

CHAPTER 5

MATING FREQUENCY OF *A. dorsata* QUEENS

When social insect queens mate with several males (polyandry) this leads to reduced relatedness among offspring and increased genetic variation among a colony's workers. Kin selection theory (reviewed in Crozier and Pamilo, 1996) suggests that the evolution of eusociality was facilitated by high intra-colonial relatedness, so reasons for the shift from monandry and high relatedness to polyandry and low relatedness are of great interest. Furthermore, each mating flight involves risks to the queen (Moritz, 1985), suggesting that the benefits of polyandry must be substantial.

Several hypotheses have been proposed for explaining the evolution of multiple mating in *Apis*. The most plausible explanations of extremely high levels of polyandry found in the genus are the so-called “**genetic variability**” (GV) hypotheses (Keller and Reeve, 1994; reviewed in Palmer and Oldroyd, 2000). The lower relatedness among nest mates caused by multiple mating of the queen is postulated to increase the reproductive success of the colony. The advantages of multiple mating for colonies of eusocial Hymenopteran are: 1) Reduction in sex investment ratio conflict between queen and workers (Moritz, 1985; Queller et al., 1993). 2) Reduced impact of the genetic load of the sex locus on colony growth due to production of diploid males (offspring being homozygous at the sex locus) (Page, 1980; Woyke, 1980). 3) Reduction in susceptibility to parasites (Sherman, Seeley and Reeve, 1988; Shykoff and Schmid-Hempel, 1991; Schmid-Hempel, 1994, 1995, 1998; Schmid-Hempel and Crozier, 1999). 4) Increased possibilities for division of labour and task performance (Oldroyd, Rinderer and Buco, 1992 a; Oldroyd et al., 1992 b;

Oldroyd et al., 1993; Page and Robinson, 1991; Robinson and Page, 1988; 1989 b; Page et al., 1995; Fuchs and Moritz, 1998). 5) Tolerance to un-preferable in environmental conditions (Crozier and Page, 1985, Fewell and Page, 1993).

The degree of polyandry varies substantially among members of social Hymenopteran. Very high levels of polyandry are only seen in honey bees of the genus *Apis* (Strassman, 2001). Within this genus, the number of matings seems to be very variable. However, the number of matings examined may depend on the techniques used to investigate mating frequency. Boomsma and Ratnieks (1996) reviewed that four methods have been used to investigate paternity in social insects: 1) direct observations of copulations; 2) sperm counts; 3) estimates of paternity based on visible genetic markers; 4) estimates of paternity based on genetic markers. Genetic methods, (usually based on microsatellite or allozyme markers), are more reliable for assessing the paternity frequency than sperm counts or direct observation. However, they cannot actually be used to assess the numbers of times a queen mates if some copulations do not result in offspring.

Cornuet, Daoudi and Chevalet (1986) evaluated the number of matings of a population of *A. mellifera mellifera* in France using allozymes. They estimated the effective number of matings to be 12.4 in this subspecies. Estoup et al. (1993; 1994) investigated the number of patriline in three different honey bees subspecies; *A. m. mellifera*, *A.m. carnica* and *A.m. ligustica*, by using microsatellite analysis. They found 7-21 patriline from the samples with an effective average of 10.15 drones per queen. The highest level of polyandry reported in *A. mellifera* is approximately 30 matings per queen for the African subspecies *A. m. scutellata* (Moritz, Kryger and Allsopp, 1996).

Microsatellite analysis has been proved to be a reliable technique for determining the genetic structure of colonies. It allows precise estimation of relatedness among individuals, and for this reason this technique has been widely used to investigate the maternity and paternity of offspring in studies of social colonies (Section 2.5.1).

Levels of polyandry reported in the 5 species of *Apis* investigated to date are variable (Table 5.1). The lowest effective mating frequency was reported in *A. florea* (Oldroyd et al., 1995; Palmer and Oldroyd., submitted). Another open-nesting site honey bees (*A. andreniformis*) had quite similar effective mating frequency (Oldroyd et al., 1997). In addition, the effective mating frequency in other cavity-nesting species (*A. cerana*, *A. koschevnikovi*) were 12.0 ± 1.6 , and 10.5 ± 8.4 , respectively (Oldroyd et al., 1998; Rinderer et al., 1998). Surprisingly, the highest number of mating frequency of the queens of *A. nigrocincta* was reported to be the highest in this genus (Palmer et al., submitted).

Table 5.1. Levels of polyandry and intra-colonial genetic relationship in the genus *Apis* (revealed by microsatellite analysis).

Species	Observed paternity frequency (Mean +SE)	Effective paternity frequency	Coefficient of relatedness	Authors
<i>A. andreniformis</i>	13.5 \pm 2.3	9.1 \pm 0.83	0.3 \pm 0.007	Oldroyd et al., 1997
<i>A. florea</i>	8.0 \pm 1.6	5.6 \pm 1.0	0.35 \pm 0.02	Oldroyd et al., 1995
<i>A. florea</i> (revised)	16.0 \pm 2.45	10.14 \pm 1.15	0.32 \pm 0.005	Palmer and Oldroyd, submitted
<i>A. cerana</i>	18.0 \pm 3.03	12.0 \pm 1.6	0.29 \pm 0.005	Oldroyd et al., 1998
<i>A. nigrocincta</i>	54.0 \pm 5.8	40.49 \pm 11.79	0.27 \pm 0.004	Palmer et al, submitted
<i>A. koschevnikovi</i>	16.3 \pm 10.5	10.5 \pm 8.4	0.31 \pm 0.03	Rinderer et al., 1999
<i>A. mellifera</i>	13.8 \pm 2.5	12.4 \pm 2.2	0.30 \pm 0.009	Estoup et al., 1994
<i>A. dorsata</i>	30.17\pm6.0	25.6\pm1.05	0.27\pm0.02	Moritz et al., 1995
<i>A. dorsata</i>	26.7\pm6.6	20.0\pm6.6	0.29\pm0.007	Oldroyd et al., 1996

Data on mating frequency of *A. dorsata* was first reported by Moritz et al. (1995). Three microsatellite primers (A14, A76 and A88) were used to determine the paternity frequency of *A. dorsata* from both single colonies and aggregated colonies collected in Malaysia and Indonesia. They found that single colonies had the higher number of patriline at 28 while 3 colonies from aggregation had the number of patriline range from 11 to 17. The mean estimate number of matings of all colonies was 30.17 \pm 5.98 (range 16-53) and the average of the effective number of mating was 25.56 \pm 11.63 (range 14.08-44.08). Similarly, Oldroyd et al. (1996) determined the mating frequency of *A. dorsata* sampled from Thailand. Queens had mated with 13-39 males (mean 26.75 \pm 5.42). The mean effective mating frequency was 19.96 \pm 6.63.

Both investigations confirmed that *A. dorsata* had the highest level of polyandry recorded at that time. However, the small sample sizes collected by Moritz et al. (1995) and Oldroyd et al. (1996) meant that they were unable to determine if this species shows extreme levels of polyandry. In general, the number of patriline is correlated with sample size. Also, in Moritz's work, the used of adult workers to determine the number of patriline may have resulted in the error in patriline estimation due to misidentification of drifted bees from adjacent colonies. In both reports, only three microsatellite loci were used in each investigation i.e. A14, A76, and A88 in Moritz's work and A14, A88, and B124 in Oldroyd's work. Hence, the estimates of mating of those queens examined may have been grossly underestimated.

In this thesis, I conducted investigations to re-examine mating frequency in *A. dorsata* to obtain more precise estimates. Firstly, to avoid drifted bees, all analyses were conducted on pupae or early adult stages. Secondly, the number of bees analysed per colony was relatively high (n=2,696 with the average 200 bees per colony). Finally, I used 4 microsatellite loci for my analyses including a new microsatellite locus cloned from *A. dorsata* and found to be highly polymorphic in that species (Parr et al., 2001).

5.1 Materials and Methods

5.1.1 Analysis of paternity

5.1.1.1 Sample collection

Thirteen fresh combs of *A.dorsata* were cut from the wild throughout Thailand in 1998-1999. Eight of these (colonies 1-8) were collected from 14 aggregated colonies in Pua district, Nan province in the north of Thailand, from a

prominent bee tree that hosts an aggregation of giant honey bees every year (Figure 5.1.; 5.2). Another five solitary colonies (colonies 9-13) were collected from various parts of Thailand (Figure 5.3). Fresh combs (Figure 5.4) were wrapped in aluminium foil and kept on ice during transportation to the Bee Biology Research Unit Chulalongkorn University in Bangkok. They were then temporarily stored in a freezer at -20°C. All samples were transported to the School of Biological Sciences University of Sydney, Australia and kept in a freezer (-70°C) until they were used for microsatellite analysis.



Figure 5.1. Fourteen colonies of *A. dorsata* aggregated on the tree.



Figure 5.2. Sample collection from aggregation.



Figure 5.3 A singly nesting colony. A piece of fresh comb was cut from this single colony for paternity determination .

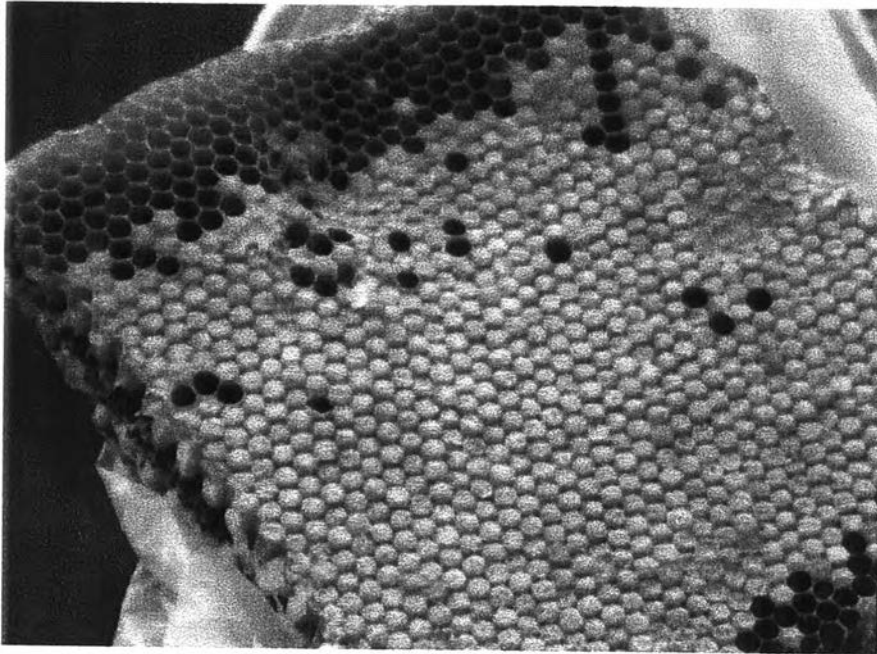


Figure 5.4 A piece of fresh comb from a colony of *A. dorsata*.

5.1.1.2 DNA Extraction

Worker pupae or early adults prior to emergence were removed from cells and kept individually in separate Eppendorf tubes. DNA extractions of samples were conducted according to methods described in Section 3.1.1.

5.1.1.3 Polymerase Chain Reaction (PCR)

Four polymorphic microsatellite primers which specific to *A. dorsata* (A14, A24, A88 and Ad3) were used to determine the maternity and paternity of those workers. DNA extractions were diluted 1:6 with distilled water. The PCR protocol is given in Section 3.1.2. Diluted templates were then added to the PCR mixture and underwent a thermocycling programme appropriate to each primer (Table 3.2 and Table 3.3). After cycling, PCR products were kept at 4 °C for electrophoresis.

5.1.1.4 Electrophoresis

PCR products of each bee was electrophoresed on an automated DNA fragment analyser (Corbett Research, Sydney). The procedure was described in Section 3.1.3.

5.1.1.5 Paternity analysis

The queen's genotype of each colony was determined using the criteria described in Section 3.2. The number of worker patriline was determined by a combination of drone alleles at four microsatellite loci.

5.1.2 Calculations

Effective mating frequency (m) is the mating frequency corrected for finite sample size. This was computed by

$$m = (n-1)/(n\sum_{i=1}^k y_i^2 - 1)$$

where: k = number of patrilines observed

y_i = observed proportion of worker sampled from the i^{th} patriline

n = number of worker examined

(Boomsma and Ratnieks, 1996; Pamilo, 1993)

Average coefficient of relatedness (G) was weighted according to the relative proportions of each subfamily. G was computed by

$$G = 0.25 + 0.5\sum_{i=1}^k p_i^2$$

where: p_i = proportion of workers in each patriline

(Boomsma and Ratnieks, 1996)

Expected frequency of non-detected patriline (dp) was calculated by

$$dp = \Pi (\Sigma qi^2)$$

where : qi = allele frequency at each of i loci

(Boomsma and Ratnieks, 1996)

Probability of not sampling a patriline was calculated by

$$(1-p)^n$$

where : p = proportion of worker represented in a patriline

n = number of worker examined

(Foster et al., 1999)

5.2 Results

The number of subfamilies found in this investigation ranged from 47-103 per colony. The effective mating frequency ranged between 26.9 and 88.5. The mean effective mating frequency of aggregation was 64.11 ± 7.388 (SE) (Table 5.2.1) while the mean effective mating frequency of queens from single colonies was 62.14 ± 10.269 (SE) (Table 5.2.2). The lowest effective mating occurred in colony 4 of the aggregation (Aggregation-4) at 28.786. The may be contribute to a single male was dominant on sperm usage in this colony.

Maternal and paternal genotypes for each colony are shown in Appendix 1.
Microsatellite image of workers was shown in Figure 5.5.

Table 5.2.1. Effective mating frequency (m), average coefficient of relatedness (G), number of subfamilies (k) and number of bees (n) of *A. dorsata* sampled from aggregated colonies examined by 4 microsatellite loci.

Colony	number of bees (n)	number of subfamilies (k)	Effective mating frequency (m)	Average coefficient of relatedness (G)
Aggregation-1	144	72	88	0.256
Aggregation-2	189	74	80.027	0.256
Aggregation-3	111	47	37.685	0.263
Aggregation-4	248	78	28.786	0.267
Aggregation-5	204	92	77.551	0.257
Aggregation-6	206	88	64.572	0.258
Aggregation-7	235	87	62.066	0.258
Aggregation-8	156	71	74.172	0.257
Mean	186.625	76.125	64.107	0.259
Standard error	16.510	5.019	7.388	0.001

Table 5.2.2. Effective mating frequency (m), average coefficient of relatedness (G), number of subfamilies (k) and number of bees (n) of *A. dorsata* sampled from single colonies examined by 4 microsatellite loci.

Colony	number of bees (n)	number of subfamilies (k)	Effective mating frequency (m)	Average coefficient of relatedness (G)
Single colony-9	176	83	88.506	0.256
Single colony-10	271	102	67.625	0.257
Single colony-11	288	80	55.623	0.259
Single colony-12	276	83	26.877	0.269
Single colony-13	192	85	67.661	0.257
Mean	240.6	86.6	62.137	0.26
Standard error	23.409	4.128	10.269	0.002

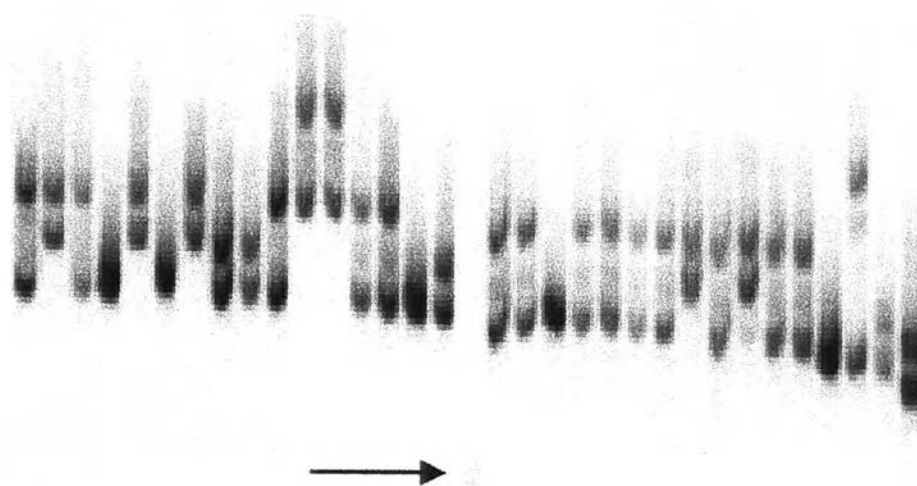


Figure 5.5 Image of microsatellite loci of workers at Ad 3 locus (resolved by polyacrylamide gel). Arrow indicates size standard at 160 bp length.

Comparisons between aggregation and non-aggregation colonies shown that there were no significant different in all parameters (number of samples (n), number of subfamily (k), effective mating frequency (m) and average coefficient of relatedness (G)) examined (Mann-Whitney U test) (Table 5.3).

Table 5.2.3. Mann-Whitney U test of the numbers of samples (n), the number of subfamilies (k), the effective mating frequency (m) and the average coefficient of relatedness (G) between aggregations and non-aggregations.

	Mann-Whitney U test	Probability	Chi-square
Number of samples (n)	31	0.107	2.593
Number of subfamily (k)	28	0.241	1.375
Effective mating frequency (m)	18	0.770	0.086
Average coefficient of relatedness (G)	21	0.881	0.022

The probability of non-detected patriline (dp) was separately calculated between aggregation and single colonies. The dp of aggregation was very low at 0.0003 while the dp of single colonies was 0.002. This means that for all 13 colonies about 2.841 patriline may carry the same genotypes and was not detected. This was very low compared to the total patriline (1,042 patriline) have been examined in this thesis.

The number of workers present in each patriline is unequal. Therefore, the probability of not sampling a patriline may be an underestimate. If only a small proportion of a colony's offspring is represented in a sample, the

probability of non-sampling is high. For example, in some patriline, only 1 worker was identified. This leads to a high non-sampling error rate (Palmer et al., submitted).

5.3 Discussion

The queen mating frequency of *A.dorsata* reported in this thesis is much higher than the preliminary estimates reported by Moritz et al. (1995) and Oldroyd et al. (1996). This is the highest number of mating of queens in this genus reported so far. The higher mating frequency reported in this thesis resulted from the higher sample size used than in the earlier studies, combined with additional microsatellite loci.

The extremely high number of mates of queens in this genus (64.11 ± 7.388 (SE) in aggregation and 62.14 ± 10.269 (SE) in non-aggregated colonies) is somewhat surprising because the mating flight of *A. dorsata* queens is very short. Queens fly from nesting sites to DCAs and fly back in the period of less than 10 minutes (Rinderer et al., 1993; Koeniger et al., 1994). This means that queens must mate several times on several flights, and each copulation must be very short. In the observation made in Borneo, the DCAs appear to be less than 700 m from nest (Koeniger et al. 1994) and this may facilitate brief mating flights. Tan et al. (1999) observed that queens of this species take several mating flights, thus permitting extremely high mating frequencies despite the brief mating flights.

Multiple mating has now been demonstrated in at least 7 species of the genus *Apis*. The extraordinarily high number of matings of queens in *A. dorsata*

is of central of interest and several hypotheses explaining the extremely high level of polyandry in *A. dorsata* are proposed.

1. *Availability of males.* *A. dorsata* colonies tend to aggregate. At DCAs near aggregations, vast numbers of males are present during the period of mating flights. Therefore, queens of this species may have greater opportunities for mating than queens from other species of *Apis*, where the number of males present at DCAs may be much lower. However, the results in this thesis showed that mating numbers of queens from singly nesting colonies and aggregated colonies is not different. Therefore, the hypothesis that the queens can mate many times due to the high number of males available at the DCAs seems to be less plausible. Further research should be carried out to observe number of males present at DCAs of this species.

2. *Parasite infestation.* Colonies that nest in dense aggregations are more likely to suffer from parasite infestation than isolated nests because both diseases and parasites can spread rapidly among the aggregated colonies. Colonies with high intra-colonial genetic variance may be more resistant to parasite infestation (Sherman et al., 1988; Shykoff and Schmid-Hempel, 1991; Schmid-Hempel, 1995, 1998; Schmid-Hempel and Crozier, 1999). Thus, selection on queens may have led to extreme levels of polyandry to maximize their genetic diversity of offspring. The occurrence of parasites in both aggregations and single colonies should be studied to get clearer investigation on effect of parasite infestation.

3. *Unfavourable environment conditions and task performance.* Seasonal migratory behaviour in this species is common. Each flight costs

workers to spend energy and time to locate new nesting sites. Also, divergence of non-preferable environment conditions during the flight and at different nesting sites may cause the new founding colonies. Workers with diverse in genotype are capable to assist in different tasks. This also involves in several workers that can tolerate to the divert of environment. Therefore, genetic diversity of workers may benefit migratory colonies.

4. *Reduce probability of inbreeding.* Increasing the number of matings reduces the probability of queens mating with brothers. Homozygosity at the sex locus of brood gives rise to diploid drones that reduce colony productivity. These diploid drones are eliminated at an early stage (Section 1.6). In *A. dorsata*, queens mate near the nest site increasing the probability of mating with brothers. Queens, in order to ensure that many of their mates are not brothers, may be selected to mate with a large number of males.

Other open nesting honey bee species, *A. florea* and *A. andreniformis* have similar mating frequencies to *A. mellifera*, *A. cerana*, and *A. koschevnikovi* which are cavity nesting species (Estoup et al., 1995; Oldroyd et al., 1995 b; Oldroyd et al., 1997; Oldroyd et al., 1998; Rinderer et al., 1998). In contrast, another cavity nesting honey bee, *A. nigrocincta* was found to have very high effective mating frequency at 40.493 ± 11.790 (Palmer et al., submitted). Hence, nesting behaviour does not explain the extremely high mating frequency of *A. dorsata*. This also does not provide the reason of difference in degree of mating frequency among the species in this genus. Therefore, it is reasonable to draw the conclusion that the occurrence of polyandry probably evolved in a common ancestor prior to the appearance of this genus (Oldroyd et al., 1998). In addition, this trait has been maintained in every species of the genus *Apis* since then.