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Lack of kin recognition in swarming honeybees (*Apis mellifera*)

Abstract Honeybee colonies reproduce by colony fission and swarming. The primary swarm leaves the nest with the mated mother queen. Further "after-swarms" can leave the nest. These are composed of virgin queens and sister workers. Since all workers in the primary swarm have the same relationship to the mother queen, kin recognition cannot have any effect on the worker distribution in the swarm. Because of polyandry of the mother queen, the after-swarm is composed of super- and halfsister workers of the virgin queen. In this case kin recognition might affect swarm composition if workers increase their inclusive fitness by preferentially investing in a supersister queen. The distribution of workers in the mother colony, the primary and the after-swarm was analyzed using single-locus DNA fingerprinting in two colonies of the honeybee (*Apis mellifera*). The colonies were composed of 21 and 24 worker subfamilies because of multiple mating of the queen. The subfamily distribution in the mother colonies before swarming was significantly different from the subfamily frequencies in the primary swarm. This indicates different propensities for swarming in the various subfamilies. The subfamily distribution was also significantly different between the mother colony and the after-swarm. There was however no significant difference between the subfamily composition of the primary and the after-swarm. The average effects of kin recognition on the distribution of the subfamilies in the two after-swarms were less than 2%. We conclude that colony-level selection sets the evolutionary framework for swarming behaviour.

Key words Kin recognition · Swarming · *Apis mellifera* · Microsatellite DNA

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Introduction

The honeybee (*Apis mellifera*) queen mates with a large number of males and stores the semen in her spermatheca. She utilizes the semen in a random fashion for fertilizing her eggs. This leads to a genetically composite colony comprising a variety of subfamilies which have different fathers (Laidlaw and Page 1984; Moritz 1986; Estoup et al. 1994). Using the pedigree coefficient of relationship G (Pamilo and Crozier 1982) workers within a subfamily are related by $G = 3/4$ (= supersisters) because of male haploidy whereas workers from different subfamilies are related by $G = 1/4$ (halfsisters). Inclusive fitness theory (Hamilton 1964 a,b) predicts that workers behaving nepotistically and investing preferentially in supersisters rather than in halfsisters should gain indirect fitness compared to workers unable to discriminate between kin and non-kin. A vast body of literature on kin recognition in honeybees demonstrates that workers indeed are able to discriminate between super- and halfsisters under certain conditions (for reviews see Getz 1991; Moritz and Southwick 1990). However, frequent doubt has also been expressed whether such a potential for kin discrimination is used by workers in a natural context and if nepotism has any impact on natural selection of honeybees (Carlin 1989; Carlin and Frumhoff 1990; Oldroyd et al. 1990; Moritz 1991). In most cases, the bees were tested under experimental conditions which were prone to artifacts. In other cases in which tests were conducted under natural conditions (e.g. Page et al. 1989, Visscher 1986) the data were barely significant.

There is no doubt that kin recognition ought to be selectively adaptive for honeybees under inclusive fitness theory. The controversy is whether the individual worker maximizes its own inclusive fitness or whether other selection pressures override the potential individual advantage of kin recognition. The strongest consequences of kin selection in honeybees ought to be observed during colony fission and swarming. During

spring the mother queen of a colony ceases egg production, reduces her ovaries to regain flight ability, and leaves the nest cavity with about half the workers and drones in search of a new nesting cavity. Often the remaining workers and a virgin sister queen leave in yet another swarm, the so-called after-swarm. Whereas the workers all have the same relatedness to their mother queen in the primary swarm, there will be super- and halfsisters workers to the queen in the after-swarm. The investment into the sister queen of the after-swarm is complete. A worker dedicates its full further life to the new queen and mistakes are irreversible because they never return to their native nest. Therefore there should be a high adaptive value if a worker can discriminate between a super- and halfsister queen when joining the after-swarm. Getz et al. (1982) conducted experiments to evaluate the kin structure in honeybee swarms. They inseminated two queens which were homozygous at the *cordovan* locus (*cd* = light brown body colour) each with semen of a wildtype (+) and a *cd* drone. This enabled them to identify workers of the patrilines by the *cd* and + phenotype respectively. In two swarms they observed that the *cd/cd* workers preferentially swarmed with the *cd/cd* mother queen. Getz et al. (1982) stated that one possible explanation for their results is that "... kin recognition is responsible for the nonrandom grouping of patrilineal lines during swarming"(p. 268; we return to this point in the discussion in detail). In an alternative explanation not invoking kin recognition Getz et al. (1982) recognized that the swarming tendency could be more strongly expressed in the homozygous *cd* workers. It is well known that there are strong racial differences in swarming behaviour in *A. mellifera*. The best known example is that of the Africanized bee in the Americas which may be replacing European honeybees because it swarms more frequently (Taylor 1977; Hall and Muralidharan 1989). Furthermore, honeybee queens naturally mate with a very large number of drones (Estoup et al. 1994; Moritz et al. 1995) which causes highly complex genotypic compositions in the hive. Moritz and Heisler (1992) showed that the potential ability of kin recognition can be found in simple choice situations with workers of two subfamilies only but decreases rapidly if the number of subfamilies in the nest increases. In this paper we study the mode of colony fission and swarming behaviour of naturally mated queens and try to identify the effects of potential kin recognition on the distribution of subfamilies in the after-swarm.

Materials and methods

Catching the swarms

Ten 20-frame colonies of *A. mellifera mellifera,* a western European race of honeybees renowned for their high swarming tendency (Ruttner 1988), were prepared for swarming by reducing the colony to one super only. The genotypic compositions of the colonies were determined from brood samples to exclude drifted individuals. To account for potential fluctuations in the subfamily composition in the colony over time, sealed brood samples of 200 workers were taken in early May about 4 weeks before the first swarm was observed. The brood sample thus reflected the genotypic composition of the colonies during the swarming season in June. We continuously observed the flight entrances of the colonies at day time to detect those that were about to swarm. When a swarm emerged, a catch net, as used by the traditional beekeepers of the Lüneburger Heide, was placed over the flight entrance until all swarming bees were caught (primary swarm with mother queen). A sample of 200 worker bees and the queen was taken from the swarm and kept at −70° C for further DNA analysis; the rest of the swarm was transferred back to the colony in order to encourage further swarming. The primary swarm sample served as null hypothesis for the swarming ability of the various subfamilies in the afterswarms. The colonies were further observed to catch the after-swarm as described above. Only two colonies produced both primary and after-swarm.

Genotyping

DNA was phenol-extracted from individual worker larvae or adult bees using routine protocols (Moritz et al. 1994). Single-locus DNA fingerprinting was used to determine the patrilines in the different samples. Four microsatellite loci (A14, A76, A29 and A107) were tested as in Moritz et al. (1995). The genotypes of the drones that fathered a subfamily were determined by comparing the queen's genotype with her worker offspring. Worker alleles that were not present in the queen were interpreted as paternal alleles. If both alleles of a worker at a given locus were identical to those of its mother queen they were not assigned to a specific patriline. In all these cases it was possible to identify the patriline by using the information from the other loci.

Results

A total of 641 workers from two colonies were genotyped in this study. The larvae sample revealed a total of 24 and 21 subfamilies in colony A and colony B respectively. The distribution was inhomogenous and some patrilines appeared at higher frequencies than others. In both colonies the subfamily frequencies in the primary swarm significantly differed from the random brood sample ($P < 0.05$, Fisher's exact test). Some subfamilies showed a greatly increased frequency in the swarm whereas other were substantially reduced (Fig. 1). Most important is the comparison between primary and after-swarm. In both colonies we found no significant difference between the subfamily distributions in primary and after swarm (not significant, Fisher's exact test).

Do swarming bees use kin recognition?

This lack of evidence for kin recognition does not necessarily mean that the workers did not use kin discrimination for their swarming decisions. Our test does not reject, but cannot verify, the null hypothesis. In order to evaluate the impact of potential kin recognition on our data set, we therefore compared the predicted frequency distribution under kin recognition with the observed
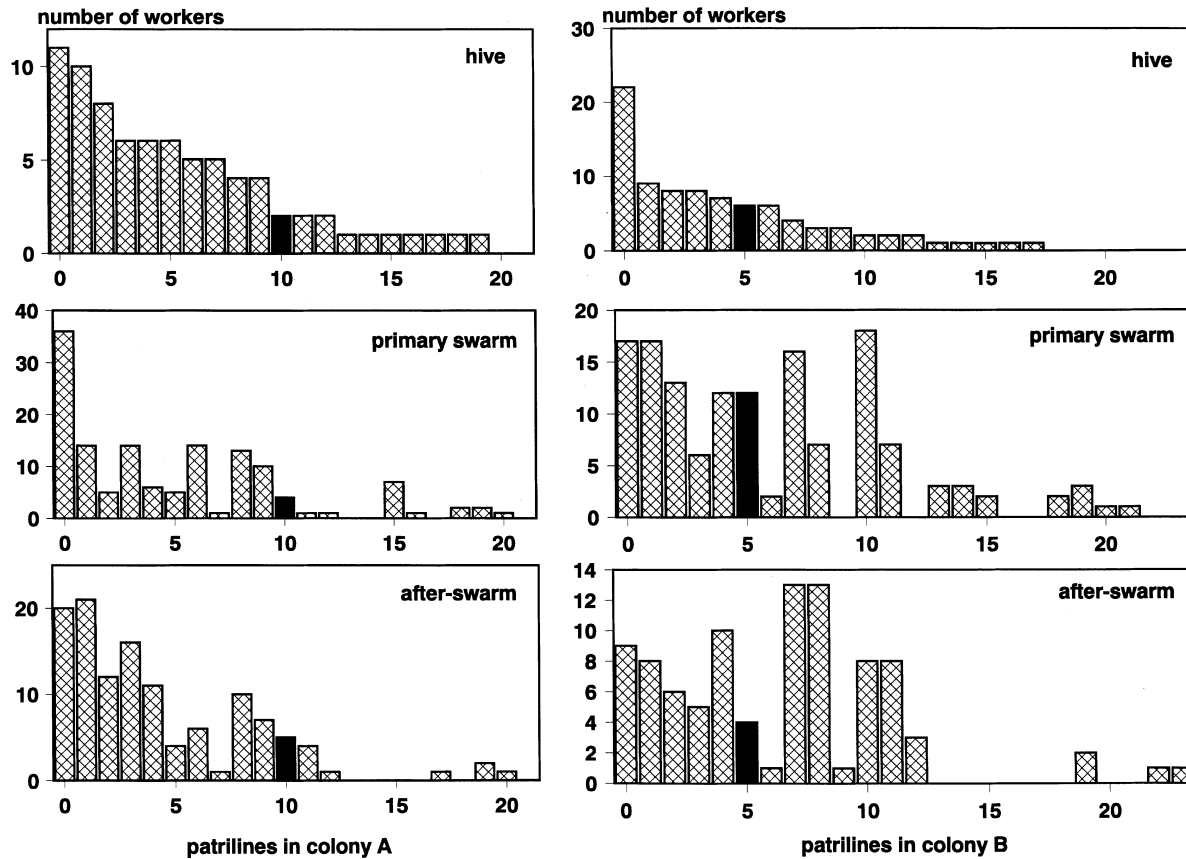
Fig. 1 Frequency distribution of the patrilines in the two colonies (A *left*, B *right*) before swarming (*top*), in the primary swarm (*centre*), and the after-swarm (*bottom*). The patrilines of the virgin queens in the after-swarm are shown in *black bars* in all figures

after-swarm subfamily distribution. We derived the predicted values from the primary swarm frequencies which we considered as a standard against which a kin recognition bias in the after-swarm could be compared.

Let us assume that the patriline composition and the propensity for workers to swarm in the colony is identical during primary and after-swarming. If workers recognize kin and use this for their swarming decision, they should all join the supersister queen. Halfsister workers should not join the swarm because by staying behind they increase their chances of investing in a supersister queen. If queens are reared at random, the chance of rearing a supersister equals the subfamily frequency f_n. This probability is clearly zero if they join the halfsister queen in the after-swarm. On average staying behind increases the relatedness between the halfsister workers and the next queen since in f_n of all cases relatedness will be $r = 3/4$ whereas in $1-f_n$ cases relatedness is $r = 1/4$. Because for any $f_n > 0$, $f_n(3/4) + 1 - fn(1/4) > 1/4$ the halfsisters should stay behind to take advantage of the possibility of becoming supersisters of the next queen to emerge. Even if they had information on the genotype of the next queen, it might be selectively advantageous to stay with a half-sister queen rather than swarm with a halfsister queen.

Vacant nesting sites are a serious constraint in honeybee life history, making swarming a risky operation.

If k is the proportion of workers which use kin discrimination to join the after-swarm, $1 - k$ of the swarming workers join the after-swarm in subfamily frequencies as observed in the primary swarm. As k approaches 1, workers of the non-queen subfamilies should avoid joining the after-swarm and only supersister workers should join the queen. The expected frequency, $\hat{f}_s$, of supersister workers (subfamily s) in the after-swarm would thus be:

$$\hat{f}_s = \frac{(1+k)f_s}{1 + kf_s - k\sum f_i} = \frac{(1+k)f_s}{1 - k + 2kf_s} \qquad (1)$$

halfsister frequencies are:

$$\hat{f}_i = \frac{(1-k)f_i}{1 + kf_s - k\sum f_i} = \frac{(1+k)f_i}{1 - k + 2kf_s} \qquad (2)$$

where:

$\hat{f}_i$ = frequency of halfsister subfamily i in the after-swarm

$\hat{f}_s$ = frequency of the supersister subfamily in the after-swarm

f_s = frequency of the supersister subfamily in the primary swarm

f_i = frequency of halfsister subfamily i in the primary swarm

k = proportion of swarming workers expressing kin recognition

We used a χ^2 analysis pooling the rare patrilines such that the expectation values were > 1 for each observation. One degree of freedom (df) is lost in the statistical analysis due to using the parameter k. This results in a total of 13 and 16 df for each colony respectively. From the lowest χ^2 values we estimated $k = 0.02$ in colony A and $k = -0.04$ in colony B (Fig. 2). Pooling the data from both colonies, we obtained an average of $k = 0$ which is our best estimate of any potential contribution of kin recognition to the fission pattern in the after-swarm. Considering the 95% confidence limits, the maximum possible proportion of workers joining the after-swarm due to kin recognition does not exceed $k_{max} = 0.06$ in colony A. Any increase in the sample variance (i.e. change in patriline frequencies during the sampling period) would increase the estimate of k_{max} overestimating the actual contribution of kin recognition.

Do non-swarming workers use kin recognition?

Another scenario could be that the workers staying behind during swarming know which queen is going to take over the old nest site. In this case one would expect differences between the genotypic composition between primary and after-swarm such that one particular patriline is under and the others are over represented. We tested this by taking a frequency decrease in the after-swarm as indication for such a mechanism. Under complete kin recognition ($k = 1$) one patriline should be completely lacking whereas the others ought to be represented as in the primary swarm. Thus the swarm is composed of a proportion of $(1 - k)$ workers with no and k workers with kin recognition. Workers of the subfamily staying behind because of kin recognition are represented at
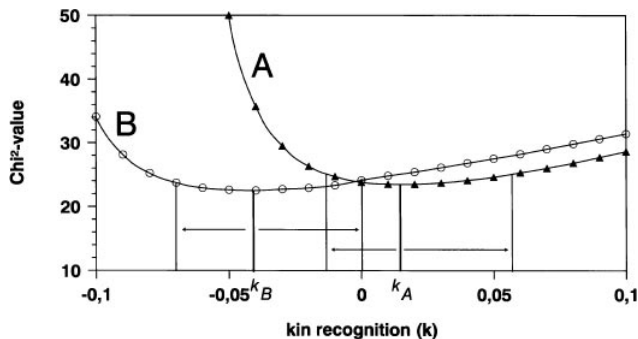
$$\hat{f}_s = \frac{(1 - k)f_s}{1 - kf_s} \qquad (3)$$

halfsisters are represented at:

$$\hat{f}_i = \frac{f_i}{1 - kf_s} \qquad (4)$$

where

$\hat{f}_i$ = frequency of halfsister subfamily i in the after-swarm

$\hat{f}_s$ = frequency of the supersister subfamily in the after-swarm

f_s = frequency of the supersister subfamily in the primary swarm

f_i = frequency of halfsister subfamily i in the primary swarm

k = proportion of workers expressing kin recognition

Using χ^2 analysis, the possibly of each colony being the one with a potentially reduced frequency in the after-swarm we could reject this hypothesis for any subfamily. Even the best fitting k value ($\hat{k} = 0.9$) yielded highly significant deviations between the observed and expected subfamily frequencies under this model ($P < 0.001$).

Do rare patrilines preferentially join the primary swarm?

It might be adaptive for workers of rare patrilines to join the primary swarm more frequently, particularly if nurse bees preferentially rear super-sister virgin queens. The chance that a reared virgin queen will be of a rare subfamily is small, even under random queen rearing. The best bet of a rare subfamily worker therefore would be to join the mother queen to which she is more closely related than to a halfsister virgin queen. Under this model there should be a negative correlation between worker frequency in the colony before swarming and the frequency difference between the prime and after-swarm. Yet we found a non significant but positive correlation of $r = 0.15$ (Fig. 3) indicating that frequent patrilines swarm more frequently in the primary than in the after-swarm opposite to the above prediction.
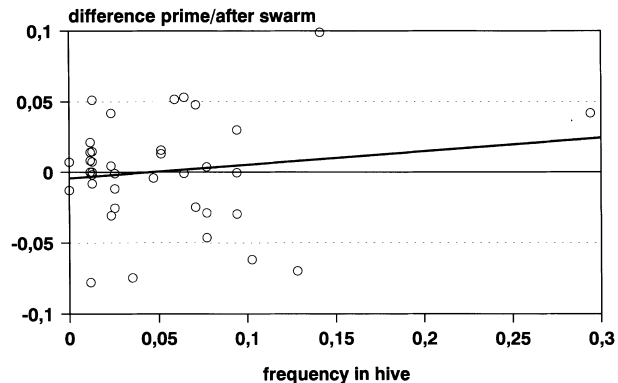

Fig. 2 Values of χ^2 (y-axis) calculated from the difference between observed and predicted subfamily frequencies in the after-swarm under different proportions of workers with kin recognition ability in the two test colonies A and B. The least-square estimates for k_A and k_B (the propensity for kin recognition in each colony) are shown with the corresponding *95% confidence limits*. The maximum value is reached in colony A with $k_{max} = 0.06$

Fig. 3 Scatter plot and regression of the difference between the subfamily frequencies in the primary (*prime*) and after-swarm on its frequency in the colony

Discussion

Variability in the subfamily composition of a honeybee colony may occur over time within certain limits. However, this cannot explain the observed subfamily frequency differences between the larval, primary and after-swarm sample in the present study. The timing of the sampling was such that the sampled workers (larvae and adults) were all from the same time window. The colonies were disease-free and there was no indication of failure of brood to develop to adults. The brood sample represents the genotypic composition of the colony during the swarming season.

The significant differences between subfamily frequencies in the primary swarm and the brood sample clearly indicates variability among the various subfamilies for the propensity of swarming behavior. Some made a very high contribution to the swarms whereas others were less active. We failed to find evidence that the workers preferentially aggregated around supersisters in the after swarm. Instead, we did find support for the hypothesis of preferential swarming of certain genotypes. We observed highly significant differences between the genotypic composition of the mother colony and the primary swarm. This may also explain the *cordovan* data in the experiments of Getz et al. (1982). It seems unlikely that the *cordovan* body colour itself has any impact on swarming behavior. However, since both queens were inseminated with one wildtype and one *cd* drone each, all genetically determined behavioral traits of that particular drone were linked with the marker gene and led to the co-segregation of swarming propensity and body color. The major difficulty with the data of Getz et al. (1982) stems from the experimental design, which is not entirely suited to address the problem of intracolonial kin recognition. They analysed only the primary swarms, where all workers have the same relationship to the mother queen irrespective to which patriline they belong. Therefore, given kin recognition has an effect of patriline distribution in swarming, it could only have had an impact on the results presented by Getz et al. (1982) if the *cd* drone was more closely related to the mother queen than the wildtype drone. To evaluate potential kin recognition mechanisms one could have analysed the remaining workers and the corresponding virgin queens. However, these data were only partially available and were not systematically studied.

The other models of potential decision-making during swarming tested also find no support in our study. This may not be surprising because they require individual workers to have an extremely good knowledge of the genotypes and the eclosion order of the virgin queens or the total genotypic composition of the colony. Both are possible but certainly much more complex than the requirements of the first hypothesis, where only a single queen needs to be evaluated. We found no evidence that workers maximize their individual fitness by making choices which ought to be most rewarding under classical inclusive fitness theory. Yet it has repeatedly been shown that workers can discriminate between kin and non-kin in experimental situations (Getz 1991). So why do workers not use their ability to discriminate among kin during swarming? They have several days between emergence of the virgin queen and take-off of the swarm to evaluate whether the new queen is a super- or halfsister. Potentially they could use this period for kin discrimination. Workers have been shown to have the physiological ability to discriminate between kin and non-kin queens on the basis of volatile odor signals (Moritz and Crewe 1988). Cuticular hydrocarbons have also been found to be potential cues for kin recognition in honeybees (Page et al. 1991; Getz and Page 1991; Arnold et al. 1995). Yet apparently, the workers use neither the discrimination ability nor the cues to increase their inclusive fitness during swarming. Instead they join the after-swarm in a similar fashion to the primary swarm.

One possible problem in interpreting the data is the fact that we returned the primary swarms back to the hives to facilitate the occurrence of afterswarms. In nature, workers will only return to the hive if the mother queen dies soon after swarming; but this is a relatively rare event. If workers are physiologically primed to swarm, and this priming is not easily reversed before afterswarming takes place, then this would cause the patriline frequency profiles (see Fig. 1) in the primary and after swarms to be more similar than would be otherwise expected. If, however, the decision by a worker to join the afterswarm is independent of its decision to join or not to join the primary swarm, then our results reflect the role of kinship considerations in swarming.

A plausible explanation can be found by considering selection pressures at other than the individual level governing the swarming process. Indeed workers may gain a greater inclusive fitness if they ensure a sufficiently large swarm rather than a genotypically correct one. Swarms with queens of rare patrilines would be doomed units if they could not recruit sufficient workers to gain enough colony strength within the brief season of the temperate climate to survive harsh winters. A failing swarm reduces fitness for all workers in the colony, because the swarm takes honey resources and reduces the worker force in the hive. Therefore, kin recognition or other non-random fission mechanisms in swarming may not be free of costs and risks. It may be counterproductive for the individual worker to be choosy and not join the afterswarm with the halfsister queen. The observed swarming strategy suggests that the indirect fitness gain by supporting a halfsister instead of a supersister queen may still be sufficient to form an evolutionary stable strategy.

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