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Genetic Evaluation of a Novel System for Controlled Mating of the Honeybee, *Apis mellifera*

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Abstract

Many apiculturally important traits of the honeybee have medium to high heritabilities and are therefore capable of strong response to selection. However, the natural mating system of honeybees makes it difficult to exclude unselected males from matings and necessitates expensive procedures like artificial insemination or isolated mating stations. By manipulating ambient light and temperature, an Australian queen breeder has developed a novel system that delays the flight time of selected queens and drones. To assess the efficacy of this "Horner system," drones and their assumed worker offspring were genotyped using microsatellite loci to test whether the workers were exclusively sired by the selected drones. The Horner system was found to provide at least 85% control of matings, equivalent to a 48% increase in the selection differential, when queens and drones are selected in a breeding program.

Key words: *Apis mellifera, instrumental insemination, isolated mating, mating flight time, microsatellite, selection differential*

In the Western honeybee (*Apis mellifera*), the heritability of honey production and disease resistance is high (Bienefeld 1986; Oldroyd et al. 1987). This results in strong response to selection for these characters and has allowed development of successful commercial breeding programs (e.g., Allan and Carrick 1988; Bienefeld et al. 2007). However, a significant impediment to successful bee breeding is adequate control of mating. *Apis mellifera* queens mate in flight (Gries and Koeniger 1996) with 7–28 drones (Estoup et al. 1994; Palmer and Oldroyd 2000), which may originate from colonies up to 15 km away (Jensen et al. 2005), and this hampers the ability of bee breeders to use selectively bred males as sires. Indeed, the lack of control over mating has been one of the most significant impediments to the success of honeybee breeding programs.

The 2 primary methods of providing control of matings are geographical isolation and instrumental insemination. Small islands (Allan and Carrick 1988) or confined valleys (Neumann et al. 1999; Jensen et al. 2005) can allow the breeding population to be effectively isolated. Instrumental insemination (Harbo 1986; Laidlaw and Page 1997) is used in bee research institutions worldwide, but its use in the commercial queen production industry has been limited because it requires specialized skills and expensive equipment. Furthermore, instrumentally inseminated queens can be inferior to naturally mated queens and have reduced life expectancy (Harbo and Szabo 1984) (but see Cobey 2007 for a review of studies that show that naturally inseminated and instrumentally inseminated queens have equal performance).

A novel system for the control of natural mating of *A. mellifera* queens has been developed by Mr Jo Horner, an Australian queen breeder in Rylstone, New South Wales. The system allows up to 240 queens to be control mated on a single day, which is far greater than can be achieved by instrumental insemination. Furthermore, the system does not require geographical isolation. Instead, Horner's system controls natural mating of queens and drones by manipulating the time that they undertake mating flights. Under natural mating, drones of *A. mellifera* start their mating flights shortly after noon and continue until 1630 or 1700 h (Koeniger et al. 2005). Males gather at drone congregation areas—specific areas in the landscape (Loper et al. 1987, Pechhacker 1994) that attract hundreds or thousands of unrelated drones (Baudry et al. 1998). Virgin queens fly to a congregation area where they mate before returning to their original colony.

To control mating, Horner delays the mating time of his selected drones and queens so that their time of flight to congregation areas is later than that of feral drones. To delay

the mating flight, mating nuclei are confined within a darkened cool room at 13–15 °C for 2 days prior to mating. The key advantages of this system over instrumental insemination are that it is technically easier and the quality of queens may be better because of the natural mating. A trolley system within the mating yard and shed allows a single operator to control the mating of up to 240 queens on the same day.

The efficacy of Horner's system in terms of the percentage of mismatings is not known. Here, we investigate the degree to which mating is successfully regulated using 2 experiments that assess the paternity of workers produced by his queens. First, we assess the system under normal operating conditions using 6 unlinked microsatellite loci, followed by a second constrained setup involving the release of drones from a single queen only, with paternity testing based on 6 linked microsatellite loci (Shaibi et al. 2008).

Materials and Methods

The Horner System

Horner encourages the prolific production of drones in his selected colonies by introducing drone combs into these colonies 40 days prior to a planned mating. Virgin queens are produced by standard methods (e.g., Laidlaw and Eckert 1962; Laidlaw and Page 1997). Prior to mating, selected drone pupae from 3 drone mother (DM) colonies are transferred to a single drone source (DS) colony to mature. The DS colonies are furnished with a queen excluder, which allows workers (but not drones) to have constant passage from the hive. Horner uses 10 such DS colonies with males from a total of 30 DM colonies for each controlled mating.

Queen pupae are introduced to standard 4-way mating nuclei (i.e., the hive box is divided 4 ways, with entrances facing in 4 directions). Each virgin is confined to her mating nucleus by a queen excluder. Two days prior to the virgins' mating flight, the colonies are placed in a darkened shed at 13–15 °C.

The day before the planned mating, the mating nuclei are taken out of the shed in the late afternoon and the queens released. This allows the queens to orientate to their mating nucleus and to learn the geography of the area. After this orientation flight, the nuclei are returned to the shed. The next day, again in the late afternoon, the mating nuclei are taken out to the precise position they had been in on the previous day. The queens and drones are then released, whereupon they fly to the drone congregation area and mate.

The precise timing of queen and drone release is determined using a sentinel hive. Horner observes the flight of drones from this hive and waits 30 min after drones are no longer seen exiting the hive before releasing his virgins and drones (ca. 1800 h).

To increase operator efficiency and the accurate positioning of mating nuclei, there are 6 rail tracks that run out of the shed into the mating yard. There are 10 hives on each track, connected by chains. When the hives are pushed out of the shed, each one ends up precisely positioned. Individual mating nuclei are marked with conspicuous colors and patterns, and the mating yard itself has large orientation cues provided.

Experiment 1: Assessment of Horner System under Normal Operating Conditions

Collection of Samples

In November 2007, Horner released 240 virgin queens and the drones from 10 DS colonies at 1800 h. For assessment of the DM genotypes, we collected 20 drones from each of the 10 DS colonies before mating—producing a total sample of 200 drones.

Six weeks after the controlled mating, we collected worker offspring of the queens. Workers ($n = 20$) were collected from each of 13 colonies. Twenty feral worker bees were also collected; 5 were collected from flowers, and 15 were collected from 3 different feral swarms (5 bees per swarm).

Extraction of DNA

DNA was Chelex extracted (Walsh et al. 1991) from the drones, worker offspring, and feral workers using one hind leg of each bee. The DNA solution was diluted 1:1 in sterile distilled water and stored at 4 °C prior to use in polymerase chain reaction (PCR).

PCR and Genotype Analysis

PCR was used to amplify the DNA of each sample at 6 microsatellite loci: A14, A107, A79, A29, A113, and B124 (Solignac et al. 2003). Microsatellites were multiplexed in a 5-µl solution containing 1 µl DNA; 1× reaction buffer (Fisher Biotech); 0.3 µM dNTPs; 0.15 µM Mg^{2+}; 0.6, 0.48, 0.52, 0.24, 0.16, and 0.4 for each primer of A14, A107, A79, A29, A113, and B124, respectively; and 0.2 U Taq Ti (Fisher Biotech). The PCR program consisted of a denaturation step of 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; followed by an extension cycle of 72 °C for 9 min. Genotyping was performed on an Applied Biosystems 3130*xl* Genetic Analyzer.

From the sample of drones from the DS colonies, we determined the allelic diversity of the 30 DM queens at each of the 6 microsatellite loci. Given that the 3 DM queens could carry a maximum of 6 alleles at any one locus, the maximum probability of not sampling a DM allele from our drone sample is $(1 - 1/6)^{20} = 0.03$. To determine whether the virgin queens had mated with Horner drones or feral drones, we determined if any progeny worker carried a paternal allele that was not identified in the DM queen allele pool. To do this, individuals from the worker offspring sample were genotyped at the same 6 loci as the Horner drones. The alleles carried by each queen mother of each group of worker progeny were then determined (maternal alleles), and the genotype of the fathering drone of each

worker was then determined by subtraction (Oldroyd et al. 1996; Oldroyd and Wongsiri 2006). We compared these paternal alleles with the previously determined DM queen genotypes. Any paternal genotype containing alleles absent from the DM queen allele pool must have been fathered by an unselected feral male. Allelic diversity in the feral population was estimated from the genotypes of the 20 feral bee samples.

Experiment 2: Assessment of Horner System under a Constrained Setup

To improve the power to detect feral drones mating with Horner's queens, a single DS colony was set up containing drones from a single DM queen. Drones and virgin queens were reared in February 2009 using the same techniques as previously. At approximately 1800 h, Horner released 240 virgin queens and the drones from the single DS colony.

Collection of Samples

Workers were collected 8 weeks after the mating, in April 2009. Ten colonies were sampled, with 96 workers collected from each colony. The DM queen was unfortunately lost and did not have sister or daughter colonies to collect relatives from for genotype determination of the lost queen. Thus, we inferred the genotype of the lost DM queen from the worker progeny of her sons.

DNA Extraction and Genotyping

A single hind leg was used to extract worker DNA using a high-salt extraction method (Aljanabi and Martinez 1997). DNA samples were air-dried and then resuspended in 50 µl 0.5× Tris–ethylenediaminetetraacetic acid buffer.

Workers were genotyped using 2 groups of linked microsatellites (Shaibi et al. 2008): UN351 and HB-SEX-01 and HB-THE-01, HB-THE-02, HB-THE-03, and HB-THE-04. PCR was performed using the conditions given in Shaibi et al. (2008). Maternal and paternal alleles for each worker were determined as in Experiment 1. The advantage of using tightly linked loci is that they facilitate identification of workers sired by the drones of a single queen. The combined loci produce 2 signature haplotypes per locus, unique (or nearly so) to the DM queen (Shaibi et al. 2008).

Results

Experiment 1

From 251 workers, 178 sires were identified, of which 19 (10.7%) were from drones carrying alleles not found in the Horner population (colony average = 8.7 ± 11.4% standard deviation).

The allelic diversity of the Horner and feral populations for Experiment 1 is given in Table 1. Of 81 identified alleles, 25% were only found in the Horner population, 41% were unique to the feral population, and 35% were common.

An inferred paternal drone genotype that contained an allele not detected in the DS population was assumed to be feral. Because of the DM genotype 0.03 nondetection error (see above), this is a conservative estimate: There is a 3% probability that workers inferred to be sired by a feral drone may in fact have been sired by a DM drone. All drone genotypes that carried a DM allele at all 6 loci were assumed to be derived from the DS population. However, 58% of all DS alleles were also identified in the feral population, giving the possibility of nondetection of feral genotypes. The probability that any one feral drone did not carry an allele unique to the feral population at any of the 6 loci examined is $\prod(1-U_i)=0.051$, where U_i is the proportion of alleles unique to the feral population at the ith locus (Table 1). Therefore, an inferred paternal drone genotype that has a DM allele at all loci has an approximately 0.05 probability of arising from the feral population. This estimate is conservative because it assumes that all alleles were at equal frequency and that all alleles present in the DM queens were also present in the feral population. The binomial probability that more than 15 of the 178 identified drones allocated to the Horner population are actually feral drones is <0.01. Therefore, we can conservatively estimate the efficacy of the Horner mating system from Experiment 1 at $(229-15)/251 = 85.3\%$.

Table 1 Allelic diversity at each of 6 microsatellite loci in a selected population of drones (Horner) and a feral population (Experiment 1)

	Number of alleles at microsatellite locus					
Allelic classification	A107	A14	A79	B124	A29	A113
Only in Horner	5	5	2	1	2	5
Only in feral	10	6	1	4	6	6
Shared	7	3	4	4	6	4
Total	22	14	7	9	14	15
Proportion of alleles unique to feral population (U_i)	0.455	0.429	0.143	0.444	0.429	0.400

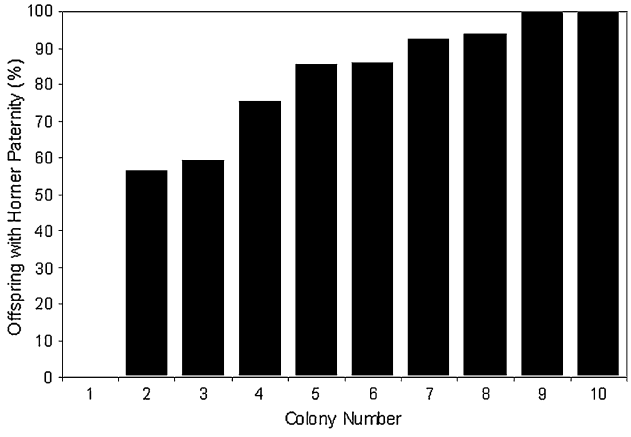

Figure 1. Contribution of 4 most frequent haplotypes to paternity of workers in 10 colonies—the 4 haplotypes are consistent with originating from a single queen.

Table 2 Allelic diversity in linked microsatellite loci used for Experiment 2 (2 sex-linked loci and 4 thelytoky-linked loci)

	Sex-linked loci		Thelytoky-linked loci			
	UN351	HB-SEX1	HB-THE1	HB-THE2	HB-THE3	HB-THE4
Number of alleles[a]	11	3	4	6	6	4

[a] For paternal haplotypes identified, see Supplementary Material online.

The number of patrilines identified per queen averaged 13.7, with a range of 8–17. With 20 patrilines, the binomial probability of not sampling more than 2 patrilines from a sample size of $n = 19$ workers is <0.05. Therefore, our observed number of patrilines is most likely underestimated by no more than 2 patrilines.

Experiment 2

Twenty different paternal haplotypes were identified in 795 workers. The 4 most frequent haplotypes accounted for 75.9% of worker paternity and were consistent with origin from a single queen. The median frequency per colony of these 4 haplotypes combined is 85.9% (Figure 1), though a single colony showed no workers with these paternities. If this colony is excluded, the 4 most frequent haplotypes account for 90.7% of worker paternity.

For an average of 80 workers genotyped in each offspring colony, the nonsampling error, assuming 16 patrilines (the average from Experiment 1 plus maximum likely sampling error), is <0.01. The cumulative probability of missing more than one patriline across all 10 colonies is <0.01. Therefore, even though individual matings cannot be identified (due to the high frequency of Horner haplotypes), our analysis has most likely identified all matings.

Given the paternal allele frequencies observed in the worker samples (Table 2 and Supplementary Material online), the probability that 2 unrelated drones carry the same haplotype (nondetection error—Boomsma and Ratnieks 1996) is 1.1×10^{-8}. The binomial probability that any number of workers carrying 1 of the 4 common haplotypes arose from another (and therefore feral) colony is $<<0.01$.

Discussion

Our genotypic survey shows that the Horner delayed mating system provides at least 85% control of honeybee mating. In contrast, Jensen et al. (2005) suggest that only 18% of open matings occur between queens and drones from the same apiary. Moritz et al. (2008) similarly found that the largest mating contribution of drones of a single genotype was 25%. Thus, the Horner system provides a highly significant increase in control of mating relative to natural mating and offers significant practical benefits over instrumental insemination or isolated mating using islands or mountain valleys.

The presence of 16 haplotypes of low frequency indicates that there were other colonies that contributed drones for mating and that the Horner system does not derive its effectiveness from geographical isolation. The area surrounding the system is densely forested, providing extensive habitat for feral colonies. Furthermore, at the time of Experiment 2, Horner had approximately 120 unregulated colonies within 5 km of the controlled mating site, many of which would have been capable of contributing drones (Jo Horner, personal communication).

Even with the absence of the DM queen genotype in Experiment 2, the results are consistent with both Experiment 1 and an expectation of low contribution of drones from any single colony in an open mated system. The difference in estimated efficacy of the system observed in the 2 experiments (85.3% vs. 75.9%) is likely influenced by the reduced number of drones released in Experiment 2—possibly as much as an order of magnitude less. If so, this would indicate that approximately 90% of the efficacy of the system arises through control of mating time, as opposed to "flooding" of the drone congregation areas with high numbers of the selected drones.

By selecting (among other traits) for color, Horner has achieved highly uniform coloration of his breeding stock. As a result, worker offspring derived from feral drones often exhibit deviations from the typical coloration. This allows Horner to effectively screen the colonies with lower mating success, further increasing the efficiency of his system.

Honeybee traits of interest to beekeepers have been shown to be of medium to high heritability (Collins et al. 1984; Oldroyd et al. 1987; Bienefeld and Pirchner 1990) and therefore capable of responding to selection. However, when only 25% of selected drones contribute to offspring in a standard open mating situation, the selection differential is significantly reduced. By using the Horner system, a 48% increase in the selection differential can be achieved, leading to substantial gains in trait improvement, when incorporated into a selective breeding program. As the Horner system does not require using isolated mating stations, instrumental insemination, or large numbers of colonies, the system is very appropriate for commercial bee breeding, providing excellent control of mating and large numbers of selected naturally mated queens.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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