Intensive Linkage Mapping in a Wasp (*Bracon hebetor*) and a Mosquito (*Aedes aegypti*) With Single-Strand Conformation Polymorphism Analysis of Random Amplified Polymorphic DNA Markers

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Manuscript received December 8, 1995
Accepted for publication April 19, 1996

ABSTRACT

The use of random amplified polymorphic DNA from the polymerase chain reaction (RAPD-PCR) allows efficient construction of saturated linkage maps. However, when analyzed by agarose gel electrophoresis, most RAPD-PCR markers segregate as dominant alleles, reducing the amount of linkage information obtained. We describe the use of single strand conformation polymorphism (SSCP) analysis of RAPD markers to generate linkage maps in a haplodiploid parasitic wasp *Bracon (Habrobracon) hebetor* and a diploid mosquito, *Aedes aegypti*. RAPD-SSCP analysis revealed segregation of codominant alleles at markers that appeared to segregate as dominant (band presence/band absence) markers or appeared invariant on agarose gels. Our SSCP protocol uses silver staining to detect DNA fractionated on large thin polyacrylamide gels and reveals more polymorphic markers than agarose gel electrophoresis. In *B. hebetor*, 79 markers were mapped with 12 RAPD primers in six weeks; in *A. aegypti*, 94 markers were mapped with 10 RAPD primers in five weeks. Forty-five percent of markers segregated as codominant loci in *B. hebetor*, while 11% segregated as codominant loci in *A. aegypti*. SSCP analysis of RAPD-PCR markers offers a rapid and inexpensive means of constructing intensive linkage maps of many species.

A new paradigm has emerged from the use of molecular genetic technology in linkage mapping, where hundreds or thousands of markers now can be mapped simultaneously in a single cross. When combined with the analysis of quantitative traits, intensive linkage mapping allows identification of the numbers and effects of genes underlying phenotypic characters (quantitative trait loci, QTL). The power and applications of QTL analysis are clear (*e.g.*, HAMER *et al.* 1993; TANKSLEY 1993; MITCHELL-OLDS 1995; PATERSON *et al.* 1995), but saturated linkage maps based on molecular markers have been developed for relatively few species. This is primarily because intensive linkage mapping can be costly and labor intensive. For example, linkage analysis of simple sequence repeat loci (SSR microsatellites) requires screening a genomic library for clones containing SSR motifs and sequence analysis to design oligonucleotide primers for PCR amplification of SSR-containing regions (DIETRICH *et al.* 1992). Mapping with restriction fragment length polymorphisms (RFLPs) via cDNA probes similarly requires construction of a cDNA library followed by Southern blotting and hybridization with the cDNA probes. Nonetheless, high density linkage maps based on SSRs and RFLPs are available for a number of eukaryotic organisms: a few insect species [*e.g.*, mosquitoes: *Aedes aegypti* (RFLP, SEVERSON *et al.* 1993), *Anopheles gambiae* (SSR, ZHENG *et al.* 1993); several Drosophila species (RFLP, O'BRIEN 1993); silkworm, *Bombyx mori* (RFLP, SHI *et al.* 1995)], agricultural crops [corn, rice, sorghum, tomato, potato (TANKSLEY *et al.* 1992; PATERSON *et al.* 1995)], vertebrates [cattle, mice, rats, humans (DIETRICH *et al.* 1992; O'BRIEN 1993; HUDSON *et al.* 1995)].

With new developments in molecular techniques, saturated linkage maps can be generated for any species in a relatively short time. Two techniques have been described recently that make it possible to amplify simultaneously many regions of a genome via PCR using short oligonucleotide primers of arbitrary sequence [AP-PCR: WELSH and MCCLELLAND 1990; random amplified polymorphic DNA (RAPD)-PCR: WILLIAMS *et al.* 1990]. Neither technique requires genomic library construction, sequencing or expensive design and construction of primers. Genetic linkage maps based upon RAPD-PCR markers have been reported for *Arabadopsis thaliana* (REITER *et al.* 1992), forest trees (TULSIERAM *et al.* 1992; NELSON and DOUDRICK 1993; GRATTAPAGLIA and SEDEROFF 1994; BYRNE *et al.* 1995; PLOMION *et al.* 1995), several agricultural crop species (ECHT *et al.* 1993; WEIDE *et al.* 1993; GIESE *et al.* 1994; KURATA *et al.* 1994; KESSELI *et al.* 1995), zebrafish (POSTLETHWAIT *et al.* 1994), two monkeyflower species (BRADSHAW *et al.* 1995), the honeybee (HUNT and PAGE 1995), and the silkworm (PROMBOON *et al.* 1995).

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The advantages of RAPD-PCR are apparent: when laboratory practices are standardized, a large number of loci can be screened in a shorter period of time and more economically than is possible for either SSRs or RFLPs (BLACK 1993). However, all molecular techniques carry with them limitations, and the same is true for mapping with RAPD-PCR. While a unique genetic locus is associated with a cDNA probe or a microsatellite primer pair, determining homology of RAPD loci among families and between species requires cross-hybridizing DNA fragments among groups (*e.g.*, GRATTAPAGLIA and SEDEROFF 1994). For mapping within species, this drawback is overcome by carefully examining segregation of alleles at a putative locus in a number of families. Approximately 90% of polymorphisms detected by separating RAPD-PCR fragments on agarose gels segregate as dominant (band present/band absent) loci, and the dominance of band-present alleles hinders phase determination in the F_2 and some backcross genotypes needed to estimate recombination frequencies. Codominant loci, where complete genotypes can be unambiguously determined, are clearly advantageous in linkage mapping and QTL analysis (TANKSLEY 1993).

Here we demonstrate that the efficiency of mapping with RAPD-PCR markers can be dramatically improved with the application of single-strand conformation polymorphism (SSCP) analysis. RAPD-SSCP reveals fine-scale variation within RAPD-PCR fragments that is not detected on agarose gels. SSCP analysis is based on the principle that electrophoretic mobilities of single-strand DNA molecules in nondenaturing gels depend upon both size and shape of the fragments. Several stable shapes or conformations are formed when secondary base pairing occurs among nucleotides on a single DNA strand. The length, location and number of intrastrand base pairs determines secondary and tertiary structure of a conformation. Point mutations that affect intrastrand interactions may therefore change the shapes of molecules and alter their mobility during electrophoresis. The SSCP technique detects 99–100% of point mutations in DNA molecules 100–300 bp in length and at least 89% of mutations in molecules 300–450 bp in length (ORITA *et al.* 1989; HAYASHI 1991; HISS *et al.* 1994; VIDAL-PUIG and MOLLER 1995).

Here we demonstrate that SSCP analysis combined with RAPD-PCR reveals more polymorphisms than are seen on standard agarose gels. More significant for mapping, SSCP analysis increases the number of codominant polymorphisms seen in RAPD-PCR products, in part because of detecting SSCPs and in part because of the increased resolution of large silver-stained acrylamide gels. We demonstrate the use of RAPD-SSCP in linkage mapping of two insect species, the haplodiploid parasitoid wasp *Bracon hebetor* and the diploid mosquito *A. aegypti*. The combination of SSCP analysis and silver staining makes linkage mapping relatively rapid, inexpensive and readily applicable to many organisms.

MATERIALS AND METHODS

Sources of insects: *Bracon (Habrobracon) hebetor* (Braconidae: Hymenoptera) is a haplodiploid parasitoid wasp that attacks the larvae of moths infesting stored grains. For more than 50 years this wasp was a model organism for studying visible mutations and the genetics of sex determination in Hymenoptera (A. R. WHITING 1961; P. W. WHITING 1932, 1934, 1943). *B. hebetor* has 2N = 20 chromosomes. Previously, 37 visible mutations were mapped to eight linkage groups that constitute 625 cM (WHITING 1961), although linkage groups have not been assigned to specific chromosomes. In recent years, the reproductive biology and behavior of this wasp have been extensively studied (COOK *et al.* 1994; ANTOLIN *et al.* 1995; ODE *et al.* 1995), and details of the wasp's life history and laboratory rearing conditions may be found in ANTOLIN *et al.* (1995) and ODE *et al.* (1995).

The *B. hebetor* used in this study originated from long-term laboratory strains that include eye and body color mutations described by P. W. WHITING (1932, 1934) and A. R. WHITING (1961). Wild-type wasps have black eyes, black sclerites on the thorax and abdomen, and a tan color. A large number of families were generated by reciprocal crosses of individuals from strains carrying mutations at the following loci: (1) *cantaloupe* (red eyes), *honey* (golden-yellow body color) and *black body* (sclerite color lacking or tan) (*c-ho-bl*); (2) *orange*^{ivory} (green eyes) (o^i); and (3) *peach* (red eyes) (*pch*). F_1 females from each cross were left unmated and given four hosts per day for oviposition for 2 weeks to produce all-male recombinant F_1 progenies. No families from *c-ho-bl* × *pch* crosses survived because of low viability of females from the *c-ho-bl* strain.

One advantage of mapping in haplodiploid organisms is that all meioses are informative among the haploid F_1 males because recessive alleles are detectable (*e.g.*, see HUNT and PAGE 1995). Five families were screened for variability generated from 10 random primers (see Table 1, all primers from Operon Technologies, Alameda, CA, except primer *Bam*HI designed by N. DUTEAU: 5′-ATGGATCCCG-3′). In the initial screening, a grandmother, two of her haploid sons, a grandfather, and three F_1 daughters were examined in each family. A linkage map was generated from one of the families, a cross between an o^i female and a *c-ho-bl* male, by carrying out additional RAPD-PCR reactions using the same 10 primers plus two others (Table 2). Genotypic analyses were carried out on the o^i grandmother, two of her haploid sons, the *c-ho-bl* grandfather, the F_1 mother, and 111 haploid recombinant F_1 male progeny. The F_1 males were scored for the four morphological markers (*c, ho, o^i, bl*) before DNA extraction.

A. aegypti, the yellow fever mosquito, has a genome that is well characterized (2N = six chromosomes). Nearly 100 loci associated with phenotypic markers, vector competence, insecticide resistance, and allozymes have been mapped during the past 35 years (MUNSTERMANN and CRAIG 1979). SEVERSON *et al.* (1993) produced an intensive linkage map of *A. aegypti* using cDNA markers.

For linkage mapping we used the red-eye strain of *A. aegypti*, which is commonly used in mapping studies because of a visible marker on each chromosome. Chromosome *I* contains a sex-determining locus (*Sex*), with a dominant male-determining allele, and a recessive mutation at the *red* locus (red eye) located 7 cM from *Sex*; chromosome *II* has a recessive mutation at the *spot* locus that disrupts formation of the large silver spots on the side of the female abdomen and largely eliminates abdominal spots in males; chromosome *III* has a recessive mutation at the black tarsus (*blt*) locus that makes hind-tarsi entirely black. Wild-type mosquitoes have distinct black and white bands on the tarsi of the hind leg. All mosquitoes were maintained as described in MUNSTERMANN and CRAIG (1989) in an insectary at the Colorado State University Arthropod Borne and Infectious Diseases Laboratory.

Fifty reciprocal crosses were made between mosquitoes from the red-eye strain (homozygous for the mutations described above) and a strain from Agbor, Nigeria. For each cross, a male and a female from each strain were isolated together as pupae and placed in individual pint cartons following emergence (P1 grandparents). Females were blood fed on mice, and an oviposition container was placed in the cage. After 48 hr, males (grandfathers) were removed to a labeled vial and stored at −70°. Following oviposition, females (grandmothers) were stored frozen with their mates. After eggs were hatched and reared, individual F_1 pupae of each sex were isolated. Four pairs of F_1 males and females from each cross were processed as described above. F_2 eggs were reared to adults, scored for eye and body color, and frozen at −70° for genotypic analysis.

Two reciprocal families were selected for analysis based on the size of the F_2 generation, one red-eye × Agbor F_2 family (RA34-3) and one Agbor × red-eye F_2 family (AR24-4). The AR24-4 family was analyzed first using three primers, and then the RA34-3 family was analyzed using 10 primers (Table 3). After the analysis by the third primer in the AR24-4 family, the DNA from this family was destroyed by nucleases probably introduced by a mineral oil overlay. Subsequently, DNA from all families was archived by storing 80% of the DNA at −70°, and the remainder was kept at 4° for daily mapping.

DNA isolation: DNA was extracted following the protocol of COEN *et al.* (1982). DNA was resuspended in 500 μl TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) for *A. aegypti* and 100 μl TE for *B. hebetor*. The majority of the DNA was archived in a −70° freezer, DNA used for PCR reactions was stored at 4°.

PCR amplification: RAPD-PCR protocols were as described in BLACK *et al.* (1992), with one μl of DNA template used in each 50 μl reaction. Each set of PCR reactions was checked for contamination by the use of a negative control (all reagents except template DNA). Samples were stored at 4° until electrophoresis.

Electrophoresis to detect SSCPs: Complete details of SSCP analysis and silver staining are described in HISS *et al.* (1994) and BLACK and DUTEAU (1996). Briefly, PCR products (5 μl of 50 μl) were mixed with 2 μl loading buffer, denatured to single strands with heat, and then plunged into ice to promote the formation of intrastrand complexes while reducing the formation of double-stranded DNA. In practice, simply mixing PCR product with loading buffer at room temperature and eliminating the heating/chilling before loading made little difference in banding patterns seen on gels. The products were electrophoresed on large (40 × 50 cm), thin (0.4 mm) glycerol (5%) polyacrylamide (5%, 2% cross-linking) gels. Electrophoresis proceeded at constant voltage (350 V) at room temperature for 16 hr (overnight), and the gels were silver stained to detect the mobility of the different DNA conformations. Six-mm sharks tooth combs were used for loading to accommodate the 6–7 μl of sample needed for each lane.

The following modifications of our published protocols have been made. Accumulation of bind silane leads to peeling of gels from the plates upon drying. The can be prevented by vigorously scrubbing plates with a grease-cutting soap or a solution of 10% NaOH and 0.2 M SDS and storing the plates in 1 M NaOH until the next use. At the beginning of the staining protocol, gels were fixed in 10% glacial acetic acid for up to 2 hr rather than 20 min to reduce background staining. Reduced overhead lighting during development also diminished background staining. Rinsing gels in water for at least 10 min after development and fixation washed away residual salts that may cloud gels after drying.

Scoring of RAPD-SSCP bands: Amplified markers were scored directly from dried gels by measuring band mobility relative to known size markers (1-kb ladder, BRL Laboratories). To estimate sizes of the amplified DNA fragments, size standards were fitted to an inverse function that relates fragment size and mobility (SCHAFFER and SEDEROFF 1981). A spreadsheet program (DOS compatible) is available from MFA or WCB4 upon request. RAPD markers were named by the primer designation followed by a period and the estimated size of the fragment. Dominant (presence/absence) markers were designated by a letter in parentheses that indicates the strain that was the source of the amplified band. Codominant markers were denoted by both letters separated by a slash.

The RAPD-PCR method generates numerous bands, some of which are not repeatable. Most of these spurious bands appear only once or twice in a family (*e.g.*, 396-bp band in Figure 1). We scored only clearly discernable bands that segregated in a predictable Mendelian pattern in the grandparents, parents, and the recombinant progeny. Further, the same RAPD primers were used on several families of each species and were found to produce repeatable banding patterns (see below). When variation in fragments are examined across three generations, it is relatively straightforward to test for Mendelian segregation of alleles even when they are multibanded SSCPs.

Data analysis: Map distances for both species were converted from recombination fractions to map units (cM) by the Kosambi mapping function (KOSAMBI 1944). Genotypes generated for *B. hebetor* haploid males were analyzed by Mapmaker/EXP 3.0b (LANDER *et al.* 1987) as an F_2 backcross, which correctly estimates the recombination fractions and LOD scores for loci in haploid families. Alleles in each male were scored as "A" (inherited from the grandmother) or "H" (inherited from the grandfather). Initial linkage groups were determined by use of the "group" command, with a maximum recombination fraction of 0.33 (40 cM) and a statistical threshold LOD score of 3.0. Order of markers was determined by three-point analysis, and the "compare" and "ripple" commands. Subsequently, unlinked markers were added to the existing linkage groups at a less stringent recombination-fraction of 0.38 (50 cM).

Offspring genotypes of F_1 and F_2 *A. aegypti* were entered into JOINMAP 1.4 (STAM 1993). MAPMAKER was not used because JOINMAP allows each marker to be analyzed according to parental genotypes, which is necessary in RAPD-SSCP mapping of diploids in an F_2 intercross. These data contain markers with dominant and codominant alleles. As a result, the numbers of parental, recombinant and uninformative genotypes, and the segregation ratios among the F_2 offspring may differ for each locus. Recessive alleles inherited through the P1 female were coded as an "A," recessive alleles inherited through the P1 male were coded as a "B." Dominant markers were coded as "C" when inherited through the P1 male or "D" when inherited through the P1 female. With codominant markers offspring were coded "A" or "B" when they had P1 female or P1 male genotypes, or as "H" when heterozygous for alleles from both P1 parents. Initially, a threshold recombination fraction of 0.38 and a LOD score of 3.0 was used to group markers, then the minimum LOD was increased to 5.0 in increments of 0.5. DRAWMAP 1.1 (VAN OOIJEN 1994) was used to plot a linkage map from this file.

RESULTS

SSCP analysis of RAPD-PCR products: Comparisons of RAPD-PCR products as they appear on SSCP gels and standard agarose gels (1.5%) are in Figures 1 and 2. These figures demonstrate common patterns found by SSCP analysis of RAPD-PCR products.

In Figure 1, several bands from *B. hebetor* that ap-

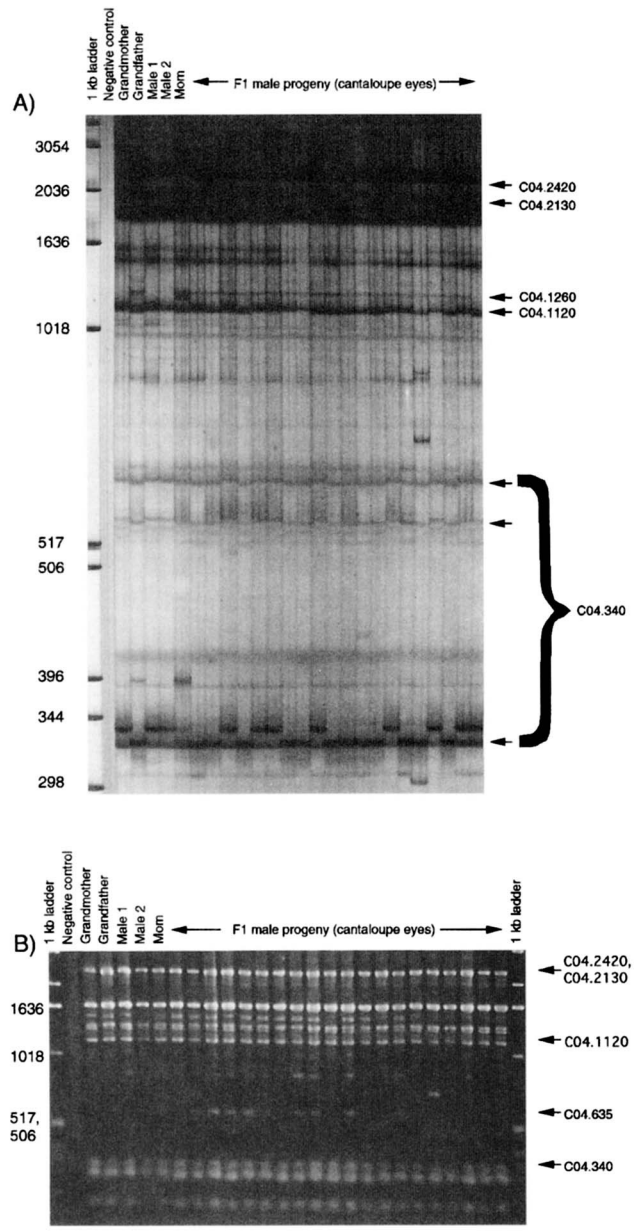

FIGURE 1.—RAPD-SSCP variability generated by primer C04 in the $o^i \times$ *c-ho-bl* family of *B. hebetor*, as seen on a nondenaturing polyacrylamide gel (A) and a 1.5% agarose gel (B). Each lane is labeled with regard to grandparents, two haploid males that are the grandmother's sons, the F_1 mother (mom), and the recombinant F_1 male progeny. The F_1 males all had the *cantaloupe* red eye color phenotype. The bracket indicates a single marker (C04.340) with two SSCP bands that have identical patterns. Two markers at the top of the gel (C04.2420, C04.2130) cannot be seen in the figure because the photograph was overdeveloped to show the lower parts of the image.

peared monomorphic on agarose gels were resolved as polymorphic markers on the SSCP gels. Locus C04.1260 resolved as a band presence/absence polymorphism, with the band absent in the grandmother (diploid, from the o^i strain) and her two haploid sons. The band was present in the grandfather (haploid, from the *c-ho-bl* strain) and the F_1 mother and segregated as 1:1 present:absent in the recombinant F_1 males. C04.1260 is 6.4 cM from o^i (Figure 4). Nineteen of 20 F_1 males in Figure 1 have the band-present allele because the recessive *cantaloupe* phenotype (red eyes) is only expressed in males who also have a wild-type allele at o^i locus (*i.e.*, the $c/+$ haplotype; males with the c/o^i haplotype have green eyes).

Two other loci in Figure 1, C04.1120 and C04.340, appeared as single bands of varying intensity on the agarose gel but clearly resolved as codominant polymorphisms on the SSCP gel. The grandmother was homozygous for slow alleles at both loci, as were her two haploid sons. The grandfather had a fast allele at both loci, the F_1 hybrid mother was a heterozygote at both loci, and the F_1 males segregated for either the fast or slow alleles at 1:1 ratios. Two additional bands that did not appear on the agarose gel were seen on the acrylamide gel (upper bands marked by arrows within the bracket in Figure 1). Fast and slow bands of both fragments were completely correlated with the dark bands at C04.340. It is likely that this set of three bands resulted from different conformations of the PCR products of a single locus (one lighter band for each strand of C04.340), and all three mapped to a single locus on linkage group III (Figure 4). Locus C04.635, which segregated as a presence/absence polymorphism on agarose gels (band-present allele from the *c-ho-bl* strain), did not appear on the SSCP gel.

Similar resolution was found by SSCP analysis of *A. aegypti*. In Figure 2, the B18.1252 locus appeared as a band of varying intensity on the agarose gel but was easily resolved into three distinct genotypes on the SSCP gel. The grandmother was homozygous for a null allele (no band), the grandfather was homozygous for a dominant allele (dark band), while both parents were heterozygous (faint band). F_2 offspring exhibited a 1:2:1 ratio of these genotypes. Similarly, locus B18.937 appeared as a band of varying intensity among F_2 offspring on the agarose gel but easily resolved into four distinct genotypes on the SSCP gel. The grandmother was heterozygous for a slow and fast allele, the grandfather was homozygous for a null allele (no band), the mother was heterozygous slow/null, and the father was heterozygous for fast/null. The complete family of F_2 offspring (not shown) exhibited a 1:1:1:1 ratio of the same four genotypes. At the B18.769 locus, a single invariant band was detected on the agarose gel, while codominant alleles and three genotypes were detected by SSCP. Both F_1 parents had identical genotypes, and genotype ratios among the F_2 occurred at a 1:2:1 ratio.

Figure 2B further demonstrates the resolution and sensitivity of the SSCP and silver staining technique. A cluster of five bands that migrated below the 390-bp size marker appeared on the SSCP gel, while on the agarose gel only a single band was faintly visible in this region. F_2 offspring in Figure 2 are arranged with respect to the red-eye phenotype, red-eye F_2 offspring shared the grandmother's genotypes at the B18.1252,

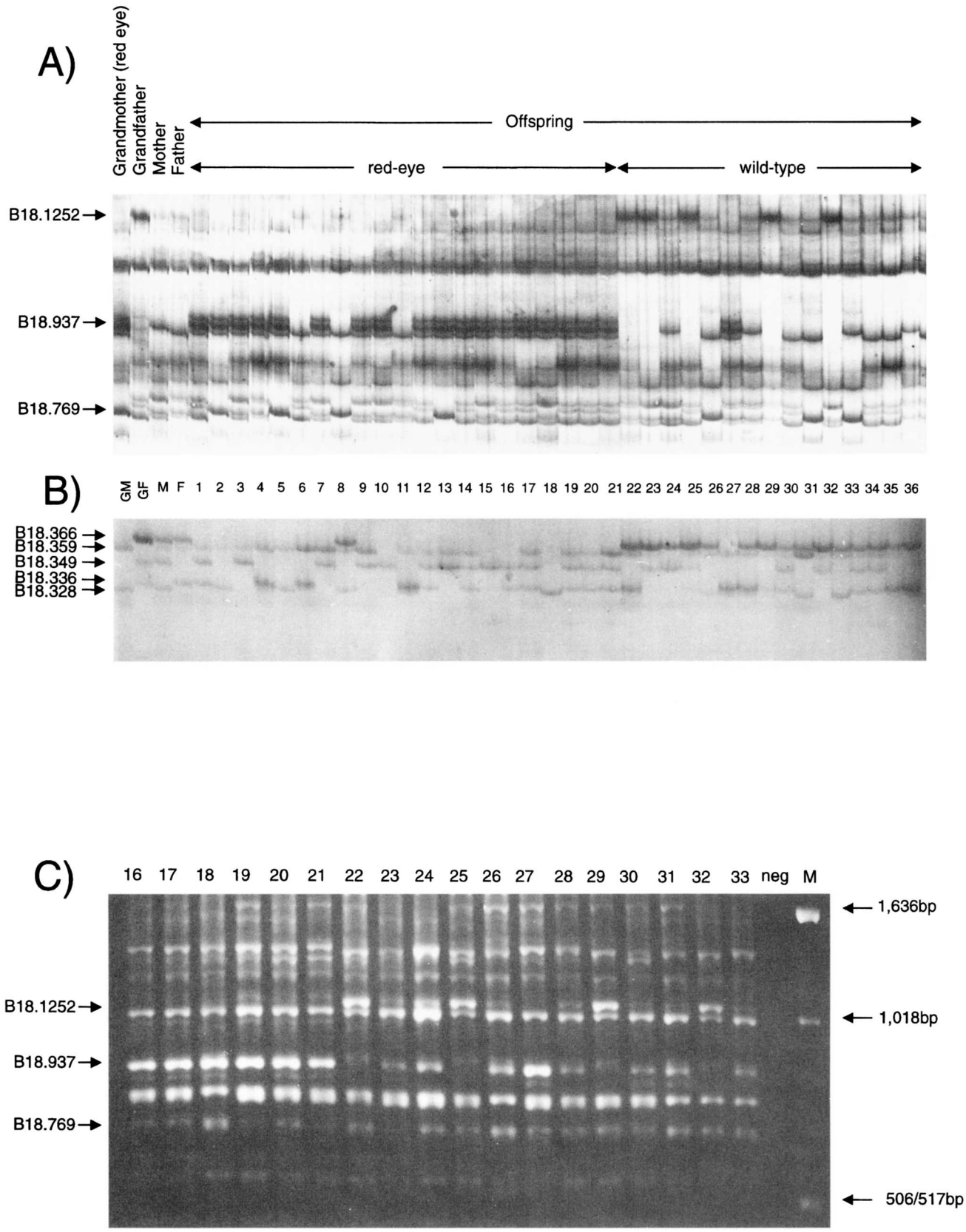

FIGURE 2.—SSCP patterns of eight markers amplified by primer B18 in family RA-34-3 of the mosquito *A. aegypti*. Each lane is labeled with regard to parents (P1 = Grandmother, Grandfather; F_1 = Mother and Father) and F_2 offspring. (A) Segregation of codominant alleles at three loci amplified by B18. (B) Segregation of dominant alleles at five markers. (C) B18 products as seen on a 1.5% agarose gel. F_2 offspring are arranged with respect to the red-eye phenotype. Bands just below the B18.1252 marker and those between B18.769 and B18.937 could not be interpreted in mendelian fashion.

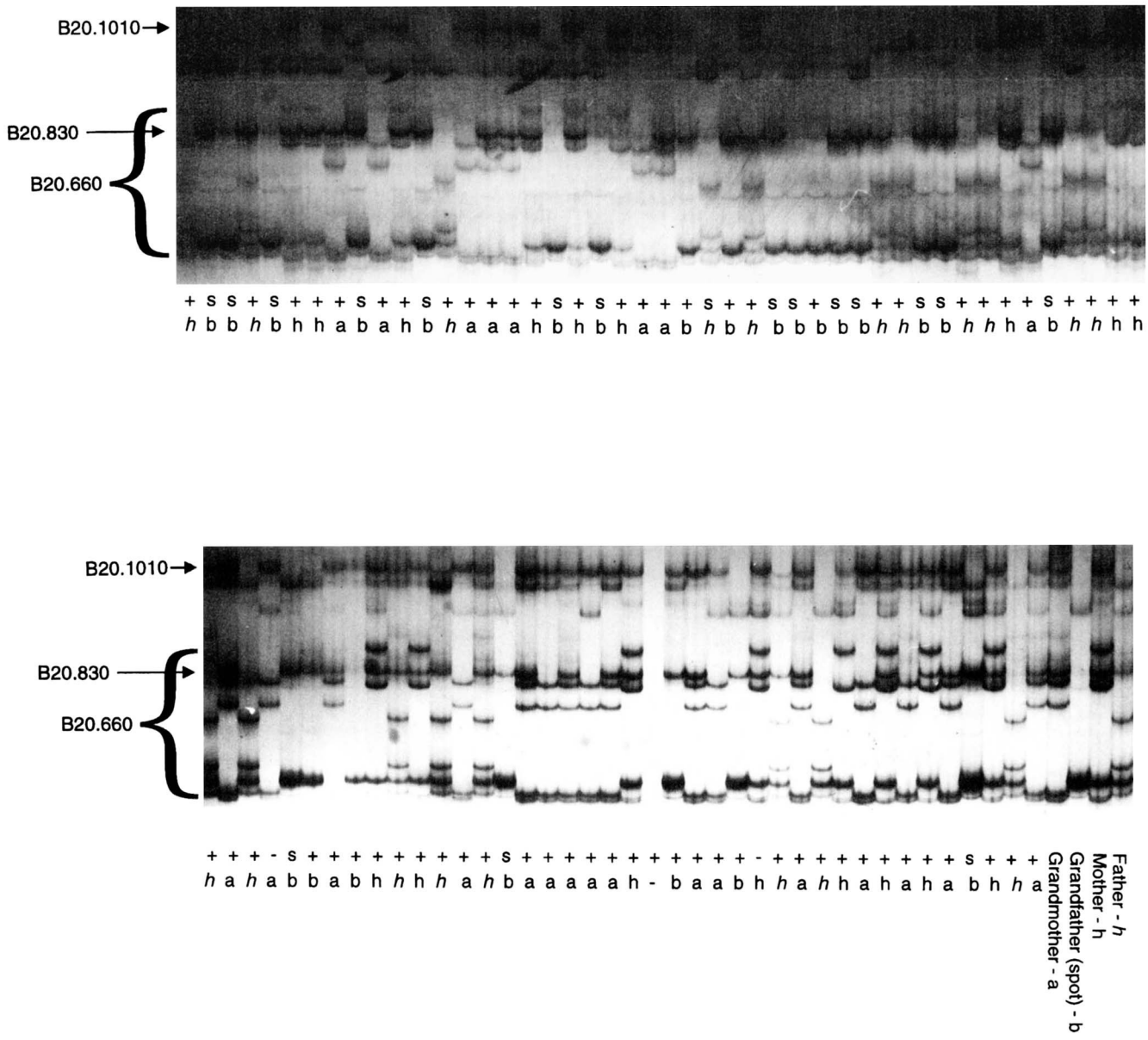

FIGURE 3.—SSCP patterns of three markers amplified by primer B20 in family AR-24-4 of the mosquito *A. aegypti*. The four lanes at the bottom right of the figure are labeled with regard to parents (P1 = Grandmother, Grandfather; F_1 = Mother and Father), and genotypes of the B20.660 locus. The P1 grandmother's banding pattern is labeled "a," the P1 grandfather's banding pattern is labeled "b," the F_1 mother's banding pattern is labeled "h," and the F_1 father's banding pattern is labeled "*h*." The grandmother was also homozygous for *spot*. Other lanes show F_2 progeny, labeled with regard to the *spot* phenotype (s, spots missing from abdomen; +, spots on abdomens; -, ambiguous spot phenotype), and the four parental B20.660 genotypes. Note that *spot* cosegregates with the B20.660 b genotype, and that both markers map to chromosome *II* (Figure 5). Segregation of two markers with dominant alleles (B20.830 and B20.1010) are also shown. The B20.830 and B20.660 genotypes segregate independently, even though bands comigrate.

B18.937 and B18.366 loci, and all loci mapped within 10 cM of the *red* locus (Figure 5).

Figure 3 shows complex SSCP patterns amplified by primer B20 in *A. aegypti*. RAPD-SSCP genotypes are presented with respect to the spot abdomen phenotype. B20.660 is an example of a codominant marker that shows a complex SSCP pattern similar to loci C04.340 in *B. hebetor* (Figure 1A), and B18.937 and B18.769 of *A. aegypti* (Figure 2A). The four parents show distinctive genotypes that occurred at a 1:1:1:1 ratio in F_2 offspring. Most F_2 individuals with the spot abdomen phenotype had the grandfather's single dark-staining band at the B20.660 locus, which was located within 15 cM of *spot* (Figure 5). In scoring the B20.660 locus, a second locus (B20.830) was identified that comigrates within the SSCP pattern of the B20.660 locus. However, both F_1 parents were heterozygous, making genotypic phase among the F_2 offspring entirely ambiguous, and B20.830 could not be mapped.

B20.1010 is a typical RAPD locus with dominant (band-present) and recessive (band-absent) genotype patterns (Figure 3). Although B20.1010 is linked to *spot* (Figure

TABLE 1

Number of RAPD-SSCP fragments amplified in five families of *B. hebetor*

Primer	Total no. of fragments amplified	No. of polymorphic fragments in each family				
		pch × *c-ho-bl*	*pch* × o^i	o^i × *pch*	*c-ho-bl* × o^i	o^i × *c-ho-bl*
A5	12	3	2	4	3	4
A20	9	2	5	5	4	5
AM7	10	1	3	3	4	5
AM10	12	1	7	7	3	3
AM11	19	7	8	10	7	7
*Bam*HI	7	2	2	1	2	1
B18	14	2	6	6	7	5
C01	9	0	3	3	2	5
C04	11	0	4	3	4	3
Q16	12	2	4	3	3	5
Total	115	20	44	45	39	43

The number of fragments differ from Table 2 because early gels were silver stained too heavily to score fragments in the 2–3 kb range. In each family, the maternal grandparent is listed first.

5), there is no clear correspondence between the SSCP pattern and the spot abdomen phenotype on the gel because only mosquitoes with the *s/B20.1010–* (parental, band absent) and *s/B20.1010+* (recombinant, band present) genotypes were informative. The remaining genotypes (*+/B20.1010–* and *+/B20.1010+*) could arise with or without recombination and were ambiguous. In Figure 3 there were 18 *s/B20.1010–* parental genotypes and no *s/B20.1010+* recombinant genotypes.

Mapping the haploid-diploid genome of *B. hebetor*: Our initial screening of the five families of *B. hebetor* indicated 11.5 consistently well-amplified fragments per primer (Table 1). The number of polymorphisms varied among families, with the most variable cross having 45/115 (39.1%) polymorphic bands. On average, we amplified 3.8 polymorphic fragments per primer (±2.2 SD) in these five families.

In the o^i x *c-ho-bl* family (Table 2), we scored 84 polymorphic RAPD markers, an average of 6.8 polymorphisms per primer. This is a greater number of polymorphisms than we scored in our initial screen because we had at first overstained the upper portions of gels. By staining less heavily (*i.e.*, by stopping the developing reaction sooner) we were able to score several more markers per primer in the 2–3 kb range without losing resolution of the smaller markers. Ten of the 12 primers generated at least five polymorphisms; 46% of polymorphic markers were codominant. With a few exceptions (see below), segregation of alternative alleles among males was 1:1, as expected in the haploid male offspring of a heterozygous female.

TABLE 2

Numbers of RAPD-SSCP markers analyzed in the o^i × *c-ho-bl* family of *B. hebetor*

Primer	No. of markers	Dominant (band presence/absence)	Codominant (size or SSCP)
A5	3	1 (1)	2
A9	5	4	1
A20	6	4	2 (1)
AM7	9	6	3 (1)
AM10	8	4	4
AM11	6	5	1
*Bam*HI	6	3 (1)	3
B18	14	9	5
C01	7	3 (1)	4
C04	9	3	6
C16	6	3	3
Q16	5	1	4
Total	84	46 (3)	38 (2)

Numbers in parentheses are markers that did not map. The linkage map generated from these markers is in Figure 4.

We determined repeatability of RAPD markers by comparing those identified in the initial screening of families with the markers subsequently mapped in the o^i × *c-ho-bl* family. Because early gels were overstained, we could only compare markers smaller than 1600 bp. The 10 primers used both in initial screening and for mapping (see Table 1) generated 51 bands smaller than 1600 bp that mapped in the o^i × *c-ho-bl* family. Of these, 37 (72.6%) were also scored during the original screening of primers and were completely repeatable.

The whole data set for the o^i x *c-ho-bl* family, including morphological traits, contained 88 markers. We placed 83 of these into four large linkage groups (≥128 cM) and nine smaller linkage groups (≤70 cM; Figure 4). Five markers did not map to any linkage group, and four of the markers were either completely linked to markers amplified by the same primer or were found to be SSCPs of a single locus (*e.g.*, upper bands of C04.340, Figure 1). The linkage map currently contains 79 loci and has a total length of 1156 cM, with an average spacing of one marker per 17.0 cM (±1.52 SE).

Two of the linkage groups, designated I and II (Figure 4), include the o^i, *c*, *bl* and *ho* eye and body color loci, and correspond to linkage groups I and II originally described by WHITING (1961). Although placement of the *ho* locus on RAPD-SSCP linkage group I

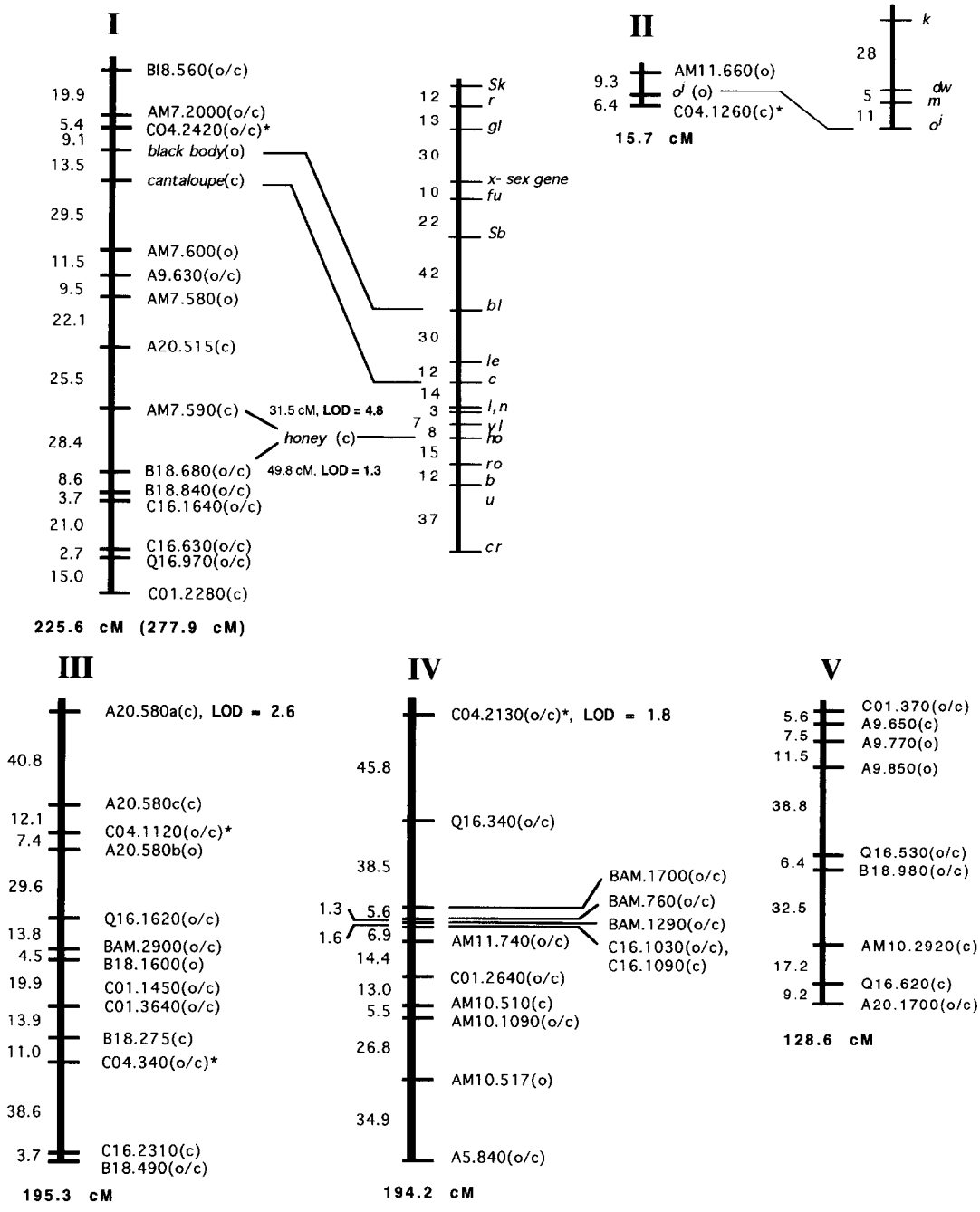

FIGURE 4.—*Bracon hebetor* RAPD-SSCP linkage groups. Groups I and II show the relationship between the RAPD-SSCP maps and the linkage maps based on morphological markers originally reported by WHITING (1961). Markers are designated by the primer name and fragment size in bp of DNA. Distances between markers (cM) are shown on the left of each group, and total linkage group length appears at the bottom of each group. An "o" following a locus indicates that the dominant allele arose from the o^i strain, while a "c" following a locus indicates that the dominant allele arose through the *c-ho-bl* strain. Codominant markers are designated o/c. SSCP patterns of markers followed by an asterisk appear in Figure 1. Markers that mapped at distances >40 cM, LOD < 3.0 have actual LOD scores shown in bold.

was problematic, resulting in significant map expansion, the orders of *bl*, *c* and *ho* are the same as on WHITING's (1961) linkage group I. Alternative alleles at the *ho* locus and surrounding markers did not segregate at the expected 1:1 ratio but segregated at a ratio closer to 3:1. For every locus on this part of the linkage group, alleles from the *c-ho-bl* strain were overrepresented, suggesting that a deleterious mutation originating from the o^i strain is located in this region of the genome.

Mapping the diploid genome of *A. aegypti*: In the two *A. aegypti* families we scored 117 RAPD markers (Table 3), yielding an average of 8.3 polymorphic markers per primer in the RA34-3 family and 7.7 polymorphic markers per primer in the AR24-4 family. For each pair of loci the number of informative F_2 individuals (*i.e.*, unambiguous parental and recombinant genotypes) were determined by a FORTRAN program (INFORM, available from WCB4 upon request) because

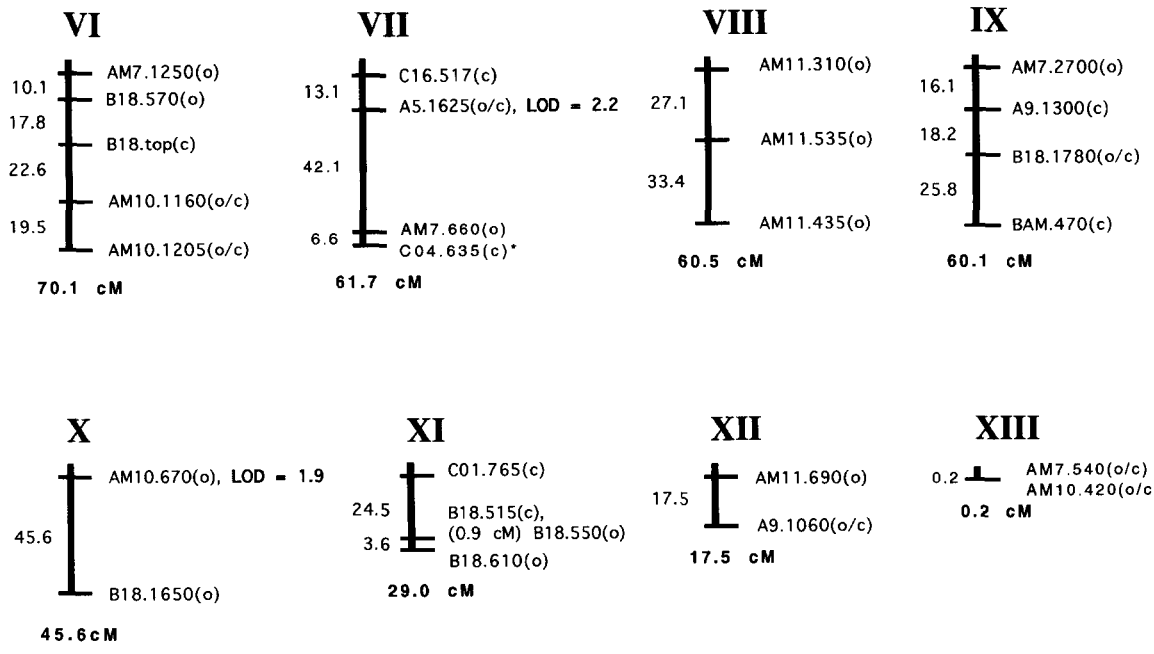

FIGURE 4.—Continued

of the large numbers of pairwise comparisons among markers [*e.g.*, (87 × 86)/2 = 3741 for the RA34-3 family]. Approximately 30% of individuals were informative for each primer. JOINMAP flags markers with segregation ratios among F_2 genotypes that depart from Mendelian expectations, but no markers were flagged in analysis of either *A. aegypti* family.

The final map (Figure 5) contains 96 markers and a total length of 168 cM, with one marker per 1.8 cM. Above the LOD threshold of 3.0, four of the 87 markers in RA-34-3 could not be joined to the map, while in AR-24-4, seven of the 26 could not be joined (Table 3). Six of the 18 (33%) RAPD-SSCP markers mapped in AR-24-4 also mapped in the reciprocal family RA-34-3. Eleven percent of the RAPD-SSCP markers segregated as codominant loci. In neither family were more than three linkage groups detected at any LOD thresholds between 3.0 and 5.5.

DISCUSSION

Figures 1–3 demonstrate the range of variation seen with SSCP analysis of RAPD-PCR products. Band presence/absence polymorphisms, which are dominant, were most common (89% in *A. aegypti*, 55% in *B. hebetor*). The use of large polyacrylamide gels combined with the greater resolution of silver staining made analysis of these polymorphisms far easier and in many cases revealed more polymorphisms than could be visualized on agarose gels. For example, marker C04.1260 in *B. hebetor* was only visible on acrylamide (Figure 1), and five B18 markers in *A. aegypti* appeared as a single faint band on agarose (Figure 2B). Codominant patterns of three types were frequently detected using large acrylamide gels. Most codominant markers segregated as fast- and slow-migrating alleles, with heterozygotes having two fainter bands (most often seen in *B. hebetor*). This increase in codominant polymorphisms is due to the greater resolution of polyacrylamide relative to agarose. Many markers that comigrated on agarose clearly resolved into two alleles on acrylamide gels. Second, multiple bands representing SSCPs of the same locus were associated with each genotype [*e.g.*, the upper bands of C04.340 in *B. hebetor* (Figure 1) and the upper and lower bands of B18.937, B18.769 and B20.660 of *A. aegypti* (Figures 2 and 3)]. Alternatively, these groups of bands could be separate tightly linked loci, but recombination between them could not be detected with the numbers of progeny tested in our crosses. Third, the relative intensity of bands varied among three genotypes, with heterozygotes having an intermediate intensity of band amplification (*e.g.*, B18.1252 of *A. aegypti*).

Loci amplified by the same primer were nonrandomly distributed. In *A. aegypti*, eight of the 11 loci amplified by primer B15 were found on chromosome *III*. In *B. hebetor* there were 60 nearest-linked pairs of RAPD loci on the 13 linkage groups, and 16 pairs of markers (28%) were amplified by the same primer. If all RAPD priming sites were randomly distributed among chromosomes, only 8.25% (1/12th, approximately) of marker pairs would be expected to be amplified by the same RAPD primer. Clustering of markers would arise if RAPDs tend to amplify regions of repetitive DNA (WILLIAMS *et al.* 1990), with primers annealing to different copies of the same repetitive motifs. This type of clustering has been reported for both RAPD and RFLP markers in tomato, lettuce and barley (WEIDE *et al.* 1993; GIESE *et al.* 1994; KESSELI *et al.* 1994).

The map of *B. hebetor* likely underestimates the complete recombinational length of the *B. hebetor* genome

TABLE 3

Numbers of RAPD-SSCP markers analyzed in two F_2 intercross families in *A. aegypti*

Marker	No. of loci	Dominant	Codominant	Proportion of informative individuals[a]
		Family AR24-4		
Visible markers	4	4	0	0.173 ± 0.171
B18	7	6 (3)	1	0.220 ± 0.106
B20	9	7 (3)	2	0.300 ± 0.094
A20	10	9 (1)	1	0.290 ± 0.139
Total	30	26 (7)	4	0.261 ± 0.127
RAPD-SSCP markers				
Dominant	22			0.284 ± 0.125
Codominant	4			0.223 ± 0.034
		Family RA34-3		
Visible markers	4	4	0	0.246 ± 0.105
B16	4	3	1	0.312 ± 0.103
A20	6	5	1	0.339 ± 0.100
B20	7	6	1	0.192 ± 0.056
B18	9	5	4	0.292 ± 0.097
B15	11	11	0	0.316 ± 0.119
C19	5	5 (1)	0	0.303 ± 0.135
C13	9	8	1	0.294 ± 0.105
C16	8	8	0	0.338 ± 0.118
C03	13	13 (1)	0	0.358 ± 0.083
C04	11	10 (2)	1	0.306 ± 0.101
Total	87	78 (4)	9	0.306 ± 0.105
RAPD-SSCP markers				
Dominant	74			0.312 ± 0.105
Codominant	9			0.270 ± 0.029

The proportion of informative individuals in a cross is the number of F_2 individuals in which the two-marker genotype could be identified as parental or recombinant, divided by total F_2 family size. This is averaged across all markers amplified by a primer. The number of markers from each primer that could not be mapped are in parentheses; the linkage map generated from these markers is in Figure 5.

[a] Values are means ± SD.

because the haploid chromosomal complement in this species is N = 10, and we found 13 linkage groups. A further indication of an underestimate is that individual markers were loosely linked (40–50 cM) to the ends of groups III, IV, VII, and X (Figure 4). Determining whether these linkages were spurious or indicate underrepresented areas of the genome will require more markers. On the other hand, an indication that this map is representative of the *B. hebetor* genome is that the map distances of the linkage groups reflect the range of physical sizes of the 10 chromosomes (five large, five small) that have been described in *B. hebetor* (TORVIK-GREB 1935). The *B. hebetor* genome is 156 megabases (Mbp) of DNA (RASCH *et al.* 1977), so that the relationship between physical size and recombinational size is currently 134 kb/cM. By adding markers from another five RAPD primers, the resolution of the *B. hebetor* map may be increased to the 52 kb/cM reported for the honeybee by HUNT and PAGE (1995).

In *A. aegypti* the recombinational map length was 168 cM, which is virtually identical to the map length of 165 cM obtained using cDNA markers (SEVERSON *et al.* 1993; D. SEVERSON, personal communication). Estimates of individual chromosomes also closely matched those from the cDNA map, with 49 cM, 60 cM and 56 cm for chromosomes *I, II,* and *III,* respectively. It is unlikely that adding more markers will increase the recombinational map size of *A. aegypti.* Given a genome size that varies from 750 to 842 Mbp (WARREN and CRAMPTON 1991), the relationship between physical and recombination distance is 4700–5300 kb/cM.

One anticipated limitation of mapping RAPD polymorphisms would be that markers mapped in one family or strain might not segregate in other crosses and that parts of each RAPD-SSCP map may be family-specific. For example, linkage group VIII in *B. hebetor* was made up of three dominant markers, all amplified by the same primer (AM11) with the band-present alleles all coming from one side of the cross. Instead, we have found that the majority of our RAPD markers are highly repeatable. At least 72% of RAPDs in *B. hebetor* were reliably amplified both in our original screens and in the family we mapped, and many of the same markers mapped in this study segregate in natural populations

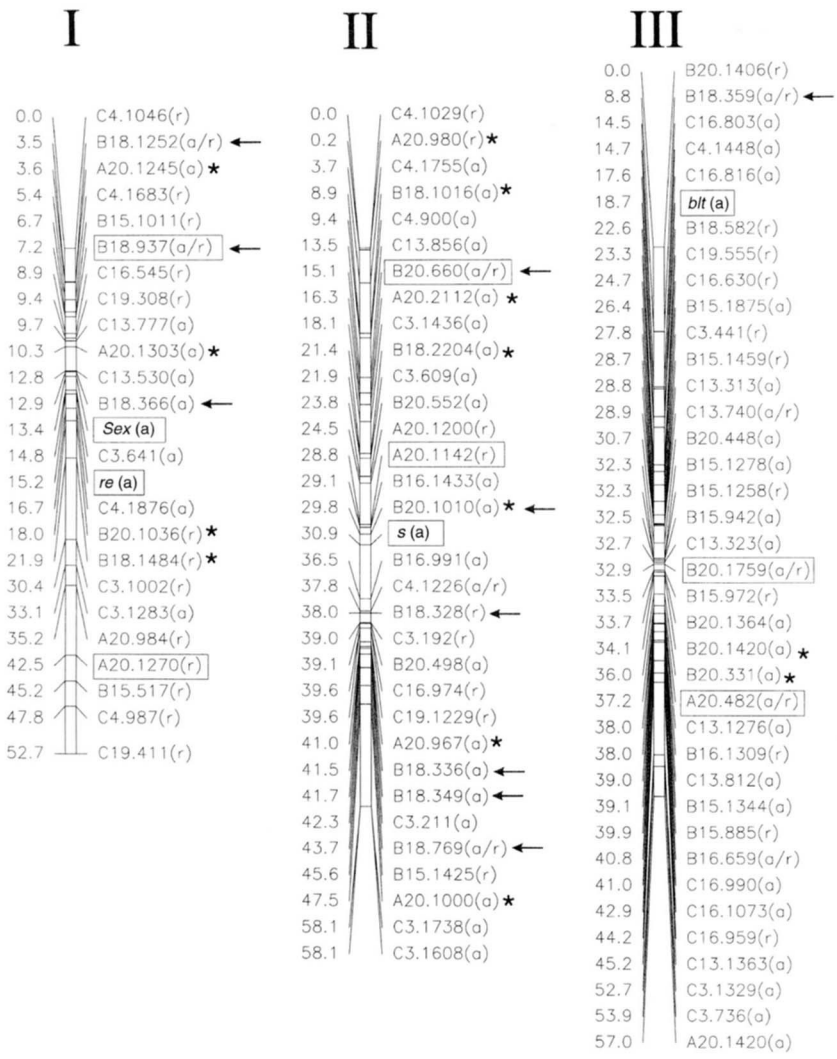

FIGURE 5.—Linkage map of 96 RAPD-SSCP loci in the mosquito *A. aegypti*. Loci that mapped in common between the two reciprocal families are in boxes. Loci mapped only in family AR24-4 are indicated with *, all other loci were mapped in family RA34-3. An "r" following a locus indicates that the dominant allele arose from the red-eye strain while "a" following a locus indicates that the dominant allele arose through the Agbor strain. Codominant markers are designated a/r. The SSCP patterns for loci indicated by arrows appear in Figures 2 and 3.

of *B. hebetor* (data not shown). One-third of the RAPD markers in *A. aegypti* mapped in family AR-24-4 also mapped to the same location in the reciprocal cross.

The ease of using RAPD-SSCP means that maps for individual families can be generated relatively quickly. Given the numbers of markers generated by each RAPD-SSCP reaction, it should be possible to make high resolution maps in relatively short amounts of time, even with uninformative individuals in crosses. In each laboratory, two workers were responsible for the results reported here. Seventy-nine markers were mapped in 6 weeks in *B. hebetor* and 83 markers mapped in 5 weeks in one family of *A. aegypti*.

Because codominant markers are most informative for QTL analysis in F_2 intercrosses (TANKSLEY 1993; MITCHELL-OLDS 1995), the increased number of codominant polymorphisms revealed by RAPD-SSCP also will make QTL analysis easier to carry out. Even dominant RAPD markers are as fully informative as codominant markers in backcrosses when the recurring parent carries the recessive alleles. Codominant markers that consistently amplify in many families may be cloned and sequenced to generate sequence tagged sites (STS) that could act as specific markers for individual linkage groups (see HUNT and PAGE 1995). STS markers are already being heavily used in genome mapping of rice (KURATA *et al.* 1994) and humans (HUDSON *et al.* 1995). STSs would be especially useful in species that do not have stocks with visible mutations. Thus, the use of RAPD-SSCP will facilitate intensive linkage mapping in a greater number of species than has been feasible until now.

We are grateful to T. BRADSHAW and G. HUNT for suggestions that clarified analysis, to D. SEVERSON and S. STACK for comments on the manuscript, and to R. SEDEROFF for providing some key references. This work was supported by a MacArthur Foundation Grant and USDA-NRI Competitive Grant 90-37363-5361 to W.C.B., and by USDA-NRI Competitive Grant 94-37312-0662 to M.F.A. and M.R.S. Early work on this project was carried out by JON CHRISTENSEN, who was funded by the Howard Hughes Medical Institute High School Scholars program at Colorado State University.

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Communicating editor: Z.-B. ZHENG