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Development of a Comparative Genetic Linkage Map for *Armigeres subalbatus* Using *Aedes aegypti* RFLP Markers

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One of the causative agents of lymphatic filariasis is the nematode parasite *Brugia malayi* that requires a competent mosquito vector for its development and transmission. *Armigeres subalbatus* mosquitoes rapidly destroy invading *B. malayi* microfilariae via a defense response known as melanotic encapsulation. We have constructed a genetic linkage map for this mosquito species using RFLP markers from *Aedes aegypti*. This heterologous approach was possible because of the conserved nature of the coding sequences used as markers and provided an experimental framework to evaluate the hypothesis that linkage and gene order are conserved between these mosquito species. Of the 56 *Ae. aegypti* markers tested, 77% hybridize to genomic DNA digests of *Ar. subalbatus* under stringent conditions, with 53% of these demonstrating strain-specific polymorphisms. Twenty-six *Ae. aegypti* markers have been mapped using an F_2 -segregating *Ar. subalbatus* population derived from a cross of strains originating in Japan and Malaysia. Linear order of these marker loci is highly conserved between the two species. Only 1 of these markers, LF92, was not linked in the manner predicted by the *Ae. aegypti* map. In addition, the autosomal sex-determination locus that occurs in linkage group 1 in *Ae. aegypti* resides in group 3 in *Ar. subalbatus*. The *Ar. subalbatus* map provides a basic genetic context that can be utilized in further genetic studies to clarify the genetic basis of parasite resistance in this mosquito and is a necessary precursor to the identification of genome regions that carry genes that determine the encapsulation phenotype.

[The composite map and sequence database information for *Ae. aegypti* markers can be retrieved directly from the *Ae. aegypti* Genome Database through the World Wide Web: <http://klab.agsci.colostate.edu>.]

Mosquito-transmitted diseases continue to cause significant human morbidity and mortality throughout the tropics and subtropics (TDR News 1994). Disease agents, including filarial worms and the protozoan species that cause malaria require a compatible mosquito host to complete their life cycles. Studies of vector competence have, until recently, relied on physiological and biochemical approaches and have provided limited understanding of the molecular events determining the success of specific mosquito–parasite interactions. A purely genetic approach could prove useful in identifying genes that confer the resistant phenotype in mosquito strains unable to transmit parasites. Genetic linkage maps recently have been constructed for two important disease vectors, *Aedes aegypti* and *Anopheles gambiae*, based on restriction fragment

length polymorphism (RFLP) markers, microsatellite markers, and random amplified polymorphic DNA (RAPD) markers (Severson et al. 1993; Antolin et al. 1996; Zheng et al. 1996). An advantage of the cDNA-based RFLP markers is that many of the sequences are conserved among mosquito species and, consequently, can be used for construction of comparative linkage maps (Severson et al. 1995a). A comparative linkage map for *Aedes albopictus*, based on *Ae. aegypti* markers, showed that linkage and gene order are completely conserved at 18 loci spanning all three linkage groups (Severson et al. 1995a).

The mosquito *Armigeres subalbatus*, mounts a rapid defense response against invading *Brugia malayi* microfilariae. This form of invertebrate immune response is known as melanotic encapsulation and results in sequestration and killing of foreign invaders in a blackened, melanin-derived capsule that effectively limits the number of parasites developing to the infective stage (Kobayashi et al. 1986; Beern-

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tsen et al. 1989). This host-parasite system has emerged as an important model for characterization of the biochemical and molecular events comprising this form of invertebrate immunity (Zhao et al. 1995; Cho et al. 1997; Liu et al. 1997); however, virtually nothing is known of the genome organization of this mosquito host.

The linkage map presented herein provides the genetic context required for further examination of the genetic basis of melanotic encapsulation. Linkage maps based on common cDNA markers also allow for the testing of hypotheses concerning orthologous quantitative trait loci (QTL) associated with vector competence (Severson et al. 1995a). Use of linkage map-based technology has resulted in the identification of mosquito QTL that influence vector competence for nematode and *Plasmodium* parasites (Severson et al. 1994b, 1995b; Zheng et al. 1997).

RESULTS

Marker Evaluation

Fifty-six *Ae. aegypti* markers were screened on bulked, *Eco*RI-digested, DNA from the J and M *Ar. subalbatus* strains, and 43 hybridized as heterologous probes (Table 1). Strain-specific RFLPs were observed for the majority of these hybridizing probes, and 23 of these markers showed no common restriction fragments between the strains (Table 1). An autoradiograph of one of these informative probes, LF198, is shown in Figure 1.

Segregation of RFLPs

Twenty-six *Ae. aegypti* markers were probed to F₂ progeny and scored with respect to the parental RFLP as either homozygous for the J or M genotype or heterozygous. Figure 2 provides an example of an F₂ segregating population probed with the chromosome 2 marker, LF115. Significant deviations from

Figure 1. Autoradiograph of genomic blot probed with LF198 to screen for unique RFLPs between pooled DNA samples representing mosquito strains. DNA was digested with *Eco*RI. (Lane 1) Japan strain; (lane 2) Malaysian strain. Size markers as determined by using *Hind*III-digested λ phage DNA markers are given.

the expected 1:2:1 ratio were observed with 42% of the loci examined in at least one of the test crosses (Table 2). Table 3 shows the observed segregation relative to sex, for the eight linkage group 3 RFLP loci. When these data were examined without respect to sex, most of these markers fitted the expected 1:2:1 ratio (Table 2).

Linkage Analysis

A linkage map was constructed by hybridizing DNA from segregating *Ar. subalbatus* individuals with 26 prescreened *Ae. aegypti* clones (Fig. 3). Linkage and linear relationships of markers on group 2 represent the best fit of data from four different crosses as determined by the JoinMap computer program (Stam 1993). Comparative information for the arrangement of these markers with respect to *Ae. aegypti* is a result of compilation of all available data. One marker, LF92, was mapped to a linkage group not predicted by the linkage arrangement observed for *Aedes*. Also, the sex determination locus, which maps to chromosome 1 in *Ae. aegypti*, is located on group 3 in this *Armigeres* cross. Finally, the linear positions of LF264, LF180, and LF203 on chromosome 2 are inverted with respect to the other group 2 markers as compared with *Ae. aegypti*. The loci mapped in *Ar. subalbatus* cover 181.5 cM, whereas

Table 1. Utility of *Ae. aegypti* Probes for Comparative Mapping with *Ar. subalbatus*

Strains	No. tested	No. hybridized	Informative loci	Mixed allele loci	Informative loci (%)
<i>Ar. subalbatus</i> Japan $\times$ Malaysia	56	43	23	20	53

Figure 2 RFLP observed in 10 segregating *Ar. subalbatus* F₂ progeny following hybridization with the *Ae. aegypti* LF115 probe. RFLP alleles representing the parental Japan strain (J) and Malaysian strain (M) are indicated. (H) Heterozygote. This locus is on chromosome 2. Size markers as determined using *Hind*III-digested λ phage DNA markers are given.

the same markers in *Ae. aegypti* cover 181.3 (D.W. Severson, unpubl.), although the spatial distribution varies among linkage groups between these two species.

DISCUSSION

Ae. aegypti cDNA markers hybridize under stringent conditions and exhibit differences in restriction fragment lengths between the two strains of *Ar. subalbatus* used in this study. Of 56 (77%) *Ae. aegypti* cDNA probes, 43 hybridized to *Ar. subalbatus* DNA, confirming the high degree of gene conservation and usefulness of heterologous mapping between different species of culicine mosquitoes (Severson et al. 1994a, 1995a). The genetic diversity between the *Armigeres* strains for these markers (Table 1) is extensive and comparable to that reported for *Ae. aegypti* markers used for mapping with *Ae. albopictus*, suggesting that there is relatively low residual heterozygosity between these two strains.

Segregation data for most RFLP loci fitted the expected Mendelian ratio (Table 2); however, there were several significant deviations. Related studies that produced a comparative map for *Ae. albopictus* resulted in significant χ^2 values for 78% of the loci examined (Severson et al. 1995b), a result attributed to an abundance of the heterozygous genotype. Deviations likely reflect the lethal effects of loci on F₂ progeny homozygous for the parental genotypes (Matthews and Craig 1989; Munstermann 1994). For the crosses presented here, this effect is most pronounced for markers on one end of chromosome 2, distal to LF115 (Fig. 3; Table 2), which ex-

hibited a preponderance of heterozygotes and paternal (J) homozygotes in three of the four crosses used to study linkage on this group.

Sex determination in the Culicinae is inherited in a manner consistent with a single autosomal gene, with maleness being the dominant allele (Gilchrist and Haldane 1947). Males are heterozygous at the sex locus and females are homozygous for the recessive genotype, ensuring that homozygous males at the sex locus will not exist. When analyzed according to sex, it is obvious that the segregation pattern does not fit a 1:2:1 ratio (Table 3), particularly when approaching the sex locus. This result is consistent with a systematic segregation distorter on linkage group 3. The deviation should disappear when data are analyzed without respect to sex (Table 2), assuming an equal number of males and females are examined. The chromosome 3 marker deviations from 1:2:1 can be attributed to an inadvertent bias toward males in production of the mapping blots.

Genetic linkage and gene order is well conserved between these two species; however, several differences were observed (Fig. 3). Chromosomal re-patterning has been studied in mosquitoes, and it was proposed that variation in linkage and linear order among mosquito groups can often be accounted for by translocations and inversions. In this case, tightly linked genes are more likely to share a chromosomal history; consequently, these genes "travel" together (Matthews and Munstermann 1994). The three central markers in *Ar. subalbatus* (LF264, LF180, and LF203) that do not map in the same linear order predicted by *Ae. aegypti* are inverted with respect to this species. One marker, LF92, maps to chromosome 2 in *Armigeres* and group 3 in *Aedes*. Database analysis of LF92 indicates a homology with the ubiquitin gene (D.W. Severson, unpubl.). Hybridization of this probe with *Armigeres* DNA is extensive, with a high degree of background signal. The only discernible polymorphism was scored. It is possible that this probe is detecting a family of genes, at several loci throughout the genome. A marker 3 cM away from LF92 in *Ae. aegypti*, LF111, occurs in the same linear order in both species (i.e., it has not been translocated with LF92).

The sex-determination locus maps to linkage group 1 in *Ae. aegypti* and group 3 in *Ar. subalbatus*, and variation in the sex-determination locus is strain-specific in another culicine genus, *Culex*. Geographic isolates of *Culex pipiens quinquefasciatus* vary in the position of this locus (Sakai et al. 1980). In another species, *Culex tritaeniorhynchus*, the sex

Table 2. Segregation of Markers in the F₂ from Five Crosses Used to Construct a RFLP Linkage Map

Marker ^a	No. of individuals ^b			χ^2 ^c
	M	H	J	
Cross 1				
Chromosome 1				
LF90	19	29	14	1.06
LF188	18	32	13	0.81
LF235	23	22	14	6.56*
LF198	19	31	12	1.58
LF178	20	30	13	1.70
LAP	22	30	11	3.98
LF204	31	23	10	18.84**
TY7	20	29	11	2.77
Scr	18	29	13	0.93
Cross 2				
Chromosome 2				
LF264	13	25	9	0.87
LF180	18	40	17	0.36
LF203	11	46	17	5.35
LF335	10	46	20	6.00*
LF92	9	44	23	7.05*
Chromosome 3				
LF377	10	41	25	6.39*
LF218	11	42	24	5.03
LF108	11	41	24	4.92
LF168	9	43	25	7.70*
LF106	12	45	18	3.96
Mall	11	48	16	6.55*
LF111	14	45	18	2.61
LF128	14	45	18	2.61
Cross 3				
Chromosome 2				
LF115	10	32	20	3.29
LF250	8	38	25	8.49*
LF335	3	32	35	29.77**
Ddc	3	36	29	20.12**
LF334	5	38	24	11.99**
LF92	3	34	24	15.26**
Cross 4				
Chromosome 2				
LF115	19	49	17	2.08
LF250	11	22	15	1.00
LF180	24	42	20	0.42
Ddc	23	47	15	2.46
Cross 5				
Chromosome 2				
LF250	8	33	12	3.79
LF203	3	30	18	10.41**
LF335	3	32	19	11.33**
LF334	3	34	17	10.89**

^aMarker loci are arranged in chromosomal order.^b(M) Malaysian strain; (H) heterozygote; (J) Japan strain.^c(*) $P < 0.05$; (**) $P < 0.01$ (loci tested for expected 1:2:1 ratio).

Table 3. Segregation of Linkage Group 3 RFLP Markers Relative to Sex

Marker ^a	Sex	No. of individuals ^b			χ^2 ^c
		M	H	J	
LF377 (35)	F	5	14	8	0.70
	M	5	27	17	6.39*
LF218 (28)	F	5	19	4	6.34
	M	6	23	20	8.18*
LF108 (16)	F	8	16	3	2.78
	M	3	25	21	13.24**
LF168 (11)	F	8	18	2	4.86
	M	1	25	23	19.78**
LF106 (6)	F	10	16	0	9.08*
	M	2	29	18	12.10**
Mall (8)	F	9	17	0	8.69*
	M	2	31	16	11.45**
LF111 (13)	F	11	16	1	7.71*
	M	3	29	17	9.65**
LF128 (23)	F	9	16	3	3.14
	M	5	29	15	5.73

^a(Numbers in parentheses) Map distance from sex determination locus.^b(M) Malaysian strain; (H) heterozygote; (J) Japan strain.^c(*) $P < 0.05$; (**) $P < 0.01$ (loci tested for expected 1:2:1 ratio).

locus segregates with chromosome 1 markers in one strain and chromosome 3 markers in another (Baker and Sakai 1976). The heredity of sex in culicine mosquitoes differs from that observed for *Drosophila* (MacDougall et al. 1995); however, it is unlikely the genetic mechanisms of sex determination and differentiation are entirely different between these two dipterans. It is likely that rather than vast differences in gene positions among species and strains of mosquitoes, genetic control is allelically based. From work in *Drosophila* it is clear that the molecular genetics of sex determination is a complex process requiring expression and interaction of many genes, and genetic variants might exist that transfer control of this cascade to a different gene in the pathway (MacDougall et al. 1995; Wilkins 1995).

The loci mapped in *Ar. subalbatus* cover 181.5 cM as compared to 181.3 cM for *Ae. aegypti*. This congruency does not reflect the variation in recombinational distances according to linkage group, with *Armigeres* markers undergoing crossovers less on linkage group 1 and more on group 2 than their *Aedes* counterparts. The haploid genome size of *Ar. subalbatus* has been estimated as containing 1.24 pg of DNA (Rao and Rai 1990) as compared to 0.81 pg for *Ae. aegypti* (Rao and Rai 1987). It is not clear why this difference in nuclear DNA content is not re-

ARMIGERES SUBALBATUS LINKAGE MAP

Figure 3 Genetic linkage map of *Ar. subalbatus* based on RFLP loci derived from *Ae. aegypti*. Map distances are given in centimorgans. Asterisks denote markers that deviate from the linkage group predicted by the *Ae. aegypti* map. Arrowheads indicate three loci segments that are inverted with respect to their position on *Ae. aegypti* linkage group 2.

flected in recombination frequencies, but recent data suggest the genome size for *Ae. aegypti* is significantly smaller than that reported by Rao and Rai (1990); similarly, it is possible that the size of the *Ar. subalbatus* genome also is smaller (D. Zaitlan, pers. comm.).

Comparative mapping can be a powerful approach to genetically characterize a previously undescribed organism, and generation of genetic linkage maps recently has provided the necessary tool to gain insight into the heritability of mosquito susceptibility to several parasite species (Severson et al. 1994b, 1995b; Zheng et al. 1997). The *Ar. subalbatus* genome is now defined in discrete units that can be used to identify non-random association of genomic regions with vector competence using QTL procedures in conjunction with crosses between parasite-resistant and -susceptible line of *Ar. subalbatus*. As data from related studies accumulate, comparative QTL studies also might elucidate common inheritance patterns associated with vector competence and suggest novel approaches for the control of vector-transmitted diseases.

METHODS

Mosquito Strains and Crosses

Two *Ar. subalbatus* strains were used for these studies. One strain originated from Japan (J), and was provided by G.B.

Craig (University of Notre Dame, South Bend IN), and the other strain was colonized in Malaysia (M) and was provided by Dr. Akio Mori (Institute for Medical Research, Kuala Lumpur). The RFLP linkage map was based on five segregating F_2 populations resulting from sibling matings of F_1 populations. The F_1 populations were generated from a mass mating between J males and M females.

Restriction Length Fragment Polymorphism Markers

All of the RFLP markers used in this study are *Ae. aegypti* cDNA clones with the exception of Scr (Sex-combs-reduced), which is a probe derived from the homeobox region of the *Drosophila melanogaster* gene (Mahaffey and Kaufmann 1987) recently mapped in the *Ae. aegypti* genome (D.W. Severson, pers. comm.). Markers were selected from throughout the *Ae. aegypti* genome, with the goal of obtaining similarly complete coverage of the *Ar. subalbatus* genome. Most of these clones represent random cDNAs (Severson et al. 1993). *Mall* and *LAP* represent known *Ae. aegypti* cDNAs—salivary gland maltase (James et al. 1989) and lysosomal aspartic protease (Cho and Raikhel 1992), respectively.

DNA Isolation, Southern Blotting, and Hybridization

DNA extracted from pools of mosquitoes representing each of the parental (J and M) strains of *Ar. subalbatus* and from F_2 individuals, was *EcoRI*-digested, Southern blotted, and hybridized according to methods described previously (Severson et al. 1993; Severson 1997). Briefly, total nucleic acids were isolated by homogenization in equal volumes of lysis buffer and phenol, followed by standard phenol/chloroform extraction (Sambrook et al. 1989). This isolate was incubated with RNase A followed by a second extraction with phenol/chloroform. *Ae. aegypti* markers initially were used to screen bulked DNA preps from each of the parental strains to verify hybridization and to identify informative loci. Ten-microgram aliquots of mosquito genomic DNA were digested with *EcoRI*, size-fractionated on 0.9% agarose gels, and blotted to Hybond (Amersham) membranes. F_2 mapping blots were prepared similarly using DNA from single individuals. Prescreening identified informative RFLP markers (Table 1) from which 26 were chosen for hybridization with (J $\times$ M) F_2 progeny for generation of mapping data. Probe insert preparation and radiolabeling were accomplished using a PCR protocol with defined primer annealing sites of the vector plasmid flanking the mosquito DNA (Severson 1997). Prehybridization, hybridization, and membrane washing procedures were conducted at 65°C in glass bottles in a commercial hybridization oven (Hybaid) (Severson et al. 1993; Severson 1997). Membranes were washed using stringent conditions (twice in $2 \times$ SSC with 0.1% NaDodSO₄ for 15 min, and twice in $0.2 \times$ SSC with 0.1% NaDodSO₄ for 15 min) before exposure to film for 3–6 days at -80°C with an intensifying screen.

RFLP and Linkage Analysis

χ^2 goodness-of-fit ratios were determined for segregation and independent assortment of alleles for all pairs of loci. Multi-point linkage analyses were performed using Mapmaker software (Lander et al. 1987) with a minimum lod threshold of 3 used to identify nonrandom association between markers.

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The Mapmaker program is unable to compile data generated from different crosses; consequently, a composite linkage map was developed for chromosome 2 and for inclusion of the sex determination locus on group 3, using the JoinMap computer program (Stam 1993). The *Ar. subalbatus* linkage map was depicted graphically using DrawMap (van Ooijen 1994). Map distances are reported as centiMorgans (Kosambi 1944).

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