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Linkage analysis of sex determination in the honey bee (*Apis mellifera*)

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Abstract A colony-level phenotype was used to map the major sex determination locus (designated *X*) in the honey bee (*Apis mellifera*). Individual queen bees (reproductive females) were mated to single drones (fertile males) by instrumental insemination. Haploid drone progeny of an F_1 queen were each backcrossed to daughter queens from one of the parental lines. Ninety-eight of the resulting colonies containing backcross progeny were evaluated for the trait 'low brood-viability' resulting from the production of diploid drones that were homozygous at *X*. DNA samples from the haploid drone fathers of these colonies were used individually in polymerase chain reactions (PCR) with 10-base primers. These reactions generated random amplified polymorphic DNA (RAPD) markers that were analyzed for cosegregation with the colony-level phenotype. One RAPD marker allele was shared by 22 of 25 drones that fathered low brood-viability colonies. The RAPD marker fragment was cloned and partially sequenced. Two primers were designed that define a sequence-tagged site (STS) for this locus. The primers amplified DNA marker fragments that cosegregated with the original RAPD marker. In order to more precisely estimate the linkage between *X* and the STS locus, another group of bees consisting of progeny from one of the low-brood viability colonies was used in segregation analysis. Four diploid drones and 181 of their diploid sisters (workers, nonfertile females) were tested for segregation of the RAPD and STS markers. The cosegregating RAPD and STS markers were codominant due to the occurrence of fragment-length alleles. The four diploid drones were homozygous for these markers but only three of the 181 workers were homozygotes (recombinants). Therefore the distance between *X* and the STS locus was estimated at 1.6 cM. An additional linked marker was found that was 6.6 cM from the STS locus.

Key words RAPD markers · Sequence-tagged site (STS) · Haplodiploidy

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Introduction

The order Hymenoptera includes about 100 000 species of insects. In this group, males are haploid and develop parthenogenetically from unfertilized eggs, but females are diploid and develop from fertilized eggs. In the wasp *Bracon hebetor*, sex is determined by a single locus, *X*, with multiple alleles; in this species, heterozygotes develop as females, hemizygotes (from unfertilized eggs) develop as haploid males, and homozygotes develop as diploid males with reduced viability (Whiting 1940, 1943; Horn 1943). In these studies, a recessive mutation, *fused*, was associated with the *X* locus, with recombination frequencies ranging from 10 to 15%. This mutation served as a marker with which to follow different *X* alleles. Individuals that are homoallelic develop as diploid males that have reduced viability and produce diploid sperm.

Diploid males have now been found in many of the superfamilies of Hymenoptera, including the most primitive one, the sawflies (Stouthamer et al. 1992; Cook 1993b; Periquet et al. 1993). The occurrence of diploid males suggests that sex is also determined in these groups by a single locus (or a few loci), and single-locus sex determination may be the ancestral condition. In the sawfly *Athalia rosae*, outcrosses were performed and half of the controlled matings between the male and female progeny produced diploid males. Matings between such diploid males and their sisters resulted in brood containing triploid females, triploid

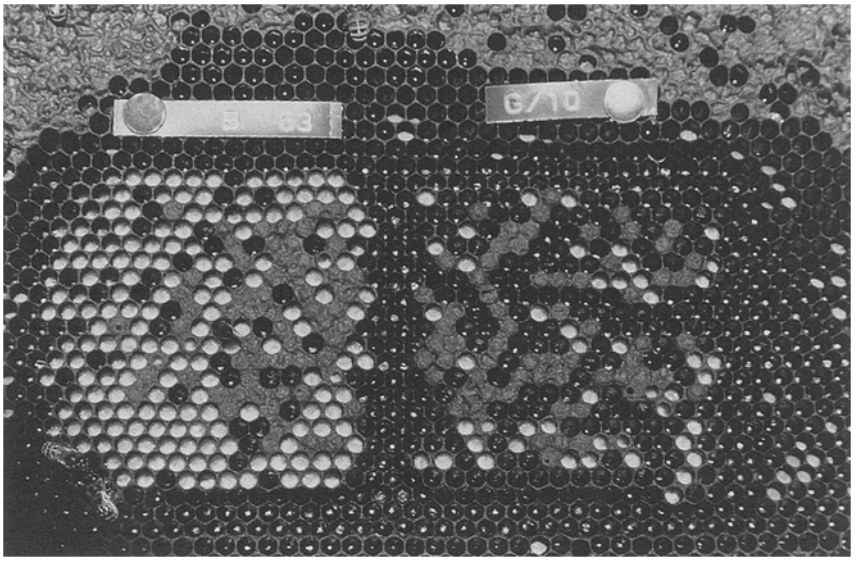

Fig. 1 The "shot-brood" colony-level phenotype is illustrated by two areas of brood. The area on the right contains the progeny of a queen that was mated to a drone that had an *X*-allele that was identical to one of her own. Half of the fertilized eggs in this patch became diploid drone larvae that were eaten by the workers, resulting in 50% brood mortality (shot-brood)

males, or only haploid males (Naito and Suzuki 1991). This is strong evidence that these sawflies have a single-locus sex determination system. However, diploid males have not been reported from the superfamily Chalcidoidea, and repeated inbreeding experiments involving one species from the Bethyloidea did not result in diploid males or inviable brood (Cook 1993a, b). A mechanism other than complementary sex determination may have arisen in these species.

Honey bees apparently have a single-locus, multiple-allele sex determination system. Mating virgin queens (reproductive females) to their "brother" drones results in a pattern of low brood viability in half of the matings, presumably due to the presence of 50% diploid drones in the diploid progeny. Sib-matings from the resultant low-viability broods produce more low-viability brood, a result that is consistent with the *Bracon* system because only two *X*-alleles were involved in these crosses (Mackensen 1951).

Males are normally haploid and have abortive meiosis, so that they transmit identical gametes to all of their progeny (Milne 1986). These gametes are of the same genotype as the unfertilized egg from which the male developed. Female development is initiated in an embryo that is heterozygous at the *X* locus, resulting in either a worker (nonreproductive female) or queen individual, depending on the nutrition the larva receives. Individuals that are homozygous at the *X* locus develop as diploid drone larvae. The workers of the colony normally eat the diploid drone larvae soon after the egg hatches, therefore adult diploid drones are not normally found in colonies (Woyke 1963a, b). However, these larvae can be rescued and adult diploid drones reared (Woyke 1969). Diploid drones have reduced testes, but in other respects appear to have a normal phenotype.

Instrumental insemination with the semen of a single drone is possible, although a queen honey bee mates naturally with about 12–17 drones [see Page (1986), for review]. If a queen is inseminated with the semen of a single drone that carries the same sex allele as one of hers, half of the progeny develop as diploid drone larvae. This results in an uneven distribution of viable larvae and empty cells in the wax brood combs, because the diploid drone larvae are eaten by the workers of the colony (Fig. 1). The appearance of many empty cells, along with an uneven age distribution of the larvae, is a colony-level trait that is called "shot-brood" by beekeepers. We will use the term "shot-brood" to refer to 50% brood mortality due to the production of diploid drones. In this paper, we report the mapping of the *X* locus of the honey bee and the production of primer sequences that define a sequence-tagged site (STS) linked to this locus.

Materials and methods

Crosses and colony evaluations

A virgin queen was instrumentally inseminated with the semen of a single, unrelated drone (a haploid male; Laidlaw 1977). An F_1 daughter queen was raised (Laidlaw and Eckert 1962) and she provided haploid sons that were used for single-drone, backcross inseminations of queens from one of the parental lines (referred to hereafter as the test queens; Fig. 2). The inseminated test queens were introduced into separate, queenless colonies.

Ninety-eight colonies were evaluated weekly for the shot-brood colony-level phenotype: uneven spatial distribution of capped and empty brood cells in the comb, and an uneven age distribution of the larvae in brood cells. A cage (6 × 9 cm) was placed over eggs that had recently been laid by the queen in worker cells. The cages allowed workers access to feed the developing larvae but the mesh was too small to allow the larger-sized queen to enter and lay eggs in empty

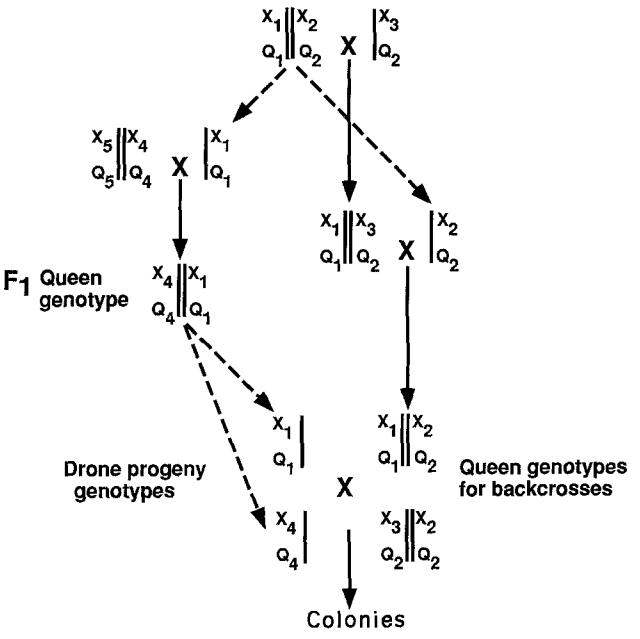

Fig. 2 Mating scheme and the inferred genotypes at the *X* and *Q* loci. Diploid genotypes represent reproductive-caste females (queens) and haploid genotypes are males (drones). Four different genotypic combinations occur (at bottom of Figure) when the drone progeny of the F_1 queen are backcrossed to the test queens, one of which resulted in shot-brood colonies due to homozygosity of the X_1 allele. *Solid lines* represent diploid progeny of a cross, *dashed lines* represent drone progeny

cells that previously contained diploid drone larvae. The proportion of cells that contained eggs was estimated, followed 5–7 days later by an estimation of the proportion of cells that contained larvae (eggs require 3 days to hatch). The date on which a queen began laying eggs and the uniformity of the laying pattern were recorded. Any diseases that might interfere with the evaluation were noted. After 2–6 weeks of observation, a colony was designated as having the shot-brood phenotype if it consistently had 50% or higher brood mortality, as determined by the above methods, and if no major disease problem existed in the colony. If at any time an area of approximately 250 identically aged larvae was observed, the colony was removed from the shot-brood category.

A few colonies did not have a large area of egg-containing, contiguous cells, due either to the egg-laying behavior of the queen or worker oophagy. Queens from these colonies were caged in queen excluder cages (12 × 12 cm) in one of two large, queenless colonies. The queens then filled the cells with eggs and were removed. The cage was left in place and the brood viability was evaluated as above (Fig. 1).

Diploid drone rearing

Four diploid drones were reared from a colony that had the shot-brood phenotype. This was done using a method similar to that of Woyke (1969). A frame containing a wax comb full of eggs was removed from the colony, wrapped in wet paper towels and placed in an incubator at 33° C until eggs hatched. Larvae were then transferred to detached queen cells containing fresh royal jelly (produced by worker bees in a colony set up for queen-rearing) and maintained in the incubator at 95% relative humidity. They were transferred to fresh cells containing new royal jelly at 12 h-intervals and were transferred to queen cells in a colony after 48 h. Then, after 1 or 2 days they were transferred to drone cells in the same colony, where they matured.

DNA extraction

Bees were frozen on dry ice and DNA was isolated. Individual bees were ground with plastic pestles in microcentrifuge tubes that contained CTAB extraction buffer (1% hexadecyltrimethyl ammonium bromide, 0.75 M NaCl, 50 mM TRIS-HCl (pH 8), 10 mM EDTA and 100 µg/ml of Proteinase K). Samples were incubated at 60° C for 2 h and extracted with phenol/chloroform and then with chloroform. One-third of a volume of 1.5 M NaCl, 50 mM TRIS-HCl (pH 8) was then added to prevent CTAB precipitation. The DNA was precipitated with one-third volume of 0.3 M sodium acetate (pH 5) and two volumes of ethanol. Following centrifugation for 10 min at 4000 × *g*, the precipitate was washed with 70% ethanol and resuspended in 10 mM TRIS-HCl (pH 7.6), 1 mM EDTA. The DNA was then quantified with a fluorometer (Hoeffer) and diluted to 3 ng/µl in 10 mM TRIS-HCl, 0.3 mM EDTA pH 7.6.

Polymerase chain reactions and linkage analysis

Random amplified polymorphic DNA (RAPD) markers were generated in polymerase chain reactions (PCR) using 10-base primers of arbitrary sequence (Operon Technologies, Alameda, Calif.). Reaction conditions were essentially the same as previously reported (Williams et al. 1990). Each primer was used singly (at 0.3–0.5 µM) in 12.5 µl reactions with 3 ng of genomic DNA from individual bees. PCR was continued for 45 cycles of: 1 min at 94° C, 1 min at 35° C, a 2 min temperature ramp to 72° C, followed by 2 min at 72° C (in a Perkin Elmer 480 Thermal Cycler). All PCR products were resolved in gels containing 0.6% agarose and 1% Synergel (Diversified Biotech, Newton Centre, Mass.).

Two hundred and sixty-five segregating RAPD markers produced from 95 of the drone fathers of the backcross colonies were tested for association with the shot-brood phenotype. Most of these markers (233) mapped to linkage groups that together span over 2100 cM (a LOD score for linkage of at least 3.0 and recombinant fraction of no more than 0.32 were the criteria for linkage). Markers that are associated with the shot-brood phenotype may be linked to the *X* allele that became homozygous in some backcross progeny (resulting in diploid drones). In order to map the *X* locus more precisely, 181 workers and 4 diploid drone siblings from a single backcross colony exhibiting the shot-brood phenotype were scored for a RAPD marker associated with this phenotype. Linkage analysis was performed with MAPMAKER software using the Kosambi mapping function [Lander et al. 1987; version 2 was provided by S. V. Tingey (E. I. duPont Nemours and Co. Wilmington, Del.)].

A PCR product was cloned by removing a portion of the band from a low-melting point agarose gel, digesting with Gelase (Epicentre Technologies, Madison, Wis.) and ligating into linearized, T-tailed Bluescript plasmid (Stratagene, La Jolla, Calif.). T-tailed vector was made by digesting the vector with *Eco*RV, and then incubating for 10 min, 72° C in PCR buffer in the presence of 0.2 mM dTTP and *Taq* polymerase (P. R. Ebert, unpublished). The ends of the cloned fragment were sequenced using the double-stranded dideoxy method (Sanger et al. 1977). The sequence information was used to design two longer primers, 21 bases in length, in order to amplify products from a single locus (hereafter called the "*Q* locus") that could be used as a sequence-tagged site (STS; Olson et al. 1989; Paran and Michelmore 1993). Primer sequences were: 5′-AGTGCAGCCAGCTACTGAGAG and 5′-AGTGCAGCCACGTGCCTGAAT. Amplifications with these primers were performed using 35 cycles consisting of: 1 min at 94° C, 1 min at 55° C, 2 min at 72° C.

Results

About twenty-five percent of the backcross colonies (25 of 98) showed the shot-brood phenotype (see Fig. 2 for mating scheme and inferred genotypes). This suggests that the matching *X* allele, designated X_1 in Fig. 2, was segregating in both the drone progeny of the F_1 queen and in the test queens to which they were mated. Thus, one-quarter of the matings involved only two sex alleles and resulted in the production of diploid drones that were subsequently removed by the workers, causing the shot-brood colony phenotype. One RAPD marker, Q16-0.58, was shared by 22 of the 25 drone fathers of the shot-brood colonies and was inherited from the recurrent parental line, suggesting linkage to the *X* locus. This marker appeared as a fragment-length polymorphism in the haploid drones. Segregation analysis indicated that another marker, R20-1.1, mapped 6.6 cM from Q16-0.58.

Segregation of the RAPD marker Q16-0.58 was followed in a second group consisting of 4 diploid drones and 181 of their diploid worker sisters. The biparental origin of the diploid drones was confirmed by three observations.

1. The four drones had abnormally reduced testes, which are a feature of diploid drone honey bees (our observations; Woyke 1986).
2. The drones also inherited codominant RAPD marker alleles from both parents that were revealed by PCR with primer K15 (Fig. 3).
3. In addition, the drones showed a tan to mottled body color and could be distinguished from their haploid, uniparental brothers that had a black body color.

Further linkage analysis indicated that black body color resulted from expression of a single recessive gene (Hunt and Page, unpublished data). We believe this gene is the major factor *black* (*bl*), reported by Laidlaw and el-Banby (1962), which Woyke proposed to have three alleles (Y, y^{ac}, y^{bl}; 1977).

Heterozygotes for marker Q16-0.58 could be distinguished by heteroduplex bands that were formed from the alternative allele fragments (Fig. 4B). The heterozygous phenotypic pattern could be generated by mixing, in the absence of polymerase activity, PCR products from drones exhibiting alternative alleles [data not shown; see Hunt and Page (1992), for a description of heteroduplex formation]. All of the worker (nonreproductive female) progeny in this shot-brood colony are expected to have identical heterozygous genotypes at the *X* locus because they all inherited the X_1 allele from their haploid father and the X_2 allele from the queen (Fig. 2). Therefore, they should also have identical heterozygous genotypes at any RAPD marker locus tightly linked to *X*, except for rare recombinants. Similarly, the diploid drones should be homozygous at

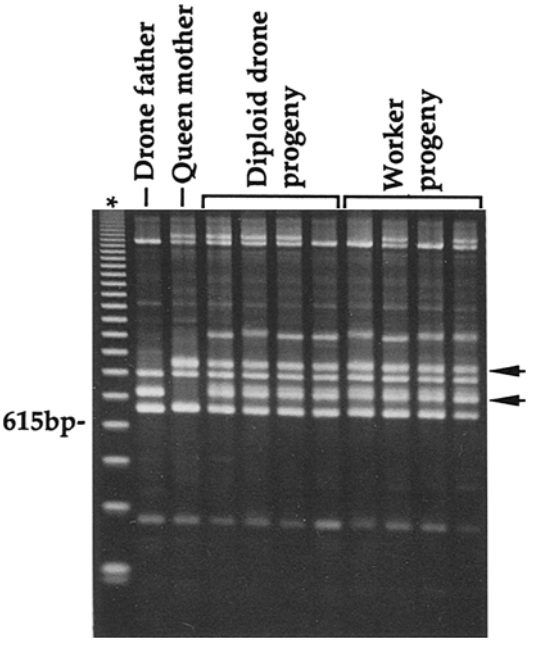

Fig. 3 PCR products from individual template DNA samples of diploid drones, their parents, and worker siblings, obtained using primer K15. The diploid drones inherited both alleles of a codominant RAPD marker (*arrows*), demonstrating their biparental nature [the 123 bp molecular weight ladder is indicated by the *asterisk*]

X, and at any tightly linked RAPD marker locus. The diploid drones were homozygous for the RAPD marker, and 178 of 181 of the workers were heterozygous. Three workers (1.6%) showed recombinant genotypes with banding patterns identical to the diploid drones (Fig. 4B; recombinants not shown in figure). A recombinant fraction of 1.6% corresponds to a map distance of 1.6 cM between *X* and the linked marker (Kosambi 1944).

The STS primers were used to generate PCR products from the *Q* locus of 60 of the drone progeny of the F_1 queen, the four diploid drones, and 109 of their sisters. All reactions amplified a single locus that cosegregated with the original RAPD marker (Fig. 4A and B). The primers were also used in reactions with some of the test queens. The marker segregated among them, with some queens exhibiting a heteroduplex pattern and some just a single band. Only three distinguishable *Q* alleles were present in the test colonies, as shown at the bottom of Fig. 2, even though four *X* alleles were present.

The *Q* locus genotypes of 15 of the test queens of shot-brood colonies were determined. Fourteen of these queens had the same heterozygous genotype at the *Q* locus. This is consistent with Fig. 2, demonstrating that, barring recombination, all of these queens must have the same *X*-allele genotype and the same *Q*-allele genotype. The one queen of a shot-brood colony that was homozygous at the *Q* locus probably

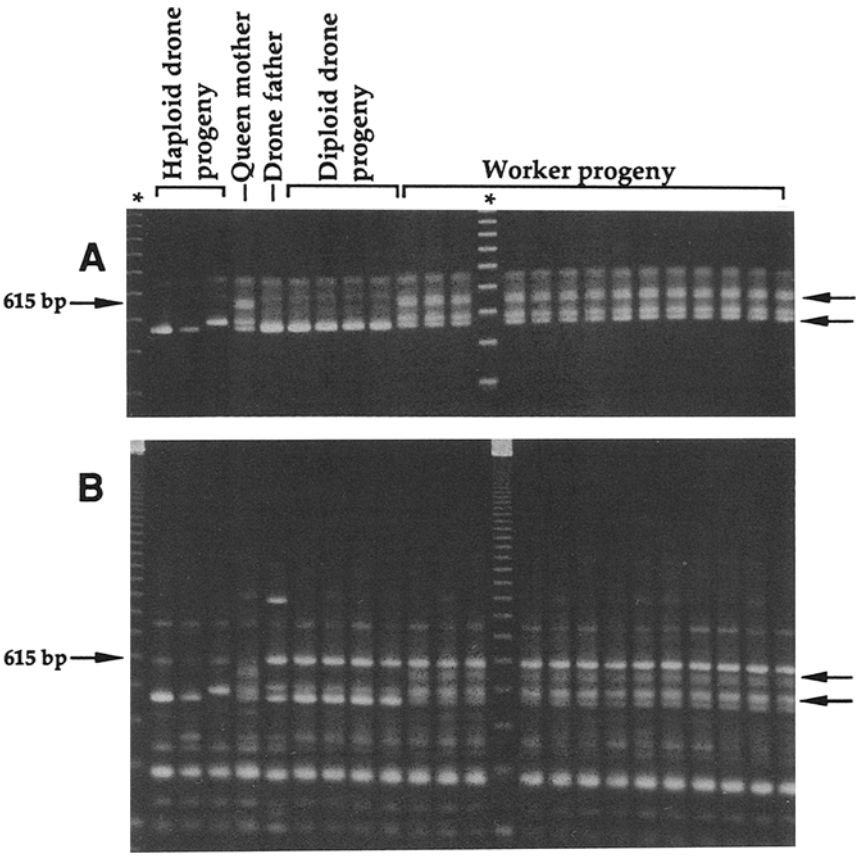

Fig. 4A, B PCR products from template DNA samples of individual honey bees taken from a colony that had 50% brood mortality due to the production of inviable diploid drones. PCR used either STS primers (**A**) or the 10-nucleotide primer Q16 (**B**). The same individuals appear in corresponding lanes of the two panels to illustrate that only a single locus is amplified by the STS primers. The pair of bands appearing as a fragment-length polymorphism (RAPD marker Q16-0.58; *lower arrow* in both panels) segregated in the haploid drone progeny of the queen. The two fragments of this fragment-length polymorphism reanneal to form heteroduplex bands in heterozygous individuals (*upper arrow* in both panels). The four diploid drone progeny of the queen were homozygous for the lower band of the fragment-length polymorphism, which all diploid progeny inherited from the drone father, but only three (not shown) of 181 worker siblings were homozygous, indicating linkage to *X*. These 3 homozygous workers were recombinants with respect to the *X* locus (molecular weight markers are indicated by the *asterisk*)

represents an error in scoring the shot-brood phenotype. In contrast, sixteen of twenty-two queens (72.7%) that headed colonies with high brood viability were homozygous at *Q*. This is not significantly different from the expected frequency of about 66% homozygotes at the *Q* locus. This expected frequency is based on the reasoning that about half of the heterozygous queens have brood with high viability (due to the 50% probability of mating with an X_4 drone), but nearly all of the homozygous queens have high brood viability.

Discussion

Our results validate previous evidence for a single locus for honey bee sex determination. Previous studies were based on a one-to-one segregation of the shot-brood phenotype (Mackensen 1951). We covered the genome at an average spacing of less than 10 cM between markers and found only one locus that mapped within the established criteria. This research also demonstrates the feasibility of mapping a gene for a colony-level trait. The trait, shot-brood, is a complex trait resulting from the interaction of individual larvae with adult workers. Larvae that are homozygous at the *X* locus produce a substance that adult workers can perceive. Larvae that produce this substance are eaten, resulting in the shot-brood colony phenotype.

The RAPD marker Q16-0.58 is closely linked to the *X* locus. Three of the 25 drone progeny of the F_1 queen appeared to be recombinant with respect to Q16-0.58 and the *X* locus, yielding a map distance of 12.3 cM. However, we based our estimate of 1.6 cM on segregation analysis of the 185 progeny of the shot-brood colony, because of the larger sample size and because data based on individual phenotypes are more robust.

One of the queens mated to an apparently recombinant drone was the only *Q*-locus homozygote observed that produced a shot-brood colony. As shown in Fig. 2, homozygotes are not expected unless there was a crossover between the *Q* locus and *X*, an unlikely event, given the tight linkage observed. It is more likely that an error was made in the determination of the phenotype of this colony. Such errors result in overestimation of the number of recombinants when using the colony-level phenotype. It also is possible that one or both of the other two colonies that were fathered by the apparently recombinant drones had been improperly scored. Difficulties arose in assigning shot-brood phenotypes when diseases such as chalkbrood and foulbrood occurred, or when the queens laid eggs in an

uneven pattern in the cells of the brood combs. In our analysis using individual phenotypes, map distance would be underestimated if there was reduced female viability due to homozygosity of genes linked to the *Q* locus. However, such a situation would require sex-limited gene expression because segregation was not skewed from 1:1 in the haploid males ($\chi^2 = 1.53$, 1 df).

The primers that define an STS linked to the *X* locus will be useful for population genetic studies. The sex determination system of the honey bee maintains many alleles in a population, due to strong selection against common alleles that result in diploid drones, and due to strong selection for rare alleles (Wright 1939; Yokoyama and Nei 1979). This selection is present in all species of Hymenoptera that have a single-locus sex determination system. In these species, the production of diploid drones is never completely eliminated but they are selected against, due to infertility (Hartl and Brown 1970; Bull 1981). Preliminary results suggest that the *Q* locus primers will be useful markers. The primers were used to amplify from the DNA of 44 unrelated European and African honey bees, resulting in many banding-pattern phenotypes, of which 26 appeared to have heteroduplex bands (our unpublished data).

Although the *Q* locus may be hundreds or thousands of kilobases from the *X* locus, this STS could serve as a starting point for cloning the locus. The eventual cloning of the *X* locus would allow studies that compare haplodiploid sex determination with sex-determining mechanisms that have already been well characterized. In *Drosophila*, sex is determined by the ratio of the X chromosomes to autosomes. Products of X-linked genes serve as counting elements of the X chromosome by forming heterodimers with proteins that are expressed maternally in excess (Cline 1988; Parkhurst et al. 1990; Erickson and Cline 1991). These helix-loop-helix proteins act as transcription factors for the master sex-determining gene *Sex-lethal* (*Sxl*). *Sxl* initiates the cascade of sex-determining genes. Although it would seem that the Hymenoptera have a very different system, it has been suggested that the products of two different *X* alleles may form an active heterodimer (Crozier 1971). It is possible that these heterodimers detect heterozygosity at the *X* locus and serve as the unambiguous signal for sex determination.

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