Oc. hendersoni* female and an *Oc. triseriatus* male yielded 159 F_2 progeny, which were scored for the morphological characters previously described. For each of the traits, a number of the larval skins could not be scored due to damage (loss of hairs, breakage of the gills, etc.). These are depicted in the distributions as 'missing'. We used multiple statistical procedures to identify QTL influencing each trait. For four traits (saddle hair branches, siphon hair branches, acus attachment and anal papillae shape) we performed IM for a binary trait, while for two traits (anal papillae index and dorsal/ventral papillae ratio) we performed composite interval mapping. However, the stringency of the experimentwise thresholds, while reducing the likelihood of declaring false positive QTL (type I error), also increases the potential for rejecting true positive QTL (type II error; Churchill & Doerge, 1994). Therefore, we also analyzed marker loci individually for an association with each trait by Fisher's exact test (Table 1). We considered markers with significant associations as identifying tentative QTL.

Progeny had an average of 4.1 saddle hair branches, with 97 (80.2%) of 121 scored having four or more branches (Figure 2a). All markers between CAT111 and ATE001 on LGIII exceeded the likelihood ratio threshold estimated for these data, indicating the presence of at least one QTL in this genome region (Figure 3). Each of these markers also had a significant association with the number of saddle hairs by Fisher's exact test. CAT129 on LGIII was also significantly associated ($p < 0.05$), suggesting the presence of a second QTL near the opposite end of LGIII (Table 1). Replacement of *Oc. hendersoni* alleles with *Oc. triseriatus* alleles resulted in a larger number of hairs present on the saddle (Table 1).

Of 123 siphon hairs examined, 97 (78.9%) showed two branches (Figure 2b). A single individual developed four branches and was excluded from the analyses. Although no likelihood ratios exceeded the estimated threshold value for declaring a QTL (Figure 3), AtL42 on LGII was significantly associated with siphon hair number by Fisher's exact test, while AtL72 on LGIII was suggestive ($p < 0.1$). Individuals homozygous for *Oc. triseriatus* alleles at the AtL42 locus were approximately 10 times less likely to have three branches than were *Oc. hendersoni* homozygotes (Odds ratio [OR] = 0.096; 95% Confidence limits [CL] = 0.015–0.621). Heterozygotes were four times less likely to have three branches (OR = 0.244; 95% CL = 0.064–0.931). Similarly, individuals homozygous for *Oc. triseriatus* alleles at the AtL72 locus were ~2.5 times less likely to have three branches than were *Oc. hendersoni* homozygotes (OR = 0.389; 95% CL = 0.092–1.650).

Table 1. Effect of substituting *Ochlerotatus triseriatus* alleles for *Ochlerotatus hendersoni* alleles at loci with a significant association with four morphological traits

Marker	LG[a]	Saddle hair branches			Siphon hair branches			Acus attachment			Anal Papillae Shape			Anal Papillae Index			
		p[b]	Odds ratio[c]		*p*	Odds ratio[c]		*p*	Odds ratio[d]		*p*	Odds ratio[e]		*p*	β Cofficient[f]	*p*	β Cofficient[f]
			Oc. triseriatus homozygous	Heterozygous		*Oc. triseriatus* homozygous	Heterozygous		*Oc. triseriatus* homozygous	Heterozygous		*Oc. triseriatus* homozygous	Heterozygous		*Oc. triseriatus* homozygous		Heterozygous
White	I							0.0447	14.4 (1.23–168.50)	1.45 (0.48–4.41)							
AtL07	I													0.0214	−0.37 (−0.68–−0.05)	0.0494	−0.18 (−0.36– −0.001)
AtL14	I													0.0004	−0.49 (−0.76– −0.22)	0.1025	–
AEGI8	II										0.0773	0.24 (0.07–0.84)	0.44 (0.14–1.33)				
AtL42	II				0.0197	0.1 (0.02–0.62)	0.24 (0.06–0.93)				0.0242	5.88 (1.30–26.51)	1.7 (0.49–5.85)				
CAT92	II										0.0545	0.1 (0.02–0.72)	0.49 (0.15–1.64)				
CAT111	IIIa	0.0023	4.09 (1.09–15.36)	5.6 (2.34–13.40)				0.0637	5 (1.24–20.12)	1.59 (0.58–4.37)							
CAT99	IIIa	0.0132	10.56 (2.55–43.70)	8 (2.56–25.01)				0.0148	5 (1.07–23.46)	1 (0.25–4.00)	0.0015	11.92 (2.18–65.15)	8.15 (2.12–31.30)	0.0063	−0.4 (−0.69– −0.11)	0.421	–
CAT296	IIIa	0.0171	5.52 (1.39–21.89)	6.21 (2.11–18.27)							0.0104	9.6 (1.86–49.48)	4.03 (1.28–12.64)	0.0396	−0.3 (−0.59– −0.01)	0.5407	–
AtL72	IIIa	0.0002	11.960 (3.61–39.59)	12.6 (3.70–42.85)	0.0627	0.39 (0.09–1.65)	1.45 (0.40–5.31)				0.002	6.69 (1.90–23.62)	8.12 (2.20–29.96)				
ATE001	IIIa	0.0102	7.52 (2.40–23.56)	6.25 (1.34–29.24)							0.0022	5.01 (1.46–17.20)	27.5 (2.62–289.13)				
LF334	IIIb							0.0096	ND	ND	0.0356	0.24 (0.04–1.45)	0.77 (0.15–4.13)				
CAT129	IIIb	0.0113	5.40 (1.41–20.59)	3.48 (1.48–8.18)				0.0202	7.92 (1.74–36.00)	1.89 (0.62–5.74)							

[a]LG = Linkage Group. The letters a and b indicate the separate linkage groups used for interval mapping analyses of LGIII (see Figure 1).
[b]*p*-Value as determined by Fisher's exact test for all traits.
[c]Fold more likely to have more saddle or siphon hair branches if indicated genotype versus homozygous for *Oc. hendersoni* alleles. Odds ratios and confidence limits (in parentheses) were manually calculated from β-coefficients obtained from proc GENMOD.
[d]Fold more likely to have an attached acus if indicated genotype versus homozygous for *Oc. hendersoni* alleles. ND = Not done; odds ratios could not be calculated due to cells with no values in the frequency table.
[e]Fold more likely to have rounded anal papillae if indicated genotype versus homozygous for *Oc. hendersoni* alleles.
[f]Change in API if indicated genotype versus homozygous for *Oc. hendersoni* alleles. Changes are not shown for non-significant genotypes.

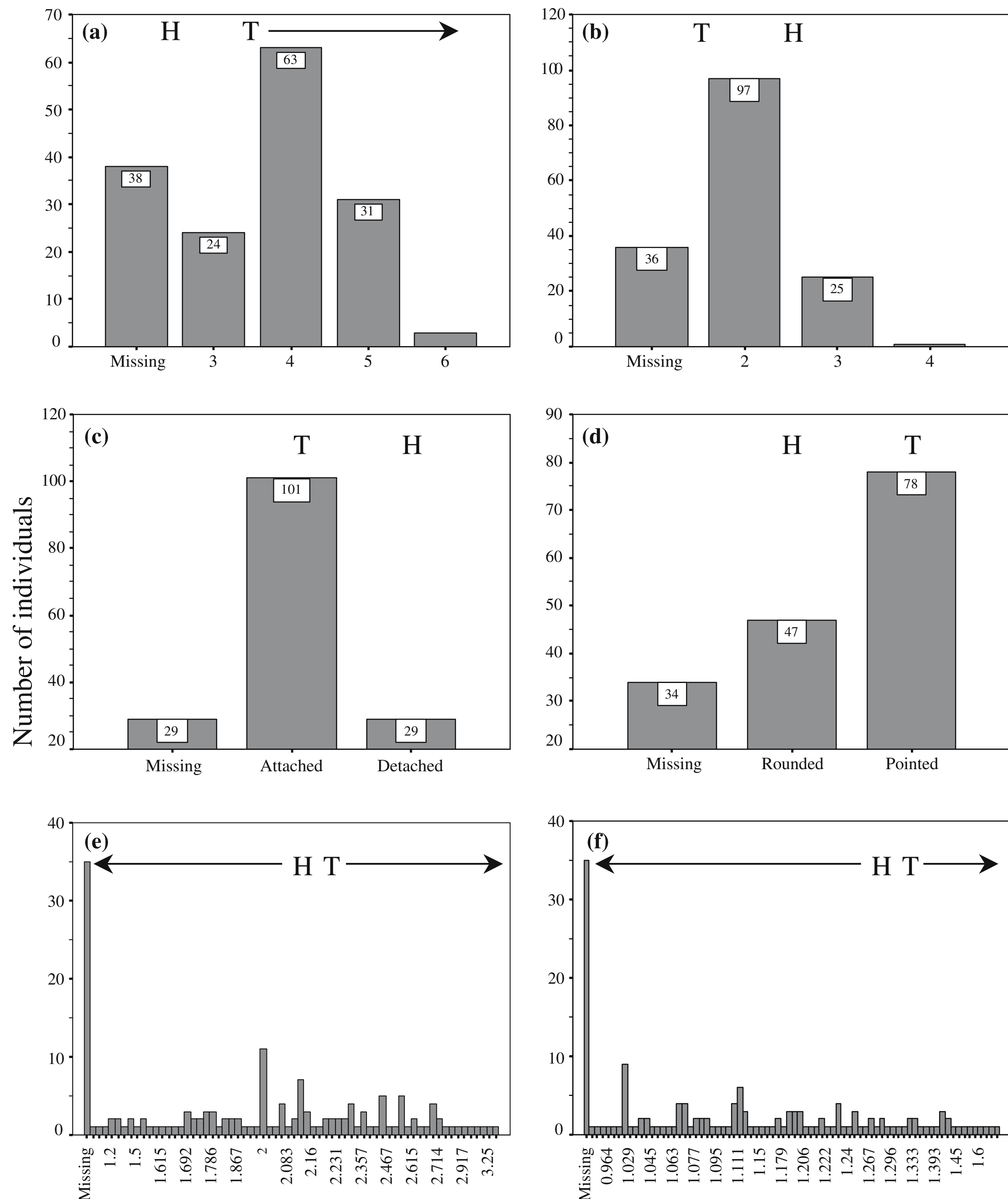

Figure 2. Distributions of the six morphological characters examined among 179 F_2 larvae from interspecific crosses of *Ochlerotatus hendersoni* females and *Ochlerotatus triseriatus* males. For each phenotype, 'missing' refers to individuals whose shed larval skin could not be examined due to physical damage. (a) Saddle hair branch number, (b) siphon hair branch number, (c) acus attachment, (d) shape of the anal papillae, (e) anal papillae index, and (f) dorsal/ventral papillae ratio. In (e) and (f), values correspond to the midpoint of every fourth interval. Species-specific phenotypes for *Oc. hendersoni* (H) and *Oc. triseriatus* (T) are shown above the bar graphs.

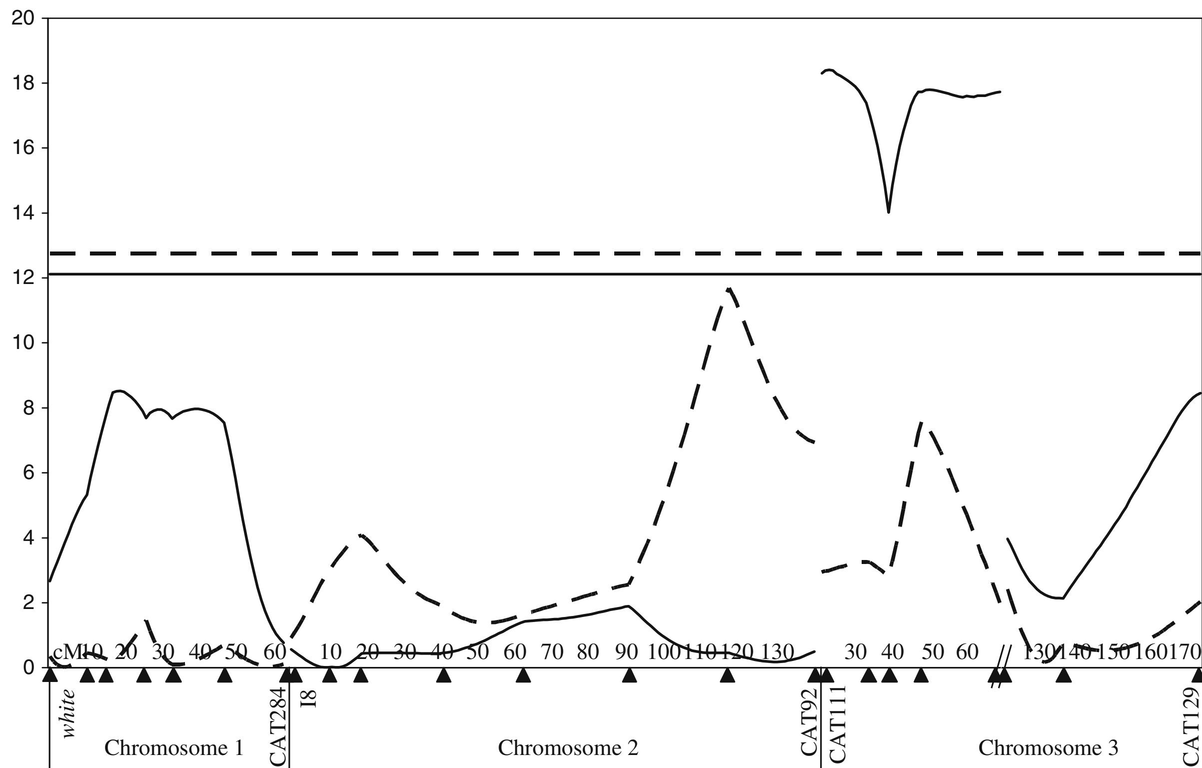

Figure 3. Likelihood ratio profiles for the number of saddle hair branches (solid lines) and siphon hair branches (dashed lines). The horizontal lines represent the estimated LR threshold values (saddle, 12.11; siphon, 12.75). Triangles correspond to positions of genetic markers used in this study (see Figure 1); two marker names are given for orientation of each linkage group. The diagonal double line indicates the location of the large interval where linkage group III was broken into two linkage groups for QTL analysis.

Heterozygotes were slightly more likely to have two branches than were homozygotes (OR = 1.448; 95% CL = 0.395–5.309).

Most individuals (101/130; 77.7%) had an attached acus (Figure 2c). The estimated threshold value was not exceeded for any interval by binary IM (Figure 4). However, Fisher's exact test identified three tentative QTL regions (Table 1). On LGI, one marker (*white*) showed a significant association with acus attachment. On LGIII, two genome regions were identified, as defined by a significant association with CAT99 and suggestive association with CAT111 at one chromosome end, and significant associations with two markers (LF334 and CAT129) at the opposite chromosome end. In all cases, with the exception of LF334 for which ORs could not be calculated due to cells with no values, addition of *Oc. triseriatus* alleles resulted in being more likely to have an attached acus (ORs range from 5.00 to 14.40) (Table 1).

Figure 2d shows the distribution of the shape of the anal papillae for this population. The interval AtL72-ATE001 on LGIII exceeded the estimated threshold for declaring a QTL by binary IM (Figure 4). Fisher's exact test identified two additional genome regions containing tentative QTL: (1) on LGII, one marker (AtL42) showed a significant association with anal papillae shape, while two markers (AEGI8 and CAT92) were suggestive of an association; and (2) on LGIII, LF334 showed a significant association (Table 1). At three loci (AEGI8, CAT92 and LF334), *Oc. hendersoni* homozygotes were four to ten times more likely to have pointed papillae than *Oc. triseriatus* homozygotes (Table 1).

For the API (Figure 2e), none of the LR estimates by composite interval mapping exceeded the 95% experimentwise threshold value (Figure 5). However, single marker analysis by linear regression identified two markers (AtL07 and AtL14) on

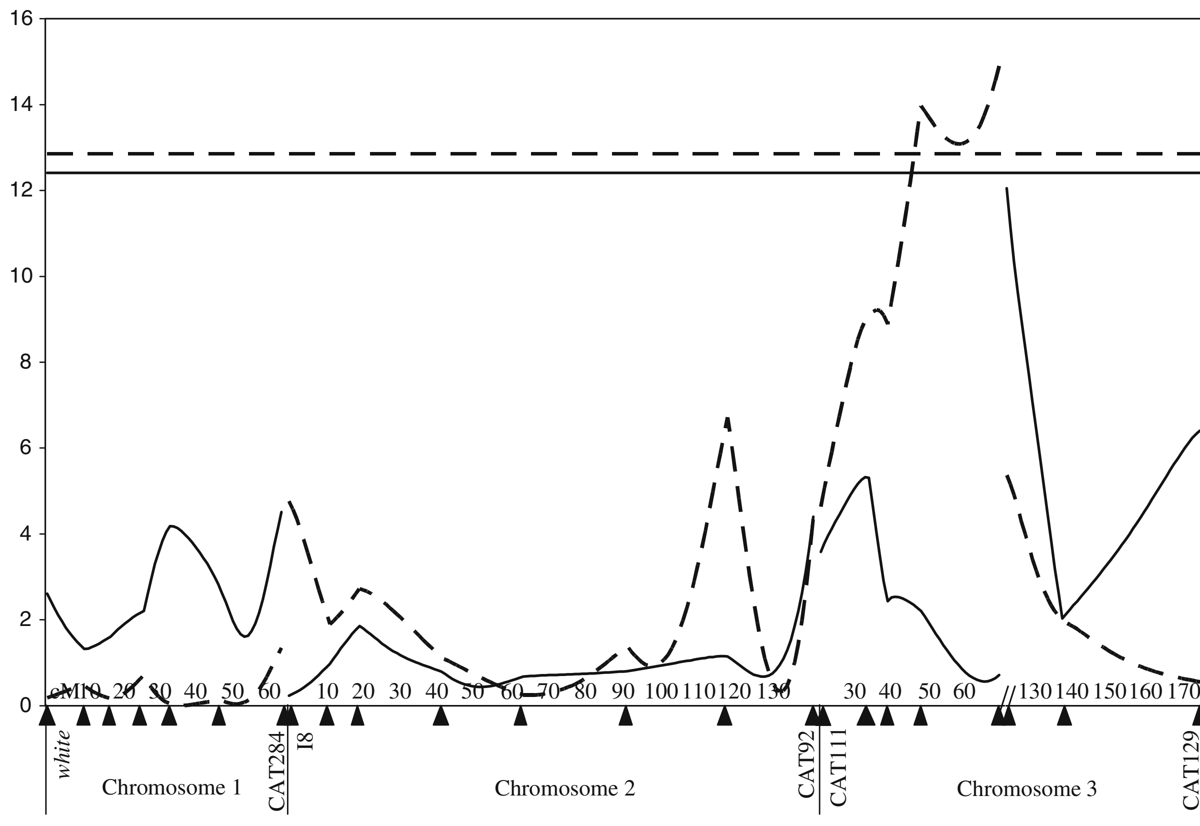

Figure 4. Likelihood ratio profiles for the acus attachment (solid lines) and shape of the anal papillae (dashed lines). The horizontal lines represent the estimated LR threshold values (acus, 12.41; papillae, 12.85). Triangles correspond to positions of genetic markers used in this study (see Figure 1); two marker names are given for orientation of each linkage group. The diagonal double line indicates the location of the large interval where linkage group III was broken into two linkage groups for QTL analysis.

LGI and two markers (CAT99 and CAT296) on LGIII that were significant for an association with the API (Table 1).

Finally, none of the LR estimates for the D/V ratio by composite interval mapping exceeded the 95% experimentwise threshold value (data not shown). The observed D/V ratios are shown in Figure 2f. Only a single marker, AtL42 on LGII, showed a significant association with the D/V ratio by linear regression.

Discussion

The evolutionary relationship between *Oc. triseriatus* and *Oc. hendersoni* has not been investigated in any depth. A series of crossing experiments among *Oc. triseriatus*, *Oc. hendersoni*, *Ochlerotatus brelandi*, and *Ochlerotatus zoosophus* found reproductive incompatibilities in at least one direction among all possible crosses (Taylor, 1987, 1988; Taylor and Craig, 1985). The former three species together form the Triseriatus group, while *Oc. zoosophus* forms a closely related, monotypic group (Zavortink, 1972). Zavortink (1972) considered *Oc. zoosophus* to be more primitive than the Triseriatus group. Within that group, *Oc. brelandi* and *Oc. hendersoni* were most closely related based on morphological characteristics. Further, isozyme analyses also appeared to recover these relationships (Munstermann, Taylor & Matthews, 1982). Matthews and Munstermann (1983) observed a genetic distance of $D=0.48$ between *Oc. hendersoni* and *Oc. triseriatus*. In contrast, a genetic distance of $D=0.15$ was obtained for the sibling anopheline species *Anopheles gam-*

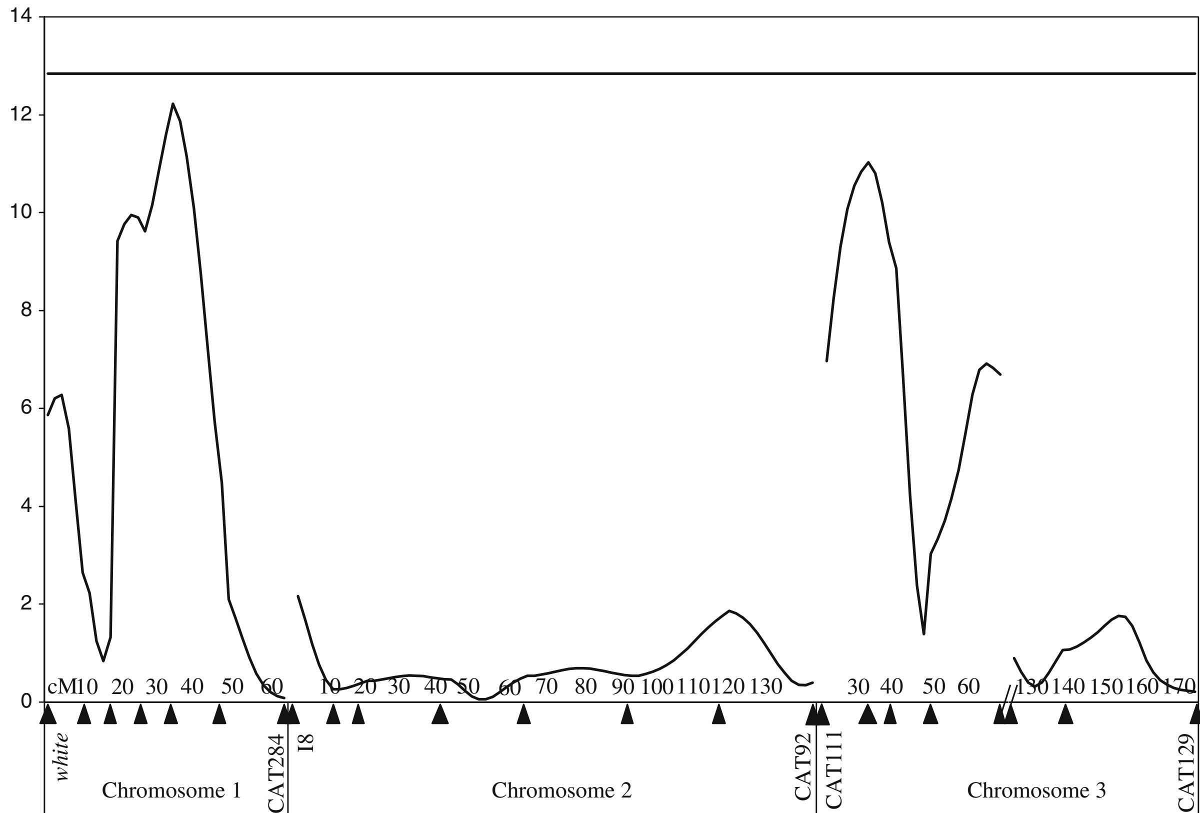

Figure 5. Likelihood ratio profiles for the anal papillae index. The horizontal line represents the estimated LR threshold value (12.84). Triangles correspond to positions of genetic markers used in this study (see Figure 1); two marker names are given for orientation of each linkage group. The diagonal double line indicates the location of the large interval where linkage group III was broken into two linkage groups for QTL analysis.

biae and *Anopheles arabiensis* (Bullini & Coluzzi, 1978). Further, meiotic abnormalities were evident in crosses between *Oc. triseriatus* and either *Oc. hendersoni* or *Oc. brelandi* (Taylor, 1987), though none was found in *Oc. hendersoni*-*Oc. brelandi* hybrids (Taylor & Craig, 1985).

Our results represent the first genetic analysis of larval characters that distinguish closely related mosquito species. For each of the six phenotypes we examined, at least one tentative QTL was identified by either interval mapping or univariate marker association. In nearly all instances, replacement of *Oc. hendersoni* alleles with *Oc. triseriatus* alleles results in the larva more closely resembling the *Oc. triseriatus* phenotype. The only exceptions are three loci (AEGI8, CAT92, and LF334) with a significant association with the shape of the anal papillae. In addition, we recently identified tentative QTL controlling both adult female body size and their ability to transmit LACV in hybrids of these species (Anderson et al., 2005). Further, although the individual QTL represent relatively broad genome regions, some genome regions, particularly both ends of LGIII and the region around the AtL42 locus on LGII, contain genes that influence several of the larval characters examined in the present study, as well as the adult female traits examined in our previous study (Anderson et al., 2005). Thus, either a small number of common genes or a larger number of linked genes controls the morphological traits we examined, although the latter seems more likely. However, using the appropriate regression models for all pairwise comparisons, we determined that only two traits, acus and API, were significantly associated by logistic regression

(β coefficient = −1.36; $p = 0.0084$). Phenotypic correlations between apparently unrelated traits are not unprecedented as, for example, in host races of pea aphids, where QTL for early fecundity (indicating resource use) and for host acceptance (a proxy for mate choice) localized to the same, or tightly linked, regions of the genome (Hawthorne & Via, 2001). In addition, although the gene *Ultrabithorax* is responsible for hindwing development in the third thoracic segment of insects (Grenier & Carroll, 2000), in *Drosophila*, this same gene also plays a role in a finer-scale trait, patterning of trichome bristles on the posterior femur of the second leg (Stern, 1998). However, most evidence for pleiotrophy or tightly linked genes involves functionally integrated morphological traits such as those investigated in this study (Leamy, Routman & Chevrud, 1999; Cai & Morishima, 2002; Conner, 2002; Westerbergh & Doebley, 2002; Cheverud et al., 2004).

Quantitative trait loci for another vector competence trait, transovarial transmission, have also been identified in *Oc. triseriatus* (Graham et al., 2003). These include one QTL on chromosome II and two QTL on chromosome III. Comparisons between QTL for transovarial transmission and QTL identified in our studies are, however, not possible as only two marker loci are common to both studies. This includes the sex determination locus on our LGI (chromosome I in Graham et al., 2003) and AtL11 on our LGII (chromosome III in Graham et al., 2003).

Two hypotheses for the differential oviposition preferences between *Oc. triseriatus* and *Oc. hendersoni* are possible, at least one of which may relate to the evolution of species-specific larval characters. First, *Oc. hendersoni* is at a competitive disadvantage to *Oc. triseriatus* for larval development when inhabiting the same breeding sites and have evolved to use a new ecological niche, thereby avoiding this competition. Indeed, competition experiments have shown that the presence of *Oc. triseriatus* larvae has a negative impact on the development of *Oc. hendersoni* (Copeland & Craig, 1992b). This is due both to *Oc. hendersoni* larvae being poor competitors with *Oc. triseriatus* larvae and to enhanced *Oc. hendersoni* susceptibility to a natural parasite of *Oc. triseriatus* (*Ascogregarina barretti* [Vavra]) (Copeland & Craig, 1992b). Either species may avoid the other through kairomonal cues present in the larval habitat. Oviposition preferences in other species have been shown to vary because of chemical cues left by predators (Angelon & Petranka, 2002; Blaustein et al., 2004) and likely, competitors.

Alternately, the difference in oviposition sites may reflect species-specific larval tolerances of dissolved tannins and other chemicals. Increased tannin concentrations have been shown to be toxic for some species, while others are highly resistant to tannin-induced mortality (Sota, 1993; Rey et al., 2000). Oviposition site selection by *Oc. triseriatus* is complex, involving site color, water color, opening orientation and size, and water chemistry (Wilton, 1968). *Ochlerotatus hendersoni* preferentially oviposits in rotholes (Copeland & Craig, 1990) or containers containing rothole water (Copeland & Craig, 1992a), which is generally characterized as relatively high in tannin content, especially when compared to pans.

While the evolutionary importance of some traits, e.g. acus attachment and the number of saddle and siphon hairs, is unclear, the morphology of the anal papillae has been shown to be important, as these organs are involved in the uptake of both anions and cations from the larval habitat (Koch, 1938; Wigglesworth, 1938; Ramsey, 1953). The size of the papillae is variable, and depends in part on the osmotic concentration of the medium in which they develop. For example, in *Aedes aegypti* (Linneaus) and *Culex pipiens* Linneaus, papillae length decreases with increasing sodium chloride concentration (Wigglesworth, 1938). Hopkins (1952) described the anal papillae of container-breeding species as being sausage-shaped and suggested that this shape was due to the low chloride ion concentrations found in such habitats, whereas those of pool-breeding species were lanceolate.

In summary, we have initiated efforts to understand the quantitative genetics of interspecific differences for four morphological characters (six phenotypes) among larvae of *Oc. hendersoni* and *Oc. triseriatus* interspecific hybrids. One or two QTL regions were identified for each trait, either by standard interval mapping or by univariate marker association. Most phenotypes were controlled, at least in part, by loci on LGIII, although some QTL were also identified on LGI and LGII, dependent on the trait. The evolutionary and ecological importance of these phenotypic differences remains to be elucidated.

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References

Anderson, J.A., J.R. Schneider, P.R. Grimstad & D.W. Severson, 2005. Quantitative genetics of body size and vector competence for La Crosse virus in *Ochlerotatus hendersoni* and *Ochlerotatus triseriatus* interspecific hybrids. Genetics 169: 1529–1539.

Angelon, K.A. & J.W. Petranka, 2002. Chemicals of predatory mosquitofish (*Gambusia affinis*) influence selection of oviposition site by *Culex* mosquitoes. J. Chem. Ecol. 28: 797–806.

Basten, C.J., B.S. Weir & Z.B. Zeng, 2001. QTL Cartographer: A Reference Manual and Tutorial for QTL Mapping. Program in Statistical Genetics . Bioinformatics Research Center, Department of Statistics, North Carolina State University.

Blaustein, L., M. Kiflawi, A. Eitam, M. Mangel & J.E. Cohen, 2004. Oviposition habitat selection in response to risk of predation in temporary pools: mode of detection and consistency across experimental venue. Oecologia 138: 300–305.

Bullini, L. & M. Coluzzi, 1978. Applied and theoretical significance of electrophoretic studies in mosquitoes (Diptera: Culicidae). Parassitologia 20: 7–21.

Cai, H.W. & H. Morishima, 2002. QTL clusters reflect character associations in wild and cultivated rice. Theor. Appl. Genet. 104: 1217–1228.

Centers for Disease Control and Prevention, 2005. http://www.cdc.gov/ncidod/dvbid/arbor/lacfact.htm.

Chambers, E.W., D.D. Lovin & D.W. Severson, 2003. Utility of comparative anchor-tagged sequences as physical anchors for comparative genome analysis among the Culicidae. Am. J. Trop. Med. Hyg. 69: 98–104.

Cheveud, J.M., T.H. Ehrich, T.T. Vaughn, S.F. Koreishi, R.B. Linsey & L.S. Pletscher, 2004. Pleiotropic effects on mandibular morphology II: differential epistasis and genetic variation in morphological intergration. J. Exp. Zoolog. B Mol. Dev. Evol. 302: 424–435.

Churchill, G.A. & R.W. Doerge, 1994. Empirical threshold values for quantitative trait mapping. Genetics 138: 963–971.

Conner, J.K., 2002. Genetic mechanisms of floral trait correlations in a natural population. Nature 420: 407–410.

Copeland, R.S. & G.B. Craig Jr. 1990. Habitat segregation among treehole mosquitoes (Diptera: Culicidae) in the Great Lakes region of the United States. Ann. Entomol. Soc. Am. 83: 1063–1073.

Copeland, R.S. & G.B. Craig Jr. 1992a. Differential oviposition by *Aedes hendersoni* and *Aedes triseriatus* (Diptera: Culicidae) in response to chemical cues associated with trechole water. J. Med. Entomol. 29: 33–36.

Copeland, R.S. & G.B. Craig Jr. 1992b Interspecific competition, parasitism, and predation affect development of *Aedes hendersoni* and *A*. triseriatus (Diptera: Culicidae) in artificial treeholes. Ann. Entomol. Soc. Am. 85: 154–163.

Doerge, R.W., 2002. Mapping and analysis of quantitative trait loci in experimental populations. Nat. Rev. Genet. 3: 43–52.

Fulton, R.E., M.L. Salasek, N.M. DuTeau & W.C. Black IV, 2001. SSCP analysis of cDNA markers provides a dense linkage map of the *Aedes aegypti* genome. Genetics 158: 715–726.

Graham, D.H., J.L. Holmes, B.J. Beaty & W.C. Black IV, 2003. Linkage mapping of quantitative trait loci conditioning transovarial transmission of La Crosse virus in the eastern treehole mosquito, *Ochlerotatus triseriatus*. Insect Mol. Biol. 12(4): 307–318.

Grenier, J.K. & S.B. Carroll, 2000. Functional evolution of the Ultrabithorax protein. Proc. Nat. Acad. Sci. USA 97: 704–709.

Grimstad, P.R., C.E. Garry & G.R. DeFoliart, 1974. *Aedes hendersoni* and *Aedes triseriatus* (Diptera: Culicidae) in Wisconsin: Characterization of larvae, larval hybrids, and comparison of adult and hybrid mesoscutal patterns. Ann. Entomol. Soc. Am. 67: 795–804.

Grimstad, P.R., S.L. Paulson & G.B. Craig Jr. 1985. Vector competence of *Aedes hendersoni* (Diptera: Culicidae) for La Crosse virus and evidence of a salivary-gland escape barrier. J. Med. Entomol. 22: 447–453.

Hawthorne, D.J. & S. Via, 2001. Genetic linkage of ecological specialization and reproductive isolation in pea aphids. Nature 412: 904–907.

Hopkins, G.H.E, 1952. Mosquitoes of the Ethiopian Region. Part I. Larval Bionomics of Mosquitoes and Taxonomy of Culicine Larvae. Adlard and Son, London.

Kitching, R.L., 1971. An ecological study of water-filled tree holes and their position in the woodland ecosystem. J. Anim. Ecol. 40: 281–302.

Koch, H.J., 1938. The absorption of chloride ions by the anal papillae of diptera larvae. J. Exp. Biol. 15: 152–160.

Kosambi, D.D., 1944. The estimation of map distance from recombination values. Ann. Eugen. 12: 172–175.

Leamy, L.J., E.J. Routman & J.M. Chevrud, 1999. Quantitative trait loci for early- and late-developing skull characters in mice: a test of the genetic independence model of morphological integration. Am. Nat. 153: 210–214.

Matthews, T.C. & L.E. Munstermann, 1983. Genetic diversity and differentiation in northern populations of the treehole mosquito *Aedes hendersoni* (Diptera: Culicidae). Ann. Entomol. Soc. Am. 76: 1005–1010.

McDaniel, I.N. & W.R. Horsfall, 1957. Induced copulation of aedine mosquitoes. Science 125: 745.

McJunkin, J.E., E.C. de los Reyes, J.E. Irazuzta, M.J. Caceres, R.R. Khan, L.L. Minnich, K.D. Fu, G.D. Lovett, T. Tsai & A. Thompson, 2001. La Crosse encephalitis in children. N. Engl. J. Med. 344: 801–807.

Meece, J.K., 2002. Quantitative genetic analyses of Plasmodium susceptibility in Aedes aegypti and preliminary genetic investigations of Plasmodium susceptibility in Anopheles gambiae s.l. Ph.D. Dissertation, University of Notre Dame, 147 pp.

Munstermann, L.E. & L.M. Wasmuth, 1985. *Aedes triseriatus*, pp. 15–24 in Handbook of Insect Rearing in edited by P. Singh & R.F. Moore. Elsevier Science, Amsterdam.

Munstermann, L.E., D.B. Taylor & T.C. Matthews, 1982. Population genetics and speciation in the *Aedes triseriatus* group, pp. 433–453 in Recent Developments in the Genetics of Insect Disease Vectors in edited by W.W. Steiner. Stipes, Champaign, IL.

Paulson, S.L. & P.R. Grimstad, 1989. Replication and dissemination of La Crosse virus in the competent vector *Aedes triseriatus* and the incompetent vector *Aedes hendersoni* and evidence for transovarial transmission by *Aedes hendersoni* (Diptera: Culicidae). J. Med. Entomol. 26: 602–609.

Paulson, S.L., P.R. Grimstad & G.B. Craig Jr. 1989. Midgut and salivary gland barriers to La Crosse virus dissemination in mosquitoes of the *Aedes triseriatus* group. Med. Vet. Entomol. 3: 113–123.

Piepho, H.P., 2001. A quick method for computing approximate thresholds for quantitative trait loci detection. Genetics 157: 425–432.

Rey, D., J.-P. David, D. Martins, M.-P. Pautou, A. Long, G. Marigo & J.-C. Meyran, 2000. Role of vegetable tannins in habitat selection among mosquito communities from the Alpine hydrosystems. C. R. Acad. Sci. III 323: 391–398.

Ramsey, J.A., 1953. Exchanges of sodium and potassium in mosquito larvae. J. Exp. Biol. 30: 79–89.

Severson, D.W., 1997. RFLP analysis of insect genomes, pp. 309–320 in The Molecular Biology of Insect Disease Vectors; A Methods Manual in edited by J.M. Crampton, C.B. Beard & C. Louis. Chapman & Hall, London.

Sota, T., 1993. Performance of *Aedes albopictus* and *A. riversi* Larvae (Diptera: Culicidae) in waters that contain tannic acid and decaying leaves: is the treehole species better adapted to treehole water? Ann. Entomol. Soc. Am. 86: 450–457.

Stern, D.L., 1998. A role of Ultrabithorax in morphological differences between *Drosophila* species. Nature 396: 463–466.

Taylor D.B., 1982. Speciation in the Aedes triseriatus species complex. Ph.D. Dissertation, University of Notre Dame, 192 pp.

Taylor, D.B., 1987. Genetic compatibility of *Aedes (Protomacleaya) triseriatus* with *A. (P.) brelandi* and *A. (P.) hendersoni* (Diptera: Culicidae). Ann. Entomol. Soc. Am. 80: 109–117.

Taylor, D.B., 1988. Hybridization of *Aedes (Protomacleaya) zoosophus* with *Ae. (Pro.) triseriatus* group species: hybrid morphology. J. Am. Mosq. Control Assoc. 4: 23–28.

Taylor, D.B. & G.B. Craig Jr. 1985. Unidirectional reproductive incompatibility between *Aedes (Protomacleaya) brelandi* and *A.* (*P.*) hendersoni (Diptera: Culicidae). Ann. Entomol. Soc. Am. 78: 769–774.

Truman, J.W. & G.B. Craig Jr. 1968. Hybridization between *Aedes hendersoni* and *Aedes triseriatus* . Ann. Entomol. Soc. Am. 61: 1020–1025.

Watts, D.M., C.D. Morris, R.E. Wright, G.R. DeFoliart & R.P. Hanson, 1972. Transmission of La Crosse virus (California encephalitis group) by the mosquito *Aedes triseriatus*. J. Med. Entomol. 9: 125–127.

Westerbergh, A. & J. Doebley, 2002. Morphological traits defining species differences in wild relatives of maize are controlled by multiple quantitative trait loci. Evol. Int. J. Org. Evol. 56: 273–283.

Wigglesworth, V.B., 1938. The regulation of osmotic pressure and chloride concentration in the haemolymph of mosquito larvae. J. Exp. Biol. 15: 235–247.

Wilton, D.P., 1968. Oviposition site selection by the tree-hole mosquito, *Aedes triseriatus* (Say). J. Med. Entomol. 5: 189–194.

Xu C., S. Xu, 2003. A SAS/IML program for mapping QTL in line crosses. Paper 235: 1–6. SUGI 28 Proceedings, Seattle.

Zavortink, T.J., 1972. Mosquito studies (Diptera, Culicidae) XXVIII. The new world species formerly placed in *Aedes* (*Finlaya*). Contrib. Am. Entomol. Inst. 8: 1–206.

Zeng, Z.B., 1993. Theoretical basis for separation of multiple linked gene effects in mapping quantitative trait loci. Genetics 90: 10972–10976.

Zeng, Z.B., 1994. Precision mapping of quantitative trait loci. Genetics 136: 1457–1468.