Quantitative Trait Loci for Honey Bee Stinging Behavior and Body Size

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Manuscript received August 21, 1997
Accepted for publication December 1, 1997

ABSTRACT

A study was conducted to identify quantitative trait loci (QTLs) that affect colony-level stinging behavior and individual body size of honey bees. An F_1 queen was produced from a cross between a queen of European origin and a drone descended from an African subspecies. Haploid drones from the hybrid queen were individually backcrossed to sister European queens to produce 172 colonies with backcross workers that were evaluated for tendency to sting. Random amplified polymorphic DNA markers were scored from the haploid drone fathers of these colonies. Wings of workers and drones were used as a measure of body size because Africanized bees in the Americas are smaller than European bees. Standard interval mapping and multiple QTL models were used to analyze data. One possible QTL was identified with a significant effect on tendency to sting (LOD 3.57). Four other suggestive QTLs were also observed (about LOD 1.5). Possible QTLs also were identified that affect body size and were unlinked to defensive-behavior QTLs. Two of these were significant (LOD 3.54 and 5.15).

THE defense of the honey bee colony is a social behavior. Colony defense is the result of the guarding and responding behaviors of worker bees (a nonreproductive female caste). "Guards" are found at the colony entrance, antennating incoming workers (Ribbands 1954). They identify and forcibly remove bees that are foreign to the nest. "Responders" fly out and sting a target, usually an intruder serving as a stimulus for defensive behavior. When a responder stings a target, the sting apparatus detaches and the bee soon dies. Alarm pheromones are released from the sting apparatus and provide additional stimuli for stinging. In a study using North American honey bees (derived from European subspecies), Breed *et al.* (1990, 1992) provided evidence for a heritable component for guarding and stinging behaviors and suggested that they are performed by different subfamilies within the colony.

An African subspecies of honey bee, *Apis mellifera scutellata*, was introduced into Brazil in 1956 and has spread rapidly throughout most of South and Central America. This subspecies is slightly smaller and much more defensive than the European honey bees that are most commonly used for beekeeping (Kerr 1967). Because of the smaller size of the *A. m. scutellata*, morphometrics and simple wing-length measurements (that correlate with overall size) have been the most common methods to distinguish them from our European honey bees. Over the years, there has been hybridization between the two subspecies but a predominantly African genotype seems to persist in tropical regions (Hall and Muralidharan 1989; Smith *et al.* 1989; Sheppard *et al.* 1991). Africanized worker bees are more likely to respond to stimuli for defensive behavior by stinging than are most European bees (Stort 1974; Stort 1975a,b,c; Collins and Kubasek 1982; Collins *et al.* 1982, 1988; Villa 1988; Guzmán-Novoa and Page 1994a). Collins *et al.* (1982) demonstrated that worker honey bees from Africanized colonies in Venezuela responded to a visual target stimulus 20 times faster than Europeans and deposited 8.5 times more stingers in the target in 30 sec.

There are several related components that can serve as measures of stinging behavior: the probability of stinging, the time to respond to a stimulus for stinging, the number of individuals recruited to sting a target, and the distance that responders will pursue the target. Stort (1975a) crossed Africanized bees with Europeans and concluded that the time to receive the first sting in a stimulus target (a small, black leather ball) showed dominant inheritance of the more reactive Africanized genotype. Guzmán-Novoa and Page (1993, 1994a) found that increasing the proportion of Africanized parentage in a colony caused an increase in the number of stings and the speed of response. But colonies consisting of all hybrid workers resembled the Africanized behavioral phenotype for the numbers of stings, indicating dominance for the colony-level response in their study in Mexico.

Social insects could be important models for identifying genes affecting colony defensive behavior, sometimes referred to as aggression. As a model organism, honey bees have some advantages compared to the vertebrate species that have most commonly been used in

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behavioral genetic studies of aggression (reviewed by Maxson 1992, 1996). Insects have nervous systems with reduced complexity. Honey bee behavioral traits that have a demonstrated genetic component include stinging (Collins *et al.* 1982; Moritz *et al.* 1987), foraging for pollen or nectar (Hellmich *et al.* 1985; Robinson and Page 1989), undertaking (the removal of dead bees from the nest; Rothenbuhler 1964) and learning (Brandes 1991; Bhagavan *et al.* 1994). Honey bees have large family sizes (usually in the tens of thousands) and haploid males (drones). A drone transmits an identical gamete to each of his several thousand worker progeny, thus facilitating progeny testing within the colony. Recently, two loci that affect foraging behavior of honey bees were identified. These quantitative trait loci (QTLs) had a major effect on the amount of pollen stored in bee hives and influenced the resource choice of individual foragers (Hunt *et al.* 1995; Page *et al.* 1995).

In the current study, we measured the speed and intensity of the colony-level stinging response in 162 colonies of backcross workers and rated several defensive behavioral traits observed during colony manipulations by a beekeeper. Wing lengths were measured as an indication of average body size of workers and drones. We show evidence for several QTLs affecting the intensity of colony stinging response and for QTLs that influence the size of workers and drones. Specific primer sequences are provided to aid in independent confirmation of results.

MATERIALS AND METHODS

Summary: Relatively nondefensive stocks of European honey bees were selected from a commercial operation in Mexico and intercrossed by single-drone insemination of queens to produce a selected European population. Very defensive Africanized stocks also were produced by collecting feral swarms, testing for defensive behavior and making crosses by single-drone insemination (Figure 1). Then, the Africanized queens were each tested for their ability to produce highly defensive hybrid workers. One of these Africanized queens was chosen to provide the haploid drone father of the F_1 queen. Drones from the F_1 queen were each individually crossed to sister queens from a single European colony. After these queens were introduced into new colonies, the colonies containing their progeny were tested for defensive behavior. The haploid drone fathers of these colonies were analyzed for the segregation of random amplified polymorphic DNA (RAPD) markers to identify associations between marker loci and colony defensive behavior.

Source of European bees: Initial selection of European stocks came from a foundation group of about 3000 colonies from a cooperating commercial apiary, Miel Vita Real, located in Ixtapan de la Sal, Mexico. Foundation colonies contained queens of European origin that were naturally mated to ten to twenty drones (Figure 1). These colonies ($\sim$250) were tested for their colony-level defensive response with the quantitative test described below. Drones and queens were raised from 40 colonies that stung the target the fewest times. Prior to making the crosses, colonies were tested with a mitochondrial DNA assay that distinguishes the African mitotype found in *A. m. scutellata* from all European races that have been found in the southwestern United States and Mexico (D. Nielsen, P. Ebert, G. Hunt, E. Guzmán-Novoa, S. Kinnee and R. Page, unpublished data). The mitotype of bees selected for crosses was consistent with a European origin. Bees were also analyzed morphometrically to insure that they had European morphology (Daly and Balling 1978; Sylvester and Rinderer 1987). Crosses were made by instrumentally inseminating virgin queens from selected European colonies with single drones from other selected European colonies. Colonies resulting from these crosses were again tested for defensive behavior, and the least defensive colony was selected to provide a daughter queen to serve as the mother of the F_1 queen.

Sources of Africanized bees: Fifteen colonies of Africanized honey bees were established from captured feral swarms (Figure 1). Colonies were tested in the same way as the European lines in order to insure African-type morphometrics, mitochondria, and behavior. Virgin queens and/or drones were raised from each of these colonies and single-pair matings were made between them by artificial insemination. A daughter queen was raised from each cross, and instrumentally inseminated with pooled European semen to establish forty test colonies. Semen pools were derived from drones that came from several related queens. The test colonies of these individual Africanized queens then contained hybrid workers that were evaluated for defensive behavior (see Mapping Population). The colony that gave the highest number of stings in the assay was chosen as the source for the single haploid drone that was used as the father of the F_1 queen.

Mapping population: An F_1 queen was produced by single-drone insemination between the selected European queen and the drone derived from the Africanized queen with the most defensive colony. This hybrid queen then provided the drones used to backcross to virgin queens from the selected European colony. As a consequence of developing from unfertilized haploid eggs, the drones of the F_1 queen represented her gametes and were used as the mapping population for determining linkage relationships. Each drone was backcrossed to a different queen from a single, preselected European colony that was unrelated to the source for the hybrid cross. Each of the European queens were supersisters because they shared the same haploid father, resulting in having at least 75% relatedness by direct descent. Because of the high relatedness of the European queens, most of the between-colony genetic variation of the QTL mapping population should have been a result of recombination and segregation of alleles in the F_1 queen. The variation between the resulting colonies was interpreted as being solely the result of the drone father's genotype. Of 313 European supersister queens that were inseminated in the backcross, $\sim$250 were introduced into separate colonies. After attrition due to loss of queens, 172 colonies that contained the backcross progeny of these queens were obtained.

Linkage mapping: The linkage map was based on the segregation of RAPD markers in 179 haploid drones which were progeny of the F_1 queen. Most of the drones (172 of them) were used as fathers of the backcross colonies. Methods for generating RAPD markers and linkage mapping were as described previously (Williams *et al.* 1990; Hunt and Page 1995; Hunt 1997a,b). All linkage group designations refer to this previously published map. Linkage group identity was confirmed by sequence-tagged sites (STSs) and segregating RAPD markers that were shared by both mapping populations. These RAPD markers were identified by observing marker fragments, generated by the same ten-nucleotide primer in two separate mapping populations that were of the same approximate size and map position relative to other common markers in both populations. RAPD markers were generated

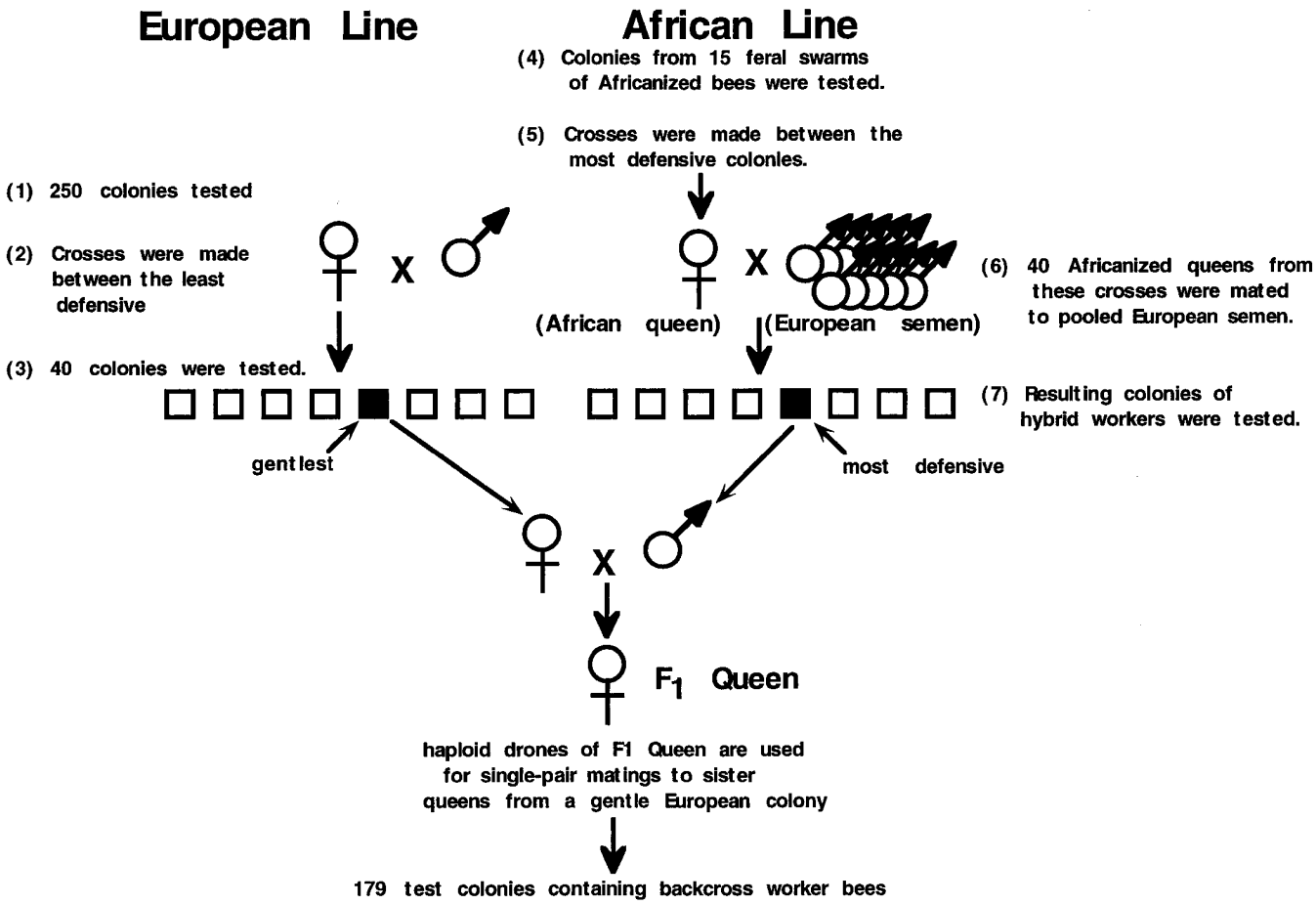


Figure 1.—Selection scheme and crosses used in this study. European honey bee queens were selected by screening 250 colonies for defensive behavior (1). Crosses were made between the least defensive colonies by raising virgin queens and mating them to single haploid drones from other gentle colonies (2). Forty progeny queens in new colonies were then tested for colony stinging behavior. A daughter queen was obtained from the queen with the gentlest colony to be the mother of the F_1 queen (3). Fifteen swarms of Africanized bees were captured, placed in hives and tested for stinging behavior (4). Crosses between the most defensive colonies (by single-drone insemination) were made (5). Virgin queens from these crosses were inseminated with pooled semen from European drones (6) and their colonies of hybrid workers were tested for stinging behavior (7). The queen with the most defensive hybrid colony was chosen to provide a haploid drone (from an unfertilized egg) as father of the F_1.

in polymerase chain reactions with 10-nucleotide DNA primers of arbitrary sequence and separated on agarose-Synergel gels (Diversified Biotech, Boston, MA). Mapmaker software was used for linkage mapping and the Kosambi mapping function was used to convert recombination frequency into map distance (Hunt 1997a). STS primers were designed by cloning RAPD marker fragments from the gel and sequencing them. These primers were used to amplify DNA from a single locus (Hunt and Page 1994). The STS primer sequences were (5′ to 3′): Q16-.58, AGTGCAGCCAGCTACTGAGAG and AGTGCAGC CACGTGCCTGAAT; N4-.245, ACCCATCACGGAAGGCAGCA TCAG and TCAAATTAAAGTCTACACTAAAAA; 25-44, AGGGC TCATTTCCAAGTAGTTTTT and TCGCGGTCACGCTATAAT CTAA; 335-.6, CCCCGAGTGGTATCCCGAGA and GACCACC CTGCCATAATACGA; O7-.76, GGGGAAAACAAACATAGATG AATAG and TGATAGTGTTGATCTGTTATGA; J20-.14, CGAC GTGGAGGACGTGGCTATTA and CCCCTGGCTGTTGTCGG TAGAT; 275-.38, AAGCCAAACATTCGTGGATAA and ATTAA GAATTAAAAGATTCAGATA; C9-.63, CTCACCGTCCTCCCC GAATC and CTCACCGTCCCAAAAATAGA; C2-.75, TCCCTG ACTTTTGAGGTTACA and AACGCGTAATTCTTTTTTTTT TTT; 275-.67, GGGCAAGCTCGATACAACAAG and GCACGA CACGCACACCGACAC; 375-.31, CGACGGAACAGTATATGA CTT and CGATTCGTAGAGGATCATAA; Q4-.62, TTTGACAT CTTTTCGGTTTT and AAAATGAAGAGGATAAATGG; 456-.81,AGGTCCCCGTGTAGAAACTAAG and CGGAGGTCCAA GATTTCTGAC; 403-1.0, GGCTGTCGAAGAAATAAAGAAA and GCTGTCAATGTAAGAGGTTGT.

Stinging behavior assay: We tested 162 of the 172 colonies containing backcross workers with a stinging behavior assay (Villa 1988; as modified by Guzmán-Novoa and Page 1993). Test colonies were maintained in standard "jumbo" hives consisting of one deep box for combs with brood and a shallow box on top (a super) for honey storage. Two weeks prior to the test, the colonies were made approximately equal in numbers of workers by removing bees and brood from the larger colonies. In this test, a black suede leather patch (the target) attached to the end of a one meter white stick was moved back and forth by hand in a rhythmic way, directly in the colony entrance. The time required for the first sting to occur was recorded for each colony. The bees were allowed to continue stinging the patch for one minute after the first sting. If no stings occurred within two minutes, the test was discontinued for that colony. All of the colonies in each apiary

were tested simultaneously to minimize behavioral interference between colonies; for example, bees of one colony stinging the targets of other colonies nearby. Test colonies were kept in nine apiaries and each colony was tested on four separate occasions.

Each backcross colony also was scored twice for other behavioral traits that differ between European and Africanized strains of bees as follows: (1) the tendency of the workers to sting during manipulations, (2) tendency to "hang" from combs, (3) tendency to "run" on the combs (a trait commonly referred to as nervousness) and (4) tendency to fly up during colony manipulations. High values for all of these traits are characteristic of Africanized bees. The top lid of the hive was carefully opened and two puffs of smoke were given to the top bars of the super frames containing wax combs. Smoke normally "calms" the bees. Then, the super box was removed and four puffs of smoke were given to the top bars of the brood frames. Four frames of brood were removed, one at a time, and inspected. The colony was then scored on a 1–5 scale for tendency to sting, hang, run, and fly. Scores for stinging behavior were based on an actual count of the number of times the bees stung the beekeeper's hands during the two hive opening events (a score of 1 = 0 stings, 2 = 1 sting, 3 = 2–3 stings, 4 = 5–10 stings, 5 = 10–20 stings. The range of average ratings for stinging was from 1 to 4.5. "Hanging" scores were based on the approximate proportion of bees hanging from the comb (1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, 5 = 100%). "Runniness" and "flying" were scored on a relative scale from 1 to 5 based on the researcher's experience.

Wing length measurements: Wings were measured to identify QTLs that affected size differences between European and Africanized bees. Twelve worker forewings were measured from each of the test colonies and an average value was assigned based on the FABIS technique (see Sylvester and Rinderer 1987). One forewing of the drone father of each colony was also measured. Wings were mounted on projection slides and projected on a flat surface such that 1 cm distance represented 50 cm when projected.

Statistical analyses: Most QTL analyses made use of the software package MapQTL (Van Ooijen and Maliepaard 1996). For parametric analyses, QTL cartographer software was also used to determine when a particular QTL met the experimentwise threshold for 95% confidence of declaring a linked QTL (Basten *et al.* 1994, 1997). This was done by permuting the phenotypic data 1000 times to calculate an empirical threshold value for the LOD score (Churchill and Doerge 1994). In this process, QTL cartographer shuffles the phenotypic data and reassigns the phenotypes to individuals at random. Then, interval mapping is used to obtain LOD scores that are calculated from the data of this shuffled data set. After 1000 permutations, it is possible to obtain an empirical probability estimate for obtaining LOD scores as high as those calculated from the actual phenotypic data and genotypes.

Parametric analyses (interval mapping) were used on forewing length and number of stings in the patch. First, standard interval mapping procedures were used to identify the major QTLs (Lander and Botstein 1989). Then, we used the restricted multiple QTL model (MQM) feature of MapQTL to fit more than one QTL at a time. This feature uses the marker closest to the QTL as a cofactor in the likelihood equation in order to account for the portion of the variance that can be attributed to that QTL. All of the cofactors for putative QTLs with LOD scores above a predetermined threshold are used, except those on the linkage group currently being scanned. The MQM can result in greater power for detecting QTLs with real effects and may increase the accuracy of QTL mapping (Jansen 1993, 1994; Jansen and Stam 1994; Zeng 1993, 1994). We used a marker as a cofactor if it was associated with a LOD score of at least 1.5. If the multifactor analysis decreased the LOD score to below 1.5, that marker was then dropped as a cofactor and the analysis was repeated. If a marker increased to LOD 1.5 in the multifactor analysis, this new marker was added to the cofactor list and the analysis was repeated until there was no further change in cofactors.

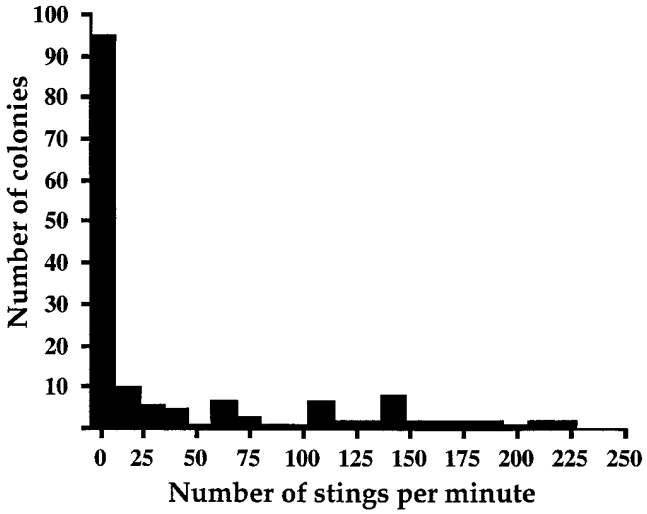


Figure 2.—Histogram for the rate of stinging of colonies containing backcross workers in the fourth defensive-behavior assay.

Only data for the number of stings in the patch of the last (fourth) defensive behavior trial was used because the number of stings increased with repeated testing. A repeated-measures analysis of variance using the marker that had the highest correlation with number of stings as the independent variable indicated a significant difference between trials for the number of stings ($P = 0.0001$). The distribution for the number of stings in the patch was nonnormal because about half of the colonies never stung the patch, or stung <10 times on any of the 4 defensive behavior trials. Other colonies stung an average of 150 times per minute, and over 200 times per minute in the last trial (see Figure 2). The nonnormal distribution of the sting data violates the assumptions of interval mapping, which is an extension of a model I analysis of variance. However, analysis of variance is a robust test, even with deviations from normality (Neter *et al.* 1990). In addition, the

Figure 3.—Possible quantitative trait loci influencing the colony stinging response. (A) *Sting-1* on linkage group IV. The significance level determined by permutation tests appears near the peak LOD score. (B) A possible QTL on linkage group III. (C) Other possible QTLs affecting colony stinging behavior. Behavioral scores (tendency to fly up, hang from comb and to sting) were made during colony inspections. Kruskal-Wallis tests at marker loci were performed to evaluate effects of QTLs on these behavioral scores ($P < 0.05$*; $P < 0.01$**; $P < 0.005$***; $P < 0.0001$******). Linkage group numbers are from Hunt and Page 1995. RAPD markers are designated by the primer names followed by approximate size of the marker fragment. Markers indicated by arrows were common to both linkage maps. Sequences for the sequence-tagged sites are designated by the "sts" prefix. Results shown are the output of multiple QTL model interval mapping (MQM).

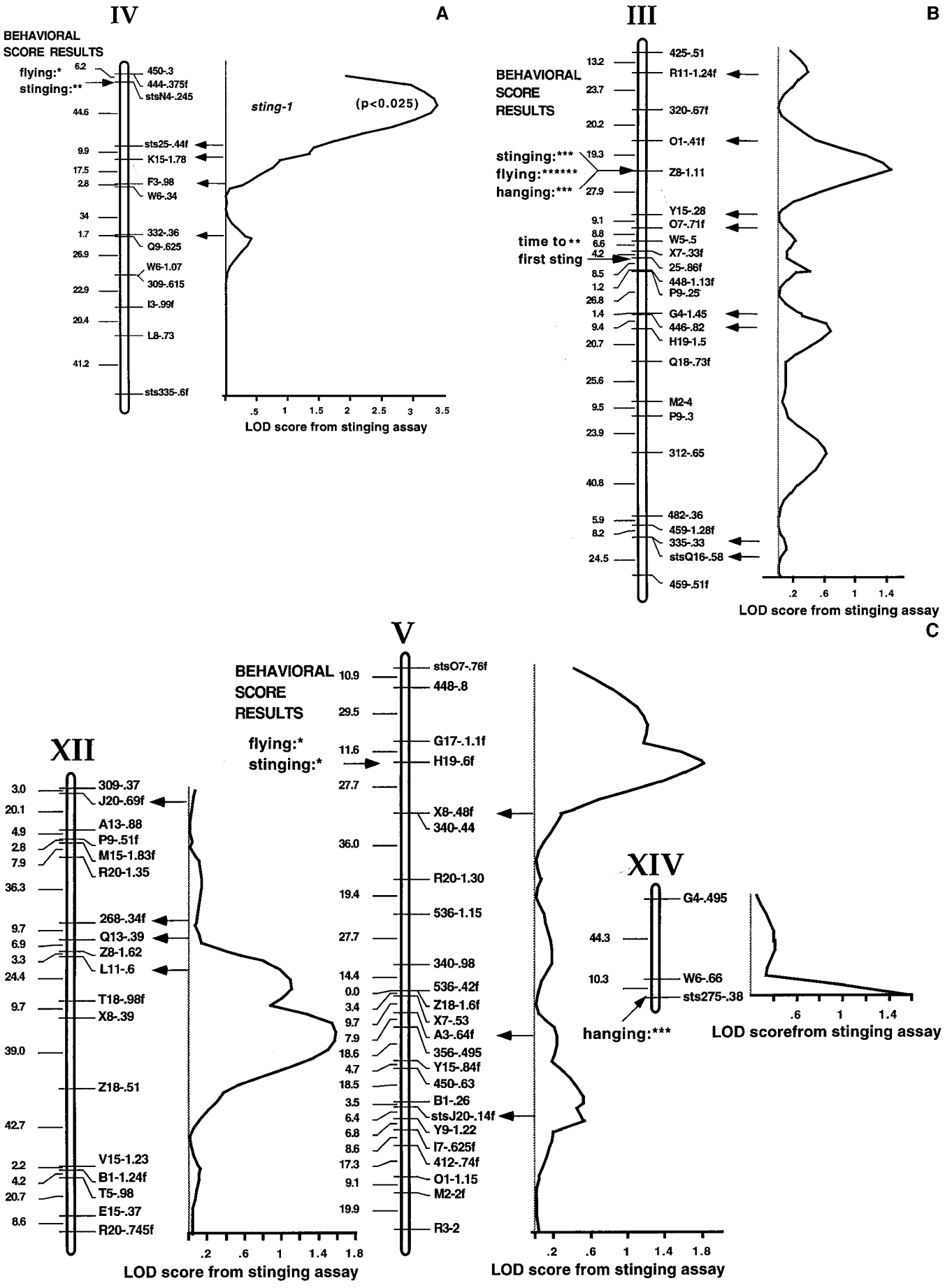

A
IV
BEHAVIORAL SCORE RESULTS
flying:*
stinging:**
6.2
450-.3
444-.375f
stsN4-.245
44.6
9.9
sts25-.44f
K15-1.78
17.5
2.8
F3-.98
W6-.34
34
1.7
332-.36
Q9-.625
26.9
W6-1.07
309-.615
22.9
I3-.99f
20.4
L8-.73
41.2
sts335-.6f
sting-1
(p<0.025)
.5 1 1.5 2 2.5 3 3.5
LOD score from stinging assay
B
III
BEHAVIORAL SCORE RESULTS
stinging:***
flying:******
hanging:***
time to** first sting
13.2
425-.51
R11-1.24f
23.7
320-.67f
20.2
O1-.41f
19.3
Z8-1.11
27.9
Y15-.28
9.1
O7-.71f
8.8
6.6
W5-.5
4.2
X7-.33f
25-.86f
8.5
1.2
448-1.13f
P9-.25
26.8
1.4
G4-1.45
9.4
446-.82
20.7
H19-1.5
Q18-.73f
25.6
9.5
M2-4
P9-.3
23.9
312-.65
40.8
5.9
482-.36
8.2
459-1.28f
335-.33
24.5
stsQ16-.58
459-.51f
.2 .6 1 1.4
LOD score from stinging assay
C
V
BEHAVIORAL SCORE RESULTS
flying:*
stinging:*
10.9
stsO7-.76f
448-.8
29.5
11.6
G17-.1.1f
H19-.6f
27.7
X8-.48f
340-.44
36.0
R20-1.30
19.4
536-1.15
27.7
340-.98
14.4
0.0
536-.42f
3.4
Z18-1.6f
9.7
X7-.53
7.9
A3-.64f
18.6
356-.495
4.7
Y15-.84f
18.5
450-.63
3.5
B1-.26
6.4
stsJ20-.14f
6.8
Y9-1.22
8.6
I7-.625f
17.3
412-.74f
9.1
O1-1.15
M2-2f
19.9
R3-2
.2 .6 1 1.4 1.8
LOD score from stinging assay
XII
3.0
309-.37
20.1
J20-.69f
4.9
A13-.88
2.8
P9-.51f
7.9
M15-1.83f
R20-1.35
36.3
9.7
268-.34f
6.9
Q13-.39
3.3
Z8-1.62
24.4
L11-.6
9.7
T18-.98f
X8-.39
39.0
Z18-.51
42.7
2.2
V15-1.23
4.2
B1-1.24f
20.7
T5-.98
8.6
E15-.37
R20-.745f
.2 .6 1 1.4 1.8
LOD score from stinging assay
XIV
44.3
G4-.495
10.3
W6-.66
sts275-.38
hanging:***
.6 1 1.4
LOD scorefrom stinging assay

TABLE 1

Results of QTL analyses for honey bee colony defensive behavior

		Stings in the patch			Other behavioral traits		
Linkage group	Marker cofactor[a]	Single QTL model LOD score[b]	Permutation *P* value[c]	MQM mapping LOD score[d]	K-W rank test for stinging[e]	K-W rank test for flying	K-W rank test for hanging
IV	stsN4-.245	3.57	<0.025	3.35	0.01	0.5	NS
III	Z8-1.11	1.55	NS[f]	1.44	0.005	0.0001	0.005
V	H19-.6f	1.73	NS[f]	1.8	0.05	0.05	NS
XII	X8-.39	1.44	NS[f]	1.56	NS	NS	NS
XIV	sts275-.38	1.85	NS[f]	1.59	NS	NS	0.005

LOD scores are reported for quantitative data on numbers of stings per minute in a target for each colony. The colonies were fathered by haploid drones from a European/Africanized F_1 queen.

[a] The RAPD or sequence-tagged-site (sts) marker used as a cofactor in the restricted multiple QTL model, or MQM feature, of MapQTL.

[b] Results from single QTL model interval mapping.

[c] Results from permuting data set 1000 times (experimentwise *P* value).

[d] Results of multiple QTL model interval mapping.

[e] Results of Kruskal-Wallis rank test at the marker for score of colony behavior during hive manipulations.

[f] NS, not significant at $P = 0.05$.

permutation tests of QTL cartographer provided an empirical threshold value that is valid for nonnormal distributions (Churchill and Doerge 1994).

The scores for the four behavioral traits rated during hive manipulations and the data on time to the first sting in the defensive-behavior assay were analyzed with the Kruskal-Wallis rank test performed by MapQTL. These analyses only evaluated effects at the marker loci. Only single-QTL models were used in these analyses.

RESULTS

Defensive response: For the number of stings in the patch, one QTL on linkage group IV was identified that met the 95% confidence threshold for controlling the experimentwise error, as evaluated by the permutation test (*sting-1*, Figure 3A). The permutation test indicated an experimentwise *P* value between 0.025 and 0.01. The LOD score for *sting-1* was 3.57 in the initial interval mapping, but decreased to 3.35 in the final analysis that fitted 3 additional sting-QTLs by using one marker for each QTL as a cofactor (multiple QTL model, or MQM mapping, see Table 1). Analysis of behavioral scores that were assigned while opening and manipulating the hives indicated that this QTL also influenced the tendency of the bees to sting the beekeeper ($P < 0.01$), and the tendency for bees to fly up as the hive was opened ($P < 0.05$).

MQM mapping indicated four other suggestive QTLs with LOD scores near 1.5. One locus on group III had a LOD score of only 1.44, but analysis of behavioral scores showed that a marker at this locus (Z8-1.11) was associated with the tendency of the bees to sting the beekeeper, to fly up, and to hang from the comb ($P < 0.005$, 0.0001 and 0.005, respectively; Figure 3B and Table 1). On group V, a possible QTL with a LOD score of 1.8 lies at marker H19-.6f. The marker genotype at this locus also had an effect both on tendency of the bees to fly up, and to sting the beekeeper ($P < 0.05$; Figure 3C). Another peak of LOD 1.56 lies close to X8-.39 on group XII, and a possible QTL (LOD 1.59) lies near the end of group XIV at the sequence-tagged-site, marker sts275-.38. This locus also may be affecting the tendency to hang from the bottom of the comb ($P < 0.005$; Figure 3C). These results suggest that stinging, hanging and flying up are correlated traits. The "stinging" QTLs may have pleiotropic effects on these other behavioral phenotypes. Overall, we did not see an increase in LOD scores when multiple QTLs were fitted to the model for analyzing numbers of stings (see Table 1, MQM results).

All of the loci influencing the numbers of stings in the defensive behavior assay had effects in the direction predicted by the parental phenotypes, with the exception of the last one mentioned, at marker sts275-.38 on group XIV. For the other four loci, the presence of the African allele at these QTLs was associated with increased numbers of stings. The same was true for the other behavioral traits that were evaluated with scores

TABLE 2

QTL analyses for honey bee body size

		Worker wing length[b]			Drone wing length[c]		
Linkage group	Marker cofactor[a]	Single QTL model LOD score[d]	Permutation *P* value[e]	MQM mapping LOD score[f]	Single QTL model LOD score	Permutation *P* value	MQM mapping LOD score
VI	R11-.43	<1.5	NS	<1.5	1.71	NS	2.56
XII	T5-.98	<1.5	NS	<1.5	1.74	NS	1.91
II	Q9-.34	<1.5	NS	2.15	<1.5	NS	1.52
II	536-.735	<1.5	NS	<1.5	1.53	NS	1.8
XI	460-.67	4.3	<0.01	5.15	1.79	NS	1.82
X	H7-.5	2.55	NS	2.2	<1.5	NS	<1.5
I	sts275-.67	2.24	<0.05[g]	3.54	<1.5	NS	<1.5

LOD scores for wing length of haploid drones (males) from a hybrid European/Africanized queen, as well as the average wing length of their diploid worker progeny.

[a] The RAPD or sequence-tagged-site (sts) marker used as a cofactor in the restricted multiple QTL model, or MQM feature, of MapQTL.

[b] Analyses based on average measurement of wings from ten backcross workers.

[c] Analyses based on measurement of wings of haploid fathers of backcross workers.

[d] Results from single QTL model interval mapping.

[e] Results from permuting data set 1000 times with simple interval mapping (experimentwise *P* value). The QTL for worker wing size on group I exceeded the threshold for the permutation with the results of MQM mapping, but permutations were done with simple interval mapping. Therefore, we are uncertain of the true experimentwise *P* value.

[f] Results from multiple QTL model interval mapping.

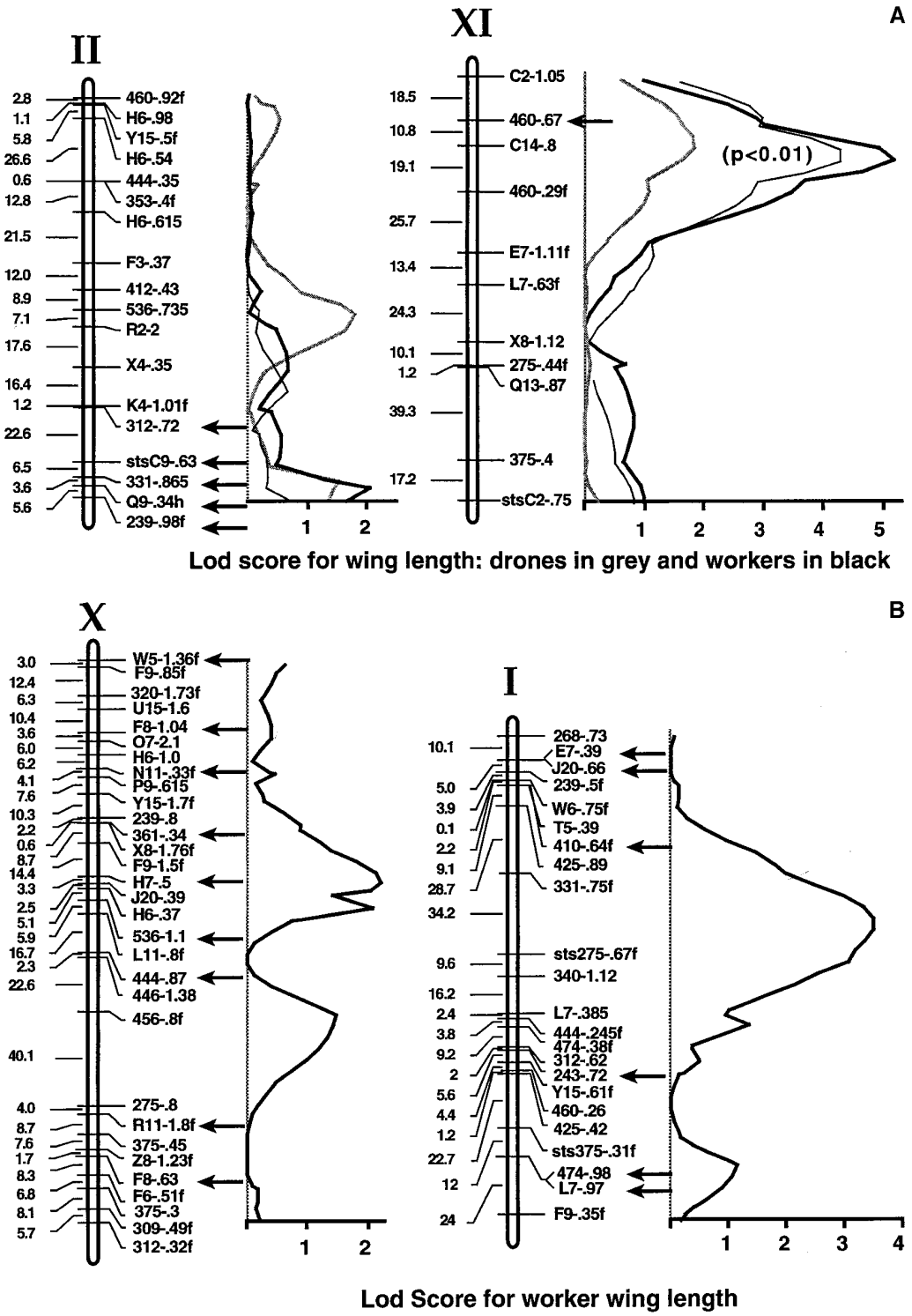
while opening the hives. African alleles increased flying, stinging and hanging. However, one exception was the effect on hanging from the comb seen on group II. For this locus, the African allele decreased the tendency to hang from the comb.

Time to sting: Time to first sting was analyzed by nonparametric tests at the markers. Loci that may have effects on the time to first sting were found at 25-.86 on group III (Figure 3A), at stsR3-.52 on group XVI and near H7-.37 on group VIII, with *P* values of 0.01, 0.01 and 0.005, respectively (last two not shown in Figures). Nonlinkage of these loci with the potential stinging-behavior loci indicates that the speed of the defensive response is independent of the intensity of the response.

Body size: All of the possible QTLs for body size (as measured by wing length) had effects in the direction that would be predicted based on parental phenotypes; the marker alleles from the Africanized parent were associated with smaller size. There were five possible QTLs that had effects on drone wing lengths and four possible QTLs that affected worker wing lengths. Two of these QTLs were found to influence the size of both workers and drones (on groups II and XI, Figure 4A and Table 2). One of the QTLs affecting worker size met the experimentwise 99% confidence level, and another met the 95% confidence threshold, according to the permutation test (on groups I and XI, Figure 4, A and B). One of these two loci had the highest LOD score of 5.15. Using a MQM increased the LOD score in this test. With simple interval mapping the LOD score for this locus was just 4.3. The other QTL (on group I) only exceeded the 95% confidence level when using MQM mapping. Therefore, we are unsure of the actual significance value at this locus because the permutations were performed with simple interval mapping. In general, there was an increase in the LOD scores with the MQM mapping of body-size QTLs. LOD scores for 3 of the 4 worker wing-length QTLs and all 5 drone wing length QTLs increased with the MQM (see Table 2 and Figure 4a). Small changes in the location of LOD score peaks were also observed with MQM mapping. For example, a minor peak for worker wing-length LOD score on linkage group II shifted toward a peak observed in drones (Figure 4A). Aside from the two significant QTLs that affected both worker and drone size, possible QTLs that influenced worker size were found on group X (Figure 4B) and two possible QTLs that influenced drone size were found on groups XII and VI (Figure 4C). There was no association observed between the wing-length QTLs and loci affecting behavior.

DISCUSSION

In the process of linkage mapping, we compared three honey bee maps that are each based on about 350 RAPD markers (Hunt and Page 1995; Page *et al.*, unpublished data). It was possible to identify linkage



Continued

groups based on observing marker fragments that were generated by the same ten-nucleotide primer, and were similar in size and map location. For example, an average of more than 4 RAPD markers were observed in common between the 9 major linkage groups presented here and the corresponding groups of the published map (Hunt and Page 1995). RAPD markers mapped in the same order in these maps. A single exception to synteny was observed with a marker near *sting-1*. In the published map, the presence of this marker (F3-.98) caused more map expansion than any other marker, suggesting errors from an incorrect order in that map. The marker did not cause map expansion in the map presented here. In addition, the current data show that

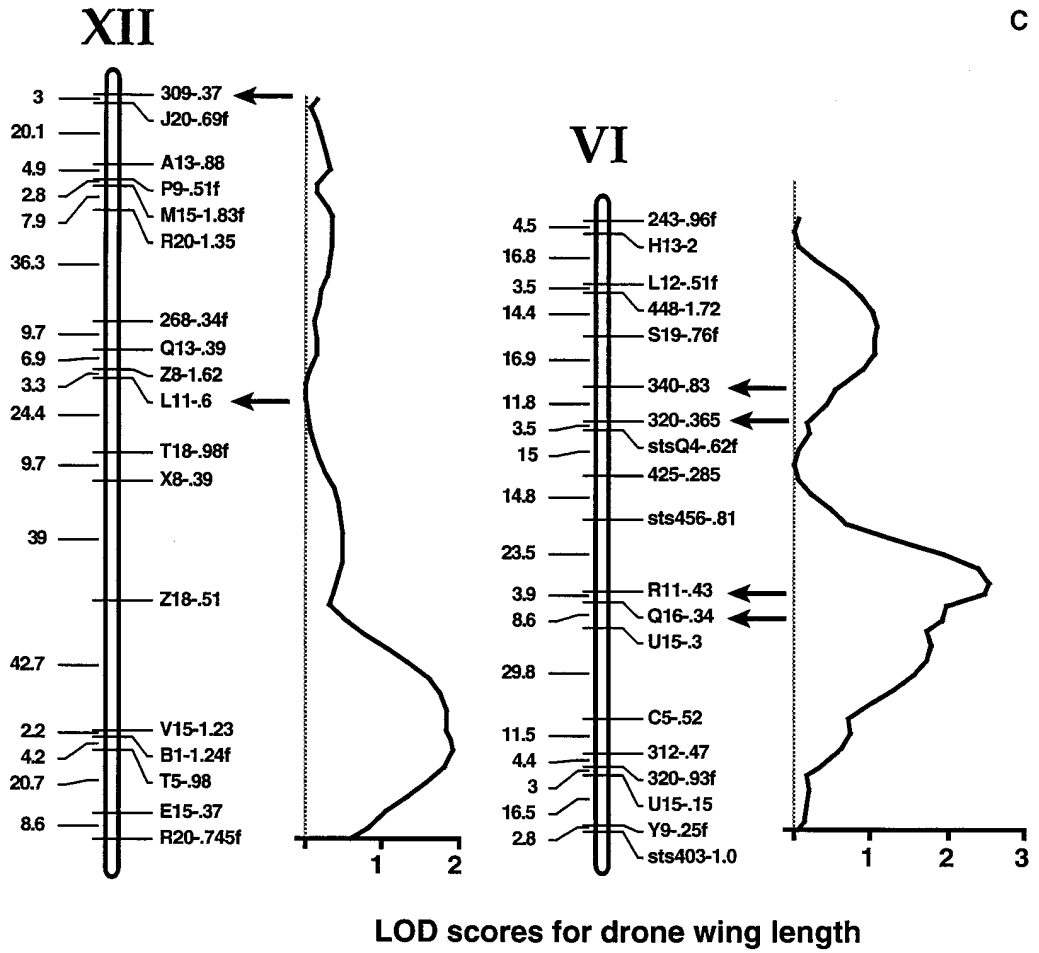


Figure 4.—Possible QTLs influencing wing length. (A) QTLs with effects on both the worker wing lengths and the drone fathers of those workers. The fine black line indicates results of standard interval mapping in workers; other lines are the results of MQM mapping. The significance level determined by permutation tests appears near the peak LOD score. (B) QTLs that only had effects on worker wing identified by MQM mapping. (C) QTLs that only had effects on drone wing length identified by MQM mapping. Linkage group numbers are from Hunt and Page 1995. RAPD markers are designated by the primer names followed by approximate size of the marker fragment. Markers indicated by arrows were common to more than one linkage map. Sequences for the sequence-tagged sites are designated with the ''sts'' prefix.

the order now presented for this marker (F3-.98) is far more likely than the previous order (a log-likelihood difference of 11.6). The new data also made it possible to detect linkage between three pairs of linkage groups from the original map. Group III is linked to XXII; X is linked to XXIV; and I is linked to XXI. The name of the longer of the two linked groups is retained here. The only linkage group (containing 3 markers) shown here that could not be identified based on RAPD markers was determined to be group XIV after we cloned a RAPD marker fragment and designed specific primers to amplify a polymorphic marker from the original mapping population. These results demonstrate that RAPD markers are a robust tool for genetic analyses.

Honey bee colony defense consists of guarding behavior and stinging behavior. But in the present study, we only considered bees that actually responded by stinging. While searching for QTLs affecting stinging behavior, we faced a statistical problem of multiple comparisons because 333 loci were tested by interval mapping and these loci are not independent of each other. In addition, the data for stings were nonnormally distributed. As an extension of analysis of variance, interval mapping should be robust in the face of deviations from normality (Harris 1975; Neter *et al.* 1990). However, the effects of the nonnormal error distribution on the actual significance level are unknown. Therefore, permutation tests were used to arrive at an empirical threshold value to control the experimentwise error rate. One locus was identified (*sting-1*) that had significant effects on the rate of stinging as measured by the colony-level defensive behavior assay. *Sting-1* exceeded the 95% confidence threshold determined by permutation tests. Four other possible "stinging" QTLs also were identified. The LOD score at *Sting-1* is close to the theoretical LOD threshold of 3.6 that should control for experi-

mentwise errors at 0.05 in the honey bee (calculated from the formula of Lander and Kruglyak 1995). The latter threshold is very stringent because it assumes many independent attempts to identify stinging-behavior QTLs. All QTLs should be considered "possible" QTLs until confirmed by an independent cross. The marker linked to *sting-1*, stsN4-.245, had significant associations with other criteria that beekeepers use to judge the defensive behavior of a colony while opening a hive; the tendency of the bees to fly up out of the hive, and the tendency to sting the beekeeper.

There was agreement between the results of interval mapping on numbers of stings and the results from nonparametric tests of behavioral scores at specific marker loci. One very suggestive QTL for stinging behavior lies near marker Z8-1.11. This marker had a significant association with scores for stinging and flying up at the beekeeper, and hanging from the bottom of the comb ($P < 0.005$, 0.0001 and 0.005, respectively). The behavioral scores recorded during hive opening are not independent of each other because scores of different behaviors were evaluated at the same time for each colony and the behaviors are related to each other. For example, if bees fly up they are probably more likely to sting and less likely to be hanging from the comb. Four of the five putative stinging-behavior QTLs were associated with markers that had significant effects on these behavioral scores (at $P < 0.05$). Only one region was identified that may influence the tendency to sting as measured by the scores, without influencing the numbers of stings in the quantitative assay. Marker 268-.64 on group XIII had significant effects on the sting rating ($P < 0.005$) but not on the number of stings in the assay (not shown in figures). Subsequent confirmation of the effects of QTLs on stinging behavior could provide a means for following the introgression of African DNA alleles affecting stinging behavior into commercial honey bee populations.

Measures of body size have been the most commonly used methods for distinguishing Africanized bees from European bees. However, morphometric techniques fail to detect intermediate and low levels of Africanization (Guzmán-Novoa *et al.* 1994). Phenotypic correlations have been found between wing length and the defensiveness of bees in managed colonies of one commercial operation in Mexico (Guzmán-Novoa and Page 1994b). These studies were conducted during the first five years of Africanization and demonstrated the usefulness of using wing length as one of the selection criteria in a breeding program. However, in areas that have been Africanized for some time this association of morphology and behavior may break down. For example, a study that looked at bees from multiple beekeeping operations in Venezuela found no correlation between morphometric characters and defensive behavior (Collins *et al.* 1994). In our study, four putative QTLs affecting worker wing length were detected. Two of these QTLs exceeded our threshold for the experimentwise 95% confidence level. But the QTLs identified for wing length were unlinked to those affecting stinging behavior. Therefore, if extensive introgression occurs between Africanized bees and European bees, morphometric characters will become less useful for selecting gentle breeding stocks.

PCR-based markers linked to *sting-1* and other possible defensive-behavior QTLs may be useful for following the introgression of genes affecting stinging behavior and for breeding gentler bees. Further studies hopefully will confirm the effects of these loci on stinging behavior, more precisely map them, and determine how each locus affects individual behavior. Behavioral and physiological assays with individual bees and colonies are needed that can be used to identify specific components of the defensive response that are influenced by specific genes. If a major-effect gene is involved in defensive behavior, it may someday be feasible to clone the gene through a map-based cloning strategy.

The authors are very grateful to Guillermo Garcia, director of Miel Vita Real, whose generous cooperation made this study possible. We thank Johan Van Ooijen for advice on statistics and Merideth Humphries for technical assistance in the lab. We thank Enrique Estrada for help in maintaining stocks of bees. We also acknowledge the help of beekeepers and researchers who volunteered their time for the defensive behavior flag test: Daniel Prieto, Angel López, Miguel Arechavaleta, Enrique Coronado, Julio López, Adriana Correa, Froylan Gutiérrez, José Calvo and Larissa García. Morphometric analyses were done by Esperanza Ochoa. This research was funded by contracts from the California Department of Food and Agriculture, United States Department of Agriculture grant 93-37302-8880, National Institutes of Health grant R29 GM 54850-01/G231NR and National Institute of Mental Health grant PHS MH53311-02.

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Communicating editor: A. G. Clark