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Anarchy in the UK: Detailed genetic analysis of worker reproduction in a naturally occurring British anarchistic honeybee, *Apis mellifera*, colony using DNA microsatellites

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Abstract

Anarchistic behaviour is a very rare phenotype of honeybee colonies. In an anarchistic colony, many workers' sons are reared in the presence of the queen. Anarchy has previously been described in only two Australian colonies. Here we report on a first detailed genetic analysis of a British anarchistic colony. Male pupae were present in great abundance above the queen excluder, which was clearly indicative of extensive worker reproduction and is the hallmark of anarchy. Seventeen microsatellite loci were used to analyse these male pupae, allowing us to address whether all the males were indeed workers' sons, and how many worker patrilines and individual workers produced them. In the sample, 95 of 96 of the males were definitely workers' sons. Given that ≈ 1% of workers' sons were genetically indistinguishable from queen's sons, this suggests that workers do not move any queen-laid eggs between the part of the colony where the queen is present to the area above the queen excluder which the queen cannot enter. The colony had 16 patrilines, with an effective number of patrilines of 9.85. The 75 males that could be assigned with certainty to a patriline came from 7 patrilines, with an effective number of 4.21. They were the offspring of at least 19 workers. This is in contrast to the two previously studied Australian naturally occurring anarchist colonies, in which most of the workers' sons were offspring of one patriline. The high number of patrilines producing males leads to a low mean relatedness between laying workers and males of the colony. We discuss the importance of studying such colonies in the understanding of worker policing and its evolution.

Keywords: anarchy, *Apis mellifera*, DNA microsatellites, social insects, worker policing, worker reproduction

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Introduction

Insect societies show great diversity in their mating systems (Boomsma & Ratnieks 1996; Strassmann 2001) and in the way reproduction is shared among colony members (Bourke & Franks 1995; Crozier & Pamilo 1996; Foster & Ratnieks 2001b; Foster *et al*. 2001). Documenting this variation among species and colonies is crucial in understanding reproductive conflicts because queen mating frequency greatly affects colony kin structure and the relatedness among female offspring (Pamilo *et al*. 1997). This in turn may influence reproductive conflicts among colony members, e.g. over the optimal sex ratio (Trivers & Hare 1976) or male parentage (Ratnieks 1988).

Honeybees, *Apis mellifera*, typically have a single queen who is the main reproductive individual within the colony. The workers cannot mate but retain functional ovaries and can lay unfertilized eggs that develop into males (Winston 1987; Page & Erickson 1988; Visscher 1989; Seeley 1995). However, the reproductive output of workers in most queen-right colonies is negligible (Visscher 1989; Ratnieks 1993; Visscher 1996). Several mechanisms are responsible for this. At a proximate level, few workers have active ovaries (Ratnieks 1993), and the presence of both the queen (Butler & Fairey 1963) and brood (Arnold *et al*. 1994) inhibits worker ovary activation. In queenless colonies, this inhibition disappears and 5–24% of workers activate their

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ovaries (Miller & Ratnieks 2001). In addition, most worker-laid eggs are eaten (policed) by other workers (Ratnieks & Visscher 1989; Ratnieks 1993; Visscher 1996; Barron *et al*. 2001). Worker policing is favoured in honeybees on relatedness grounds because *Apis* queens typically mate with multiple males (Estoup *et al*. 1994; Oldroyd *et al*. 1997; Palmer & Oldroyd 2000). As a result, honeybee workers are on average more related to the queen's sons than to their sister workers' sons, and they benefit by worker policing as this causes the rearing of queen's sons rather than the less related workers' sons (Ratnieks 1988; Barron *et al*. 2001).

Although worker policing normally ensures that few worker sons are reared in queen-right *A. mellifera* colonies, many males are worker-derived in 'anarchistic colonies'. Anarchistic colonies are very rare, ≈ 1 colony per 1000–10 000 (Barron *et al*. 2001). Although there is no overt difference in the appearance of workers' and queen's sons within a colony, anarchistic colonies can be easily detected in managed hives when a queen excluder is used to confine the queen to the lower hive boxes. The co-occurrence of male brood above the excluder and brood of both sexes, and the queen, below the excluder strongly suggests anarchy. The kin structure of two naturally occurring anarchistic colonies from Australia has been described (Oldroyd *et al*. 1994; Montague & Oldroyd 1998). In both colonies, the workers were the offspring of a single queen mated to many males, as is typical, but only one patriline of workers produced the majority of the workers' sons (98% in one, Oldroyd *et al*. 1994 and 84–92% in the other, Montague & Oldroyd 1998).

Here we provide a detailed genetic analysis of a naturally occurring anarchistic colony of *A. mellifera* from Britain. We used 17 polymorphic DNA microsatellite loci to distinguish between workers' sons and queen's sons, and between the offspring of different worker patrilines and even individual workers. In contrast to the two Australian anarchistic colonies, our results show that at least 8 of the 16 worker patrilines produced males. Our results also show that many individual workers produced these males. In addition, because the queen was not the mother of any of the males reared above the queen excluder, our data show that queen eggs or larvae were not transferred from below the queen excluder.

Materials and methods

In April 1999, a novice beekeeper from Widnes, UK reported a honeybee colony, *Apis mellifera*, with brood above the queen excluder to an Internet newsgroup on beekeeping for advice as to what was going on. One of us (FR) visited the beekeeper and confirmed that it was a queen-right colony with brood of both sexes below the excluder but only male brood (many eggs, and hundreds of larvae and pupae) above the excluder. The queen had been marked with a paint dot, which indicated that she had been reared before 1999 and therefore that the colony had not been queenless at any time in the previous few months. If the colony had been temporarily queenless and had recently been requeened, worker reproduction could have been caused by the absence of the queen (Winston 1987; Page & Erickson 1988; Miller & Ratnieks 2001). The colony bore all the hallmarks of anarchy. This was only the second naturally occurring anarchistic colony that FR had seen in 18 years of beekeeping during which he has inspected more than 1000 colonies with queen excluders. The beekeeper donated the colony for research and it was transported to the laboratory apiary.

The colony had a healthy egg-laying queen, brood of both sexes and ≈ 30 000 workers. New male brood continued to be observed above and below the queen excluder during the spring. On 26 May 1999 frames of brood were taken from below and above the queen excluder and kept in a freezer. Samples of worker and male pupae were taken from the frame below the excluder and male pupae were taken from the frame above the excluder. Pupae rather than adult bees were collected to exclude bees that drifted from adjacent colonies, which can represent as many as 89% of the adult drones and 14% of the adult workers (Neumann *et al*. 2000). By sampling workers and males at the same time, the workers in the sample were younger than those that laid the eggs that gave rise to the sample of male pupae. However, because sperm use by *A. mellifera* queens becomes consistent a few months after mating (Estoup *et al*. 1994; Franck *et al*. 1999) and because the queen was at least 8 months old, the patriline proportions in the colony at the sampling time should be comparable with the proportions at the time the male eggs were laid.

DNA from the heads of 214 pupae ($n = 94$ workers, $n = 96$ males from above the excluder, $n = 24$ males from below the excluder) was extracted using phenol (Bruford *et al*. 1998). Polymerase chain reaction (PCR) amplifications were used to amplify 17 microsatellite markers (Table 1) previously developed for *A. mellifera* and *Bombus terrestris* (Estoup *et al*. 1994, 1995; Baudry *et al*. 1998). PCRs were performed with a Hybaid thermal cycler in a 10-μL volume containing 10–50 ng DNA, 1.0 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.05 U of *Taq* DNA polymerase (Thermoprime plus, Advanced Biotechnologies), in the manufacturer's buffer at a final concentration of 20 mM $(NH_4)_2SO_4$, 75 mM Tris–HCl pH 9.0 and 0.01% (w/v) Tween. The reaction profile for each locus was 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, annealing temperature (Table 1) for 30 s, and 72 °C for 30 s. The forward primer of each marker was 5′-end-labelled with a fluorescent phosphoramidite (NED, 6-FAM or HEX). The PCR products were visualized on an Applied Biosystems (ABI) 377 DNA sequencer using an internal size-standard (R500 GENESIZE). Because of the size and dye differences

Table 1 The 17 DNA Microsatellite markers used. Markers were isolated from *Apis mellifera* except B124 which was isolated from *Bombus terrestris*

Marker set	Locus	Fluorescent label	T_a (°C)	No. alleles	Size range (bp)	Heterozygosity
1	*A107*[a]	Hex	60	7	165–186	0.742
	A113[b]	6-FAM	60	4	203–227	0.667
	A24[b]	Ned	55	4	96–106	0.095
	A35[a]	Hex	57	4	114–125	0.624
	A43[a]	6-FAM	55	3	126–139	0.326
	A76[a]	Ned	58	8	230–308	0.947
	A88[b]	Ned	55	2	143–151	0.447
	B124[a]	Hex	55	7	218–242	0.691
2	*A14*[a]	6-FAM	58	8	219–255	0.946
	A28[a]	Ned	58	2	131–137	0.731
	A29[a]	6-FAM	54	8	134–163	0.737
	A7[a]	Hex	58	4	110–132	0.558
3	*Ap14*[c]	Ned	62	4	134–148	0.839
	Ap16[c]	Hex	52	2	143–157	0.042
	Ap19[c]	6-FAM	56	7	134–146	0.916
	Ap33[c]	Hex	54	9	226–253	0.958
	Ap37[c]	6-FAM	56	3	188–193	0.589

Multiplexing and labelling with one of three fluorescent dyes allowed us to run the 17 loci in 3 marker sets on an ABI 377 automated DNA sequencer.
T_a annealing temperature.
The markers used were published by [a]Estoup *et al*. (1994), [b]Estoup *et al*. (1995) and [c]Baudry *et al*. (1998).
Numbers of alleles and heterozygosities, calculated with CERVUS (Marshall *et al*. 1998) are given based on this colony alone (n = 94 workers).

between the PCR products for the 17 loci we were able to multiplex them in three different sets of markers (Table 1). The gels were analysed using ABI GENESCAN software (Version 3.1) and GENOTYPER DNA fragment analysis software (Version 2.5).

Many markers had to be analysed to obtain a clear picture of the colony kin structure. For example, when trying to determine the mother of a male there are several possibilities (e.g. the queen and workers of different patrilines) and these potential mothers all have many genes identical by descent because they are related. Males are haploid and each male inherits one or the other of his mother's two alleles at each locus. Because workers are all daughters of the queen, a worker's son inherits a queen-derived allele at a locus with a probability of 0.5. When this happens, that locus is uninformative in assigning the male to a particular patriline, and it also makes the male indistinguishable from a queen's son. This causes a large proportion of the workers' sons to be indistinguishable from queen's sons when only a small number of marker loci are used. Even when a male inherits a paternal allele it may not be possible to assign the male as a worker's son, if the paternal allele is the same as one or both of the queen's alleles at that locus. When assigning worker's sons to their maternal patriline, the fact that the males fathering the different patrilines can share alleles with the queen and between each other makes the assignment of maternity to different patrilines more difficult.

Results

Kinship of worker offspring

The microsatellite markers used were highly polymorphic with 2–9 different alleles per locus (mean 5.11) detected across all the males and workers analysed. Heterozygosities were calculated using CERVUS (Marshall *et al*. 1998) and ranged from 0.042 to 0.958 (Table 1).

We inferred the genotype of the queen from the workers' genotypes. If the queen is heterozygous at a locus then the workers will have one of two maternal alleles with approximately equal frequency. If the queen is homozygous then all the workers will carry the same maternal allele. The genotype of each worker's father was then determined by subtraction and the total number of fathers and their relative paternity determined.

In total, 16 patrilines (named A–P) were found in the 94 workers. The large number of loci used and their high variability means that it is unlikely that we failed to find any fathers due to genetic nondetection. However, because of the large number of fathers it is possible that unsampled rare patrilines were present. By analysing 94 workers, any male who contributed to 3% or more of the offspring has a > 95% probability of being represented in the sample (Boomsma & Ratnieks 1996). No undetected patrilines appeared in the workers' sons, which further suggests that

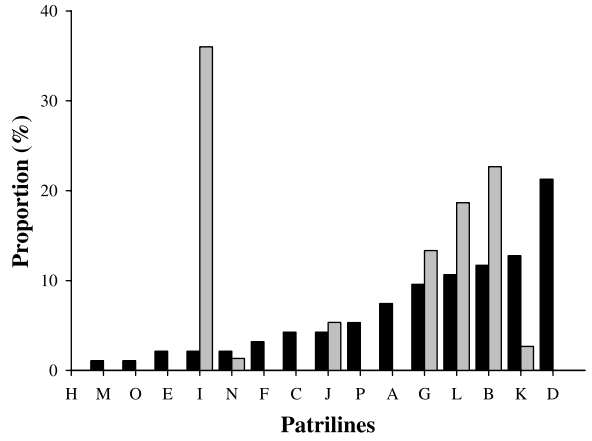

Fig. 1 Patriline distribution of workers (n = 94, black bars) and workers' sons (n = 75 assigned to specific patrilines, grey bars) from above the queen excluder. Assigned males from below the excluder are not included, as they constitute another independent sample.

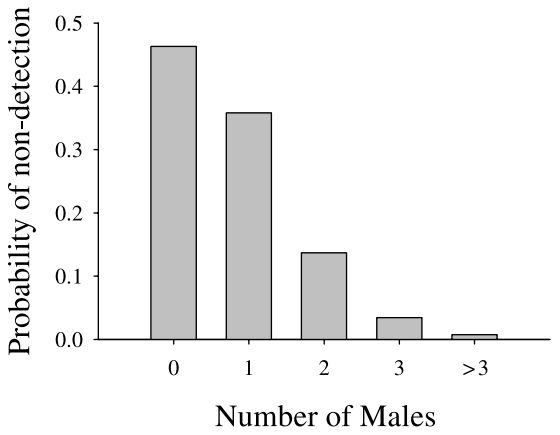

Fig. 2 Probability of not detecting worker's sons in a sample of 96 males calculated from a binomial distribution with n = 96 and P = 0.992.

we sampled all patrilines. Because the workers were not equally frequent among patrilines (Fig. 1) the effective mating frequency (Me) is 9.05 (Starr 1984; Boomsma & Ratnieks 1996), and 9.85 if corrected for sample size (Pamilo 1993). This corresponds to a mean relatedness among workers of 0.30 (Pamilo 1993).

When genotyping individual bees, there is a risk of mistyping the individuals and of mutations. This is mostly problematic for patrilines represented by one or two workers or patrilines for which the implied paternal genotype only differs at one or two loci from that of another father. When this occurred, new PCRs were performed on the individuals and run on new gels to check for correct typing. This leaves the possibility of mutations. In our sample, most paternal genotypes differed at more than three loci. However, four patriline pairs differed at fewer than three loci. Patriline J and P differ at one locus but are represented by four and five workers, respectively. Patriline O, represented by a single worker, differs from patriline K at two loci, and it is unlikely that two mutations would occur at the same time. Patriline N, represented by two workers, differs from patriline E at one locus but it is unlikely that the same mutation occurred twice in the two N individuals. Patriline H, represented by a single worker only differs from patriline D at one locus. There is a chance that this is due to a mutation. We chose to include this worker from patriline H as belonging to a distinct patriline in the results. However, combining D and H into one patriline would not change the results in a significant way as the mean relatedness among workers would become 0.307 as opposed to 0.305 with 16 patrilines. Given the high mating frequency of honeybee queens and unequal sperm use, it is highly possible to find patrilines represented only by 1 worker in a sample of 94.

Males from above the queen excluder

Workers' sons or queen's sons? The detection of workers' sons is made difficult by the fact that, at any locus, the son of a worker inherits his mother's paternal allele only half the time. In addition, the father of the egg-laying worker may also share an allele with the queen at a given locus, which leads to this locus being uninformative for the whole patriline. The probability, P, of being able to detect a worker's son of a given patriline is

$$P = 1 - 0.5^{l}$$

where l is the number of informative loci for this patriline, that is the number of loci where the father's allele is different from both the queen's alleles. These probabilities ranged from 93.75% (patriline A) to > 99.9% (patrilines B, C, L, N) (Table 2). A mean detection probability can be calculated (Foster & Ratnieks 2001a) as

$$P = \sum_{i=1}^{n} p_i (1 - 0.5^{l_i})$$

where n is the number of patrilines, p_i is the proportional representation of the ith patriline and l_i is the number of informative loci analysed for the ith patriline. This probability was 99.2% in the study colony. Of the 96 males from above the queen excluder, 95 were positively identifiable as workers' sons because each carried at least 1 paternal marker. The remaining male could not be assigned. However, with a detection probability of 99.2% the probability that at least 1 of 96 workers' sons will have no paternal allele is high ($1-0.992^{96} = 0.537$; Fig. 2). It is therefore fully consistent with the genetic detection probabilities that the unassigned male is also a worker's son.

Table 2 Genotypes of the queen and father of each worker patriline (A–P) detected using 17 microsatellite markers (n = 94 workers)

	A107	A113	A24	A35	A43	A76	A88	B124	A14	A28	A29	A7	Ap14	Ap16	Ap19	Ap33	Ap37	No. workers	No. alleles different from queen	Prob. detection	Mean relatedness of workers to workers' sons	Mean relatedness of workers to all males
Queen	174	215	96	114	126	230	143	222	225	137	141	110	134	143	134	238	193					
	188	221	96	119	126	288	151	237	235	137	155	132	142	143	142	242	193					
A	165[i]	227[i]	96	119	126	230	143	218[i]	235	137	155	132	134	143	134	250[i]	193	7	4	0.9375	0.125	0.193
B	168[i]	221	96	123[i]	126	232[i]	143	222	230[i]	131[i]	163[i]	110	140[i]	143	140[i]	253[i]	89[i]	11	10	0.9990	0.182	0.219
C	171[i]	203[i]	96	123[i]	126	244[i]	143	242[i]	230[i]	131[i]	143[i]	110	140[i]	143	134	245[i]	189[i]	4	11	0.9995	0.125	0.193
D	172[i]	203[i]	96	119	138[i]	244[i]	143	222	230[i]	131[i]	141	110	134	143	138[i]	250[i]	193	20	8	0.9961	0.125	0.193
E	186[i]	221	104[i]	125[i]	126	234[i]	143	222	221[i]	137	139[i]	110	134	157[i]	146[i]	247[i]	188[i]	2	10	0.9990	0.125	0.193
													142									
F	174	221	106[i]	119	126	234[i]	143	239[i]	230[i]	137	134[i]	110	134	143	144[i]	226[i]	189[i]	3	8	0.9961	0.125	0.193
							151					132	142									
G	174	215	96	114	126	238[i]	143	222	237[i]	131[i]	139[i]	110	140[i]	143	140[i]	236[i]	188[i]	9	8	0.9961	0.158	0.208
H	174	203[i]	96	119	138[i]	244[i]	143	222	230[i]	131[i]	141	110	134	143	138[i]	250[i]	193	1	7	0.9922	0.125	0.193
							151						142									
I	186[i]	221	104[i]	114	126	244[i]	143	227[i]	230[i]	137	157[i]	110	134	143	140[i]	226[i]	189[i]	2	9	0.9980	0.215	0.234
				119																		
J	174	221	96	114	126	259[i]	151	242[i]	230[i]	137	139[i]	110	140[i]	143	144[i]	245[i]	193	4	7	0.9922	0.138	0.199
K	174	221	96	114	126	308[i]	143	218[i]	237[i]	131[i]	139[i]	110	140[i]	143	140[i]	236[i]	188[i]	12	9	0.9980	0.132	0.196
L	176[i]	215	96	114	139[i]	238[i]	143	222	255[i]	131[i]	141	119[i]	148[i]	143	136[i]	234[i]	188[i]	10	10	0.9990	0.172	0.214
M	176[i]	215	96	114	126	244[i]	143	222	219[i]	137	161[i]	117[i]	134	143	134	236[i]	193	1	6	0.9844	0.125	0.193
		221		119			151								142							
N	186[i]	221	104[i]	125[i]	126	234[i]	143	227[i]	221[i]	137	139[i]	110	134	157[i]	146[i]	247[i]	188[i]	2	11	0.9995	0.128	0.194
O	174	221	96	114	126	308[i]	143	218[i]	230[i]	131[i]	143[i]	110	140[i]	143	140[i]	236[i]	188[i]	1	9	0.9980	0.125	0.193
P	174	221	96	114	126	259[i]	151	242[i]	230[i]	137	139[i]	110	140[i]	143	144[i]	242	193	5	6	0.9844	0.125	0.193

i. The marker is informative for the given patriline.

When two alleles are given in a father's genotype cell, it means that they had an allele identical to the queen and it was not possible to distinguish between the two possibilities.

Probability of detection gives the probability that a son of a worker of that patriline could be distinguished from a queen's son.

Table 3 Assignment of the 96 males sampled from above the queen excluder to their mother's patriline

Mother workers' patrilines	No. males
B	17
G	10
I	27
J	4
K	2
L	14
N	1
B or C	*1*
D or H	***1***
G or K	7
G or L	2
J or P	*6*
C, D or H	***1***
B, C, F or I	*1*
B, C, D, G, H, K, L or O	*1*
Not assigned	1
Total	96

Some of the males could not be assigned to a single patriline and could have been the offspring of workers of several patrilines, as indicated.
Bold Italics: Possible patrilines are different from the patrilines definitely involved in male production which means at least one additional patriline produced males.
Italics: both definitely male producing patrilines and others are possible.

How many worker patrilines produced the males? Each worker's son inherits between 0 and 17 paternal alleles from his mother. (The actual number follows a binomial distribution with $P = 0.5$ and $n = 17$, assuming unlinked loci and fair meiosis.) These paternal alleles allow us to determine which patriline a mother belongs to. However, because different fathers that mated to the same queen can have the same allele at a locus, the number of informative loci for assigning the mother worker's patriline is fewer than 17. We were able to determine the exact patriline origin of 75 of the 96 males reared above the excluder. Seven of the 16 patrilines produced males (Fig. 1), but not in equal proportions (χ^2; $P < 0.01$) or in proportions similar to their representation in the workers (χ^2; $P < 0.01$) (Fig. 1). The effective number of patrilines contributing to male production was 4.21, considering only the 75 patriline-assigned males. In the 21 males who could not be assigned to a precise patriline, 6 other patrilines (C, D, F, H, P, O) could have produced males and one of them (patriline D or H) definitely produced at least one male (Table 3). Thus, at least 8 patrilines were producing males, only 7 of which could be named. In addition, it is possible that several of the 6 other patrilines also produced males.

How many individual workers produced the males? Because males are haploid all the workers in one patriline inherit the same paternal alleles. These alleles therefore provide no information about whether two males from the same patriline of workers had the same or different mother workers. But maternal alleles (i.e. from the queen via a worker mother) can provide this information when the queen is heterozygous. A worker's son inherits the queen allele with a probability of 0.5 per locus. Workers can inherit one of two alleles per locus from their mother queen if she is heterozygous. If two workers of the same patriline have two different maternal alleles at a given locus and their father's allele is different from the queen's, their respective sons will inherit different detectable queen alleles from them at this locus. In this situation, it is possible to say that male offspring of the same patriline (via the paternal alleles) have different mothers. By examining all the informative loci in the males from one patriline, the minimum number of workers that could have produced these males can be estimated by finding the minimum number of unique combinations of maternal alleles. This method shows that at least 19 workers produced the 75 males, with at least 5 in patriline I and 5 in patriline B.

Males from below the queen excluder

Using the same methods, 11 of the 24 males from below the queen excluder were positively identified as workers' sons. The probabilities that 1 or > 1 of the remaining 13 males are workers' sons are 9.4% and 0.45%, respectively. The queen was therefore most likely the mother of 54% (probability 90%) of the males sampled from below the excluder. The 95% binomial confidence interval for this proportion is $\pm 1.96\,\sigma/\sqrt{n}$, which is ±4.1% (Sokal & Rohlf 1995). If an additional male was a worker's son (probability 9.4%), the proportion would be 50 ± 4.1% (95% CI). In the colony, the presence of the queen excluder meant that workers had access to more drone cells than the queen and therefore workers produced ≈ 75% of the male brood throughout the colony. In the absence of the excluder, it is likely that competition for cell space between workers and the queen would have brought the proportion down to the 54% observed below the excluder.

Relatedness of workers to males

Assuming that all fathers are unrelated to each other and to the queen, the relatedness between workers of non-reproducing patrilines and workers' sons is 0.125. The relatedness between workers from anarchistic patrilines and worker-produced males was slightly higher, 0.128 for patriline N up to 0.215 for patriline I (Table 1), or 0.159 on average. The mean relatedness between all workers and worker-derived males was 0.143. If we consider that the

queen is the mother of 54% of the males in the colony, as suggested from the sample from below the excluder, we can also estimate the mean relatedness of the workers to all males produced in the colony. We used this estimate because it seems closer to what the proportion would have been if the queen excluder were not present in the colony. For nonanarchistic workers, this is 0.193. For anarchistic patrilines, it ranges from 0.194 for patriline N to 0.234 for patriline I (Table 1), with a mean of 0.208. If all workers are considered, the relatedness is 0.201. If the estimate of 75% of worker-derived males in the colony had been used, the mean relatedness of anarchistic patrilines to all workers would have been 0.227, which is still below 0.25. Patriline I produced the most males, 36% of the worker's sons. From the molecular data we determined that at least five workers of patriline I produced these males. If these workers were the only ones to reproduce in patriline I and did so equally, the mean relatedness of these individual workers to all males would be 0.238. Clearly then, there are no relatedness gains to the anarchistic workers, as 0.238 is still below 0.25, the relatedness to brothers.

Discussion

The genetic analyses confirm the field diagnosis of anarchy by showing that the workers were producing many of the colony's males. The analysis of the worker pupae demonstrated that 16 patrilines were present, that the effective paternity was 9.85 and that the mean relatedness was 0.30. This is a typical figure for *Apis mellifera* in which multiple paternity is the rule (Estoup *et al*. 1994; Oldroyd *et al*. 1997).

The 17 loci gave us the necessary power to show that all but 1 of the 96 males from above the queen excluder were definitely workers' sons. Because 1% of the workers' sons could not be distinguished from the queen's sons, the remaining male was probably also a worker's son. This shows that the presence of drone brood above the queen excluder is indeed indicative of worker laying, and that workers do not merely transfer queen's sons, eggs or larvae, from below the excluder. Previous studies (Ratnieks 1993; Ratnieks *et al*. 2002) had implicitly made this assumption, and our study shows this to be reasonable.

Our results show that workers' sons were also being reared below the queen excluder making it highly unlikely that worker reproduction was caused by the isolation of workers above the excluder and away from the normal inhibition of ovary activation caused by the queen and her brood. Approximately half the males being reared below the excluder were workers' sons. This confirms that anarchy is a distinct reproductive syndrome in honeybee colonies rather than simply a manifestation of worker reproduction caused by the use of a queen excluder (Montague & Oldroyd 1998).

The minimum estimate of the number of workers that produced the 75 males assigned to patrilines was 19. This shows that multiple workers were responsible for male production in each of the 8 male-producing patrilines. In both patrilines I and B, which produced 27 and 17 males, respectively, at least 5 workers contributed to the production of the males. Therefore, male production was not monopolized by just a few individual workers. Nineteen is probably a great underestimate of the actual number of mother workers for two reasons. First, we only analysed a sample of the males being reared. Second, the genetic methods did not always allow us to distinguish among mothers within the same patriline.

In contrast with the two other previously described naturally occurring anarchistic colonies studied (Oldroyd *et al*. 1994; Montague & Oldroyd 1998), the males in our colony were sons of many worker patrilines. Eight of the 16 patrilines detected in the 94 workers analysed were also detected in the 96 workers' sons analysed. Even though some of these patrilines produced few males (Fig. 1), the effective number of mother patrilines, 4.21, was well above 1 and approximately half the effective number of patrilines, 9.85. Different patrilines varied significantly in their production of males, and also differed from their numerical representation in the worker sample. For example, patriline I produced 36% (27/75) of the males but represented only 2.1% (2/94) of the workers, whereas patriline K, which represented 12.8% (12/94) of the workers, produced only 2.7% (2/75) of the males (Fig. 1). In other patrilines (G, L, B), male production is more in line with the number of workers in the patriline. From 6 to 8 of the patrilines produced no males. This variation among patrilines in male production provides further evidence for a genetic component to anarchistic behaviour (Oldroyd & Osborne 1999).

The use of many highly polymorphic DNA microsatellite loci allowed us to make a clear but necessarily incomplete picture of male production in the study colony. Importantly, our data show that the transfer of eggs across the queen excluder either does not occur or is of negligible importance, thereby confirming that studies examining eggs laid above the queen excluder indeed demonstrate worker-laying. This is the first naturally occurring anarchistic colony to be studied with many worker patrilines producing males. This has previously been observed only after active selection for anarchistic reproduction (Oldroyd & Osborne 1999). In our colony, the presence of many anarchistic patrilines suggests that the trait is, in part, maternally inherited, although the differences in male production among patrilines suggest that the fathers also influenced the phenotype of their daughters. In other words, the anarchistic phenotype may be influenced both by maternally and paternally derived genes, as is expected in diploid genetics. For example, patrilines may not share the same threshold values for signals that normally inhibit ovary

activation. Similar differences between patrilines have already been demonstrated for oviposition and oophagy in queenless colonies (Robinson *et al*. 1990). Finding a naturally occurring colony displaying such a trait confirms that anarchy has a complex genetic determinism (Oldroyd & Osborne 1999) and that both the maternal and paternal genotypes have an influence on the anarchistic phenotype, whereas the two Australian anarchists with only one patriline producing males (Oldroyd *et al*. 1994; Montague & Oldroyd 1998) might have suggested a predominant effect of paternally transmitted genes.

As a result of the high number of patrilines producing males, the mean relatedness of workers in anarchistic patrilines to the males being reared in the colony was below 0.25. Thus, not even the anarchistic workers benefited from worker reproduction. Anarchistic behaviour ceases to be beneficial even to anarchist patrilines when there are more than two effective anarchistic patrilines in the colony. In the study colony, anarchistic workers do not increase their fitness by reproducing and only their father's genes, which would not otherwise be present in the males produced, benefit from the worker reproduction caused. Anarchy is therefore costly for the workers of the colony and, as worker policing theory predicts, should be selected against by policing genes. However, it should be noted that anarchistic workers have a fitness advantage over nonanarchists within an anarchistic colony. When an anarchistic colony occurs the egg-layers will always have higher relatedness to the colony's males than the non egg-layers. The anarchistic trait is akin to a selfish gene (Hurst *et al*. 1996) that spreads at a cost to its host, which in this case is the whole colony. Why anarchy does not readily spread to high frequencies in the population remains a puzzle (Barron *et al*. 2001). But part of the answer is suggested by this study: if the anarchistic gene does not cause any relatedness gains to the workers carrying it, modifiers will soon control worker reproduction, returning the population to the normal state of worker sterility in the presence of queen and brood.

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