SHORT COMMUNICATION

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Seven suggestive quantitative trait loci influence hygienic behavior of honey bees

Abstract In 1964, Walter Rothenbuhler proposed a two-gene model to explain phenotypic variance in the remarkable behavior in which honey bee workers remove dead brood from their colonies. Rothenbuhler's model proposed that one locus controls the uncapping of brood cells containing dead pupae, while a second controls the removal of the cell contents. We show here, through molecular techniques and quantitative trait loci (QTL) linkage mapping, that the genetic basis of hygienic behavior is more complex, and that many genes are likely to contribute to the behavior. In our cross, we detected seven suggestive QTLs associated with hygienic behavior. Each detected QTL controlled only 9–15% of the observed phenotypic variance in the character.

Introduction

The term hygienic behavior is used to describe a two-step process carried out by some worker honey bees upon detection of dead or diseased larvae or pupae in brood comb. The process first involves 'uncapping' (elimination of the wax cap covering the brood cell), followed by 'removal' of the corpse inside (Spivak and Gilliam 1998). To explore the genetic control of this behavior, Rothenbuhler (1964) performed an elegant backcrossing experiment. First he crossed a strongly hygienic line with a non-hygienic line. The F_1 hybrid colonies were non-hygienic, and these colonies were backcrossed to the hygienic line. The backcross colonies segregated into four behavioral phenotypes, as would be expected from two independently assorting Mendelian genes. From these data, Rothenbuhler (1964), inferred that the two components of hygienic behavior are each controlled by an independent locus. Workers that are homozygous recessive (*uu*) at the 'uncapping' locus, uncap cells containing dead pupae whereas workers that are *Uu* or *UU* fail to uncap dead cells. Workers that are *rr* at the 'removal' locus remove dead pupae from uncapped cells, whereas *Rr* and *RR* individuals do not remove dead brood. Thus expression of the behavior requires workers to be homozygous recessive at both loci (*rruu*).

Examples of quantitative variance in behavior and artificially induced single-gene mutants that affect the behavior of animals abound (Yamamoto et al. 1997). However, with one exception (Rinderer and Beaman 1995), also from honey bees, no naturally occurring variation in a complex behavior of a higher organism has been suggested to be under such strict genetic control as hygienic behavior of honey bees. Thus Rothenbuler's results are often cited in textbooks as one of our best examples of a complex behavior under the control of simple Mendelian genetics (e.g., Herskowitz 1977; Ridley 1995). Although Rothenbuhler's two-gene model has been challenged (Moritz 1988), it is still widely believed that hygienic behavior of honey bees is controlled by a few Mendelian genes of large affect.

Materials and methods

Crossing

To investigate Rothenbuhler's two-gene model of hygienic behavior using modern genomics we repeated his crossing scheme by instrumentally inseminating (Harbo 1986) a queen from a hygienic line with a male from a non-hygienic line (Spivak and Gilliam 1998). We then reared a queen from this cross to produce an F_1 queen. Sons of this queen should have been segregating for any loci associated with hygienic behavior.

Individual sons of this F_1 queen were then used to artificially inseminate (*n*=90 successful inseminations) individual queens reared from the putative recessive parent (based on Rothenbuhler's model) – the hygienic queen.

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Hygienic behavior bioassays

Colonies of workers arising from the 90 backcrossed queens were then scored in a hygienic behavior bioassay (Spivak 1998; Spivak and Reuter 1998) on 1–3 separate occasions over three consecutive weeks in summer 1998 at a location north of Kurrajong, NSW, Australia. Colonies were housed in six frame nucleus colonies and were generally populous due to excellent breeding conditions during the preceding month. During the assay, there was a light nectar flow.

Each colony received two scores, one for expression of the uncapping phenotype only and one for expression of overall hygienic behavior (uncapping + removal). The scores were the proportion of about 100 dead brood cells that were uncapped or removed after 48 h (Spivak 1998; Spivak and Reuter 1998).

Linkage mapping

We extracted DNA from 119 sons of the F_1 queen (including the 90 successful fathers) using a CTAB lysis method (Hunt 1997). Molecular markers were generated from these DNA samples using RAPD (Williams et al. 1990; Hunt 1997), and microsatellite loci (Estoup et al. 1993) and a sequence tagged site (Hunt et al. 1995). We also scored each male for one of two possible alleles at the allozyme locus malate dehydrogenase (Nielson et al. 1994). Combined, these analyses produced 482 scorable segregating markers.

Full multipoint linkage analysis of these 482 markers across the 119 males using the Kosambi mapping function of Mapmaker v 3.0 (Lander et al. 1987) was used to produce a new linkage map of the honey bee using methods appropriate to haplo-diploid social insects (Hunt and Page 1995; Hunt et al. 1995, 1999). A LOD score of 3.0 and a recombination fraction of 0.34 were used as the criteria for linkage.

Quantitative trait loci analysis

Complete phenotypic (i.e., successfully scored on three occasions) and molecular data were available for 63 segregating colonies. In association with the map generated from the 119 males, these data were analyzed using MapQTL v 3.0 software in order to identify any quantitative trait loci (QTLs) (Jansen 1993; Jansen and Stam 1994) associated with hygienic behavior.

Results

A numerical score based on the average level of hygienic behavior exhibited over the triplicate assays was assigned to each colony. We defined the following phenotypic classes: hygienic, >95% of dead brood removed in 48 h; non-hygienic, <50 dead brood cells removed in 48 h; intermediate, 50–95% of dead brood removed in 48 h (Spivak 1998).

We assessed the repeatability of the traits uncapping and total hygiene (i.e., proportion of dead brood removed) across the three assays (n=90 colonies) as a measure of the upper bound of heritability (Falconer 1981). The proportion data were first normalized using an arcsine $\sqrt{x}$ transformation. The repeatability, r, of uncapping was 0.54±0.061 (±SE) (Becker 1984) and the repeatability of overall hygienic behavior was 0.57±0.059.

Linkage analysis produced a linkage map of 358 marker loci over 25 linkage groups, spanning a total distance of 3,406.2 cM. The average distance between each marker was 9.5 cM. Loci that could not be successfully joined to linkage groups, or that generated unacceptably large linkage maps, were discarded. Based on this total map size and number of linkage groups, the LOD score that is declared significant for a genome-wide significance level of 5% is 3.3 (van Ooijen 1999).

QTL analysis for overall hygienic score identified six suggestive and one significant QTL associated with linkage groups 2, 4, 5, 6, 13, and 22 (Fig. 1, Table 1). QTLs located on linkage groups 2, 4, 6, and 22 were associated with the uncapping scores only (Table 22). Two loci were in common; two were independent (Tables 1 and 2). This may suggest that QTLs linked to markers 242_0.24, 172_0.5f, 395_0.7 and G17_1.0 influence the removal aspect of hygienic behavior but not uncapping.

Individually, each QTL is of small effect, with each explaining only 9–16% of the variance observed (Tables 1 and 2). Collectively, the putative QTLs identified here

**Table 1** Putative QTLs for hygienic behavior in honey bees: overall hygienic behavior

Linkage group	Nearest marker	LOD score	% Variance explained
2	336_0.71	2.91	13.1
4	397_0.9f	3.04	14.5
5	242_0.24	2.07	8.9
6	172_0.5f	1.72	9.3
13	395_0.7	2.14	9.2
15	G17_1.0	2.17	9.2
22	123_1.0	3.37[a]	15.2

[a] Significant LOD score for a genome-wide significance level of 5%

Table 2 Putative QTLs for hygienic behavior in honey bees: uncapping only

Linkage group	Nearest marker	LOD score	% Variance explained
2	336_0.71	2.68	12.0
4	397_0.9f	3.08	15.8
6	U15_0.75f	1.90	10.4
22	123_1.0	2.95	13.6

Fig. 1 LOD Score profiles of seven QTLs controlling hygienic behavior of honey bees. Profiles are shown for overall hygienic behavior (*solid line*) and uncapping behavior only (*gray line*) in seven linkage groups. LOD scores are given on the *y* axis. A LOD score of 1.5 was considered indicative of a linked QTL. *Codes* refer to the RAPD primer used to obtain the marker. Loci commencing with a *numeral* were obtained from the University of British Colombia. Loci commencing with a *letter* refer to primer kits obtained from Operon Technologies, California. *Numerals after the underscore* are the primer within the kit. Size polymorphisms are followed by the letter '*f*'. All others are presence/absence polymorphisms. The nearest marker to each putative QTL is shown in *bold*
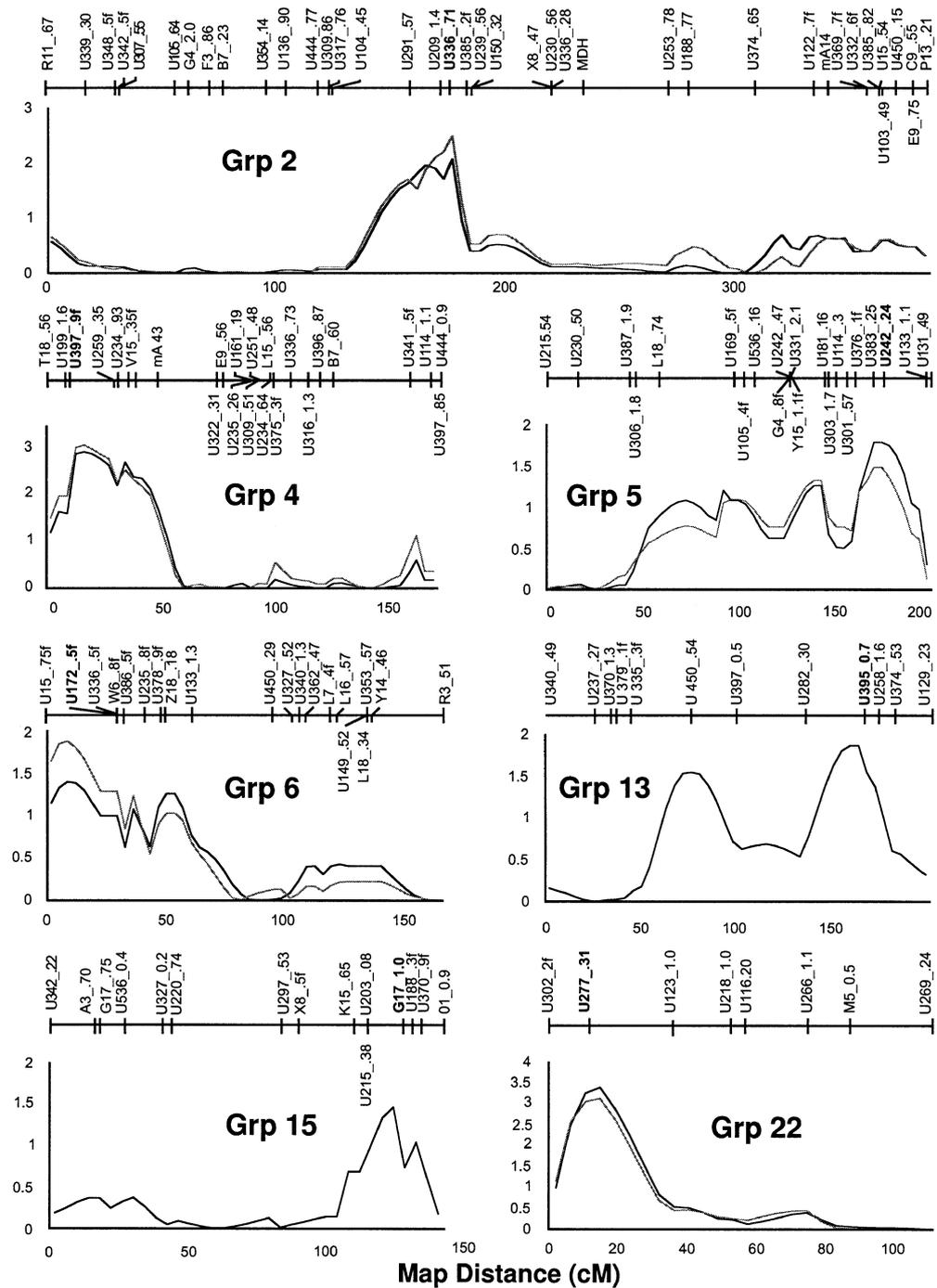
explain 79.4% of the observed variance in the expression of hygienic behavior.

Discussion

Our study revealed six markers that show suggestive linkage (van Ooijen 1999) to loci that contribute to overall phenotypic variation in honey bee hygienic behavior, and one (123_1.0) that showed strong linkage,

with a significant LOD of 3.37. These markers await confirmation in independent crosses or by other means.

We expected to find two (Rothenbuhler 1964), or perhaps three (Moritz 1988), QTLs of major effect on hygienic behavior. However in our experiment we did not find any evidence for such genes. Instead we found that the behavior seems to be inherited in a more quantitative manner. Thus although our quantitative genetic analysis of repeatability and the QTL analysis confirm Rothenbuhler's findings that hygienic behavior has a strong

genetic component (as expected by the success of selection for the character), we cannot confirm the existence of two genes of major effect that control hygienic behavior. It may be, however, that in the actual cross performed by Rothenbuhler, only two loci of large effect were segregating. Moreover, our assay is very different from that performed by Rothenbuhler. He used brood that had been naturally killed by the bacterium *Paenibacillus larvae*, and allowed the bees up to 16 days to remove the dead brood. The standardized test we used utilizes freeze-killed brood and is performed over 48 h. Thus our results are not directly comparable with those of Rothenbuhler.

The number of linkage groups in a saturated map should theoretically match the number of chromosomes in an organism (Heckel 1993). The haploid number of the honey bee is 16, so the map generated in this study contains nine gaps, which are likely to span distances greater than that equating to the maximum recombination fraction of 0.34 used in the creation of the map. The map published by Hunt and Page (1995), which was also based on segregating RAPD markers, covered 3,110 cM on 26 linkage groups. Taking into account the ten gaps in their map, and a total physical size of 178,000 kb (Jordon and Brosemer 1974), Hunt and Page (1995) estimated the total genome size of the honey bee to be 3,450 cM, with a ratio of physical size to recombinational size of 52 kb/cM. If it is similarly assumed that the nine gaps in the map we generated are at least 34 cM each, then we estimate the genome size to be 3,712 cM, with a ratio of physical size to recombinational size of 48 kb/cM. This size supports the claim of Hunt and Page (1995) that there is a very high recombinational rate in honey bees.

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