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Levels of polyandry and intracolonial genetic relationships in *Apis florea*

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Abstract DNA was extracted from worker and drone pupae of each of five colonies of the dwarf honey bee *Apis florea*. Polymerase chain reactions (PCR) were conducted on DNA extracts using five sets of primers known to amplify microsatellite loci in *A. mellifera*. Based on microsatellite allele distributions, queens of the five colonies mated with at least 5–14 drones. This is up to 3 times previous maximum estimates obtained from sperm counts. The discrepancy between sperm count and microsatellite estimates of the number of matings in *A. florea* suggests that despite direct injection of semen into the spermatheacal duct, either *A. florea* drones inject only a small proportion of their semen, or queens are able to rapidly expel excess semen after mating. A model of sexual selection (first proposed by Koeniger and Koeniger) is discussed in which males attempt to gain reproductive dominance by increasing ejaculate volume and direct injection of spermatozoa into the spermatheca, while queens attempt to maintain polyandry by retaining only a small fraction of each male's ejaculate. It is shown, at least in this limited sample, that the effective number of matings is lower in *A. florea* than in *A. mellifera*.

Key words Polyandry · Microsatellite · Multiple mating · Relatedness · *Apis*

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Introduction

Haplo-diploidy produces high relatedness among female offspring of singly-mated hymenopteran queens. Evolution of eusociality was probably facilitated by this high relatedness (Hamilton 1964; Pamilo 1991). However, in many of the advanced eusocial species, multiple mating (polyandry) occurs, which causes a reduction in average relatedness (Page and Metcalf 1982). Given appropriate cost-benefit coefficients (Crozier 1979), eusociality can be maintained despite multiple mating, but the reasons for the evolutionary shift are of great interest (Page 1980; Page and Metcalf 1982; Cole 1983; Crozier and Page 1985).

It has been postulated that multiple mating is adaptive because:

1. Genetically diverse colonies may be able to tolerate a wider range of environmental conditions, perhaps by increased polyethism (Oldroyd et al. 1992 a, b) or the potential for increased caste differentiation (Crozier and Page 1985), or by increased tolerance to pathogens (Sherman et al. 1988; Shykoff and Schmid-Hempel 1991a, b).
2. Multiple mating eliminates the possibility of a queen mating with a single drone carrying the same sex allele as herself (Page 1980; Crozier and Page 1985; Ratnieks 1990). Because of the method of sex determination in Hymenoptera, such matings result in a 50% reduction in brood viability (Woyke 1963).
3. Multiple mating can reduce conflict between workers and queens over the preferred sex ratio (Moritz 1985; Pamilo 1991).

Understanding the evolution of polyandry requires good estimates of the number of matings in a broad range of species. Of interest is not only the actual number of copulations, but the number and proportion of paternities represented in the workers i.e. the "effective" number of matings (Oldroyd and Moran 1983) or the "effective promiscuity" (Starr 1984). This will often differ from the number of copulations because of variance in the number of spermatozoa among males, sperm competition (Harbo 1990), the "last male advantage" (Moritz 1986) and possibly sperm clumping at least in younger queens (Taber 1955) (but see Kerr et al. (1980), Crozier and Brückner (1981), Page and Metcalf (1982) and Laidlaw and Page (1984) who all argue or show that sperm clumping is minimal in *A. mellifera*).

The ideal technique for estimating the "effective" number of matings is the use of "microsatellites" (Estoup et al. 1994). Among eusocial Hymenoptera, microsatellite loci have been reported for honey and bumble bees (Estoup et al. 1993, 1994, 1995), a vespid wasp (Choudhary et al. 1993) and three ants (Evans 1993; Hamaguchi et al. 1993; Chapuisat 1994). Microsatellites are sections of DNA that consist of tandem repeats of short motifs such as $(CT)_n$ (Tautz 1989; Queller et al. 1993). Because of high mutation rates, the lengths of microsatellite sequences tend to be highly variable among individuals. If regions of DNA flanking a microsatellite region are sequenced, then primers for the polymerase chain reaction (PCR) can be synthesised. PCR amplifications conducted with these primers on DNA extracts of experimental individuals result in variable length products (alleles), which can be easily discriminated by electrophoresis on a denaturing acrylamide gel. Estoup et al. (1993) and Queller et al. (1993) provide detailed accounts of the cloning and selecting of microsatellite sequences.

The dwarf honey bee *Apis florea* is native to south Asia including Borneo and Java, and as far west as Iran and Oman (Ruttner 1988). Very little is known of its mating biology (Ruttner 1988; Crane and Walker 1993). Koeniger et al. (1989) found that *A. florea* drones from Thailand had on average 0.44 ± 0.04 (SD) million sperm (n = 8 individuals), while mated laying queens had 0.90 ± 0.39 million sperm in their spermathecae (n = 7). Two queens which had recently returned from mating flights had 0.72 and 0.48 million sperm in their spermathecae. Woyke (1993) reported that six mated *A. florea* queens from Poona, India had 0.90 ± 0.46 million sperm in their spermathecae. These results strongly suggest that *A. florea* queens typically mate two or three, perhaps up to four times. Koeniger et al. (1989) found no mating sign associated with newly mated *A. florea* queens, and provided good evidence that semen is injected directly into the spermatheca during mating. However, the notion that the semen of only two to four drones is present in the spermatheca of most queens should be treated with some caution. As Koeniger et al. (1989) point out, there remains the possibility that *A. florea* queens mate many times, ejecting excess semen, but retaining a genetic contribution from each male. Such a process is known to occur in *A. mellifera* (Koeniger and Koeniger 1991). There are also the possibilities that the drones do not inject all their semen, or that the species has several queens per colony.

We used microsatellite genetic markers in order to precisely determine the effective number of matings, the paternity and maternity of worker and drone offspring, and the average genetic relatedness within five colonies of *A. florea*. We discuss the evolution of polyandry in the genus *Apis* in the light of these new data.

Materials and methods

Collections

Worker pupae were collected from each of five *A. florea* colonies located in water mimosa trees on river flats of the Ping river near Lampang (18.15 N 99.31 E) in northern Thailand. Drone pupae were collected from the two colonies in which they were present. Colonies are common in this area. Six searchers found these colonies in less than 1 h despite extremely dense vegetation. Small sections of brood comb containing c. 100 pupae were wrapped in aluminium foil and placed in liquid nitrogen for transport to the laboratory where they were stored at −70° C. Use of brood rather than adults eliminates the possibility of bees drifting among colonies contributing to the results.

On beginning this project we assumed that *A. florea* mates one to four times (Koeniger et al. 1989). The expected number of patrilines $E(k)$ in a sample of size n from a colony of k equally represented patrilines is given by:

$$E(k) = k - [(k-1)^n / k^{n-1}] \quad (1)$$

(Cornuet and Aries 1980). This equation shows that if $k = 6$ (considered a maximum on the basis of Koeniger et al. 1989), the expected number of patrilines in a sample of size 24 is 5.9, providing a very good chance of observing all patrilines. We therefore sampled 24 bees per colony except for colony 1, where we sampled 71 bees in case the estimate of Koeniger et al. (1989) was grossly low.

DNA extraction and polymerase chain reactions (PCR)

DNA was extracted from individual pupae by the method of Crozier et al. (1991) and resuspended in 50 µl of Tris-EDTA (10 mM Tris-HCl, 1-mM EDTA, pH 8.0) buffer. PCR amplifications were performed using five sets of primers which are known to amplify microsatellite sequences in *A. mellifera* (Estoup et al. 1994). For each primer pair, one primer was radio-actively end-labelled. In a total reaction volume of 10 µl, the γ-phosphate from ^{33}P-dATP (Dupont) was transferred to the 5'-terminus of primer-2, using T4 polynucleotide kinase (Promega). The reaction contained 70 mM Tris-HCl, 10 mM $MgCl_2$, 0.2 µM primer, 5 µl ^{33}P-dATP, and 4 units of polynucleotide kinase. The reaction was incubated for 30 min at 37° C and stopped by heating to 90° C for 2 min.

Aliquots (1 µl) of 1/10 dilution sample DNA were amplified using primers (one end-labelled) and PCR temperature profiles specified in Estoup et al. (1994) and Table 1. PCR reactions were performed in a total volume of 10 µl containing 0.167 mM of each dNTP, 1 µg BSA, 0.4 µM unlabelled primer, 0.02 µM labelled primer, 1X Promega reaction buffer and 0.4 units of Promega Taq

Table 1 Polymerase chain reaction (PCR) conditions and primer sequences for five microsatellite loci used to detect paternity in *Apis florea*

Locus	Primers	Annealing temperature (°C)	$MgCl_2$ (mM)	Number of cycles
A8	5'CGAAGGTAAGGTAAATGGAAC 5'GGCGGTTAAAGTTCTGG	51	1.5	30
A76	5'GCCAATACTCTCGAACAATCG 5'GTCCAATTCACATGTCGACATC	58	1.5	25
A88	5'CGAATTAACCGATTTGTCG 5'GATCGCAATTATTGAAGGAG	50	1.5	30
A107	5'CCGTGGGAGGTTTATTGTCG 5'GGTTCGTAACGGATGACACC	50	1.5	25
B124	5'GCAACAGGTCGGGTTAGAG 5'CAGGATAGGGTAGGTAAGCAG	55	1.5	30

polymerase. PCR products were run on standard 6% polyacrylamide sequencing gels with M13 control DNA sequencing reactions run on the same gel as size standards. Microsatellite alleles were scored as fragment lengths in base pairs.

Analyses

Where possible, queen genotype was determined for each locus for each colony. When an allele was present in every worker, the queen was considered homozygous for that allele. When every worker carried one of two alleles, the queen was assumed heterozygous for those two alleles (Estoup et al. 1994).

Paternal alleles of each worker were deduced by subtraction (Estoup et al. 1994). That is, the paternal allele of each worker is that allele not carried by the queen. Where a queen is heterozygous at a particular locus, paternity at that locus is uncertain for all workers with the same genotype as the queen. That is, one cannot tell if a particular allele is paternal or maternal in origin. Our approach to these workers was to leave the paternity unassigned, and later pool all unassigned genotypes that could potentially be the same.

When two worker bees are sired by the same drone, and provided that the parents are neither related nor inbred, the pedigree coefficient of relatedness, G, between them is 0.75, while the relatedness between two half-sisters is 0.25 (Crozier 1970). We therefore computed the average coefficient of relatedness, R_c, (Laidlaw and Page 1984) weighted according to the relative proportions of each subfamily in our samples:

$$R_c = \sum_{i=1}^{k} \{([0.75p_i] + [0.25(1-p_i)])p_i\} \quad (2)$$

where p_i = the relative frequency of the ith subfamily and k is the number of subfamilies.

Of interest for sociobiological questions is not so much the number of matings, but the number and proportion of drones that are represented in the worker cohort at any one time. The effective number of matings (m), is most easily computed from Starr (1984):

$$m = 1 \Big/ \sum_{i=1}^{k} p_i^2 \quad (3)$$

Results

We observed 5–14 patrilines in the five colonies studied (Table 2). Colony 1 had the highest number of patrilines but this estimate is based on a larger ($n = 71$) worker sample than that for the other four colonies. The distribution of workers among patrilines was unequal in colony 1 ($\chi^2_{13} = 34.6$ $P < 0.01$).

Because the number of alleles was relatively small in the colonies studied, unambiguous assignment of queen genotype could not be made in 4 of the 25 cases. For two of these ambiguous cases the distribution of alleles in the drone progeny were used to confirm queen genotype. For the remaining two cases (locus B124 in colony 3 and locus A8 in colony 5), the queen genotype could not be resolved and is left unassigned in Table 2.

Mean average relatedness ($R_c \pm$ SE) for the five colonies was 0.35 ± 0.02 while the mean effective number of matings (m) was 5.65 ± 1.04 (Table 3).

There is no evidence of polygyny in the five colonies examined. A single heterozygous queen can produce only two kinds of gametes. Therefore, polygyny would be unambiguously detected by more than two kinds of homozygous workers in any one colony. No more than two kinds of homozygous individuals were detected in the 875 worker/locus combinations examined.

The males examined were almost certainly progeny of queens, not workers. Of the two colonies which had drone pupae, [17 males (colony 3) and 7 males (colony 5)], all males carried a queen allele at each microsatellite locus.

Discussion

These results demonstrate that our *A. florea* queens mated with at least five drones and almost certainly many more. The observed number of patrilines is likely to be a considerable underestimation for colonies 2–5 where the sample size was inadequate to detect more than five equally distributed patrilines (Eq. 1). Since patrilines are not equally distributed (at least in colony 1 which is the only colony for which a valid χ^2 test can be performed), we almost certainly did not detect rare patrilines in these colonies. Further, we may have underestimated the number of drones in the mating in those cases where heterozygous queens mated drones carrying an identical allele. This bias is small however, as in one half of cases our assignment would be correct, and pooled assignments would usually be correctly

Table 2 Genotypes (microsatellite length in base pairs) of queens and paternal drones for 5 microsatellite loci in 5 colonies of *A. florea*. (/indicates genotypes where paternal and maternal alleles cannot be distinguished)

	Microsatellite locus A8	A76	A88	A107	B124	Observed number of worker bees
Colony 1						
Queen allele 1	166	197	144	110	192	
Queen allele 2	166	195*	140	112	192	
Drone 1	166	195/197	140/144	106	190	6
Drone 2	166	195/197	140/144	110/112	192	6
Drone 3	166	195/197	142	106	190	2
Drone 4	166	195/197	142	110/112	190	1
Drone 5	166	197	138	106	192	2
Drone 6	166	197	142	106	192	5
Drone 7	166	197	142	110/112	192	8
Drone 8	168	195/197	138	106	192	2
Drone 9	168	195/197	142	106	190	8
Drone 10	168	195/197	142	106	192	15
Drone 11	170	195/197	138	106	192	4
Drone 12	170	195/197	144	106	192	4
Drone 13	170	197	140/144	118	192	2
Drone 14	172	195/197	142	110/112	192	6
					Total	71
Colony 2						
Queen allele 1	166	197	142	106	194	
Queen allele 2	168	197	142	112	194	
Drone 1	166	195	138	106/112	190	1
Drone 2	166	197	138	106/112	194	2
Drone 3	166	197	142	110	190	1
Drone 4	166/168	197	142	106/112	192	6
Drone 4	166/168	197	142	106/112	194	2
Drone 6	166/168	197	142	110	192	5
Drone 7	170	199	140	110	192	1
Drone 8	178	197	142	112	194	1
Drone 9	178	199	142	106/112	194	5
					Total	24
Colony 3						
Queen allele 1	166	197	140	106	194	
Queen allele 2	172	199	134	120	192/194	
Drone 1	166/172	197/199	142	106/120	192/194	7
Drone 2	168	197/199	142	106/120	192/194	7
Drone 3	168	197/199	142	112	192/194	3
Drone 4	170	197/199	144	106/120	192/194	2
Drone 5	178	197/199	138	112	192/194	5
					Total	24
Colony 4						
Queen allele 1	168	197	142	102	192	
Queen allele 2	168	197	142	108	194	
Drone 1	166	197	142	102/108	190	2
Drone 2	166	197	144	102/108	192/194	5
Drone 3	168	197	142	102/108	190	4
Drone 4	168	197	142	102/108	192/194	3
Drone 5	168	197	144	102/108	192/194	6
Drone 6	170	197	144	102/108	190	4
					Total	24
Colony 5						
Queen allele 1	168	197	142	102	190	
Queen allele 2	166/168	197	144	108	190	

* This queen might also be 197/197. We assumed 197/195 because the 195 allele is rare in the population. The paternity analysis is unffected by the assumption made.

Table 2 (Continued)

	Microsatellite locus A8	A76	A88	A107	B124	Observed number of worker bees
Drone 1	166/168	197	138	102	190	1
Drone 2	166/168	197	142/144	102	190	11
Drone 3	166/168	197	142/144	102/108	188	5
Drone 4	166/168	197	142/144	106	192	1
Drone 5	166/168	197	142/144	106	190	2
Drone 6	166/168	197	144	102	192	3
					Total	23

Table 3 Observed (k) and effective (m) number of matings and average coefficient of relatedness (R_c) for five colonies of *A. florea*

Colony	Number of matings Observed	Effective	Average relatedness
1	14	9.42	0.30
2	9	5.88	0.33
3	5	4.23	0.37
4	6	5.43	0.34
5	6	3.29	0.40
Mean (± SE)	8.0 ± 1.64	5.65 ± 1.04	0.35 ± 0.02

assigned by alleles at other loci. Finally, because the number of alleles observed in the samples was quite low (cf. Estoup et al. 1994), some males may have been indistinguishable on the basis of genotype. Our results therefore indicate that the previous maximum estimates obtained from sperm counts (Koeniger et al. 1989; Woyke 1993) are an underestimate.

Estimates of the number effective number of matings in *A. mellifera* is in the range 6.6–17.9 (Estoup et al. 1994) compared to 3.3–9.4 observed for *A. florea* in this study. Therefore, the effective number of matings appears to be lower in *A. florea* than in *A. mellifera*. (These data need to expanded to a broader range of subspecies and environments before they can be generalised.)

When data are acquired for other *Apis* species, we may be able to better resolve competing arguments for the evolution of the high levels of polyandry observed in the genus. Arguments for the evolution of polyandry based on the genetic load imposed by the sex locus predict mating by at least six drones, but no benefit from a significantly larger number of drones (Shaskolsky 1976; Page 1980; Ratnieks 1990). Conversely, arguments relating to worker/queen conflict over sex allocation (Queller 1993) or the fitness benefits of increased intracolonial genetic variance (Crozier and Page 1985) might predict a larger number of matings.

Based on the *a priori* assumptions that polyandry increases fitness of queens and colonies, Koeniger and Koeniger (1990) proposed models for the evolution of the two dichotomous mating strategies of honey bees.

1. In the cavity nesting species (*A. mellifera* and *A. cerana*), drones were initially selected to produce large amounts of spermatozoa in order to try and obtain reproductive dominance. In response, queens evolved mechanisms to transfer only a small percentage of each male's sperm to the spermatheca.
2. In the dwarf species (*A. andreniformis* and *A. florea*), males were selected to obtain reproductive dominance by direct injection of semen into the spermatheca. Subsequent colony-level selection for polyandry caused males to evolve small ejaculate volume leaving room in the spermatheca for sperm from subsequent drones.

Our present results suggest that these models should be modified. *A. florea* drones contain about 0.5 million spermatozoa in their seminal vesicles, while queens have only 1 million spermatozoa in their spermathecae (Ruttner 1988; Koeniger et al. 1989; Woyke 1993). Therefore, *A. florea* queens do not receive the full complement of spermatozoa from each of the 5–14 drones with which we have shown they mate. Our mating frequency data show either that queens are able to expel the excess semen before returning to the nest, or that each drone injects only a small proportion of the semen he produces. *A. florea* queens recently returned from mating flights have virtually no (i.e. 0–15) spermatozoa in their oviducts (Koeniger et al. 1989). It seems unlikely that queens could expel all excess semen in the few minutes between copulation and return to the nest. Therefore we suggest that drones do not inject all their sperm. This hypothesis could be tested by examining the seminal vesicles of newly mated drones, though this may be difficult. Drones may have evolved so that they do not inject all their semen, but producing less of it would seem more efficient. More plausible is the possibility that drones are prevented from injecting all their sperm by some structure or behavior of the queen during copulation. Therefore we suggest that, because of the fitness benefits of reproductive dominance, *A. florea* males are under selection for the production of high numbers of spermatozoa. On the other hand, queens that retain only some of the semen of each particular drone in their spermathecae increase their fitness by increased polyandry. One objection to this argument is that while *A. florea* drones are of similar size to *A. cerana*, they produce far less sperm (Koeniger et al. 1993).

Oldroyd et al. (1994b) demonstrated behavioural polymorphisms between eight genotypic classes of

A. florea workers revealed by restriction fragment length polymorphisms. However, they were unable to distinguish between the possibilities of polygyny (multiple queens) and polyandry (multiple males). Our present results demonstrate that polyandry and monogyny are usual in the species. Therefore it seems likely that the eight genotypic classes observed by Oldroyd et al. (1994b) were indeed the progeny of different drones.

Drone production by workers in normal queen-right *A. mellifera* colonies occurs (Page and Erickson 1988; Visscher 1989), but is usually kept at a low level by mutual policing behavior (Ratnieks 1988; Ratnieks and Visscher 1989), although the policing system is not perfect (Page and Erickson 1988) and occasionally fails (Oldroyd et al. 1994a). We found no evidence for worker reproduction in the *A. florea* colonies studied, although the sample size was small.

In conclusion, we have shown that queens of *A. florea*, mate with at least 5 drones and probably many more as shown in colony 1. This high level of polyandry generates intra-colonial genetic relationships similar to that found in *A. mellifera* of around 0.3. *A. mellifera* and *A. florea* are phylogenetically diverse species (Alexander 1991; Willis et al. 1992), with widely differing ecological ranges (Ruttner 1988). Data from other *Apis* species are urgently needed to determine the range of mating frequencies, and possibly resolve the competing (or perhaps complementary) hypotheses for the evolution of polyandry in eusocial Hymenoptera.

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