THE ROYAL SOCIETY

Relatedness among honeybees (*Apis mellifera*) of a drone congregation

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The honeybee (*Apis mellifera*) queen mates during nuptial flights, in the so-called drone congregation area where many males from surrounding colonies gather. Using 20 highly polymorphic microsatellite loci, we studied a sample of 142 drones captured in a congregation close to Oberursel (Germany). A parentage test based on lod score showed that this sample contained one group of four brothers, six groups of three brothers, 20 groups of two brothers and 80 singletons. These values are very close to a Poisson distribution. Therefore, colonies were apparently equally represented in the drone congregation, and calculations showed that the congregation comprised males that originated from about 240 different colonies. This figure is surprisingly high. Considering the density of colonies around the congregation area and the average flight range of males, it suggests that most colonies within the recruitment perimeter delegated drones to the congregation with an equal probability, resulting in an almost perfect panmixis. Consequently, the relatedness between a queen and her mates, and hence the inbreeding coefficient of the progeny, should be minimized. The relatedness among the drones mated to the same queen is also very low, maximizing the genetic diversity among the different patrilines of a colony.

Keywords: honeybee; microsatellites; lod scores; mating behaviour; relatedness; panmixis

1. INTRODUCTION

In honeybees (*Apis mellifera*), drones do not collect nectar or pollen. Neither do they participate in the defence or the keeping of the hive; mating with young queens is their only known function. Drones take their first flights between five and eight days after emergence (Ruttner 1966). These first flights are short and supposedly serve for orientation (Drescher 1969). About ten days after emergence, they begin to perform mating flights, which can last longer than 30 min (Witherell 1971). When atmospheric conditions are favourable, in late spring and summer, drones can perform several mating flights in a single afternoon. During the mating flights, drones join a congregation area (Zmarlicki & Morse 1963) where they remain flying in wide loops until they return to the colony to feed (Ruttner 1974). Congregation areas have usually a diameter of 30–200 m. The height of drone flights is 15–40 m above the ground. Several thousand drones participate in the congregation, which is formed irrespective of the presence of a queen (Ruttner 1966). A congregation area has a limited spatial extension and drones are not attracted by a queen flying outside the area (Ruttner & Ruttner 1966).

The distribution of drone congregation areas has been studied with balloons carrying tethered or caged queens and by radar (Gary 1963; Loper *et al*. 1987). It has been shown that several drone congregation areas can be found within the flight range of an apiary (Zmarlicki & Morse 1963; Ruttner & Ruttner 1966). A range of mating places is hence available for drones and queens. The location of drone congregation areas remains constant for several years. The orientation mechanism that drones and queens use to find the congregation areas is not well understood, although the light distribution and the shape of the horizon probably have some influence (Pechhacker 1994). When a queen approaches a congregation, drones chase her, forming a comet-like swarm in her wake. Several drones copulate successively in flight with the queen (Gries & Koeniger 1996) and die immediately after. The total number of matings of a queen has been estimated by different methods to range from 7 to 20 (Taber 1954; Woyke 1955; Adams *et al*. 1977; Estoup *et al*. 1993*a*).

The study of drone congregations is difficult because drones fly high up in the air. Although it is generally assumed that numerous colonies delegate drones to these congregations (Ruttner & Ruttner 1972; Page & Metcalf 1982), experimental data on the number of colonies and the relative contribution of each participating colony have seldom been collected. The composition of drone congregations has several important consequences for the genetic structure of honeybee colonies. The number of colonies represented in a congregation influences the

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relatedness between a queen and her mates and hence the inbreeding level of colonies, as well as on the relatedness between the mates of a queen, which affects genetic diversity within colonies. Genetic markers can be used to estimate the number of colonies represented in a congregation, which is equivalent to determining the number of groups of brothers in a sample. We conducted these analyses using microsatellite DNA loci which, given their hypervariability, are ideal markers to determine the relatedness relationships among individuals (Queller & Goodnight 1989; Blouin *et al*. 1996).

2. MATERIALS AND METHODS

(a) *Biological material and DNA extraction*

Two samples of honeybees (*Apis mellifera carnica*) were collected at Oberursel (near Frankfurt, Germany). The first sample (congregation sample) consisted of 142 drones taken at random from 2123 drones captured within 30 min at a height of 40 m using a helium-filled balloon and pheromone drone trap, after Williams (1987). The second sample (apiary sample) comprised 18 groups of four young drones (to avoid drift between colonies) from as many colonies belonging to five apiaries situated in the vicinity of the congregation area. DNA was extracted from bee heads with a phenol–chloroform extraction, followed by ethanol precipitation, as described by Kocher *et al*. (1989) and modified by Garnery *et al*. (1993).

(b) *DNA amplification and genetic analysis*

We selected 20 unlinked loci among the most polymorphic ones in the species (Estoup *et al*. 1993*b*). Drones were genotyped at 20 (congregation sample) or 12 (apiary sample) unlinked microsatellite loci (accession number of primers in EMBL: AJ229000–AJ229039). A sample of 10 µl of the polymerase chain reaction (PCR) mixture, using incorporation of [α-^{33}P]dATP (Estoup *et al*. 1993*b*), was processed through 30 cycles consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 52–62 °C (depending on the locus), and elongation for 30 s at 72 °C. A sample of 2 µl of each reaction was run on 6% polyacrylamide sequence gels. Gels were dried and exposed to X-ray films for 24–48 h. Number of alleles per locus, allele frequencies, expected heterozygosity per locus and linkage disequilibria between loci were calculated in the congregation sample using GENEPOP software (Raymond & Rousset 1995).

(c) *Lod score*

Several statistics are commonly used to test relatedness between individuals with microsatellite markers (Thompson 1975; Queller & Goodnight 1989; Chakraborty & Jin 1993); we chose the lod score (Morton 1995), a statistic based on a likelihood ratio. Because of drone haploidy, there are only two possibilities at a given locus: two drones do or do not share the same allele. The probability that two drones share the same allele A_i (frequency p_i in the population) is $p_i(1+p_i)/2$ if they are brothers, and p_i^2 if they are unrelated. The ratio of these two probabilities ($R_S=(1+p_i)/2p_i$) is the 'likelihood ratio' of the two drones being brothers as opposed to being unrelated, knowing that they have the same genotype at the locus under study. The probability that the two drones have different alleles, A_i and A_j (j being any allele but i), is $p_i(1-p_i)/2$ if they are brothers and $p_i(1-p_i)$ if they are unrelated. So the corresponding likelihood ratio is $R_D=0.5$.

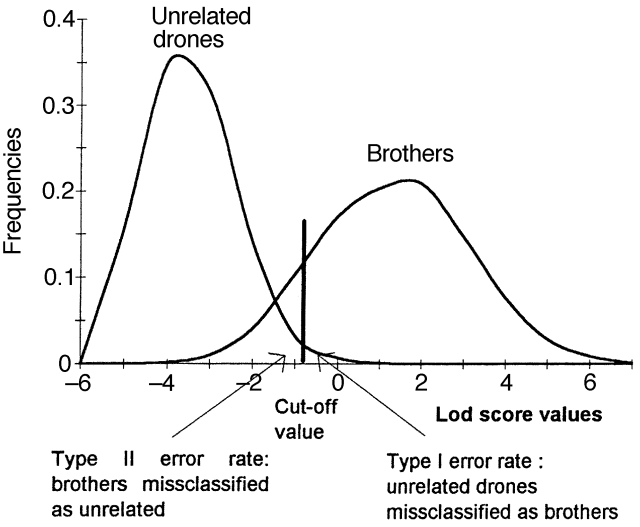

Figure 1. Criterion for the classification of relatedness between pairs of drones. The type I error is the proportion of unrelated drones that are misclassified as brothers because of their lod score being above the cut-off value. The type II error rate is the proportion of brothers that are misclassified as unrelated drones because of their lod score being under the cut-off value.

For several loci, the likelihood ratio is the product of likelihood ratios at each locus, provided that loci are genetically independent and that there are no linkage disequilibria between loci. Lod scores are the decimal logarithms of likelihood ratios, so that multilocus lod scores are sums of lod scores over all loci.

(d) *Simulations*

To be able to use lod scores to determine relationships between drones taken pairwise, the distribution of lod scores among brothers and among unrelated drones must be determined. Computing these distributions, using actual allele frequencies of the population under study, is possible but the number of genotype combinations to examine can be enormous (more than 10^{34} in the present study), and it is much more effective to perform simulations. Actual allele frequencies were used to randomly generate pairs of unrelated drones and pairs of brothers, assuming Hardy–Weinberg equilibrium and no linkage disequilibria between loci. The simulation process was validated by comparing the lod-score distributions in the apiary sample where individuals have known relatedness (the drones within colonies are brothers and the drones from different colonies are unrelated) with the lod-score distributions in corresponding simulated samples of brother and unrelated drones. The apiary sample comprises 72 drones taken in 18 colonies (four drones per colony). This represents $18\times6=108$ pairs of brothers and $[(72\times71)/2]-108=2448$ pairs of unrelated drones. The simulated sample consisted of 5000 simulated unrelated drones and 5000 simulated brothers generated with the allele frequencies of the apiary sample.

(e) *Choice of a threshold value for the test*

We need a threshold value to decide whether two drones are brothers or unrelated. The distribution curves of lod scores for brother pairs and unrelated pairs (figure 1) are always overlapping. The consequence is that any threshold value will produce two kinds of erroneous assignments: unrelated individuals classified as brothers (type I error) and brothers

Table 1. *Number of alleles and expected heterozygosity (H_e) per locus in the congregation sample*

locus	*B124*	*A79*	*A113*	*A107*	*A7*	*Ap34*	*A14*	*A29*	*Ap36*	*A76*
number of alleles	12	11	9	18	13	8	12	24	17	50
H_e	0.734	0.846	0.599	0.901	0.809	0.728	0.476	0.910	0.730	0.972
locus	*A8*	*Ap43*	*Ap33*	*Ap1*	*Ap12*	*Ap19*	*Ap55*	*Ap37*	*Ap16*	*Ap14*
number of alleles	7	11	14	18	3	6	12	7	5	11
H_e	0.609	0.724	0.855	0.681	0.635	0.645	0.664	0.505	0.520	0.805

classified as unrelated individuals (type II error). These two error types are linked and will depend on the threshold value, e.g. increasing the threshold value will decrease type I error and increase type II error. We are not interested in the fate of a particular pair of drones, but rather in having the best possible estimation of the number of pairs of each kind (brothers versus unrelated). The distribution of lod score observed in the congregation sample indicates that pairs of unrelated drones were much more frequent than pairs of brothers. For this reason, rather than choose a threshold value that equalizes the two types of error rates, we instead chose the numbers of the two types of misclassified individuals: N_1, the number of unrelated drones misclassified as brothers, and N_2, the number of brothers misclassified as unrelated. N_1 and N_2 were calculated as follows:

$$N_1 = \alpha N_P (1 - P_B),$$

$$N_2 = \beta N_P P_B,$$

where α is the type I error of the test, β is the type II error (figure 1), N_P is the total number of pairwise comparisons, and P_B is the proportion of brothers in the sample. The cut-off value of the test was chosen in order to satisfy the equality $N_1 = N_2$, which results in:

$$\alpha(1 - P_B) = \beta P_B. \quad (1)$$

The parameter P_B was estimated by adjusting the observed distribution of lod scores in the sample (D_{obs}) with a combination of the two theoretical distributions for brothers and unrelated individuals. The latter distributions were determined by simulating 300 000 brother pairs (simulated distribution for brothers, D_{sb}) and 300 000 unrelated pairs (simulated distribution for unrelated drones, D_{su}). We looked for the value of P_B that minimizes the deviation between D_{obs} and $[P_B D_{sb} + (1 - P_B) D_{su}]$, using the χ^2 criterion.

Giving the theoretical distributions (D_{sb} and D_{su}), type I and type II errors are known functions of the threshold value (t), e.g. $\alpha = f(t)$ and $\beta = g(t)$. Then the value of t is obtained by solving (numerically) the equation: $f(t)(1 - P_B) = g(t) P_B$. From t, we get the values of α and β.

3. RESULTS

(a) *Genetic variation and simulations*

The number of alleles and the genetic diversity in the congregation sample are given for each locus in table 1. The number of alleles ranges between 3 and 50, with an average of 13.4 and the genetic diversity ranges between 0.48 and 0.97 with an average of 0.71. Among the 190 pairs of loci, only 11 pairs presented significant linkage disequilibrium at the 5% significance level, which is only a little above the expected number by chance in case of equilibrium ($190 \times 0.05 = 9.5$).

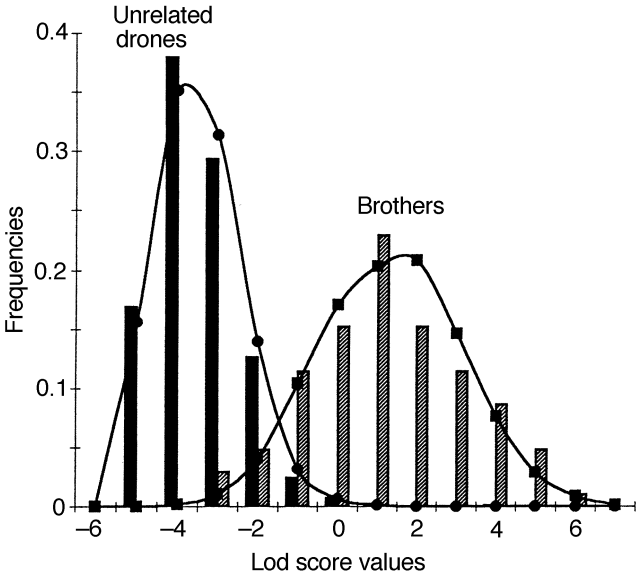

Figure 2. Observed (bars) and simulated (curves) distributions of lod score for brothers and unrelated drones. The observed distributions have been calculated with the drones of the apiary sample (the 18 groups of 4 brothers) for which parentage relationships are known (blacks bars represent unrelated drones and grey bars represent brothers). The simulated distributions have been calculated with 5000 unrelated drones and 5000 brothers, using the allele frequencies of the apiary sample.

Figure 2 presents the distribution of lod scores for the brothers and the unrelated drones of the apiary sample (the 18 groups of four brothers). The simulated distribution of lod scores for brothers and unrelated drones, computed with the allele frequencies of the apiary sample, is represented on the same figure.

(b) *Threshold value and brotherhood analysis*

The proportion of brother pairs among the 10 011 drones pairs of our sample was estimated to be $P_B = 0.00646$. The resulting threshold value was $t = 1.776$. The type I and type II errors associated with this threshold value were $\alpha = 0.0009$ and $\beta = 0.1428$, which corresponds to *ca.* nine pairs of each type (brothers and unrelated individuals) misclassified in the other category. The 10 011 tests were performed and 65 pairs of drones had a lod score above 1.776. Among them, 13 pairs of

Table 2. *Expected and observed numbers of groups of brothers (assuming a Poisson distribution)*

(The value of the χ^2 associated with these numbers is 1.056, which is not significant.)

number of drones by which a colony is represented	expected numbers	observed numbers
1	78.29	80
2	23.31	20
3	4.63	6
4	0.69	1
total	106.92	107

individuals occurred in only one of the 65 pairs, and 52 in more than one pair. Grouping individuals that had a lod score above 1.776 with at least one other drone of the group, we obtained eight groups of three to seven drones. The question of determining whether or not all the drones of a group are brothers can be approached by looking at the number of different alleles present at each locus. If no mutation occurred, there should be no more than two different alleles in all the males of a group of brothers, as they all have the same mother. Thus, each group of drones was analysed in the following way: all possible subgroups (including three to seven drones) were evaluated for the number of alleles present at the 20 loci. Rejecting all combinations that resulted in more than two alleles at one or more loci, we finally kept one group of four brothers, six groups of three brothers and 20 groups of two brothers. The 80 remaining drones had no brothers in the sample and were taken as groups of one individual. The total number of these groups was equal to $N_c=1+6+3+20+80=107$, which is the estimated number of colonies represented in the sample of 142 drones.

(c) *Number of colonies in the congregation*

If we assume that all colonies send, on average, the same number of drones to a congregation area, the number of drones from a colony that are present in a sample approximately follows a Poisson distribution. More precisely, given that we can only detect colonies for which at least one drone is present in the sample, the number of groups obtained above will follow a truncated Poisson law. Noting λ the parameter of the Poisson law, the probability of a colony being represented by i drones in the sample is equal to $\lambda^i e^{-\lambda}/n!(1-e^{-\lambda})$. The log-likelihood of the sample is equal to:

$$\log(L)=N_c[\lambda-\log(1-e^{-\lambda})]+N_d\log(\lambda)+C,$$

where C is a constant independent of λ, and N_d is the number of drones in the sample ($N_d=142$). Deriving with respect to λ, and equating to zero, we obtain a maximum-likelihood estimate of λ, $\hat{\lambda}=0.5954$.

The observed and expected numbers for the different classes of the Poisson distribution are given in table 2. The value of the χ^2 associated with these numbers (1.056) is not significant. As the parameter λ is the mean of the Poisson distribution, it follows that a colony is on average represented by λ drones in the sample of 142 drones. Hence the total number of colonies (N_t) sending drones to the congregation is such that $N_t\lambda=142$. Taking the estimate $\hat{\lambda}$ for the true value of the parameter provides an estimated total number of colonies of 238, with a 95% confidence interval of (192–304) calculated using the maximum-likelihood method.

4. DISCUSSION

(a) *Test of parentage*

Parentage testing is a well-established field of research and it is widely used in several areas of ecology and evolution. Resolving individual relationships is of importance for the study of mating behaviour and genetic dispersal, the management of captive-breeding programmes for endangered species and the study of the evolution of social behaviour. Until recently, relatedness studies were limited by the availability of informative genetic markers. Accurate estimations were not possible at the individual level, except when pedigree data were available, and one could only estimate the average relatedness within a group of individuals (Pamilo & Crozier 1982; Queller & Goodnight 1989). The recent development of hypervariable DNA loci in many species has ameliorated this problem.

For this study, we had to resolve the precise parentage relationship that existed between two individuals. The accuracy with which pairs of individuals can be classified in different relatedness categories by a parentage test increases with the number of loci and their heterozygosity. Microsatellites, which are polymorphic and abundant co-dominant markers, are ideal markers to determine parentage relationships between individuals (Queller *et al*. 1993; Blouin *et al*. 1996). A small number of microsatellite is generally adequate to distinguish between first-order relatives and unrelated individuals (Brookfield & Parkin 1992). The detection of smaller relatedness differences is also feasible with microsatellites if enough loci are available (Queller *et al*. 1993).

In our case, the situation was very favourable as we used 20 microsatellite loci chosen for their high heterozygosity to distinguish between brothers and unrelated individuals. We also developed a powerful test, as the type I and the type II error rates were respectively 0.0009 and 0.14 (Brookfield & Parkin (1992) consider a test to be powerful as soon as the sum of the two types of error is below 0.5). However, we had some difficulties to estimate the exact number of brothers in our sample. This can be explained by two main factors. First, the parentage relationships between the drones of the congregation were tested using the assumption that two drones could only be either brothers or unrelated. (This hypothesis was quite likely, as in honeybees, drones develop from unfertilized eggs and have no father. Furthermore, drones die very shortly after mating and hence no generation overlap is possible. Few parentage relationships are therefore possible between drones.) However, drones can also be cousins if their mothers are related. In our study, the 'cousin' relationship was not considered. The existence of cousins could explain the existence of 'groups' of drones, where several drones were linked by high lod scores but were not always brothers. Second, we had to perform more than 10 000 parentage tests. This very high number of individual tests markedly reduces the power of the

global test (when n tests are performed, each with an error rate of α, the total number of errors is $n \times \alpha$). These two kinds of problems will always be encountered when trying to determine the precise parentage relationships among numerous individuals in a natural population.

(b) *Simulation assumptions*

Our simulation process was based on the assumptions of no linkage disequilibria between loci and no deviation from Hardy–Weinberg proportions. The absence of significant linkage disequilibria between loci has been verified directly in the congregation sample. As drones are haploid, the Hardy–Weinberg proportions could not be tested for. However, the close agreement between simulated and observed distributions of lod score for brothers and unrelated drones (figure 2) indicates that this unverified assumption of the simulation, and the simulation process itself, were correct.

Natural colonies of honeybees are rare in Germany. Most of the colonies represented in the congregation are probably commercial colonies and it is common beekeeping practice to requeen colonies with sister queens. The presence of numerous cousin drones would have caused an underestimate of the total number of colonies contributing to the congregation.

This number has been calculated using the assumption that all colonies contributing drones are equally represented in this congregation. The very small value of χ^2 associated with the observed and calculated numbers of the Poisson distribution suggest that this assumption was probably correct. It is noteworthy that, if the colonies had not equally contributed to the congregation, the value of 238 colonies represented in the congregation would again have been an underestimate.

(c) *Consequences of congregation structure*

The number of colonies represented in the congregation (238) is very high. This value is in good agreement with the observations of N. E. Gary (personal communication to Page & Metcalf (1982)), who reported that drones from 200 different colonies were present in a congregation. Assuming that the mean flight distance of drones is 2.5 km (Ruttner & Ruttner 1972) and that the density of colonies is about 20 km^{-2} around Oberursel (N. Koeniger, unpublished data), then most of the colonies of the region had delegated drones to this congregation. The first genetic consequence of numerous colonies contributing equally to the congregation is that a queen has an equal chance of insemination by a male from each colony within the recruitment perimeter of the congregation. Consequently, honeybees represent probably one of the most completely panmictic system possible among terrestrial animals.

Estoup *et al*. (1993*a*) have used ten microsatellite loci to study the genetic structure of a honeybee colony from the southeast of France. The distribution of paternal alleles in the colony worker sample and in the local honeybee population was not significantly different. These observations are consistent with our results on drone congregation structure, i.e. the males that fecundate a queen are a representative sample of the local population.

Honeybees are highly sensitive to inbreeding. Physiology, morphology and behaviour are negatively affected by inbreeding (Brueckner 1978). Furthermore, mating between a queen and a related drone increases the probability of offspring being homozygous at the sex locus, leading to the production of diploid drones that are killed by workers shortly after hatching and hence represent a heavy genetic load for a colony (Mackensen 1951; Woyke 1963; Brueckner 1978). The very high number of colonies represented in a congregation makes the copulation of a queen with one of her brothers very unlikely ($p = 0.061$ for a queen mated 15 times), avoiding inbreeding and fatal homozygosity at the sex locus for her progeny.

Using the same molecular tools, it has been recently shown that, during the mating flight(s), a queen copulates with as many as 7–20 drones (Estoup *et al*. 1993*a*), a figure slightly higher than those previously reported (Page 1986). Several hypotheses have been proposed for the evolution of polyandry (Crozier & Page 1985; Boomsma & Ratnieks 1996). All assume that polyandry is needed to enhance the genetic diversity within colonies. If the mates of a queen were closely related, the amount of genetic variation given by polyandry would be very small. However, the very high number of colonies represented in a congregation makes the mating between a queen and two or more brothers unlikely. Consequently, the average relatedness between the mates of a queen expected from the familial structure of drone congregations is very low (0.002). In conclusion, polyandry associated with the composition of drone congregations ensures maximal genetic diversity within colonies.

The sociobiological consequences are also of importance (Visscher 1998). Honeybee workers have reduced but functional ovaries and, in certain conditions, may lay unfertilized eggs from which drones develop. However, with few exceptions (Oldroyd *et al*. 1994), the workers of a queen-right colony are sterile. If the queen has been fertilized by numerous unrelated drones, the average relatedness between a worker and the sons of another worker is lower than the relatedness between a worker and the sons of a queen. In this case, workers should prevent each other from laying eggs: the so-called workers 'policing' (Ratnieks 1990; Oldroyd *et al*. 1994). Thus, the composition of the drone congregation helps avoid potential conflicts between workers for reproduction and contributes to the stability of the hive.

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