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Kin structure and colony male reproduction in the hornet *Vespa crabro* (Hymenoptera: Vespidae)

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Abstract We estimated queen mating frequency, genetic relatedness among workers, and worker reproduction in *Vespa crabro flavofasciata* using microsatellite DNA markers. Of 20 colonies examined, 15 contained queens inseminated by a single male, 3 colonies contained queens inseminated by two males, and 2 colonies contained queens inseminated by three males. The genetic relatedness among workers was estimated to be 0.73 ± 0.003 (mean \pm SE). For this high relatedness, kin selection theory predicts a potential conflict between queens and workers over male production. To verify whether males are derived from queens or workers, 260 males from 13 colonies were genotyped at four microsatellite loci. We found that all of the males were derived from the queens. This finding was further supported by the fact that only 33 of 2,990 workers dissected had developed ovaries. These workers belonged to 2 of the 20 colonies. There was no relationship between queen mating frequency and worker reproduction, and no workers produced male offspring in any of the colonies. These results suggest that male production dominated by queens in *V. crabro flavofasciata* is possibly due to worker policing.

Key words Mating frequency · Relatedness · *Vespa crabro* · Worker reproduction · Microsatellites

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Introduction

In social insects, the kin structure of colonies is a central factor influencing social behavior among individuals (Hamilton 1964; Crozier and Pamilo 1996). For monogynous hymenopteran species, the mating frequency of a queen and the number of males contributing to her insemination are the main determinants of relatedness among workers in the colony. In colonies of social Hymenoptera, male production is undertaken by queens or unmated workers. Kin selection theory predicts that in colonies governed by singly mated queens, workers will compete with the queen for the production of males, because the relatedness (r) of a worker to her own offspring (0.50) is greater than the relatedness to male offspring of her full sister (0.375) or to male offspring of the mother queen (0.25). However, this theory predicts that as the mating frequency of queens increases, the potential for conflict between queens and workers will decrease. An increase in queen mating frequency enhances the proportion of workers that are half sisters for a worker and reduces the average genetic relatedness among workers. Because the relatedness of a worker to the male offspring of her half sister (0.125) is lower than the relatedness to the male offspring of the mother queen (0.25) worker reproduction should be inhibited by mutual policing (Ratnieks 1988), which will consequently allow only queens to monopolize male production. Therefore, worker policing will evolve more readily in monogynous colonies headed by multiply mated queens than in colonies headed by singly mated queens (Ratnieks 1988). In the European honeybee *Apis mellifera*, workers detect and destroy worker-derived eggs (Ratnieks and Visscher 1989). Workers may gain a genetic benefit from rearing only queen's sons by mutual policing under the condition of a high degree of polyandry (Ratnieks and Reeve 1992).

Kikuta and Tsuji (1999), nevertheless, demonstrated that even in monogynous colonies headed by a singly mated queen, worker reproduction of males was inhibited by worker policing. Three hypotheses are proposed to explain worker policing in monogynous and monandrous

hymenopteran species. First, worker policing may evolve to prevent a decrease in the genetic relatedness among workers due to frequent gamergate replacement. Second, worker policing will evolve despite high relatedness among workers if worker reproduction reduces colony productivity (Ratnieks 1988; see also Cole 1983). Third, worker policing may function to promote the optimal resource allocation between colony maintenance and reproduction for the next year. In Vespinae, no queen replacement occurs, and the colonies are monogynous and have an annual life cycle with the production of reproductives at the end of summer. These conditions suggest that the second hypothesis is plausible.

In queen-right colonies of *Vespula* wasps, worker reproduction and ovarian development of workers are likely to occur in species with large nests (Ross 1985; Goodisman et al. 2002). Foster and Ratnieks (2000) found that in colonies of the Swedish species *Dolichovespula saxonica saxonica*, in which singly mated and multiply mated queens are found, worker reproduction occurs when the paternity frequency of the colony is low (<2). However, no worker reproduction has been observed in queen-right colonies of the Japanese species, *D. saxonica nipponica* with smaller colony size (J. Takahashi et al., unpublished data).

V. crabro is widely distributed in Eurasia (Matsuura and Yamane 1990; Archer 1992) and consists of six subspecies, which are distinguished mainly based on coloration (Archer 1992). The Japanese subspecies *V. crabro flavofasciata* has the largest colony size (c. 1,500–4,500 cells) among the six subspecies, while the subspecies *V. crabro gribodi* in Britain has the smallest colony size (c. 600–3,300 cells; Matsuura and Yamane 1990; Archer 1993). These facts lead to the hypothesis that workers of the Japanese *V. crabro flavofasciata* are likely to reproduce when genetic relatedness among them is high and when colony productivity is not influenced by worker reproduction. This study primarily aims to test this hypothesis.

Recent developments in DNA microsatellite techniques enable the estimation of mating frequency, kin structure, and worker reproduction in social Hymenoptera. In Vespinae including *Vespa*, *Provespa*, *Dolichovespula*, and *Vespula*, genetic markers have been developed (Thorén 1998; Daly et al. 2002; Hasegawa and Takahashi 2002), and the kin structure and worker reproduction have been reported for 11 species (Ross 1986; Thorén 1998; Foster et al. 1999, 2001; Foster and Ratnieks 2000, 2001; Goodisman et al. 2002; Takahashi et al. 2002). Using microsatellite DNA analysis, Foster et al. (1999) revealed that queens of *V. crabro gribodi* mate with a mean of 1.11 males. Although this result suggests a potential conflict between queens and workers over male production, in their later report Foster et al. (2000) found that workers of *V. crabro gribodi* do not produce males in queen-right colonies. To elucidate the effects of relatedness among workers and colony size on worker reproduction in queen-right colonies, we determined kin structure and colony male production in *V. crabro flavofasciata* with many large colonies by using microsatellite DNA analysis and compared the results with those obtained in *V. crabro gribodi* with small colonies. This is the

first comparative study of queen mating frequency and colony male production between subspecies with different colony sizes.

Materials and methods

Sample collection

Twenty colonies of *V. crabro flavofasciata* were collected in Kurohime, Nagano Prefecture and in Myokou, Arai, and Joetsu, Niigata Prefecture, from August to November 1999 by stuffing the nest entrance and infusing about 30 ml of diethyl ether into the nest. All individuals from each colony were brought to the laboratory where the absence or presence of the foundress queen was determined. For microsatellite DNA analysis, 20 pupal workers from each colony and 20 males from each of the 13 colonies in which males were present were collected and preserved in 99% ethanol before they were stored at -20°C . All adult workers from each colony 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 μl of 5% Chelex (Bio-rad) resin at 95°C for 10 min. All polymerase chain reactions (PCRs) were performed in a total volume of 10 μl containing 1.0 μl (about 10 ng) of template DNA, 0.2 μM of primer, 400 μM of dNTP mix, 1.0 μl of $10\times$ reaction buffer, 1.5 mM MgCl_2 , and 0.05 units Taq polymerase (Takara). All PCRs 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 $52\text{--}58^{\circ}\text{C}$, and 30 s at 72°C (Hasegawa and Takahashi 2002). The PCR products were analyzed with 6% polyacrylamide sequence gels by a manual sequencer (FMC) and then visualized by silver stain.

Data analysis

We estimated the regression relatedness (b), inbreeding coefficients (F), and allele frequencies using the Relatedness 4.2 computer program (Goodnight and Queller 1994). Colonies were equally weighted, and standard errors and t -tests were calculated by jackknifing over colonies (Queller and Goodnight 1989). Pedigree relatedness (g_{ww}) for each colony was inferred from worker genotypes over the four loci (Boomsma and Ratnieks 1996):

$$g_{\text{ww}} = 0.25 + 0.5 \sum p_i^2 \quad (1)$$

where $\sum p_i$ is the average of the sums for the squared proportional i th fathering males. The effective mating fre-

quency (M_e) for a queen was calculated according to Starr (1984):

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

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 error (Boomsma and Ratnieks 1996): sampling and detection errors. The probability of nonsampling of a specific paternal genotype becomes higher as the number of workers analyzed becomes smaller. We assume that estimates of intracolony paternity will be kept at acceptable levels by genotyping 20 workers from each colony. For the 20 workers analyzed, the probability of nonsampling of such a rare male is negligibly small. Rare males have a small effect on effective mating frequency, and nonsampling error was calculated according to Foster et al. (2001). We used the procedure of Foster et al. (1999) to estimate the probability of nondetection of worker's sons. The emergence probability of male offspring of workers was calculated according to the method of Foster et al. (2001). The male offspring of workers can be detected because 50% of the alleles are transmitted from workers. For unlinked loci (one to four loci per colony), the total number of assignable males in a sample can be estimated from the formula of Foster et al. (2001).

Dissection of workers' ovaries

For the 20 colonies, up to 70% of the workers in each colony were dissected and their ovarian development was quantified. Developmental stages of ovaries were scored for a total of 2,990 adult workers using Yamane's index (1974).

Table 1. Number of alleles, allele frequency, and expected heterozygosity (H_E) of four microsatellite loci for *Vespa crabro*

Locus	Number	Allele frequency					H_E
		a	b	c	d	e	
VMA-1	5	0.122	0.256	0.201	0.278	0.143	0.70
VMA-3	4	0.322	0.286	0.287	0.105		0.63
VMA-9	4	0.199	0.350	0.149	0.302		0.67
VMA-12	3	0.302	0.248	0.450			0.60
Mean	4						0.63

Table 2. Mating frequency, genetic relatedness among workers, and worker male production in two subspecies of *V. crabro*

Species	Effective mating frequency	Range of mating frequency	Regression relatedness	Laying workers	Workers' sons	Reference
				(%)	(%)	
<i>V. crabro flavofasciata</i>	1.13	1–3	0.73	1.1	0	Present study
<i>V. crabro gribodi</i>	1.11	1–3	0.69	1.0	0	Foster et al. 1999, 2000

Results

Variation at microsatellite loci for *V. crabro flavofasciata*

High allelic variation was observed in the 20 colonies (Table 1). The VMA-1, VMA-3, VMA-9, and VMA-12 microsatellite loci had allelic numbers of 5, 4, 3, and 4, respectively, and the mean expected heterozygosity for all loci was 0.63.

Mating structure

Of the 20 colonies examined, 15 had queens inseminated by a single male, 3 colonies had queens inseminated by two males, and 2 colonies had queens inseminated by three males (Fig. 1). In the multiply mated colonies, the fathering male ratio was 17:3, 15:5, and 16:4 for two-paternity colonies, and 15:3:2 and 16:3:1 for three-paternity colonies. The mating structure of *V. crabro flavofasciata* is characterized by the fact that each queen mates with one to three males with sperm utilization being biased to one male in the multiple-paternity colonies. The major paternity of these colonies was not significantly different from the estimates for worker genotypes (Table 2). The inbreeding coefficient was, over the four loci, significantly different from zero ($F = 0.0187$), which is expected for a randomly mating system. The nondetection error for colony levels (d_c) ranged from 0.0010 to 0.0012, and that for a population level (d_p) was very low at 0.001 over the 20 colonies (Table 2). Therefore, rare paternity failed to be detected just at 0.1%, suggesting no effect on our results.

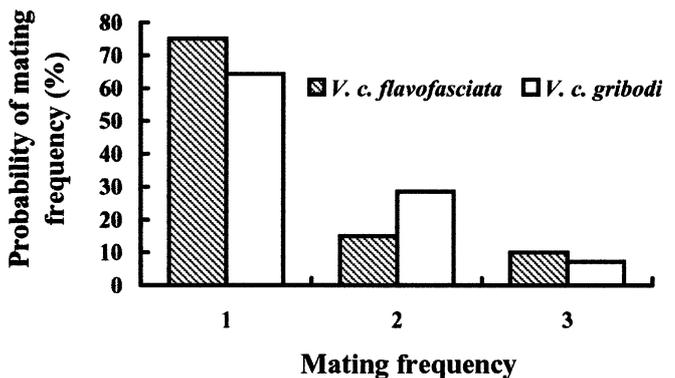


Fig. 1. The mating frequency of two subspecies of *Vespa crabro*. The data on *V. crabro gribodi* are from Foster et al. (1999, 2000)

Genetic relatedness among workers

The regression coefficient for relatedness among nestmate workers was 0.724 ± 0.0029 (mean \pm SE) when averaged over the 20 colonies. On the other hand, the pedigree relatedness among nestmate workers was 0.708, agreeing well with the regression coefficient for relatedness (Table 2). The result suggests (a bias on paternity frequency) that paternity is dominated by a certain male in the multiple-paternity colonies.

Worker oviposition

Of the 2,990 dissected adult workers from 20 colonies, a total of 33 workers from two queen-right colonies had developed ovaries (Table 2). However, all males had alleles peculiar to the genotypes of the queens at all loci. Therefore, even though workers had developed ovaries, all the males were clearly the offspring of queens, indicating that none of the workers were able to reproduce successfully. The number of assignable males (N_a) was 231, which means that if worker oviposition contributes to the production of more than 1% of the males in a colony, the probability of failing to detect worker oviposition is lower than 9.81%. Workers with developed ovaries were found in two colonies in which the queens were doubly mated. Therefore, the presence of ovipositing workers was not correlated with genetic relatedness among workers.

Discussion

This study shows that *V. crabro flavofasciata* queens characteristically mate with only one male and that colonies are headed by a single queen. Similarly, Foster et al. (1999) reported that *V. crabro gribodi* queens mate once to three times with a mode of single paternity. Effective mating frequency in *V. crabro flavofasciata* (1.13) is lower than the observed mating frequency (1.35) because of sperm dominance, up to 77%, by one male in colonies headed by multiply mated queens. The mean estimate for the effective mating frequency of *V. crabro gribodi* is 1.11 (Foster et al. 1999), which does not differ significantly from that in *V. crabro flavofasciata*. Similarly, the proportion of multiply mated queens does not differ between the two subspecies. Furthermore, the regression coefficient of relatedness among workers is estimated to be 0.724 for *V. crabro flavofasciata* and 0.69 for *V. crabro gribodi* (Foster et al. 1999).

Haplo-diploid sex determination is typical in the Hymenoptera (ants, bees, wasps, and sawflies); diploid males often arise from inbreeding through complementary sex determination (CSD). The diploid male is sterile and has a fitness cost at an individual colony level (Cook and Crozier 1995). In 1 of 13 colonies of *V. crabro gribodi* in Britain, 10 males were found to be diploid (Foster et al. 2000). However, we found no diploid males in the 260 *V. crabro flavofasciata* males examined in this study. Our

results indicate that the estimate of inbreeding coefficient (F) from 20 colonies is not significantly different from zero. This low level of inbreeding could explain the lack of diploid males in *V. crabro flavofasciata*.

These results show that the founder population of *V. crabro flavofasciata* generally consists of monogynous, singly mated queens that breed randomly. Therefore, the kin selection theory predicts that a conflict should arise between queens and workers over male production (Ratnieks 1988); workers will gain genetic benefits through producing sons. This theory further hypothesizes that worker reproduction should be hindered by worker policing if the coefficient of relatedness among workers is less than 0.25 (Ratnieks 1988). However, genotypic analysis of the 260 males from 13 colonies revealed that all the males were derived from the queens and not from workers. This was corroborated by the fact that only 33 of the 2,990 workers exhibited ovarian development. Therefore, we found no positive correlation between genetic relatedness of workers and worker reproduction. Furthermore, the difference in colony size between the two subspecies had no effect on worker reproduction. The low level of worker reproduction in *V. crabro flavofasciata* is contradictory to our prediction based on colony size and relatedness among workers. In the case of eusocial insects, Kikuta and Tsuji (1999) first reported that workers of the monogynous and monandrous ant *Diacamma* sp. selectively eliminated eggs produced by queen and other workers, showing that worker policing could evolve even in monogynous, singly mated eusocial Hymenoptera. To explain this phenomenon, Kikuta and Tsuji (1999) suggested that worker policing evolves despite a high kin structure if worker reproduction reduces colony productivity. Contrary to our expectations, a high genetic relatedness among workers and large colony size in *V. crabro flavofasciata* were not responsible for worker reproduction. Therefore, we conclude that the lack of worker reproduction in *V. crabro* can be explained by the same mechanism as in *Diacamma* sp.; that is, the cost to colony productivity may outweigh the genetic benefit from producing worker's sons. To clarify the relationship between colony size, relatedness of workers, and worker reproduction, it is necessary to study species with a larger variation in colony size than that found in *V. crabro*. Vespine wasps are greatly different from other social insects (ants, bees, and other wasps) in having a large intraspecific variation in colony size (Matsuura and Yamane 1990; Takahashi et al. 2002). Therefore, the Vespinae includes species that are suitable for verifying costs and benefits of worker reproduction and policing.

Foster et al. (2002) observed that *V. crabro* workers could discriminate eggs laid by workers from eggs laid by the queen and eliminated worker eggs in queen-right colonies. These results suggest that male production in colonies of *V. crabro flavofasciata* is also dominated by the queen via worker policing and that worker oviposition occurs only when a colony becomes queenless.

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