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Mating frequency and mating system of the polygynous ant, *Leptothorax acervorum*

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Abstract

Multiple mating by queens (polyandry) and the occurrence of multiple queens in the same colony (polygyny) alter patterns of relatedness within societies of eusocial insects. This is predicted to influence kin-selected conflicts over reproduction. We investigated the mating system of a facultatively polygynous UK population of the ant *Leptothorax acervorum* using up to six microsatellite loci. We estimated mating frequency by genotyping 79 dealate (colony) queens and the contents of their sperm receptacles and by detailed genetic analysis of 11 monogynous (single-queen) and nine polygynous colonies. Results indicated that 95% of queens were singly mated and 5% of queens were doubly mated. The corrected population mean mating frequency was 1.06. Parentage analysis of adults and brood in 17 colonies (10 monogynous, 7 polygynous) showed that female offspring attributable to each of 31 queens were full sisters, confirming that queens typically mate once. Inbreeding coefficients, queen–mate relatedness of zero and the low incidence of diploid males provided evidence that *L. acervorum* sexuals mate entirely or almost entirely at random. Males mated to queens in the same polygynous colony were not related to one another. Our data also confirmed that polygynous colonies contain queens that are related on average and that their workers had a mixed maternity. We conclude that the mating system of *L. acervorum* involves queens that mate near nests with unrelated males and then seek readoption by those nests, and queens that mate in mating aggregations away from nests, also with unrelated males.

Keywords: Hymenoptera, mating system, microsatellite, polygyny, relatedness, social insect

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Introduction

Studies of eusocial Hymenoptera provide some of the most powerful tests of Hamilton's (1964) kin selection theory (Bourke & Franks 1995; Crozier & Pamilo 1996; Queller & Strassmann 1998; Chapuisat & Keller 1999). Accurately testing the theory requires a detailed knowledge of species' mating systems and the genetic structure of their colonies (e.g. Ross 2001). In particular, information is required on the mating frequency of queens and the relatedness of coexisting queens, because variation in both these traits affects variation in relatedness among colony members (Pamilo 1991a,b). The determination of queen mating frequency and colony genetic structure has been greatly assisted by the development of highly variable, polymerase chain reaction (PCR)-based, molecular markers such as microsatellites (Pamilo *et al*. 1997; Queller & Strassmann 1998; Ross 2001). Microsatellites have enabled fine-scale analysis of parentage and relatedness of individuals within colonies (e.g. Bourke *et al*. 1997; Chapuisat *et al*. 1997; Evans 1998; Herbers & Mouser 1998; Giraud *et al*. 2000) and the analysis of mating frequency by direct genotyping of sperm stored by queens after mating (e.g. Gertsch & Fjerdingstad 1997; Chapuisat 1998; Krieger & Keller 2000; Sanetra & Crozier 2001; Tay & Crozier 2001).

Leptothorax acervorum is a facultatively polygynous ant species, that is, one whose populations contain colonies headed by a single queen (monogyny) or multiple queens (Buschinger 1968; Chan & Bourke 1994; Heinze *et al*. 1995b). Previous genetic studies, employing molecular markers such as allozymes, mitochondrial DNA (mtDNA) or microsatellites, have shown that queens resident in

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polygynous colonies are typically related to one another (Douwes *et al*. 1987; Stille *et al*. 1991; Stille & Stille 1992; Heinze 1995; Heinze *et al*. 1995a,b; Bourke *et al*. 1997; Chan *et al*. 1999). High relatedness of female offspring in monogynous colonies (Seppä *et al*. 1995; Heinze *et al*. 1997) and in the offspring of queens isolated in the laboratory (Heinze *et al*. 1995b), and observations of female mating behaviour (Felke & Buschinger 1999), suggest that queens mate singly. Furthermore, inbreeding coefficients not significantly different from zero are consistent with random mating (Stille *et al*. 1991; Stille & Stille 1993; Seppä *et al*. 1995; Bourke *et al*. 1997; Heinze *et al*. 1995a,b, 2001).

Field observations suggest that at least some *L. acervorum* sexuals mate in large mating aggregations situated away from nests (e.g. Franks *et al*. 1991). It seems very unlikely that queens visiting these aggregations are capable of returning to their natal nests (Douwes *et al*. 1987; Bourke & Franks 1991; but see Foitzik & Herbers 2001). Therefore, combining the genetic and field data suggests that some newly eclosed queens mate with unrelated males near the natal nest and then return to it, where they are readopted (so giving rise to polygyny with multiple, related queens), whereas other queens disperse to mating aggregations, mate and then leave the aggregations to initiate new colonies elsewhere (e.g. Douwes *et al*. 1987; Franks *et al*. 1991; Bourke & Heinze 1994; Bourke & Franks 1995). Mating near the nest plausibly occurs in *L. acervorum* because in mating aggregations queens pheromonally 'call' for males from positions on the ground (Franks *et al*. 1991) and in a Spanish population identical to or very closely related to *L. acervorum* queens have been observed 'calling' next to laboratory nests, mating, then returning to the nest (Felke & Buschinger 1999).

However, much remains that is unknown about the mating system of *L. acervorum*. Specifically, mating frequency has not been investigated directly and current conclusions about the degree of inbreeding may be erroneous because F_{IS} statistics calculated using molecular markers such as allozymes, which typically exhibit low levels of variation, suffer from low power in detecting inbreeding (Cole & Wiernasz 1997; Ross 2001). To investigate the mating system of *L. acervorum* more rigorously, we genotyped mated, colony queens, their sperm receptacles (spermathecae) and samples of brood and adults from monogynous and polygynous colonies using up to six highly variable microsatellite loci. We also present new data on the sociogenetic structure of the two types of colony.

Materials and methods

Field collection and sampling

Colonies of *Leptothorax acervorum* were collected from a facultatively polygynous population in Santon Warren, Thetford Forest, Norfolk, UK on 13 and 20 June 1995 ('SD95', $n = 96$ colonies; Chan *et al*. 1999), 2 June 1996 ('SD96', $n = 24$ colonies) and on 3 and 10 June 1999 ('SD99', $n = 46$ colonies). For details of collection methods see Chan & Bourke (1994). Colonies were removed from twigs within 2–3 days of collection and transferred to artificial nests in the laboratory. SD95 and SD96 colonies were kept under standard rearing conditions until after sexuals (new queens and males) had emerged (Bourke 1991; Chan & Bourke 1994; Chan *et al*. 1999). SD99 colonies were frozen at −70 °C immediately after removal from their twigs.

Dealate queens (queens that have shed their wings and are potential colony queens) from a subset of colonies arbitrarily selected from the SD95 sample ($n = 15$ queens from seven polygynous colonies), the SD96 sample ($n = 52$ queens from 11 monogynous and 5 polygynous colonies) and the SD99 sample ($n = 119$ queens from 19 monogynous and 16 polygynous colonies) were dissected to assess their ovarian development and mating status (Bourke 1991) and to obtain paternal DNA from sperm stored in the spermatheca for the determination of mating frequency. Polygynous colonies were defined as those containing more than one mated, dealate queen. Some dealate queens were found to be nonreproductive virgins and therefore yielded no DNA from the spermatheca. In mated queens, because of the small size of the spermathecae (≈ 100 μm in diameter) we did not attempt to isolate sperm (Evans 1993; Gertsch & Fjerdingstad 1997), so making the contamination of sperm with queen-derived tissue a possibility. Regardless of this, we refer to DNA extracted from spermathecae as sperm DNA.

A subset of 20 colonies (one queenless, 10 monogynous and nine polygynous) from the SD99 sample was selected for more detailed genetic analysis (Table 1). The selected colonies were ones that produced sexuals and, in the case of polygynous colonies, that had between two and eight resident queens. Previous studies at Santon Warren showed that > 75% of polygynous colonies had queen numbers within this range (Chan *et al*. 1999). As all 20 colonies had been frozen whole immediately after removal from the nest, their social and genetic structure determined by genotyping was that at the time of collection from the field.

Molecular genetic methods

We extracted DNA from sperm and eggs by proteinase K digestion in 1× TE buffer (60 μL 1× TE; 5 μL of 20 mg/mL proteinase K). DNA from adults or pupae was extracted either by proteinase K digestion in 1× TE (300 μL 1× TE; 25 μL of 20 mg/mL proteinase K) or by using a commercially available kit (QIAmp Mini Kit™, Qiagen). For the TE/proteinase K method eggs, sperm, pupae and adult ants were digested with proteinase K at 70 °C for

Table 1 Summary of microsatellite genotyping from the 20 SD99 colonies whose sociogenetic structure was investigated in detail. All individuals were genotyped at six microsatellite loci except where parentheses to the right of the count indicate otherwise, e.g. 7 (4), seven individuals were typed at four loci. If individuals were not genotyped at the same number of loci, the range is given. New workers (NW) were either worker pupae or recently emerged (callow) workers whereas old workers (OW) were adult at the time of collection

Colony code	Mated dealate queens (DQ)	Sperm-athecae (DQS)	Unmated dealate queens (UDQ)	Adult males (M)	Alate queens (NQ)	New workers (NW)	Old workers (OW)	Eggs (E) and first instar (small) larvae (SL)	Total
Monogynous/queenless colonies									
SD99.59	1	1	0	3 (4)	0	0	9 (4)	63 (1–4)	77
SD99.60	1	1	0	11 (4)	0	0	9 (4)	42 (2–4)	64
SD99.67	1	1	2	0	16	16	15	58 (1–3)	109
SD99.69	0	0	2	15	15	15	15	36 (2–6)	98
SD99.71	1	1	0	22 (4–6)	15	15	15	70 (1–6)	139
SD99.73	1	1	20	23 (4–6)	15	15	15	42 (1–6)	132
SD99.81	1	1	2	20 (4–6)	15	15	15	61 (1–6)	130
SD99.83	1	1	0	21 (4–6)	15	15	15	58 (1–6)	126
SD99.85	1	1	0	23 (4)	15	15	15	33 (1)	103
SD99.87	1 (4)	1 (4)	1 (4)	0	0	0	0	57 (1–3)	60
SD99.103	1	1	1	12	15 (4)	15 (4)	15	39 (1–6)	99
Polygynous colonies									
SD99.53	4	4	2	7 (4)	15 (5)	15	15	47 (1–4)	109
SD99.54	4	3	3	6	21	19	15	79 (2–4)	150
SD99.55	3	3	0	16	15	15	15	71 (1–6)	138
SD99.61	2	2	0	16	0	15	15	70 (1–6)	120
SD99.64	2	2	0	12	0	15	15	58 (2–6)	104
SD99.74	4	4	0	7	3	15	15	42 (2–6)	90
SD99.78	3	3	0	19	1	16	15	48 (2–6)	105
SD99.94	8	8	0	0	5	15	10	82 (1–6)	128
SD99.96	3 (4)	3 (4)	0	6 (4)	0	0	0	50 (1–4)	62
Total	43	42	33	239	181	246	253	1106	2143

1–2 h and then heated to 100 °C for 10 min to denature the proteinase K prior to a PCR.

SD95 samples were typed at the loci *LXAGA1*, *LXAGA2* (Bourke *et al*. 1997) and *LXGT223* (Hamaguchi *et al*. 1993); SD96 at *LXAGT1*, *LXAGA1*, *LXAGA2* (Bourke *et al*. 1997), *LXGT223* and *L18* (Foitzik *et al*. 1997); and SD99 at *LXAGT1*, *LXAGA1*, *LXAGA2*, *LXGT218*, *MYRT3* (Evans 1993) and *L18*. Levels of genetic variation ranged from 40 alleles (expected heterozygosity, $H_E = 0.96$) at *LXAGT1* to two alleles at *LXGT223* ($H_E = 0.46$). Four of the seven loci used (*LXAGA1*, *LXAGA2*, *LXAGT1* and *L18*) had > 10 alleles and H_E values > 0.80. Not all SD99 samples were typed at all six loci, with each individual being typed at an average of 3.75 loci (range 1–6) (Table 1). SD96 and SD99 samples were genotyped using an ABI PRISM™ 373 automated sequencer whereas SD95 samples were genotyped using radioactive isotopes as described in Bourke *et al*. (1997).

Inbreeding coefficients and relatedness

We tested for linkage disequilibrium between loci with the program GENEPOP (Raymond & Rousset 1995; web version at http://wbiomed.curtin.edu.au/genepop/) using data on genotypes of mated, dealate queens from the SD96 and SD99 samples, with random sampling of a single queen per colony (n = 16 and 31 queens, respectively). Inbreeding coefficients and regression relatedness (Queller & Goodnight 1989) were estimated using the programs RELATEDNESS 4.2 and RELATEDNESS 5.07 (Goodnight Software: http://gsoft.smu.edu/GSoft.html). Population allele frequencies were estimated separately for SD96 (n = 52 queens) and SD99 (n = 67 queens) from mated, dealate queen genotypes. To calculate inbreeding coefficients (F_{IS}), colonies were weighted equally. Standard errors were estimated by jackknifing and coefficients were tested for departures from hypothetical values using two-tailed t-tests. F_{IS} and relatedness (R) were not calculated for SD95 as too few dealate queens (n = 15 queens from seven colonies) were genotyped to allow reliable estimation of population allele frequencies.

Estimation of queen mating frequency

Mating frequency was assessed in two ways. First, we compared a queen's genotype with that of her sperm DNA.

Table 2 The probability of not detecting double mating given knowledge of the queen's genotype (column 1) and where single mating has been inferred from queen-spermatheca genotyping. It was assumed that queen alleles co-amplified with sperm alleles so that the spermatheca genotype was mixed (column 2) and that mating was random. Errors were calculated for queen-spermatheca pairs for each locus where the mating type was such that a male allele was identifiable (superscript*). Mating types in which a male allele would not have been identifiable (e.g. AA × A) are not shown and are omitted from calculations as this might have arisen from amplification failure. The total nondetection probability was estimated as the product of the per-locus estimates. p_A, p_B, p_C = frequency of alleles A, B and C, respectively

Queen's genotype (PCR amplification from head and thorax)	Mixed genotype (dealate queen and sperm alleles from spermatheca)	Possible genotypes of males that would allow double mating to be undetected	Probability of nondetection of double mating per case	Sum of probabilities of nondetection of double mating
AA	AB*	Male 1: A, Male 2: B	$p_A p_B$	$2p_A p_B + p_B^2$
		Male 1: B, Male 2: A	$p_B p_A$	
		Male 1: B, Male 2: B	p_B^2	
AB	ABC*	Male 1: C, Male 2: C	p_C^2	$p_C^2 + 2p_C (p_A + p_B)$
		Male 1: C, Male 2: A	$p_C p_A$	
		Male 1: A, Male 2: C	$p_A p_C$	
		Male 1: C, Male 2: B	$p_C p_B$	
		Male 1: B, Male 2: C	$p_B p_C$	

Second, we compared genotypes of dealate queens with genotypes of their putative progeny in the 20 SD99 colonies investigated in detail.

Sperm DNA genotyping

In over 50% of sperm DNA amplifications, alleles of the same size as those amplified independently from queens (from head and thorax samples) were amplified from sperm DNA. This presumably occurred because of contamination of sperm DNA by queen DNA (e.g. Krieger & Keller 2000). Because of this, an allele amplified from sperm DNA was only designated as male-derived when it differed in size from alleles amplified independently from queen DNA. Thus double-mating was inferred if two alleles, of different size to the queen's, were found at one or more loci.

Genotyping of sperm DNA gives rise to three sources of nondetection error: (i) a male mated to a queen may share the same genotype as another male mated to her; (ii) contamination of sperm DNA with queen DNA means that male alleles of the same size as the queen's may be masked by the queen's alleles; and (iii) if there is unequal contribution of sperm by males, the minority male's alleles may fail to amplify during PCR. Errors (i) and (ii) depend on allele frequency distributions and the genotype of the queen, whereas type (iii) errors cannot be calculated directly from queen and sperm genotypes. However, typing of experimentally mixed sperm in other ant species suggests that minority male sperm contributions > 10% can be detected by sperm typing (Gertsch & Fjerdingstad 1997; Chapuisat 1998). In view of this, we ignored type (iii) errors and calculated nondetection probabilities of type (i) and (ii) for each queen–sperm DNA pair. Pedersen & Boomsma (1999a) recently outlined a general method for estimating effective mate number. However, as queen alleles were often amplified along with putative paternal alleles in *L. acervorum*, we developed a method that explicitly deals with this problem (Table 2). Like Pedersen & Boomsma (1999a), we concentrated on estimating the probability of incorrectly classifying a doubly mated queen as singly mated because our data indicated that the frequency of multiple mating was low, and where we identified multiple mating it was always compatible with two mates and never with three or more.

Comparison of mother–offspring genotypes

We independently inferred queen mating frequency by reconstructing maternal sibships using the genotypes of dealate queens from the 20 selected SD99 colonies and their putative female progeny (OW, NQ, NW and female eggs; Table 1), using the program KINSHIP 1.1.2 (Goodnight & Queller 1999; http://gsoft.smu.edu/GSoft.html). We tested the likelihood of a full-sister relationship between progeny against the likelihood of a half-sister relationship, setting each mated, dealate queen within a colony as the hypothetical mother of all potential female progeny in that colony. Pairs of individuals generating significant likelihood ratios ($P < 0.01$) were grouped together into full sisterhoods using KINSHIP's sort algorithm, with individuals excluded from being daughters of the putative mother being assigned a likelihood of zero. We then checked paternal alleles identified from groups of full sisters (defined as the single universally shared allele) against putative paternal alleles identified from sperm DNA. The mating frequency of queens was assessed by

counting the number of full sisterhoods, and therefore number of mates, attributable to each queen.

Statistical analysis

Unless stated otherwise, we considered statistics to differ significantly from critical values at the 5% level of significance. In cases of multiple tests performed on the same data, we used sequential Bonferroni corrections (Rice 1989). We report inbreeding and relatedness coefficients ±1 SE.

Results

Inbreeding coefficients

No significant linkage disequilibrium between loci was detected in either the SD96 or the SD99 samples. In the SD96 sample, the average inbreeding coefficient (F_{IS}) over the four loci typed was 0.037 ± 0.080 for monogynous colonies ($n = 11$ queens from 11 colonies), 0.118 ± 0.037 for polygynous colonies ($n = 41$ queens from five colonies) and 0.062 ± 0.057 for the whole population ($n = 52$ queens from 16 colonies). In the SD99 sample, the average inbreeding coefficient over five loci typed was -0.119 ± 0.046 for monogynous colonies ($n = 15$ queens from 15 colonies), 0.112 ± 0.041 for polygynous colonies ($n = 52$ queens from 16 colonies) and 0.010 ± 0.039 for the whole population ($n = 67$ queens from 31 colonies). Following Bonferroni correction, none of these inbreeding coefficients was significantly different from zero (all $P > 0.015$, corresponding to overall alpha = 0.05), although there was a trend for heterozygote excess in monogynous colonies in SD99 and homozygote excess in polygynous colonies in both SD96 and SD99.

Queen relatedness and sociogenetic structure of 20 SD99 colonies

In the single queenless SD99 colony and the 10 monogynous SD99 colonies whose detailed sociogenetic structure was examined, genotypes of workers and female brood were consistent with all individuals being offspring of a singly mated queen (see below). In the 10 monogynous colonies, the compatible mother was the dealate queen that was resident in the colony. The compatibility of recently laid female eggs in the queenless colony (SD99.69) with being full sisters of older female brood suggested that a once-mated mother queen had been lost from the colony shortly before or during collection. Mean relatedness among old workers in monogynous colonies was 0.707 ± 0.029 ($n = 135$ workers from 10 colonies). Following Bonferroni correction, neither this value nor any value in individual colonies differed significantly from that expected (0.75) among full sisters (all $P > 0.014$, corresponding to overall alpha = 0.05).

In the nine polygynous colonies, average relatedness between dealate queens within colonies was 0.210 ± 0.029 ($n = 32$ queens), which was significantly greater than zero ($t = 7.26$, d.f. = 8, $P < 0.001$) and significantly less than 0.75 ($t = 18.69$, d.f. = 8, $P < 0.001$). However, colonies varied greatly in queen–queen relatedness (range, −0.093 to 0.876). In the colony with the highest queen–queen relatedness, all queens had genotypes compatible with full sisterhood, and in the one with the next highest relatedness (SD99.74) the genotypes suggested that the four dealate queens consisted of one maternal queen and three daughter queens who were all full sisters of one another. We calculated an overall estimate for mean dealate queen relatedness in the SD99 sample by adding data from a further seven polygynous colonies, genotyped as part of the mating frequency analysis. This estimate was 0.256 ± 0.022 ($n = 52$ queens from 16 colonies), which was also significantly higher than zero ($t = 11.7$, d.f. = 15, $P < 0.001$) and significantly less than 0.75 ($t = 22.6$, d.f. = 15, $P < 0.001$). We compared this estimate with that calculated from the SD96 data (0.483 ± 0.049, $n = 42$ queens from five colonies), the estimate of Bourke *et al*. (1997) from samples collected in 1993 (0.48 ± 0.08), and that calculated by Heinze *et al*. (1995a) from colonies collected in 1991–92 (0.26 ± 0.09). We found that queen relatedness varied significantly across years (specifically 1991/1992 and 1999 vs. 1993 and 1996; all significant $P < 0.001$, corresponding to overall alpha = 0.05). Combining data from samples in which queen relatedness values were available from individual colonies, i.e. from 1993 (Bourke *et al*. 1997), SD96 and SD99, we found no significant relationship between relatedness among dealate queens and queen number (Spearman's rho = 0.172, $P = 0.373$, $n = 29$ polygynous colonies).

In the eight SD99 polygynous colonies with data, the mean relatedness among old workers was 0.278 ± 0.026 ($n = 115$ workers). Following Bonferroni correction, both this value and all values in individual colonies proved to be significantly < 0.75 ($P < 0.001$, corresponding to overall alpha = 0.05). As expected from this, relatedness among old workers was significantly higher in monogynous colonies than in polygynous ones (0.707 vs. 0.278, $t = 122.2$, d.f. = 16, $P < 0.001$).

Queen-spermatheca amplification and mating frequency

The frequency with which a queen's mate's allele was amplified from sperm DNA and was identifiable as a male allele varied among loci (range, 18.6–46.0%). However, in 63% (79/125) of queen-spermatheca amplifications, alleles were identified, at one locus or more, that differed in size from the queen's alleles and so were assumed to belong to the queen's mate or mates. In 95% (75/79) of these a

maximum of one male allele per locus was identifiable, a result compatible with a minimum estimate of one mating per queen. In 5% (4/79) of cases two male alleles were amplified at one locus or more (in two cases at one locus; in one case at two loci; in one case at three loci), which was compatible with a minimum of two matings per queen. No cases were found with three or more putative male alleles. All four doubly mated queens were from polygynous colonies. One was from the SD96 sample and three were from the SD99 sample and occurred in two separate colonies (none of which belonged to the sample of 20 SD99 colonies investigated in detail). There was no significant difference in the proportion of doubly mated queens from monogynous (0/15) or polygynous (4/64) colonies (Fisher's exact test, $P = 0.423$).

For singly mated queens, the average probability of not detecting a second male (Table 2) was 0.01 (range, 6.2×10^{-13} to 0.198). The wide range of nondetection errors reflected variation among queens in the number of loci at which male alleles were identified. The low average nondetection error indicated that fewer than one doubly mated queen (0.748 in a sample of 75 queens) was incorrectly classified as singly mated. The corrected mean mating frequency for the population was therefore 1.06 matings per queen ($n = 79$ queen–mate pairs from 42 colonies).

Mother–offspring analysis of mating frequency

In the KINSHIP analysis of the female progeny of the 20 SD99 colonies, the mean numbers of progeny attributed to individual queens were, for queens of monogynous colonies ($n = 10$), 34.8 eggs (range, 2–49) and 39.4 adults or pupae (range, 7–65) per queen. These represented all female progeny typed in these colonies (i.e. no typed progeny were unattributable to the colony queen). For queens of the polygynous colonies ($n = 7$), the mean numbers of progeny attributed to individual queens were 9.8 eggs (range, 4–25) and 6.6 adults or pupae (range, 0–21) per queen. In these colonies, the overall fraction of female progeny assigned to queens was 73% (total n female progeny typed = 466). The unassigned potential progeny almost certainly occurred because of queen turnover (Bourke *et al*. 1997). Across all colonies, 31 full-sister groups were identified, and each such group was assigned uniquely to the single potential mother queen in its colony in the case of monogynous colonies ($n = 10$ queens) or to one of the potential mother queens in its colony in the case of polygynous colonies ($n = 21$ queens). Correspondingly, within all 31 maternal sibships only one patriline was identified, suggesting that each queen was singly mated. In the two polygynous colonies with the highest relatedness among dealate queens (with three and four queens, respectively), it was not possible to unambiguously assign brood because of the high fraction of alleles shared between queens. However, in neither colony was there evidence for multiple mating, as a maximum of three putative male alleles per locus was identifiable from female eggs in each colony, which was equal to or one fewer than the number of queens per colony.

For 20 of the 31 queens used in the mother–offspring analysis, data were available from the sperm DNA typing. In all cases the genotype of the queen's mate reconstructed from the full sisterhood of progeny matched the allele amplified from sperm DNA. This confirmed that no multiply mated mothers not detected by amplification of sperm DNA were present in this sample.

Relatedness of queens to their mates and among males mated to queens in the same colony

Relatedness of queens to their mates, and among males mated to queens from the same colony, was estimated using the pedigree data from the 31 SD99 queens used in the mother–offspring analyses, rather than from sperm genotypes. We used only pedigree data because the identification of a male allele from sperm DNA depended on its size differing from that of the queen's alleles; male alleles identical to the queen's, as a result of putative inbreeding, would therefore have been systematically undetected. Mean relatedness of queens to their mates did not differ significantly in queen–mate pairs from either monogynous colonies ($R = -0.149 \pm 0.050$, $n = 10$ queen–mate pairs from 10 colonies) or polygynous colonies ($R = -0.015 \pm 0.056$, $n = 21$ queen–mate pairs from seven colonies) ($t = 1.392$, d.f. = 28, $P = 0.175$), so colony types were pooled. Overall queen–mate relatedness ($R = -0.058 \pm 0.038$, $n = 31$ queen–mate pairs from 17 colonies) was not significantly different from zero ($t = 1.529$, d.f. = 30, $P = 0.137$). Furthermore, relatedness among males mated to queens from the same polygynous colony ($R = 0.083 \pm 0.064$, data from male mates of 21 queens from seven colonies) was not significantly different from zero ($t = 1.285$, d.f. = 20, $P = 0.213$).

Diploid males

In Hymenoptera with complementary sex determination, individuals heterozygous at the sex-determining locus or loci develop as females, whereas those homozygous or hemizygous develop as males (Cook & Crozier 1995). Inbreeding, by increasing homozygosity, should therefore increase the production of diploid males (Cook & Crozier 1995). In this study, only three of the 239 adult or pupal males (1.3%) typed had one or more heterozygous microsatellite loci and were therefore diploid males (sampled from the 20 SD99 colonies selected for detailed genetic analysis). All occurred in a single, polygynous colony (SD99.53; Table 1).

Discussion

Queen relatedness and sociogenetic structure of colonies

Our results confirmed previous studies that have found queens of *Leptothorax acervorum* living together in polygynous colonies to be, on average, related to one another. In addition, evidence from one colony (SD99.74) suggested the coexistence of reproductive daughter queens alongside their reproductive mother. These findings support the view that polygyny in *L. acervorum* typically arises by the adoption of daughter queens into their natal colony (see Introduction). Average relatedness of queens in polygynous colonies in the Santon Warren population varied significantly across years (range, ≈ 0.25–0.5), but the reasons for this are unclear. If queens outbreed, and polygyny arises purely by the adoption of daughter queens, relatedness among queens within colonies is predicted to be negatively correlated with colony queen number (Keller 1995). However, we found no relationship between queen number and the relatedness of queens in polygynous colonies of *L. acervorum*. Likewise, Heinze *et al*. (1995b) found no relationship between queen number and relatedness among old workers in polygynous *L. acervorum* colonies from a population in Germany. These results could have stemmed from colonies adopting a certain fraction of unrelated queens (cf. Goodisman & Ross 1997), as suggested by both the occurrence of colonies with very low queen relatedness (this study) and by other studies showing the presence of maternally unrelated queens within *L. acervorum* colonies (Stille & Stille 1992). They could also have occurred because, even with daughter readoption, the predicted association might be obscured by high variation, both among colonies and temporally, in the numbers of queens adopted and in degree of queen turnover (Bourke *et al*. 1997).

We found that the genetic structure of monogynous and polygynous colonies differed markedly. In the SD99 sample, old workers within monogynous colonies were all full sisters and were significantly more related than workers in polygynous colonies, which were significantly less related than full sisters. These results were in agreement with those of Heinze *et al*. (1995a), who also found, in the same *L. acervorum* population, that workers were significantly more highly related in monogynous ($R_{OW} = 0.50 \pm 0.06$) than in polygynous colonies ($R_{OW} = 0.28 \pm 0.09$). However, overall worker relatedness in monogynous colonies in the study of Heinze *et al*. (1995a) was significantly lower than that expected among full sisters, presumably because of queen turnover in some of these colonies (cf. Bourke *et al*. 1997). Our finding that old workers in polygynous colonies were significantly less related than full sisters suggested that they were the offspring of multiple mothers (given single mating). This matched the similar conclusions of Heinze *et al*. (1995a) and Bourke *et al*. (1997) for, respectively, worker and sexual progeny.

Mating frequency of queens

Typing of the spermathecal contents of queens and analysis of mother–offspring pedigrees, along with the correspondence between the two, showed conclusively that most queens (95%) in the Santon Warren population were singly mated and that double mating was rare. This conclusion confirms the previous findings from this and other populations of *L. acervorum* (see Introduction) by means of a larger dataset and a more robust methodology. The confirmation that nearly all *L. acervorum* queens in the study population are singly mated supports the assumption of the parentage analysis conducted by Bourke *et al*. (1997). Studies have shown that other species within *Leptothorax* and allied genera likewise typically mate once (Bourke *et al*. 1988; Foitzik *et al*. 1997; Foitzik & Herbers 2001) and that many species of ant have mating frequencies close to one (Boomsma & Ratnieks 1996; Strassmann 2001).

It has been hypothesized that multiple mating by queens is beneficial because it increases genetic variation within colonies (e.g. Keller & Reeve 1994; Boomsma & Ratnieks 1996; Schmid-Hempel & Crozier 1999). Keller & Reeve (1994) suggested that if this is so, and multiple mating also incurs costs, there should be a negative relationship between polygyny and polyandry. Our results do not support the genetic variability hypothesis for multiple mating as the frequency of multiple mating did not differ between monogynous and polygynous colonies. Recent studies of mating frequency in other polygynous ants have found either no correlation between mating frequency and queen number (*Formica paralugubris*, Chapuisat 1998), or a positive correlation (*Myrmica sulcinodis*, Pedersen & Boomsma 1999b).

Degree of inbreeding

Our findings suggest that inbreeding is rare or absent in *L. acervorum*, in agreement with previous investigations of the study population (Heinze *et al*. 1995a; Bourke *et al*. 1997). First, the overall inbreeding coefficients calculated from both the SD96 and SD99 samples were not significantly greater than zero. Second, typing of queens and their spermathecal contents showed that queens and their mates were unrelated in both monogynous and polygynous colonies. Third, diploid males, whose presence is an indicator of inbreeding, occurred at very low frequency. In a German *L. acervorum* population, diploid males were likewise very rare (0/231 males typed; Heinze *et al*. 1995b), although they have been detected at higher frequency in other *Leptothorax* species (Loiselle

et al. 1990; Herbers & Grieco 1994; Foitzik & Heinze 2000). Despite these findings, we cannot exclude the possibility of a low level of inbreeding in queens forming polygynous colonies. Evidence for this came from the trend for queens from polygynous colonies to exhibit both higher inbreeding coefficients than queens from monogynous colonies and a higher level of queen–mate relatedness.

Mating system

In facultatively polygynous populations of *L. acervorum*, the co-occurrence of related queens within nests and observations of mating aggregations suggest a mixed mating strategy among queens involving either dispersal from the nest to the mating aggregation or mating near the nest followed by readoption (see Introduction). Our confirmation that queens in polygynous colonies are, on average, related is support for this scenario. Our findings also suggest that males disperse before mating to mate either in the aggregation or near (unrelated) nests. The first piece of supporting evidence comes from our data suggesting a lack of extensive inbreeding despite the high probability that all queens seeking readoption in their natal colonies mate near the natal nest. Second, there was no significant difference in queen–mate relatedness between monogynous and polygynous colonies, even though, on average, queens in the two types of colony are likely to have mated in aggregations and near nests, respectively. Third, we found that males mated to different queens within the same polygynous colony were not significantly related, again despite the likelihood these matings occur near nests. (The small amount of inbreeding that is suggested by trends in the data to occur among queens in polygynous colonies is then likely to be present because a few queens seeking readoption do mate with nestmate males.) *L. acervorum* resembles some other polygynous ants (e.g. *Myrmica rubra*, Seppä & Walin 1996; *Rhytidoponera* sp. 12, Tay & Crozier 2001) in showing a lack of relatedness between males mated to different queens of the same colony, but differs from others (e.g. *Myrmica sulcinodis*, Pedersen & Boomsma 1998). This feature of *L. acervorum* further suggests that related *L. acervorum* males do not compete with one another for mates (local mate competition), again unlike males in some species (*Myrmica sulcinodis*, Pedersen & Boomsma 1998). The inferred lack of local mate competition in *L. acervorum* is consistent with the assumptions of analyses of sex allocation in the study population (Chan & Bourke 1994; Chan *et al*. 1999). Studies of local population structure in *L. acervorum* have shown that there is little structure at nuclear allozyme loci in contrast to the clustering exhibited by mitochondrial haplotypes (Douwes *et al*. 1987; Stille & Stille 1992, 1993). This suggests that gene flow is largely mediated by males and again matches the conclusion that males disperse widely.

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This work is part of a larger project aimed at investigating reproductive conflicts in polygynous ant societies. Rob Hammond was the post-doc on this project and he is interested in the evolutionary genetics of social insects and conservation genetics. Andrew Bourke's research focuses on the behavioural ecology of social insects, and in particular the role of kin-selected conflict in their evolution. Mike Bruford is interested in the fine-scale genetic analysis of socially structured and threatened populations.