Binary Trait Loci That Influence Honey Bee (Hymenoptera: Apidae) Guarding Behavior

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ABSTRACT This study was conducted to detect binary trait loci (BTLs) that influence guarding behavior of individual honey bees, *Apis mellifera* L., and to locate genetic markers that are associated with these BTLs on genetic maps derived from guard bees from two reciprocal backcross colonies. Samples of guards and control bees were taken from two backcross colonies derived from a defensive colony and a gentle colony. Amplified fragment length polymorphism (AFLP) markers were produced from DNA samples of guards. Two genetic maps were generated, one for each type of colony. A chi-square goodness-of-fit test was performed for each marker in the map to look for deviations from the 1:1 segregation of the markers. For those markers that significantly deviated from the 1:1 ratio in the guards, AFLPs were generated from two samples of control bees, one for each type of backcross. Those markers that showed a skewed segregation pattern in the guards but not in the controls were analyzed with a 2×2 chi-square to test for associations between the markers and the expression of the trait. Ten markers were associated with guarding behavior (five in each backcross). The 10 markers represented seven putative BTLs that influence honey bee guarding behavior. One of the BTLs represents a QTL that was previously detected in analysis of colony-level stinging response, others represent new loci specific to the behavior of individuals guarding the colony entrance.

KEY WORDS honey bee, binary trait loci, guarding behavior, defensive behavior, division of labor

Honey bees, *Apis mellifera* L., are highly social, polyandrous insects that exhibit age-based division of labor. However, a number of studies have shown that genetic variation among the workers in a colony is another factor that influences division of labor (Frumhoff and Baker 1988, Robinson and Page 1988, Breed et al. 1990, Trumbo et al. 1997, Giray et al. 2000, Page et al. 2000, Guzmán-Novoa et al. 2002, Arechavaleta-Velasco et al. 2003).

An important evolutionary characteristic of honey bee colonies is the ability to defend their nest. Colony defense primarily involves two distinctive behaviors: guarding and stinging. Guards are workers that patrol the entrance of the hive. Guards are very active and inspect bees as they enter the hive. Inspection leads to the recognition of nest mates and the rejection of non-nest mates (Moore et al. 1987, Breed et al. 1990). They also actively respond to other insects, animals, or objects that approach the entrance of the nest. Guards are specialists, only a small proportion (≈10–15%) of the bees in a colony will ever perform guarding behavior during their life (Moore et al. 1987). Guards also play an important role when a honey bee colony initiates a defensive response by stinging intruders. Arechavaleta-Velasco and Hunt (2003) found that during stinging behavior tests only a small proportion of the guards in a colony reacted by stinging. But the intensity of the colony defensive response was influenced by the presence and the number of guards in the colony, and it was positively correlated with the proportion of guards that stung.

Guarding behavior at the colony level is influenced by both the genetic makeup of the colony and the environment (Butler and Free 1952, Ribbands 1954, Breed and Rogers 1991, Downs and Ratnieks 2000, Arechavaleta-Velasco and Hunt 2003, Hunt et al. 2003). The genotype of an individual bee influences the probability that she behaves as a guard in a colony. Robinson and Page (1988) found that the proportion of subfamilies in a sample of bees that behaved as guards was different than that of other groups of bees in the colony. Recent studies showed that quantitative trait loci (QTLs) affecting stinging behavior that were mapped as a colony trait (Hunt et al. 1998) influence the expression of guarding behavior of individual bees (Guzmán-Novoa et al. 2002, Arechavaleta-Velasco et al. 2003). In one of these studies, it was found that guards were genetically different from other types of bees in the colony, including nurses, foragers, and stingers, based on the allelic frequencies of four sequence tag sites (STSs) linked to stinging behavior QTLs (Arechavaleta-Velasco et al. 2003).

The expression of guarding behavior of an individual bee is assayed as a discrete variable, so it can be considered a binary trait. At any one time, an indi-

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vidual bee either behaves as a guard or not. Binary traits can be associated with genetic markers to detect and locate genomic regions that influence these traits (Spielman et al. 1993, Beebe et al. 1997). The concept of binary trait loci (BTLs) has been used to describe these genomic regions (McIntyre et al. 2001). BTLs can be detected using 2 by 2 chi-square tests to establish associations between genetic markers and a binary trait (Wilcox et al. 1996, McIntyre et al. 2001).

The objectives of this study were to 1) detect BTLs that influence guarding behavior of individual honey bees and to 2) locate genetic markers associated with these BTLs on maps by using amplified fragment length polymorphisms (AFLPs) derived from guard bees of two reciprocal backcross colonies.

Materials and Methods

Experimental Colonies. Two honey bee colonies were used as parental sources, one classified as high defensive and one classified as low defensive, based on their relative defensiveness evaluated by a rating method (Arechavaleta-Velasco and Guzmán-Novoa 1996, Hunt et al. 1998, Guzmán-Novoa et al. 2002). A queen was reared from the defensive colony and was artificially inseminated with the semen of three of her brothers. This inbreeding step performed in the defensive source lineage was done to help ensure more genetically uniform F1 queens. A daughter queen reared from this cross was inseminated with the semen of a drone from the gentle colony. From this daughter queen, 12 hybrid queens were reared and divided into two groups. Six queens were single-drone artificially inseminated with drones from the defensive colony, and six queens were single-drone artificially inseminated with drones from the gentle colony to produce two types of colonies composed of backcross workers. The F1 queens that headed the backcrossed colonies shared the same queen mother and drone father. As a consequence of these matings, the queens were super-sisters that had an average genetic relationship of at least 0.75. Therefore, all the F1 queens inherited the same alleles from their low defensive drone father (Arechavaleta-Velasco et al. 2003, Arechavaleta-Velasco and Hunt 2003).

Each queen was introduced into a small colony consisting of three frames of brood, two frames of honey, four frames of empty comb and ≈1.5 kg of bees. The colonies were kept in a single brood chamber of standard Langstroth hives in the same apiary. All colonies were managed the same way for a period of 60 d to allow time for workers in the colony to be replaced by daughters of the inseminated queens. Two colonies were selected, one from each type of backcross, based on their relatively high defensive behavior.

Behavior Assays. Each of the two selected colonies was observed for a period of 30 min, and bees performing guarding behavior at the entrance of the hive were marked on their thorax with a dot of enamel paint. A bee was identified as a guard if it patrolled the entrance of the hive and actively approached and inspected incoming foragers. Different colors were used for each colony to ensure that bees were not drifting between colonies. Twenty-four hours later, marked bees that continued to guard were collected with a pair of forceps and each one was introduced into a 1.5-ml plastic tube. The tubes with sampled guards were immediately placed on dry ice and kept at −80°C until the DNA extraction was performed. This procedure was repeated on five different occasions, allowing a 24-h period between collecting guards and marking new guards.

A random sample of nurse bees was taken as a control from each of the two backcrossed colonies as soon as the guards were collected. Control bees were sampled by removing frames from the interior of the brood nest. The bees found on the frames were collected with a pair of forceps and introduced into 1.5-ml plastic tubes and placed in dry ice. The samples were kept at −80°C until DNA analysis was performed.

Genetic Analysis. DNA was extracted from individual guards and control bees from each of the backcross colonies and from the drone father of each colony plus the drone father of the F1 queens. DNA extraction involved grinding the bees in lysis solution (1% hexadecyltrimethyl-ammonium bromide, 50 mM Tris pH 8.0, 10 mM EDTA, 1.1 M NaCl), followed by phenol/chloroform extraction and ethanol precipitation of the DNA (modified from Hunt 1997). The DNA of each individual bee was quantified with a fluorometer and diluted to a final concentration of 14 ng/μl in double distilled water.

AFLP markers (Vos et al. 1995) were produced using the AFLP analysis system II kit (Invitrogen, Carlsbad, CA). Genetic markers were generated from DNA samples of 94 guards from each of the two types of backcross colonies, the drone father of each colony and the drone father of the F1 queens. To produce the AFLP markers, the DNA of each individual bee was digested with two restriction endonucleases, *Eco*RI and *Mse*I, to generate restriction fragments. The DNA fragments were ligated to *Eco*RI and *Mse*I adapters that function as primer binding sites that flank template DNA for amplification. Polymerase chain reaction (PCR) was performed in a first amplification reaction (preamplification) by using an *Eco*RI primer 5′ AGA CTG CGT ACC AAT TC and a *Mse*I primer 5′ GAT GAG TCC TGA GTA AC. The PCR conditions were 20 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min.

The products of the preamplification were diluted and used as template for a second set of PCR called selective amplification by using two more primers: an *Eco*RI primer containing two selective nucleotides that was previously labeled using ^{33}P, and an *Mse*I primer containing two selective nucleotides. The PCR was performed under the following conditions: one cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; followed by 12 cycles of denaturing at 94°C for 30 s, annealing at 65°C for 30 s, with a ramp time of 5 min to reach the extension temperature of 72°C for 1 min. The annealing temperature was decreased by −0.7°C per cycle during the 12 cycles, giving a touch down phase of 13 cycles. This was followed by 22 cycles at

Table 1. Genetic markers that showed deviations from the expected 1:1 segregation pattern in the guards but not in the control bees of the gentle backcross colony, based on a chi-square goodness of fit test

Locus	Linkage group	Guards					Controls				
		n	Genotypes		χ^2	*P*	*n*	Genotypes		χ^2	*P*
			A	H				A	H		
A32.150	3	92	22	70	25	<0.001	96	45	51	0.4	NS
A42.135	6	90	34	56	5.4	0.02	94	49	45	0.2	NS
A31.090X	9	92	64	28	14.1	<0.001	69	38	31	0.7	NS
A16.330bX	10	91	20	71	28.6	<0.001	95	39	56	3.0	NS
A31.240	10	93	25	68	19.9	<0.001	75	37	38	0.0	NS
STSA64.084	12	94	35	59	6.1	0.014	95	49	46	0.95	NS
A28.190X	12	87	21	66	23.3	<0.001	95	41	54	1.8	NS
A31.330b	14	89	59	30	9.4	0.002	72	40	32	0.9	NS
A28.105X	14	92	56	36	4.3	0.038	96	44	52	0.7	NS
A16.175	17	93	35	58	5.7	0.017	95	53	42	1.3	NS

NS, not significant.
A and H represent the two types of marker alleles.

94°C for 30 s, 65°C for 30 s, and 72°C for 1 min. Twenty-eight selective amplifications were performed for each sample, by using four *Eco*RI primers and eight *Mse*I primers. The *Eco*RI primers differ in the last two bases at the 3′ end: E1, -AA; E2, -AC; E3, -AG; and E4, -AT. The *Mse*I primers differ in the last two bases at the 3′ end: M1, -CAA; M2, -CAC; M3, -CAG; M4, -CAT; M5, -CTA; M6, -CTC; M7, -CTG; and M8, -CTT.

Selective amplification products were separated on a 42 by 36 cm 6% denaturing polyacrylamide gel with 1× Tris borate-EDTA. The gel was transferred to chromatography papers and dried in a gel dryer. The gels were placed in a cassette with X-ray film for 48 h, and the autoradiographs were scored manually on a light box. Marker names were assigned as follows: the letter "A" was used to indicate that the marker is an AFLP, followed by a two-digit number separated by a dot from a three-digit number. In the two-digit number, the first digit corresponds to the *Eco*RI primer used in the selective amplification and the second digit represents the number of the *Mse*I primer. The three-digit number after the dot indicates the size of the amplified fragment in base pairs.

Five STSs linked to stinging behavior QTLs were also used to generate genetic markers (Hunt et al. 1998, Guzmán-Novoa et al. 2002, Arechavaleta-Velasco et al. 2003). PCR was used to amplify the STSs under the following conditions. The DNA was initially denatured at 94°C for 30 s; followed by five cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min; followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min; followed by a final extension for 72°C of 8 min. The reaction products were run on 2.5% agarose gels.

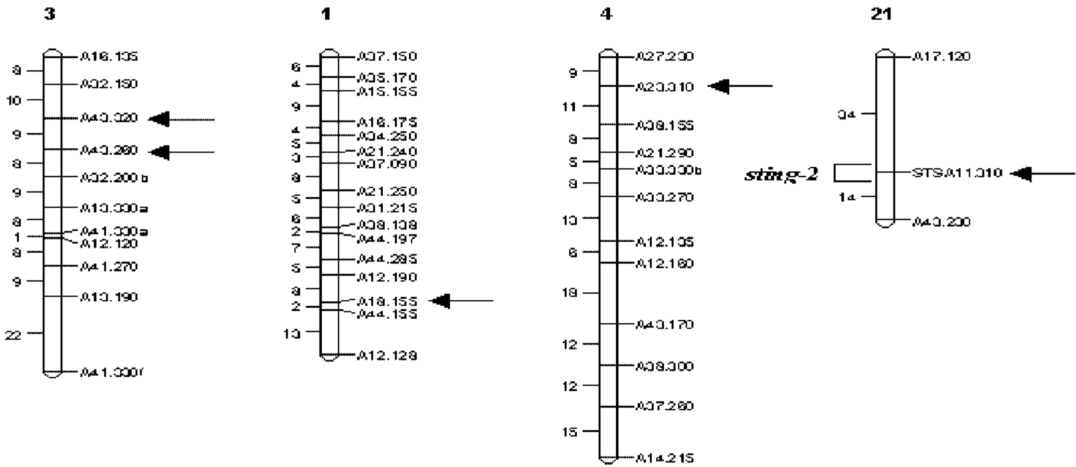
The primer sequences for the STSs were as follows: STS A17.080 5′ TGG TGG AAG GTT TGT ATA TTC G and AAG TTT CTT ACC ACG AGC CTG T; STS A11.310 5′ ACT TTT GAG GCG AAG AGG AAT AC and CTT GTC CAC GAC GAT TAC TTT TC; STS A64.084 5′ ATC CAG AGG ATT GAT CTC GAT G and TGC AAC ATT TGT CTC TGT GAT G; STSN4P88K14 5′ GAA ATT GTT GAC GCG AAA GAC and GTT GTA ACG GAA GAT TGG AAG G; and STSR25.44 5′TGC CGG TCA CGC TAT AAT CTA A and AGG GCT CAT TTC CAA GTA GTT TTT.

Linkage and Statistical Analysis. Linkage analysis was performed with Joinmap 3.0 software (Van Ooijen and Voorrips 2001), coding the data as a backcross. The mapping procedure for each linkage group used the following parameters: a minimum LOD value of 3.0 for linkage, a maximum recombination between markers of 0.40, and a maximum jump in the goodness-of-fit of 5.0. The Kosambi function was used to estimate genetic distances from the recombination fractions (Kosambi 1944).

To detect BTLs that influence guarding behavior of individual bees, the sample of 94 guards of each backcross were scored for polymorphic markers and a chi-square goodness-of-fit test was performed for each marker in the map to look for deviations from the 1:1 segregation of the genotypes that would be expected for a colony composed of backcross workers. For those markers that significantly deviated from the 1:1 ratio in the guards, AFLPs were generated from two samples of 94 control bees, one for each of the two types of backcross colonies after the procedure described above. Those markers that showed a skewed segregation pattern in the guards but not in the controls were analyzed with a 2 by 2 chi-square to test for associations between the AFLP markers and the expression of guarding behavior to detect BTLs that influence the trait.

Table 2. Genetic markers associated with BTLs that influence guarding behavior in honey bees of the gentle backcross colony, based on a 2 × 2 chi-square test for independence

Locus	Linkage group	χ^2	*P*
A32.150	3	9.82	0.0017
A16.330b	10	6.95	0.0084
A31.240	10	8.05	0.0045
A28.190	12	6.50	0.010
A16.175	17	5.51	0.018



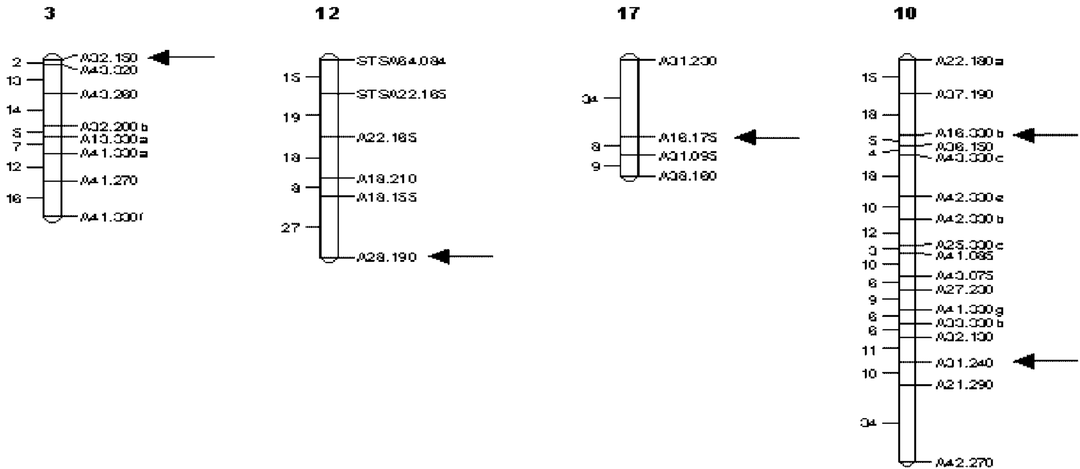


Fig. 1. Genetic markers associated with BTLs that influence the expression of guarding behavior mapped in two reciprocal backcross colonies.

Results

In the gentle backcross, 10 of the mapped markers showed deviations from the expected 1:1 segregation pattern in the genotypic frequencies of guards, but not in the control bees (Table 1). Five of these markers showed a significant association with genomic regions affecting the task of guarding based in a 2 by 2 chi-square test (Table 2). These five markers were located in four different linkage groups, suggesting that five putative BTLs influence guarding behavior in the gentle backcross (Fig. 1).

In the defensive backcross, 14 of the mapped markers showed deviations from the 1:1 ratio in the genotypic frequencies of guards but not in the genotypic frequencies of control bees (Table 3). Five of these markers were significantly associated with genomic regions influencing guarding behavior (Table 4). These five markers were located in four linkage groups, suggesting the presence of four putative BTLs that influence guarding behavior in the defensive backcross (Fig. 1).

Discussion

Results of this study indicated that 10 markers were associated with the expression of guarding behavior. Five of these were found in the defensive backcross and five in the gentle backcross. Nine of the markers were AFLPs and one was an STS. None of the markers were associated with the behavior in both backcross colonies. This could be due to chance segregation of the marker alleles because colonies used for the study were not derived from inbred lines. Another possible explanation is that there are dominant effects of the

Table 3. Genetic markers that showed deviations from the expected 1:1 segregation pattern in the guards but not in the control bees of the defensive backcross colony, based on a chi-square goodness of fit test

Locus	Linkage group	Guards					Controls				
		n	Genotypes		χ^2	P	n	Genotypes		χ^2	P
			A	H				A	H		
A18.155	1	79	57	22	15.5	<0.001	89	40	49	0.9	NS
A12.128	1	92	34	58	6.3	0.012	88	42	46	0.2	NS
A43.320	3	93	37	56	3.9	0.048	89	52	37	2.5	NS
A43.260	3	93	31	62	10.3	<0.001	89	52	37	2.5	NS
A23.310	4	92	71	21	27.2	<0.001	90	45	45	0	NS
A22.220	5	90	58	32	7.5	0.006	88	44	44	0	NS
STSA17.080	9	96	60	36	6.0	0.014	90	45	45	0	NS
A18.331b	13	84	53	31	5.8	0.016	89	46	43	0.1	NS
A21.330a	13	86	55	31	6.7	0.01	82	44	38	0.4	NS
A21.320	19	89	34	55	5	0.025	82	37	45	0.8	NS
A22.190	20	91	56	35	4.8	0.028	88	45	43	0.1	NS
A12.148	20	91	57	34	5.8	0.016	88	42	46	0.2	NS
STSA11.31	21	94	60	34	7.2	0.007	92	44	48	0.17	NS
A43.230	21	93	32	61	9	0.003	89	42	47	0.3	NS

NS, not significant.
A and H represent the two types of marker alleles.

BTLs linked to the markers affecting the allelic frequencies observed in the backcross colonies. However, two markers, A43.320 and A43.260, that were associated with guarding behavior in the defensive backcross and one marker, A32.150, that was associated with the behavior in the gentle backcross, mapped to the same region of linkage group 3. The map distance between A32.150 and A43.260 was 15 cM in the gentle backcross and 19 cM in the defensive backcross (Fig. 1). This suggests that these three markers are linked to one BTL influencing guarding behavior in both backcrosses. Another AFLP marker, A18.155 that mapped into linkage group 1 in the defensive backcross was associated with the trait. In the gentle backcross colony, marker A18.155 mapped to linkage group 12 and was placed 27 cM from A28.190 that was associated with the behavior in this backcross. These two markers could be linked to one BTL influencing guarding behavior, assuming linkage group 1 is actually syntenic with linkage group 12 in the other backcross. Therefore, we have identified a total of at least seven putative BTLs affecting guarding behavior in the two backcross colonies, two of which were detected in both colonies.

Of the seven BTLs detected, six were associated with AFLPs markers and these represent new genomic regions found in this study that influence the expression of guarding behavior of individual bees. The one other BTL detected is associated with marker STSA11.310.

Table 4. Genetic markers associated with BTLs that influence guarding behavior in honey bees of the defensive backcross colony, based on a 2 × 2 chi-square test for independence

Locus	Linkage group	χ^2	P
A18.155	1	11.61	0.0006
A43.320	3	5.6	0.0108
A43.260	3	10.56	0.001
A23.310	4	13.38	0.0002
STSA11.310	21	4.2	0.040

This STS is linked to a defensive behavior QTL (*sting-2*) that was mapped as a colony trait by Hunt et al. (1998). The BTL found in this study and *sting-2* represent the same genomic region. A previous study, using the same samples of bees, was conducted to confirm the effect of three defensive behavior QTLs (*sting-1*, *sting-2* and *sting-3*) on the expression of guarding and stinging behaviors of individual bees. The previous study also included samples of stingers and foragers, and confirmed that *sting-1*, *sting-2*, and *sting-3* influenced the expression of guarding behavior of individual honey bees using a chi-square goodness-of-fit test to look for deviations in the genotypic frequencies of the STS markers linked to the QTLs (Arechavaleta-Velasco et al. 2003). The results of the current study show that only *sting-2* was associated with guarding behavior under the more stringent chi-square test for independence. Because this study was designed for detecting, instead of confirming, genomic regions associated to the specific task of guarding, we used first a goodness-of-fit test to identify markers that deviated from the expected allelic proportions in the guards and then used the more stringent 2 by 2 chi-square test for independence and one group of control bees, which in this case were a random sample of bees collected from the frames that contain brood removed from the center of the nest. In the previous study, a goodness-of-fit test was used to test for the effect of the QTLs that were previously detected by using the phenotypic data from 172 backcross colonies derived from Africanized and European honey bees (Hunt et al. 1998).

We used the random sample of nurse bees as controls in the current study, because they represent a clearly different behavioral group of bees that in general are younger than guards. Nurse bees are old enough to survive any problem of developmental viability due to genetic effects but are not old enough to perform any task outside of the colony, so they are not exposed to predators or environmental factors that

could affect the proportion of the two expected genotypes.

Our findings indicate that specific regions of the honey bee genome influence guarding behavior and that genetic variation among members of the same family could influence the probability that an individual bee performs a particular task in the colony. Other studies have found QTLs that affect behavioral traits in the honey bee (Hunt et al. 1995, Page et al. 1995, Hunt et al. 1998, Page et al. 2000, Chandra et al. 2001, Lapidge et al. 2002). Some of these QTLs, mapped as colony traits, have been found to influence the expression of specific behaviors of individual bees (Page et al. 2000, Guzmán-Novoa et al. 2002, Arechavaleta-Velasco et al. 2003). All but one of the studies relied on colony phenotypes for QTL detection, the study conducted by Chandra et al. (2001) found QTLs affecting the associative learning performance in individual drones.

The approach followed in this study, to analyze guarding behavior as a binary trait, provided us with a practical method to measure the behavior at the individual level and allowed us to identify guards that performed the behavior for at least two consecutive days. The genetic and statistical methods that we used were powerful enough to detect genetic markers from genomic regions associated with guarding behavior. Other models besides a binary model could be used to study this highly specialized task. Guarding is a complex behavior and is likely that is controlled by several genes and is affected by environmental factors.

Our study is the first to detect BTLs influencing a behavior in individual workers and shows that this approach can be used to find genomic regions associated with behavioral traits of bees within a colony. Studies conducted to identify behavioral QTLs in bees have relied mostly on colony level phenotypes because it was believed that larger genetic effects would be observed if the behavioral traits of many genetically similar individuals were measured as a group. However, the analyses of guarding behavior of individual bees in this study not only confirmed the effects of a stinging behavior QTL originally detected based on whole colony response but also identified new loci that influence the behavior. In one instance, our results on linkage group 3 detected the same region in both backcrosses, which provides corroboration for the effect of this BTL. In this study we have detected and mapped genomic regions influencing the probability that a bee behaves as a guard, one of the multiple tasks performed by the workers in a colony. BTL mapping could provide a way to identify loci and eventually genes that regulate traits involved in honey bee social behavior.

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