

Mating structure and male production in the giant hornet *Vespa mandarinia* (Hymenoptera: Vespidae)

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Abstract

Queen mating frequency, genetic relatedness between workers and worker reproduction were estimated in *Vespa mandarinia* by microsatellite DNA markers. Of 20 colonies examined, eighteen contained queens inseminated by a single male and two colonies contained queens inseminated by two males. The estimated effective number of matings was 1.03 ± 0.023 (mean \pm SE) with 85% of the offspring of the two multiply-mated queens being sired by one of the two males. The genetic relatedness between workers was 0.738 ± 0.008 , which was almost identical to the predicted value of 0.75 under monogyny and monandry. For this low paternity, kin selection theory predicts a potential conflict between queens and workers over male production. To learn whether males are derived from queens or workers, 400 males from 20 colonies were genotyped at four microsatellite loci. We found that queens produced all males. This finding was confirmed by the observation that 4,317 dissected workers had not developed ovaries. There was no relationship between queen mating frequency and the frequency of worker reproduction, and workers did not produce any male offspring. These results strongly suggest that male production dominated by queens in *V. mandarinia* is possibly due to worker policing.

Key words: Mating number; relatedness; *Vespa mandarinia*; worker reproduction; microsatellites

INTRODUCTION

Colony kin structure is a central parameter for testing kin selection of social insects (Crozier and Page, 1985; Boomsma and Ratnieks, 1996; Crozier and Pamilo, 1996). The mating frequency of a queen and the number of queens are the main determinants of genetic colony structure, especially in eusocial Hymenoptera because genetic colony structure potentially affects the outcome of the conflict over sex ratio and male production between queens and workers. Several hypotheses have been proposed as to why queen mates multiply instead of apparent decrement of genetic interests for workers to rear broods (Strassmann, 2001).

Kin selection theory predicts that in monogynous colonies, workers and queens will compete

for male production because the relatedness (r) of a worker to her son (0.5) is greater than to nephews (0.375) or brothers (0.25). However, as the mating frequency of queens increases, the potential conflict over genetic interests between queens and workers diminishes because the relatedness of workers to nephews becomes smaller than to brothers, resulting in the evolution of worker policing (Woyciechowski and Lomnicki, 1987; Ratnieks 1988). An increase in queen mating frequency enhances the proportion of workers that are half sisters and reduces the average genetic relatedness between workers. Since the relatedness of a worker to the male offspring of a half sister (0.125) is lower than the relatedness to the male offspring of the foundress queen (0.25), worker reproduction should be inhibited by mutual policing (Ratnieks,

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1988), allowing the queens to monopolize male production. Therefore, worker policing is expected to evolve more readily in monogynous species with polyandrous queens than with monandrous queens (Ratnieks, 1988).

Nevertheless, Kikuta and Tsuji (1999) demonstrated that worker production of males was inhibited by worker policing even in monogynous and monandrous colonies, suggesting that worker policing is selected for, despite workers incentive to produce their own sons under high worker relatedness, if worker reproduction reduces colony productivity (Cole, 1986; Ratnieks, 1988).

Vespinae is an excellent group to test relationships between worker reproduction and paternity frequency because all members have similar annual life cycles and simple social structure, and the four genera (*Vespa*, *Provespa*, *Dolichovespula* and *Vespula*) include relatively few species for which phylogenetic relationships have been extensively investigated (Matsuura and Yamane, 1990; Boomsma and Ratnieks, 1996; Foster and Ratnieks, 2001). The relationship between worker reproduction and paternity frequency has been extensively investigated so far in *Dolichovespula maculata*, *D. media*, *D. sylvestris*, *D. norwegica*, *D. saxonica* (Foster et al., 2001), *D. arenaria* (unpublished data in Foster and Ratnieks, 2001), *Vespula germanica* (Ross, 1985; Goodisman et al., 2002), *V. maculifrons* (Ross, 1985, 1986), *V. vulgaris* (Foster and Ratnieks, 2001) and *V. squamosa* (Ross,

1986). Two genera of Vespinae, *Dolichovespula* and *Vespula*, have been studied for sociogenetic structure in some detail (Table 1). Queens of *Vespula* generally mate multiply and have effective mates of 1.9 to 7.1. In multiply mated queens, sperm are mixed and used at random over a long period of time (Ross, 1986). *Dolichovespula* societies are characterized by low effective number of mates of 1 to 1.35 and by worker male production. This is particularly interesting because Foster and Ratnieks (2000) investigated the facultatively polyandrous wasp *Dolichovespula saxonica* and found that workers produce males when worker relatedness within a colony is high (>0.5). By contrast, in the genus *Vespa*, sociogenetic structure has only been investigated in *V. crabro* (Foster et al., 2000; Takahashi et al., 2004) and *V. ducalis* (Takahashi et al., 2002). *V. crabro* workers have been shown to police worker eggs in queen-right colonies (Foster et al., 2002).

In *Vespa*, genetic markers have been developed (Thóren, 1998; Hasegawa and Takahashi, 2002) and the colony genetic structure has been reported for three species (Foster et al., 1999; Takahashi et al., 2002, 2003, 2004). Microsatellite DNA analyses showed that queens of *V. crabro*, *V. ducalis* and *V. analis* generally mate with one male (Foster et al., 1999; Takahashi et al., 2002, 2003, 2004). Although these results suggest a potential conflict between queens and workers over male production in these species, workers in queen-right colonies of *V.*

Table 1. Sociogenetic structure in Vespinae wasps. All estimates are based on genetic markers, either allozymes (A) or DNA microsatellites (M).

Species	Colonies	Effective mate	Paternity range	Worker's son (%)	Marker	Reference
<i>Vespa crabro gribodi</i>	14	1.11	1–3	0	M	Foster et al. (1999, 2000)
<i>Vespa crabro flavofasciata</i>	20	1.13	1–3	0	M	Takahashi et al. (2003)
<i>Vespa ducalis</i>	20	1.00	1	0	M	Takahashi et al. (2002)
<i>Vespa analis</i>	20	1.05	1–2	0	M	Takahashi et al. (2004)
<i>Dolichovespula media</i>	10	1.08	1–2	7	M	Foster et al. (2002)
<i>Dolichovespula maculata</i>	10	1.00	1	21	M	Foster et al. (2002)
<i>Dolichovespula arenaria</i>	20	1.09	1–4	17	M	Ratnieks and Boomsma (unpublished data)
<i>Dolichovespula sylvestris</i>	10	1.15	1–2	10	M	Foster et al. (2002)
<i>Dolichovespula norwegica</i>	10	1.08	1–3	3	M	Foster et al. (2002)
<i>Dolichovespula saxonica</i>	10	1.35	1–3	35	M	Foster et al. (2002)
<i>Vespula germanica</i>	55	2.35	1–7	—	A and M	Ross (1985); Goodisman et al. (2002)
<i>Vespula maculifrons</i>	30	7.14	—	0	A	Ross (1985, 1986)
<i>Vespula vulgaris</i>	17	1.90	1–4	0	M	Foster and Ratnieks (2001)
<i>Vespula squamosa</i>	17	3.33	—	0	A	Ross (1986)

crabro and *V. ducalis* have been shown to produce no males (Foster et al., 2000; Takahashi et al., 2002, 2003).

V. mandarinia is a common, giant hornet of the mainland and islands of East Asia (Archer, 1995). Males of *V. mandarinia* are often observed aggregating around the entrance holes of nests and attempt to mate with queens that emerge from the nests. Observations in Japan suggest that *V. mandarinia* queens mate with only one male (Matsuura and Yamane, 1990). If this is true, worker male production should occur as predicted the under relatedness framework. The aim of this study was to confirm and quantify the results of previous field observations on *V. mandarinia* using DNA microsatellite genotyping and to clarify the relationship between paternity frequency and worker reproduction in queen-right colonies.

MATERIALS AND METHODS

Sample collection. During October 1999, 20 colonies of *V. mandarinia* were collected from a forest near Noda City in Chiba Prefecture, Japan (13°20'N, 20°45'E). All nests were located within a 70 km² area and collected at night by blocking the nest entrance and adding 50 ml of diethyl ether to the nest. For microsatellite DNA analysis, 20 pupal workers and 20 adult males were collected from each of the 20 colonies and preserved in 99% ethanol before they were stored at -20°C. All adult workers were stored at -20°C and later their ovarian development was assessed.

DNA extraction and microsatellite DNA analysis. Microsatellite DNA analysis was conducted using four microsatellite primers (Hasegawa and Takahashi, 2002). DNA extraction was based on the method of Walsh et al. (1991). Template DNA was extracted from individuals by boiling macerated tissue in 400 μ l of 5% Chelex (Bio-rad) resin at 95°C for 10 min. All polymerase chain reactions (PCR) were performed in a total volume of 10 μ l containing 1.0 μ l (about 10 ng) of template DNA, 0.2 μ M of primer, 400 μ M of dNTP mix, 1.0 μ l of 10X reaction buffer, 1.5 mM MgCl₂, and 0.05 units Taq polymerase (Takara). All PCR reactions were performed as follows: after one denaturing step of 3 min at 94°C, the samples were processed through 30 cycles consisting of 30 s at 94°C, 30 s at 52°C to 58°C, and 30 s at 72°C

(Hasegawa and Takahashi, 2002). The PCR products were analyzed by 6% polyacrylamide sequence gels with a manual sequencer (FMC) and then visualized by Silver stain.

Data analysis. The regression relatedness (b), inbreeding coefficient (F), and allele frequencies was estimated by the Relatedness 4.2 computer program (Goodnight and Queller, 1994). Colonies were equally weighted, and the standard errors and t -test were calculated by jack-knifing over colonies (Queller and Goodnight, 1989). The average coefficient of pedigree relatedness (g_{ww}) was inferred from worker genotypes over the four loci for each colony:

$$g_{ww} = 0.25 + 0.5 \sum_{i=1}^k p_i^2 \quad (1)$$

where p_i is the relative frequency of the i th paternity and k is the number of fathering males for each colony (Laidlaw and Page, 1984). The effective mating frequency (M_e) was calculated according to Starr (1984) as:

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

where p_i is the proportional contribution of the i th fathering male, and k is the number of fathering males for each colony. Estimates of queen mating frequencies are affected by two sources of errors: non-sampling and non-detection (Boomsma and Ratnieks, 1996). The risk of overlooking one or more rare paternal genotypes is high when the number of workers analysed is small. We assumed this risk was reduced to acceptable levels by the analysis of 20 workers from each colony. In any event, rare males have only a small effect on the effective mating frequency.

The procedure of Foster et al. (1999) was used to estimate the non-detection error probability. Patriline are indistinguishable when the genetic marker loci have only low levels of polymorphism. The probability of two patrilines in a population having identical genotypes at analyzed all loci is:

$$d_p = \prod (\sum q_i^2) j \quad (3)$$

where q_i is the allele frequencies at the i th locus and j is the number of loci (Foster et al., 1999). However, these estimates assume that alleles of paternal and maternal origin can be distinguished.

The male offspring of a worker can be detected because 50% of her alleles are transmitted. The emergence probability of workers' male offspring in the i th colony (p_i) can be calculated as:

$$p_j = \sum_1^n p_i 1 - 0.5^{l_i} \quad (4)$$

where n is the number of patriline in the colony, p_i is the proportional representation of the i th patriline and l_i is the number of informative loci analyzed at the i th patriline (Foster et al., 2001). The expected number of males that are derived from workers (number of assignable males, N_a) in a sample can be calculated as $\Sigma(P_j N_j)$ where N_j is the number of males analyzed for the j th colony. If workers produce x proportion of the males, the probability of not sampling any worker-produced males is then $(1-x)^{N_a}$. For unlinked loci, the total number of assignable males in a sample can be estimated from the formula in Foster et al. (2001).

Dissection of workers ovaries. For the 20 colonies, up to 90% of the workers in each colony were dissected and their ovarian development was quantified. Developmental stages of ovaries was scored for a total of 4,317 adult workers using Yamane's index (1974). All samples were compared with the ovarian condition of five laying workers from queen-less colonies collected during early August from the same population.

RESULTS

Variation at microsatellite loci for *V. mandarinia*

High allelic variation was observed in the 20 colonies (Table 2). The VMA-3, VMA-4, VMA-6 and VMA-7 microsatellite loci had allelic numbers of 5, 3, 4 and 4, respectively, and the mean observed heterozygosity for all loci was 0.62.

Mating structure

Of the 20 colonies examined, 18 had queens inseminated by a single male and two colonies had queens inseminated by two males (Table 3). In the doubly-mated colonies, the fathering male ratio was 17:3 for both colonies. Effective number of matings was 1.03 ± 0.023 (mean \pm SE). The mating structure of *V. mandarinia* is thus usually characterized by single, at most double, mating by the queen and by sperm utilization biased to one male in a doubly-mated queen (Table 3). The inbreeding coefficient was not significantly different from over the four loci zero ($F = 0.0187 \pm 0.001$). The non-detection error for colony levels (d_n) ranged from 0.001 to 0.074, and that for the population level (d_p) was low at 0.024 over the 20 colonies (Table 3). Therefore, rare paternity failed to be detected at 2%, suggesting no effect on our results.

Genetic relatedness between workers

The regression relatedness between nestmate workers was 0.703 ± 0.033 (mean \pm SE) when averaged over the 20 colonies. Similarly, the pedigree relatedness between nestmate workers was 0.738, which is in good agreement with the regression relatedness (Table 3). These results suggest that *V. mandarinia* is genetically monogynous and monandrous, and the relatedness between females is maintained at high levels because the frequency of double mating is low and one of the two males predominantly sired offspring if queens mated with two males.

Worker oviposition

The number of workers of 20 colonies was 220.7 ± 97.2 (mean \pm SD). This value was similar to that reported by Matsuura and Yamane (1990). All of the 4,317 dissected adult workers had less developed ovaries than did laying workers of queen-less

Table 2. Number of alleles (n), allele frequency and observed heterozygosity (H_o) of four microsatellite loci for *V. mandarinia*

Locus	n	Allele frequency					H_o
		a	b	c	d	e	
VMA-3	5	0.109	0.453	0.121	0.195	0.123	0.72
VMA-4	3	0.100	0.286	0.614			0.53
VMA-6	4	0.444	0.334	0.139	0.084		0.67
VMA-7	4	0.138	0.602	0.226	0.034		0.57
Mean	4						0.62

Table 3. Colony-level data on mating frequency, relatedness between workers, proportional contribution of mate males and non-detection error (d_n) of *V. mandarinia*

Colony no.	Mating number		Pedigree relatedness	Proportional contribution of mate males	Non-detection error (d_n)
	observed	effective			
9902	1	1.00	0.75	—	0.005
9903	1	1.00	0.75	—	0.015
9904	1	1.00	0.75	—	0.006
9906	1	1.00	0.75	—	0.056
9910	1	1.00	0.75	—	0.021
9912	2	1.34	0.63	0.85, 0.15	0.074
9913	1	1.00	0.75	—	0.013
9950	1	1.00	0.75	—	0.001
9951	1	1.00	0.75	—	0.021
9972	1	1.00	0.75	—	0.014
9973	1	1.00	0.75	—	0.015
9976	1	1.00	0.75	—	0.001
9977	1	1.00	0.75	—	0.056
9982	1	1.00	0.75	—	0.011
9983	2	1.34	0.63	0.85, 0.15	0.003
9984	1	1.00	0.75	—	0.034
9985	1	1.00	0.75	—	0.035
9986	1	1.00	0.75	—	0.018
9987	1	1.00	0.75	—	0.074
9988	1	1.00	0.75	—	0.011
Mean	1.10	1.03	0.738	—	0.024
S.E.	0.688	0.023	0.0083	—	0.0052

colonies. In addition, all males had those alleles which were found at any locus in the queens. The number of assignable males (N_a) was 321, which indicates if worker oviposition contributes to the production of 1% of the males in a population, the probability of failing to detect males by workers is equivalent to 4%. These lines of evidence show that the queens produced all males in these colonies. Workers with developed ovaries were not found in all colonies where the genetic relatedness between workers was similar to full sister (0.75).

DISCUSSION

We found that *V. mandarinia* queens typically mate with only one male and that colonies are headed by a single queen. Similarly, Matsuura and Yamane (1990) reported that *V. mandarinia* queens mate once or twice. These findings agree with previous field observations that monogyny is typical for temperate hornets including *V. mandarinia* (Matsuura and Yamane, 1990). Effective mating frequency in *V. mandarinia* (1.03) is lower than the

observed mating frequency (1.10) because of predominant usage of sperm (up to 85%) from one male of the two males. The mean estimate of the effective mating frequency is 1.11 for *V. crabro* (Foster et al., 1999; Takahashi et al., 2004), 1.0 for *V. ducalis* (Takahashi et al., 2002), and 1.1 for *V. analis* (Takahashi et al., 2003), which are not significantly different from that of *V. mandarinia* (1.03). Furthermore, the effective mating frequency remains very low at 1.03, because the paternity of workers is skewed between the two mating partners. The mean effective numbers of matings in *V. crabro*, *V. ducalis* and *V. analis* are 1.11, 1.00 and 1.02, respectively (Foster et al., 1999; Takahashi et al., 2002, 2003, 2004). Consequently, these four species have a similar frequency of multiple paternity. Similarly, the proportion of multiply-mated queens does not differ between the three species. The regression coefficient of relatedness between workers was estimated to be 0.724 for *V. crabro*, 0.69 for *V. ducalis* (Takahashi et al., 2002) and 0.74 for *V. analis* (Takahashi et al., 2003). The pedigree relatedness between nest mate workers in

V. mandarinia (0.74) was slightly higher than in *V. crabro* (0.69) (Foster et al., 1999, 2000) and similar to that of *V. ducalis* (0.75) and *V. analis* (0.74) (Takahashi et al., 2002, 2003). However, the average genetic relatedness of *V. mandarinia* nest mate workers was not significantly different from that of other *Vespa* species (Tables 1, 3).

Haplo-diploid sex determination is the rule in the Hymenoptera (ants, bees, wasps and sawflies), though diploid males often arise from inbreeding through complementary sex determination (CSD). The diploid male is sterile and has a fitness cost at the colony level (Cook and Crozier, 1995). Foster et al. (2000) reported diploid males in *V. crabro* in Britain. However, we found no diploid males in 400 *V. mandarinia* males examined in this study. Our results indicate that the inbreeding coefficient (F) estimated from 20 colonies is not significantly different from zero. This low level of inbreeding might explain the lack of diploid males in *V. mandarinia*.

These results show that *V. mandarinia* foundress queens are generally monogynous and monandrous under random mating. Therefore, kin selection theory predicts conflict between queens and workers over male production (Ratnieks, 1988) with workers gaining genetic benefits by producing males. The theory also predicts that worker reproduction should be hindered by worker policing if the coefficient of worker relatedness is less than 0.50 (Ratnieks, 1988). However, genotypic analysis of 400 males from 20 colonies of *V. mandarinia* showed that all the males were queen-derived. This was corroborated by the fact that none of the 4,317 workers showed ovarian development. Thus, we found no positive correlation between worker genetic relatedness and the frequency of worker reproduction. The absolute lack of worker reproduction in *V. mandarinia* contradicts our prediction based on worker relatedness. No worker offspring have been detected in two other monogynous taxa (*V. crabro* and *V. ducalis*) (Foster et al., 2000; Takahashi et al., 2002, 2004). Kikuta and Tsuji (1999) reported that workers of the monogynous and monandrous ant *Diacamma* sp. selectively eliminate eggs produced by other fellow workers. To explain this phenomenon, Kikuta and Tsuji (1999) suggested that worker policing in *Diacamma* sp. evolved despite high levels of relatedness between workers if worker reproduction reduced colony

productivity (Cole, 1986; Ratnieks, 1988) and that mutual policing leads to the evolution of self-inhibition of male production. Contrary to our expectations, high genetic relatedness between workers in *V. mandarinia* did not lead to worker reproduction. The lack of worker reproduction and ovarian development in *V. mandarinia* workers may be explained by the same mechanism as in *Diacamma* sp. (cost to colony productivity outweighs genetic benefit from worker reproduction). Even in the two populations of *V. crabro* that contained workers with fully developed ovaries none of the adult males were worker sons (Foster et al., 2000; Takahashi et al., 2004). Foster et al. (2002) observed that *V. crabro* workers can discriminate eggs laid by workers from those laid by the queen and selectively eliminate worker eggs in queen-right colonies. These results suggest that the monopoly of male production in colonies of *V. mandarinia* is also inhibited by worker policing and that worker reproduction occurs only when a colony becomes queenless.

Evolutionary factors high incidence of polyandry have repeatedly been reviewed (Crozier and Page, 1985; Boomsma and Ratnieks, 1996; Crozier and Pamilo, 1996), but the cause of polyandry remains unclear. Strassmann (2001) suggested that multiple mating in eusocial hymenopteran queens is a rare and derived trait in taxon, including the Vespinae, where single matings are ancestral. The genus *Pogonomyrmex* is one of three ant genera with multiply-mated queens (Gadau et al., 2003). Evidence of mating frequency data and phylogenetic information suggest that multiple matings in *Pogonomyrmex* species is the ancestral state of *Pogonomyrmex sensu* (Gadau et al., 2003). There is, however, very little information about conflict between queens and workers in *Pogonomyrmex* ants. In contrast, vespine species have very similar biologies, such as annual life cycle, morphologically distinct queen and worker castes, monogyny (Matsuura and Yamane, 1990) and reproductive conflict, such as matricide and male production (Bourke, 1994; Foster and Ratnieks, 2001). These features facilitate cross-species comparisons. Hypotheses about the evolution of polyandry and policing can be tested with the vespine wasps.

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