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Kevin R. Foster · Perttu Seppä · Francis L.W. Ratnieks
Peter A. Thorén

Low paternity in the hornet *Vespa crabro* indicates that multiple mating by queens is derived in vespine wasps

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Abstract Queen mating frequency was studied in the European hornet, *Vespa crabro*, by analyzing four DNA microsatellite loci in 20 workers from each of 14 nests. Queens were found to be predominantly singly mated (9/14), although double (4/14) and triple mating (1/14) also occurred. For most multiply mated queens, paternity was significantly biased with the majority male fathering on average 80% of the female offspring. The population-wide effective mating frequency was therefore low (1.11), and sister-sister relatedness high (0.701 ± 0.023 SE). Low effective mating frequency in *Vespa*, in combination with data from other vespines, suggests that high paternity frequency is derived in the group. Some problems with the non-detection of fathers, where the queen was not sampled or shared alleles with males, are analyzed.

Key words Paternity · DNA microsatellites · *Vespa* · Relatedness · Non-detection

Introduction

The family structure of animal societies is central to their social behavior (Hamilton 1964; Crozier and Pamilo 1996), particularly patterns of reproductive cooperation and conflict (Trivers and Hare 1976; Pamilo 1991a, 1991b; Ratnieks and Reeve 1992). This tenet, formally kin selection theory (Maynard Smith 1964), has been used to make behavioral predictions in a wide variety of animal taxa (e.g. Emlen and Wrege 1988; Packer et al. 1991; Grosberg et al. 1996) including social insects (Crozier and Pamilo 1996).

In single-queen eusocial Hymenoptera, queen mating frequency, specifically the number of males contributing to paternity (Boomsma and Ratnieks 1996), is the key determinant of family structure. Increased paternity frequency is predicted to reduce potential queen-worker conflict over sex allocation (Trivers and Hare 1976; Benford 1978), male production (Ratnieks 1988), and queen killing by workers (Bourke 1994), but to cause potential nepotistic conflict among workers of different patrilines (Getz 1981; Ratnieks and Reeve 1992; Bourke and Franks 1995; Crozier and Pamilo 1996 review the theory). Determination of queen mating frequency is, therefore, pivotal in the study of reproductive behavior.

Although numerous studies of paternity in eusocial Hymenoptera have been made (reviewed by Page 1986; Boomsma and Ratnieks 1996; Crozier and Pamilo 1996), more data are needed. This is partly to take advantage of new techniques, particularly DNA microsatellites, which provide greater power in detecting paternity than was typically available with protein allozymes (Queller et al. 1993). In addition, more data are needed to characterize paternity adequately in the highly diverse social Hymenoptera (Boomsma and Ratnieks 1996). One specific need is for concentrations of data in specific taxa to give a better picture of the relationship between paternity frequency and reproductive behavior in a phylogenetic context.

The vespine wasps are a taxon in which paternity data should be particularly informative (Ratnieks 1988; Boomsma and Ratnieks 1996). They have a well-supported phylogeny (Carpenter 1987) which, combined with the relatively small size of the group [*Vespa* (23 species), *Provespa* (3 species), *Dolichovespula* (13 species), and *Vespula* (22 species)], should make it possible to produce a largely complete evolutionary tree of paternity data to relate this to colony reproductive characteristics. The basic biology of the Vespinae is similar, typically with an annual monogynous paper nest, a morphologically distinct queen caste and workers

K.R. Foster (✉) · F.L.W. Ratnieks
Department of Animal and Plant Sciences, University of Sheffield
Western Bank, Sheffield, S10 2TN, UK
e-mail: bop97krf@shef.ac.uk, Fax: +44-114-2220002

P. Seppä · P.A. Thorén
Department of Conservation Biology and Genetics
Uppsala University, Box 7003
S-75007 Uppsala, Sweden

that are unable to mate (Ross and Matthews 1991). However, variation in queen mating frequency between species has been recorded (Ross 1986; Thorén et al. 1995; Thorén 1998; F.L.W. Ratnieks and J.J. Boomsma, unpublished data). In addition, differences in key aspects of reproductive behavior have been observed, including variation in the incidence of worker laying and associated queen-worker aggression (Greene et al. 1976; Reed and Akre 1983; Ross 1986), split sex ratios (Greene et al. 1976; F.L.W. Ratnieks and J.J. Boomsma, unpublished data), matricide (Ishay 1964), and occurrence of a queen pheromone (Ikan et al. 1969). This variation in mating frequency and conflict behavior among species with otherwise similar life history traits makes comparative study of the Vespinae particularly interesting.

Hornets, *Vespa*, are an important vespine genus both in terms of the number of species and as the most basal member of the taxon (Carpenter 1987). However, there are no studies of paternity frequency. Therefore, we performed a DNA microsatellite study on the most widely distributed member of the genus, *Vespa crabro*. The results show clearly that paternity is low in *V. crabro*.

Methods

Study organism and sample collection

V. crabro was collected during July and August 1997 from nests located in the New Forest, Hampshire, England, where it is relatively abundant (Nixon 1982). *V. crabro* is the only hornet found in Britain and northern Europe. Nests were collected from an area of approximately 20 × 20 km. The entire nest was collected in four cases. For the other nests, only a sample of workers was collected. Collection of the whole nest was rarely possible due to the habit of *V. crabro* of nesting in cavities, such as hollow trees. In addition, the New Forest is a nature reserve, so collecting large numbers of nests was avoided for conservation reasons. Entire nests were only removed when a nest had to be destroyed because its location caused significant human disturbance.

Molecular methods

Nineteen DNA microsatellite loci previously designed for *Vespula rufa* (Thorén et al. 1995) were screened for use in *V. crabro*. Four variable loci amplified reliably (Rufa 5, 13, 15, 18) and a further two (Rufa 2 and 6) less well. Genetic variation at the four reliable loci was studied in 20 workers from each of 14 nests. Additionally, the queen was analyzed in the four nests that were collected. DNA extraction and PCR used standard methods (see Thorén et al. 1995). A 'touchdown' PCR was carried out from 56 to 46 °C (Don et al. 1991), using ^{33}P-α-dATP in internal labeling. PCR products were separated in 6% polyacrylamide sequencing gels, and visualized by autoradiography.

Statistical methods

Worker regression relatedness (b), inbreeding (F), and allele frequencies were estimated from the worker genotype frequency data following Queller and Goodnight (1989), using the Relatedness 4.2 program (Goodnight and Queller 1994). The program calculates standard error estimates for b and F by jackknifing across nests.

Pedigree estimates

Direct estimates of paternity frequency were made by inspecting worker genotypes across the four loci for each nest. This was used to produce a second estimate of relatedness (r) assuming outbreeding and to estimate sperm bias when multiple paternity occurred. The population effective mating frequency (M_e) was estimated after Starr (1984)

$$M_e = \frac{n}{\Sigma(\Sigma(p_i^2))_j} \quad (1)$$

where p_i is the proportional contribution of the ith male in the jth nest for n nests.

Non-sampling error

The probability of not sampling female offspring from a father who actually has paternity was kept to acceptable levels by the analysis of 20 workers from each nest. When 20 workers are sampled, a male fathering a proportion p of the offspring will be sampled with probability $1 - (1- p)^{20}$. For example, the non-sampling probabilities of 50% and 10% paternity are 9.5×10^{-7} and 0.12 for 20 offspring, respectively. For males with very low paternity contributions, the non-sampling probability is significant. Such males, however, have a small effect on effective mating frequency (Eq. 1) so that not sampling them introduces only a small error in estimates of relatedness.

Non-detection error

Estimates were calculated at both the population (d_p) and nest (d_n) level. The probability of two males in a Hardy-Weinberg population having identical genotypes at all loci studied and thus having indistinguishable offspring is:

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

where q_i denotes the allele frequencies at each of j loci (Boomsma and Ratnieks 1996). However, such an estimate assumes that paternally and maternally transmitted alleles can be distinguished. In our data set this was not always possible because the queen genotype was not always known. As a result Eq. 2 can underestimate the non-detection error. Nest-level estimates of non-detection error allow this problem to be corrected for and additionally reveal the magnitude of non-detection error per nest. Three corrections were required to account for ambiguity in paternal genotype, each corresponding to a particular situation (Appendix).

Results

Allelic diversity

Genetic variation at the four microsatellite loci studied was moderate, with three to six alleles per locus and a mean expected heterozygosity across all loci of 0.60 (Table 1).

Estimates of relatedness

Worker nestmates were related by $b = 0.749 \pm 0.035$ (SE), exactly that expected from full sisters. However, high relatedness is partly caused by a low degree of inbreeding: $F = 0.087 \pm 0.052$. By adjusting for

Table 1 Genetic variation in the microsatellite marker loci studied, where n is the number of alleles detected and H_e the heterozygosity at each locus

Locus	n	Allele frequencies	H_e
5	5	0.318, 0.305, 0.265, 0.102, 0.011	0.73
13	3	0.509, 0.455, 0.036	0.53
15	6	0.5, 0.186, 0.161, 0.116, 0.036, 0.002	0.67
18	4	0.7, 0.128, 0.095, 0.076	0.48
Mean	4.5		0.60

inbreeding (Pamilo 1984, 1985) worker relatedness drops to 0.702 (b*).

Pedigree estimates

Genotype inspection revealed single paternity in 9 nests, double paternity in 4 nests, and triple paternity in 1 nest (Table 2). Single maternity gave the most parsimonious solution for all genotype arrays and in the four nests collected only a single queen was present. In the 5 multiple-paternity nests, the majority male fathered 54%, 70%, 85%, 95%, and 95% of the brood, with the latter 4 significantly different from equality (binomial probability < 0.05). The population effective mating frequency (Eq. 1) was 1.11. Pedigree worker relatedness (r) was 0.701 $\pm$ 0.023. This, as expected, agrees with the inbreeding-adjusted regression estimate (b*).

Statistical power of analysis

Using Eq. 2 and the allele frequencies observed, the population estimate of non-detection error was 0.02 suggesting that a second father is not detected only 2% of the time. The mean from the more conservative nest-based estimates was greater at 7 $\pm$ 2% (SD). Importantly, the non-detection error is still low. Altogether 20 males were detected and the expected number of males not detected in all 14 nests was 1.0 (Σd_{en}). Thus the combination of sampling and non-detection error may lead to a slight underestimate of M_e but the effect is relatively minor and does not qualitatively change the conclusion that the effective paternity is close to one.

Table 2 Nest-level data on paternity, paternity bias, pedigree sister-sister relatedness (r), and non-detection error. Non-detection error estimates include the corrections detailed in the Appendix (* indicates that the queen was analyzed and so corrections 2a and 2b (Appendix) were not required)

Nest number	Number of fathers detected	Percent contribution of majority males	Sister-sister relatedness	Non-detection error (d_n)
2	1	–	0.75	0.12
3	1	–	0.75	0.04
6	2	70	0.54	0.08
7	1	–	0.75	0.05
8	2	95	0.70	0.11
12	2	95	0.70	0.07
14	2	55	0.50	0.23
15*	1	–	0.75	0.01
17*	3	85, 10	0.62	0.01
18*	1	–	0.75	0.004
19*	1	–	0.75	0.05
21	1	–	0.75	0.06
22	1	–	0.75	0.13
23	1	–	0.75	0.01

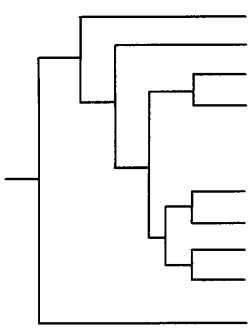
Discussion

Behavioral studies on *V. crabro* have consistently shown monogyny (Matsuura and Yamane 1990). However, nest usurpation by 'piratical' queens is reported (Nixon 1983) leading to the possibility of offspring from multiple queens in one nest. Multiple matrilines were not apparent in the workers analyzed suggesting, as expected, that successful nest takeovers are rare (Nixon 1983, 1986) or that usurpation occurs early in the season so that any daughters of a first queen were dead at the time of sampling.

The majority of *V. crabro* nests analyzed revealed single paternity. Multiple mating occurred in 5 of 14 nests although the effective mating frequency remained low at 1.11. This is because of biased paternity with the majority male on average having 80% of paternity. However, the paternity of the majority male varied considerably, being nearly equal to that of the other male in one nest, but significantly biased in the other four.

With small non-sampling and non-detection errors, the data provided by this study give a reliable estimate of paternity frequency in *V. crabro*. In a study on *V. crabro* pheromones, Batra (1980) observed multiple copulations of queens. However, the exact copulation frequency was not recorded. Matsuura and Yamane (1990) report that queens of the Japanese hornet *Vespa mandarinia* rarely copulate more than once. These observational data are consistent with the results of this study, although observations of copulation are often an unreliable predictor of paternity (Boomsma and Ratnieks 1996). *V. crabro* fits into the single-to-multiple (s–m) category for paternity proposed by Boomsma and Ratnieks (1996), although the paternity bias means that the offspring relatedness of 0.7 is higher than expected for this category (0.6–0.65). In ants, single-to-multiple represents a moderately frequent paternity category accounting for 3 out of the 19 species reviewed (Boomsma and Ratnieks 1996).

Paternity frequency data are now available for the three most diverse genera of the Vespinae, *Vespa*, *Dolichovespula*, and *Vespula,* including three of the species groups in *Vespula*. In combination with Carpenter's (1987) phylogeny, this allows some inferences to be made on the evolution of paternity frequency in the group. High effective mating frequencies (M_e) have been shown in all three *Vespula* species groups for which there are data (Ross 1986; Thorén et al. 1995).



		Effective paternity	Paternity range	No. broods analyzed	Reference
Vespa	*crabro*	**1.11**	1-3	14	this study
Provespa	no data				
D. maculata grp	*media*	**1**	1	3	Thorén 1998
D. norwegica grp	*saxonica*	**4.81**	4-11	7	Thorén 1998
	arenaria	**1.09**	1-3	20	Ratnieks and Boomsma, unpublished data
V. vulgaris grp	*maculifrons*	**7.14**	-	30	Ross 1986
V. koreensis grp	no data				
V. rufa grp	*rufa*	**-**	⩾2	1	Thorén et al. 1995
V. squamosa grp	*squamosa*	**3.33**	-	17	Ross 1986
Polistinae					
Polistes	*bellicosus*	**1**	1	18	Field et al. 1998
	chinensis	**1.26**	1-2	20	Miyano and Hasegawa 1998
	jadwigae	**1.04**	1-2	67	Tsuchida 1994
	annularis	**≈1***	1-2	9	Peters et al. 1995

Fig. 1 Phylogeny of paternity frequency in the Vespinae. Data from *Polistes* are shown for outgroup comparison. The phylogeny follows Carpenter (1987) and recognizes his species groups (*grp*) within *Dolichovespula* and *Vespula* (* based on sperm data)

Dolichovespula shows a mixed pattern with paternity close to one in two species (*Dolichovespula arenaria*: F.L.W. Ratnieks and J.J. Boomsma, unpublished data, *D. media*: Thorén 1998) but high paternity in *D. saxonica* (Thorén 1998). With a low mating frequency in *Vespa*, parsimony suggests that high paternity is a derived, and hence recent, character in the Vespinae (Fig. 1). This prediction is supported by outgroup comparison with the Polistinae where studies consistently show paternity frequencies near one (Tsuchida 1994; Peters et al. 1995; Goodnight et al. 1996; Field et al. 1998; Miyano and Hasegawa 1998). The data suggest multiple origins to high paternity within the Vespinae. A single origin is only possible if *Dolichovespula* is polyphyletic, which seems unlikely as 12 autapomorphies unite the genus (Carpenter 1987). Using Carpenter's phylogeny, the most parsimonious solution is of two origins to high paternity, one at the base of *Vespula* and one within the *D. norwegica* group.

Several aspects of reproduction in social Hymenoptera depend on paternity frequency. Low paternity frequency should lead to queen-worker conflict over worker laying (Ratnieks 1988), sex allocation (Trivers and Hare 1976), and queen killing by workers in annual nests (Ratnieks 1988; Bourke 1994). The phylogeny (Fig. 1) suggests that these conflicts are ancestral in the Vespinae with multiple mating leading to decreased queen-worker conflict and increased social coherence in the more derived *Vespula*. Behavioral data seem to support this trend with the occurrence of queen killing in *Vespa* (Ishay 1964) and worker reproduction in *D. arenaria* (Greene et al. 1976; F.L.W. Ratnieks and J.J. Boomsma, unpublished data) in contrast with the *Vespula squamosa* and *vulgaris* groups where worker reproduction (Ross 1986) and matricide (Akre et al. 1976) have not been observed. High paternity in *D. saxonica* and the observation of worker reproduction in *V. consobrina* (*V. rufa* group, Akre et al. 1982) are potential exceptions since these are atypical of their respective genera. Further data would be valuable, particularly on worker reproduction in *D. saxonica* and paternity frequency in *D. consobrina* and other species in the *V. rufa* group. Currently the only data for this group is from eight workers from a single colony (Thorén et al. 1995).

The Vespinae give the clearest indication to date that high paternity is a derived trait associated with low intracolony conflict. This pattern seems to extend to the eusocial Hymenoptera in general with the highest paternity for bees found in the honeybee *Apis* (Estoup et al. 1994), and ants in the leafcutter, *Acromyrmex* (Boomsma et al. 1998), both well known for their highly derived eusociality.

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Appendix

Nest-level non-detection error estimates

The genotypes of the father males for each nest provide estimates of non-detection error at the nest level (Pamilo 1982). For every male there is a specific probability that a randomly selected second male has the same genotype across all loci. This is simply the product of the frequencies of the alleles possessed by the focal male. For a haploid male with allele frequencies i, j, k, l at four loci, the probability that a second male would possess the same genotype d_n is:

$$d_n = i \times j \times k \times l \quad (3)$$

Corrections for ambiguity in identification of paternal alleles

Equation 3 assumes that the paternal alleles can always be identified in daughter progeny and that the only source of ambiguity in assignment of paternity is when two males share the same multilocus genotype. For the data set analyzed, three additional sources of ambiguity arose.

Correction 1: a known heterozygote queen and male share an allele

In this situation, non-detection can occur two ways: (1) the second male has the same allele as the first male (basic non-detection) or (2) the second male has the queen's unique allele (Table 3). Pamilo (1982) corrected for this in estimating non-sampling and non-detection error for a single biallelic locus. Only non-detection is considered here to allow application to multilocus, multiallele systems, which would otherwise be difficult (Boomsma and Ratnieks 1996). The probability of non-detection (d_1) at an affected locus is:

$$d_1 = \underbrace{a}_{\text{basic non-detection}} + \underbrace{b(0.5)^f}_{\text{queen-masking effect}} \quad (4)$$

where a is the frequency of the first male's allele (A), corresponding to i, j, k, or l from Eq. 3, and b is the frequency of the queen allele not shared with the first male (B). Queen-masking also requires that the queen donates only her A allele. The probability of this is 0.5 for each offspring, hence the term $(0.5)^f$ where f is the number of offspring of a B male sampled. For this study, f was set to 1, corresponding to the probability that a B male with 5% (1/20) paternity is not detected. d_1 is used in place of i, j, k, or l in Eqs. 3 or 5 for any affected loci.

Correction 2: queen genotype unknown

For 10 of 14 nests, the queen genotype could only be inferred from female offspring genotypes, resulting in two novel problems in detecting a second male.

(a) With two worker genotypes present at a locus, e.g., AC, BC, there are two possible parental combinations that produce the observed pattern, one reflecting single paternity, e.g., AB × C, and the other double paternity. e.g., CC × A, B [Table 4(a)]. It is possible, therefore, that two males with different genotypes have mated with the queen but still remain unresolvable, contrary to the assumptions of Eq. 3. This reduces the number of useful detecting loci by one, as any such locus is uninformative. Detection requires a second locus at which the males differ [Table 4(b)]. If one such locus occurs in a worker sample, the non-detection error must be calculated without it. Several affected loci can be accounted for by calculating the non-detection error discounting one locus at a time, and taking the mean. For three affected loci in a four-locus analysis:

Table 3 Possible paternal genotypes resulting in non-detection when the colony queen is heterozygous AB and shares an allele with the first male detected A. The first example is the basic non-detection scenario. In the second example, non-detection occurs when the queen transmits her A allele as only BB progeny reveal the second male

Queen genotype	Male genotypes	Male 1 progeny	Male 2 progeny
AB	A, A	AA, AB	AA, AB
AB	A, B	AA, AB	*BB*, AB

Table 4 (*a*) Offspring genotype combination that could reflect single or double paternity. Possible parental genotypes: queen BC, male A (single mating) or queen AA, males B and C (double mating). (*b*) Resolution of a double mating, if present, is only possible if males have different alleles at an additional locus. Detection then occurs through the correlation of worker genotypes across loci (B with E, C with F) indicating the non-recombinant genotypes of haploid males

Offspring	(a)	(b)
1	AB	DE
2	AC	DF
3	AC	DF
4	AB	DE
5	AB	DE
6	AC	DF
7	AB	DE
8	AC	DF
9	AC	DF
10	AB	DE

$$d_n = \frac{(ij + ik + jk)}{3} l \quad (5)$$

with i, j, and k as the allele frequencies of the male allele at the affected loci assuming single mating. This compares to a non-detection error of $ijkl$ without the correction (Eq. 3). Equation 5 assumes an equal probability that each locus will 'detect' a double mating, which in reality is dependent on allele frequency. However, this effect is difficult to quantify, as the probability that any one locus detects double mating interacts with the probability of double mating in the population, which for the purposes of error estimates is an unknown. Assuming that each affected locus detects a double mating with equal probability in general produces the most conservative estimate. Accounting for allele frequency would cause the loci with the most effect on non-detection to be discounted least, lowering the non-detection estimate. The exception to this occurs if applying Eq. 4 reorders the magnitudes of non-detection (i, j, k, l) at the loci. This is in practice rare and the resulting estimate is still conservative relative to Eq. 3.

(b) If all workers are heterozygous at a locus, it is unknown which allele is paternally and which is maternally derived. This can be accounted for by weighting the two alternative estimates of non-detection error by the relative probability that each allele is paternally derived. For worker genotypes AB of allele frequencies a and b, the probability that the queen is AA and male is B is a^2b versus b^2a for queen BB and male A. The *relative* probability that allele B is male derived is then $a^2b/(a^2b + b^2a)$ and so the probability of non-detection at an affected locus (d_2) equals:

$$d_2 = \frac{a^2b}{a^2b + b^2a} b + \frac{b^2a}{a^2b + b^2a} a = \frac{2ab}{a + b} \quad (6)$$

where, as for d_1, d_2 is used in place of simple allele frequency (i, j, k, or l) in Eqs. 3 or 5.

Non-detection error for each nest was calculated by classifying ambiguities at all affected loci and applying these corrections. In the polyandrous queen nests, corrections 1 and 2a were required. The latter is only required when the suspected queen alleles [B and C in Table 4(a)] are found in only one patriline, as the queen genotype remains ambiguous. This is likely with high paternity bias.

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