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Investigation of the population genetic structure and mating system in the ant *Pheidole pallidula*

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Abstract

The origin of eusociality in haplo-diploid organisms such as Hymenoptera has been mostly explained by kin selection. However, several studies have uncovered decreased relatedness values within colonies, resulting primarily from multiple queen matings (polyandry) and/or from the presence of more than one functional queen (polygyny). Here, we report on the use of microsatellite data for the investigation of sociogenetic parameters, such as relatedness, and levels of polygyny and polyandry, in the ant *Pheidole pallidula*. We demonstrate, through analysis of mother–offspring combinations and the use of direct sperm typing, that each queen is inseminated by a single male. The inbreeding coefficient within colonies and the levels of relatedness between the queens and their mate are not significantly different from zero, indicating that matings occur between unrelated individuals. Analyses of worker genotypes demonstrate that 38% of the colonies are polygynous with 2–4 functional queens, and suggest the existence of reproductive skew, i.e. unequal respective contribution of queens to reproduction. Finally, our analyses indicate that colonies are genetically differentiated and form a population exhibiting significant isolation-by-distance, suggesting that some colonies originate through budding.

Keywords: ants, isolation-by-distance, microsatellite data, polyandry, polygyny, social organization

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Introduction

Kin selection theory (Hamilton 1964a,b) predicts that the frequency of sterility can increase if the direct fitness cost of bearing that trait is less than the weighted benefit in indirect fitness (dispensed to relatives). In other words, eusocial Hymenoptera workers might be sterile because the probability of survival of the copies of their genes is higher when they help in the rearing of their sisters than if they were to produce their own offspring. Workers are indeed closely related to the brood they rear because of their haplo-diploid sex-determining system (Crozier 1971). Two factors primarily influence relatedness values among individuals within a colony: the occurrence of multiple mating (polyandry) and of multiple reproductive queens (polygyny) (Ross 2001). Polyandry and polygyny yield a decrease in the mean intracolonial relatedness and, hence, reduce the mean indirect fitness gained by workers from raising sexuals in the colony (Bourke & Franks 1995; Crozier & Pamilo 1996).

Most explanations for the evolution of multiple-mating emphasize the selective benefits of increased genetic diversity within colonies, in terms of (i) increasing resistance against pathogens (Sherman *et al*. 1988; Schmid-Hempel & Crozier 1999); (ii) increasing colony performance through task partitioning among workers (Page 1986; Page *et al*. 1995); (iii) reducing queen–worker conflicts (over reproduction) through reduction of relatedness asymmetries (Ratnieks & Boomsma 1995); or (iv) limiting the genetic load due to production of sterile diploid males (Crozier & Page 1985; Pamilo *et al*. 1994). Additional hypotheses suggest that queens attempt to mate with multiple males in order to acquire enough sperm to fertilize all their eggs (Cole 1983; Fjerdingstad & Boomsma 1998). Although an

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average of as many as 6.76 mates per queen have been reported in *Pogonomyrmex occidentalis*, most ant species are monandrous (Boomsma & Ratnieks 1996; Strassmann 2001).

On the other hand, polygyny is common in many ant species and has been shown to affect greatly the colony kin structure (Crozier & Pamilo 1996; Herbers & Stuart 1996; Seppä & Walin 1996; Ross *et al*. 1997). Several hypotheses have been proposed to account for the occurrence of polygynous colonies (Keller 1993, 1995; Bourke & Heinze 1994), including (i) low survival of solitary founding colonies, and (ii) increased productivity in polygynous colonies. Recent molecular studies on polygynous species revealed that the number, and relatedness among breeding queens as well as their relative reproductive successes can greatly vary among colonies, populations and species (reviewed in Bourke & Franks 1995; Keller 1995; Crozier & Pamilo 1996). Moreover, colony structure may vary over time as a consequence of queen turnover, i.e. replacement of old queens by newly inseminated immigrant queens (Evans 1996; Bourke *et al*. 1997; Goodisman & Ross 1999; Pedersen & Boomsma 1999a; Foitzik & Heinze 2000; reviewed in Heinze & Keller 2000).

Polygyny is associated with specific reproductive and social traits such as loss or limitation of mating flights (i.e. sexual forms mating within or close to the native colony), colony budding, and polydomy (multiple nests per colony) (Bourke & Franks 1995; Ross & Keller 1995; Pamilo *et al*. 1997). The impact of variation in queen number on mating and/or dispersal strategies has been the focus of several studies, because it has important consequences on the genetic structure at the colony and population levels (Seppä & Pamilo 1995; Chapuisat *et al*. 1997; Tay *et al*. 1997; Chapuisat & Keller 1999b; Giraud *et al*. 2000).

Among the family Formicidae, *Pheidole* is one of the most speciose and widespread genera, with 898 species distributed world-wide (Hölldobler & Wilson 1990; Bolton 1995; Wilson 2002). The species *Pheidole pallidula* occurs throughout the Mediterranean region. Analyses of allozyme data (Aron *et al*. 1999) previously suggested that the species exhibits facultative polygyny (i.e. both monogynous and polygynous colonies coexist in a same population) and probably monandry. However, this study did not allow precise determination of (i) the queen mating frequency in each colony, (ii) the relative proportions of monogynous and polygynous colonies within the population under study, and (iii) the number of females in polygynous colonies.

Among the 391 microsatellite loci that we isolated from *P. pallidula*, we chose 22 loci for investigating population genetic structure and mating systems in the same population as that investigated in Aron *et al*. (1999). Fifteen of these loci showed levels of polymorphism adequate for an accurate characterization of polygyny and polyandry parameters. We estimated within-colony genetic relatedness, the number of matrilines, and the queen effective mating frequencies within colonies. In addition, because polygyny is frequently associated with limited dispersal of female sexuals and production of new nests by budding, we tested for a possible correlation between geographical and genetic distances among colonies (i.e. isolation-by-distance). The markers characterized here are of potential use in other species of *Pheidole*.

Materials and methods

Field collection and sampling

Colonies of *Pheidole pallidula* are common in arid areas, on sunny slopes with low vegetation density. Colonies extend underground on rocky soils and may contain up to 18 000 individuals. In this species, sterile individuals are divided into two morphologically distinct castes: large-headed majors (usually called soldiers) and small-headed minors. There is no over-wintering brood; queens lay eggs from early March to early September. An early-laid fraction of these eggs gives rise to male and female sexuals that hatch at the end of June/early July, whereas another fraction later develops into workers (Bontpart 1964). Sexuals of *P. pallidula* reportedly engage in large mating flights (Bontpart 1964), but the mode of colony foundation remains unknown.

Twenty-six colonies of *P. pallidula* were sampled from Bruniquel (Tarn et Garonne, France) on 29 June and 1 July 1999, i.e. just before the nuptial flight. The site was described in Aron *et al*. (1999) and was approximately 180×170 m. Large samples of minors, majors, males and winged females, but no reproductive queen, were collected. In summer, queens are very difficult to excavate because they are located in the deeper parts of the colony. Individuals belonging to each sex and caste were stored at −20 °C in 95% ethanol.

Isolation and characterization of microsatellite sequences

Pheidole pallidula genomic DNA was partially digested with the enzyme *Sau*3A and a fraction ranging from 500 to 2000 base pairs was isolated after electrophoresis in agarose. The fragments corresponding to the selected fraction were purified, ligated into a zero background vector (Invitrogen), and transferred to *Escherichia coli* DH5α competent cells. The library was first screened with a $(CA)_{14}$ radio-labelled oligonucleotide probe then with a mix of seven radio-labelled probes specific to di-, tri-, and tetra-nucleotide repeats. Positive clones were sequenced on both strands following the manufacturer's protocols (dRhodamine Cycle Sequencing, electrophoresis on 377 sequencer; Applied Biosystems). The full sequence of each positive clone was fed into a β-version of an in-house

program (Van Belle and Milinkovitch, unpublished data) that both identifies any string of ([2] … [N])$_j$ nucleotides (where $j > 3$) through the use of a finite state automaton, and designs optimal specific primers flanking the repeated sequence.

Polymerase chain reaction (PCR) amplification of microsatellite loci

Individual ants were ground in digestion solution (100 mM NaCl, 50 mM Tris, 1 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulphate, and 200 μg/mL proteinase K) and incubated for 2 h at 55 °C. DNA was purified through phenol–chloroform extractions and ethanol precipitation following standard protocols. A subset of 22 loci (chosen on the basis of repeat length and theoretical annealing temperature of primers) was tested for levels of polymorphism within *P. pallidula* populations (12 winged females randomly chosen from the sample collected in summer 1999) using fluorescent nucleotide ([F]dNTP, ABI). PCR were carried out in a 10-μL volume: 10 mM Tris–HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 μM of each primer, 1.5 U *Taq* polymerase, 1 μL of genomic DNA (about 10 ng), and 2.5 μM of [TAMRA]dUTP or 0.625 μM [R6G]dUTP or 0.625 μM [R110]dUTP (ABI). After an initial denaturing step of 6 min at 94 °C, the PCR consisted of 35 cycles of 25 s at 94 °C, 40 s at the annealing temperature (see Table 1), and 40 s at 72 °C, followed by a final extension step of 2 min at 72 °C (MJ Research PTC-200 thermocycler). Loci estimated as having sufficient levels of polymorphism among the 12 test individuals were typed in all individuals using 5′ end-labelled primers. Amplified fluorescent fragments were visualized on 5% polyacrylamide/6 M urea sequencing gels using an automated 377 ABI sequencer.

Population structure

F-statistics and allele frequencies were estimated from worker genotype frequency data using the programs RELATEDNESS 4.2c (Queller & Goodnight 1989) and GENEPOP 3.2a (Raymond & Rousset 1995). Differences in allelic distributions between minor and major nestmate workers were calculated as described by Raymond & Rousset (1995); a global test across all loci was carried out using GENEPOP 3.2a (Fisher's method). Because comparisons were conducted across the 26 colonies, tests were corrected for multiple comparisons with Bonferroni adjustments (Rice 1989). The mating structure was estimated using the inbreeding coefficient *F*, which measures the deviation of observed genotype frequencies from expected Hardy–Weinberg equilibrium frequencies: $F_{IS} = 1 - (H_O/H_E)$, where H_O and H_E are the observed and expected frequencies of heterozygotes, respectively. Deviation from Hardy–Weinberg equilibrium in the population was tested at each locus according to the method of Li & Horvitz (1953) modified by Seppä (1992): $\chi^2 = (k-1)n\,F_{IS}^2$ and $df = k(k-1)/2$, where k is the number of alleles and n is the sample size. The sample size was equal to $n = c(0.75/r_w)$, where c is the number of colonies sampled and r_w is the relatedness among nestmate workers.

Isolation-by-distance was investigated by plotting modified F_{ST} [i.e. $F_{ST}/(1 - F_{ST})$] coefficients between pairs of colonies against the logarithm of geographical distances (Slatkin 1993; Rousset 1997). The significance of the Pearson correlation coefficient between genetic differentiation and geographical distance was assessed with Mantel tests with 2000 permutations (Mantel 1967), using GENEPOP 3.2a. Moreover, we also used RELATEDNESS 4.2c to investigate whether groups of adjacent nests had nonzero interrelatedness.

Inbreeding and regression relatedness among colony members (r) were assessed using RELATEDNESS 4.2c and 5.0.7 (Queller & Goodnight 1989), weighting all colonies equally. Standard errors were obtained by jackknifing over colonies.

Mating frequencies

To estimate the number of mating events per queen, 21 additional colonies were collected from Bruniquel on 10 March 2000. At this time of year (early spring), the first sunny days follow relatively cool nights; in these conditions, queens and workers tend to migrate towards the superficial parts of the colony.

Queen mating frequencies of the ant *P. pallidula* were estimated by sperm-typing and by mother–offspring combination. These two estimates are complementary (Chapuisat 1998): seminal fluid analysis avoids not detecting multiple matings because of finite offspring sample size and/or temporal fluctuations in the contributions of mates (Boomsma & Ratnieks 1996; Sundström & Boomsma 2000), while mother–offspring analysis takes into account the relative effective contribution of each male under polyandry (Gertsch & Fjerdingstad 1997).

Sperm-typing. To isolate sperm DNA, queens from the 21 colonies were dissected, the spermatheca was withdrawn and placed in a drop of digestion solution (cf. above). The spermatheca envelope was ruptured with forceps, the seminal fluid was collected using a micropipette, resuspended in 100 μL of digestion solution, and stored at −20 °C. As males of Hymenoptera are haploid, all sperms of a single male present the same single allele for a given locus. Sperm typing can leave multiple mating undetected when two males have the same genotype at all studied loci or one male contributed more than 90% of sperm (Gertsch

Table 1 Microsatellite loci developed for the ant *Pheidole pallidula*

Locus	Core repeat (sequenced allele)	Size (bp)	Size range (bp)	N_a	f	H_O	H_E	T_{ann} (°C)	Primers (5′–3′)	GenBank accession number
Ppal-03	$(GT)_{22}$	118	94–122	6	0.571	0.429	0.681	62	F: GCGTGCGTTGTGTATGTGTAT R: CAGCCGTGCTCCTGTATCAG	AF426745
Ppal-08	$(TG)_{21}$	108		—	—	—	—	55	F: AATTCGGATCTTCTTCACTG R: AATATATCAACGCGAATGTT	AF426746
Ppal-12	$(AC)_{18}$	124	95–124	9	0.278	0.556	0.895	63	F: AGAGGAACGGCGTGTAATGC R: GTATACGGAACGCGTACGTGT	AF426747
Ppal-15	$(CA)_{17}T(AC)_4$	128		—	—	—	—	62	F: CGAATTCGGATCCACCCTTTATAT R: TTCTTGCCGACCTCTGGGAT	AF426748
Ppal-33	$(TG)_{30}$	129	106–122	9	0.214	1.000	0.934	61	F: CGATACGGACGTGTGTTAGA R: CATGCAGCACCACAAGGTGA	AF426749
Ppal-35	$(TC)_9(TG)_{16}(AG)_9$ GAA$(AG)_4$	157		—	—	—	—	58	F: CTCTAGATGCATGCTCGAGC R: GGGAACTAAGATAATGCAGTTTAG	AF426750
Ppal-42	$(CA)_{28}$	125		—	—	—	—	50	F: ATCATTATTCCATGCATTGT R: TTTTTCAGAATTTTTTATCA	AF426751
Ppal-48	$(TG)_{24}(AG)_4$	130	110–125	3	0.571	0.429	0.626	51	F: TTTTGAATGATATTACGTATT R: AAGAACTTGTATCAACGTAA	AF426752
Ppal-50	$(GT)_{19}$	120		—	—	—	—	64	F: GGCCCTCTAGATGCATGCTCG R: GGGAGTGACGAACCGTGGGG	AF426753
Ppal-56	$(AC)_{17}(AT)_{11}$ GAATT$(TA)_5$	136		—	—	—	—	55	F: CCGTAAACCCTTTCATTAAT R: AGTTTAATAAGTAGCTACCTCA	AF426754
Ppal-58	$(GC)_9(GT)_{17}$	129	109–122	7	0.285	0.857	0.890	58	F: AATTATTGCCCGGGAGTGTA R: TAATTCTACAAACGAAACGCA	AF426755
Ppal-68	$(TG)_{20}$	114	104–113	4	0.375	0.750	0.821	56	F: ACGATCGAAAACTTTTTATAA R: GTGCCGTCTTTTAAGAATAA	AF426756
Ppal-69	$(CA)_{23}(TA)_4$	142	124–151	12	0.222	0.667	0.935	52	F: AAGCTCCATTTTAAATTTAT R: GAGACATCCCGTGTATATAC	AF426757
Ppal-73	$(CA)_{23}r$ °CGC$(AC)_2$ AA$(AC)_8(AT)_2A(CG)_4$	167	136–150	8	0.313	0.500	0.858	63	F: GTCCTTCGTGAGTCGAAACG R: GGAAAGCGTCCATTGTAAATCCTG	AF426758
Ppal-77	$(AT)_7(GT)_{43}$ACG CAGGTACAGAGA $(GA)_4$	193	106–170	9	0.167	0.500	0.955	64	F: TTCGTGCTTCTCCTGTGGCTTTATA R: GTTCTCGCGCTTCGACACAG	AF426759
Ppal-83	$(TG)_{24}$	136	110–136	7	0.500	0.333	0.739	53	F: AGAATGACGACTCAGATTAA R: AATTAACGTTTGTGAGAGAA	AF426760
Ppal-84	$(CT)_4T(TC)_{19}$	131	104–128	8	0.250	0.625	0.892	63	F: TTCCCCAATAATGAACATTCCCCGA R: TCGCACGGAAACGAGGAGAG	AF426761
Ppal-01T	$(GGA)_5(GGT)_3$ GGAG$(AGA)_{24}$	177	156–194	11	0.167	0.667	0.941	64	F: CCCCTTTCGCTGCAGGTCAA R: AGATACGCTTCCGTCTTACGC	AF426762
Ppal-09T	$(TAA)_4TAG(TAA)_{11}$ CAATAGTAAC $(AAT)_3$AAAAAT AAC$(AAT)_6$	164	154	1	1.000	–	–	61	F: CAAAAGGCGTCACGTCACAA R: AACACATAGTGGCCTAGAACCG	AF426763
Ppal-16T	$(CA)_6$TACACACAT $(AC)_5$AATAAAGAC $(TTA)_{15}$	167	159–183	8	0.417	0.667	0.848	59	F: CTTTCCGTGTGATTGTGCAC R: ATTTCAAATATAGTATGCCCAGC	AF426764
Ppal-19T	$(TTA)_{13}$	117	93–119	7	0.333	0.778	0.837	59	F: GAATTTCAGATGCACCGCTTA R: ATTTTTCGCTTATTTTCCTGC	AF426765
Ppal-26T	$(AAT)_{13}$	105	125–134	4	0.400	0.800	0.733	63	F: CGCGCCGTCATAAATAAAATTG R: TCGAGTGCTTCGGGATTCTCC	AF426766

The size of the sequenced clone, the observed size range, the number of alleles (N_a), the frequency of the most common allele (f), and the estimates of observed (H_O) and expected (H_E) heterozygosities are based on 12 winged females collected at Bruniquel (France) in 1999. The annealing temperature (T_{ann}) is given for each primer pair.

& Fjerdingstad 1997). We estimated the probability of two males bearing the same allele at each locus using data from workers only (Pamilo 1993):

$$\prod_j \sum_i p_{ij}^2$$

where p_{ij} is the population frequency of allele i at locus j; or males only (Krieger & Keller 2000):

$$\prod_j m_j$$

where m_j stands for the male frequency of allele m at locus j.

When no double mating is detected, the upper limit of double mating frequency (D_{est}) can be estimated by assuming that a $N + 1$th sampled queen would have mated with two males (Krieger & Keller 2000):

$$D_{est} < \frac{1}{(N+1)\left(1 - \prod_j \sum_i p_{ij}^2\right)}$$

where N is the sample size, and p_{ij} is the population frequency of allele i at locus j. Krieger & Keller (2000) proposed an alternative procedure using genotypes of males only. In this situation, the upper limit of the proportion of doubly mated queens is

$$D_{est}^* < \frac{N}{(N+1)\left(N - \sum_k^N \prod_j m_{jk}\right)}$$

where m_{jk} is the male allele frequency of allele m at locus j for the male k, and N the sample size.

Mother–offspring combination. Twelve colonies were set up in the laboratory under summer conditions (Passera 1980). They were examined weekly and the worker pupae produced were removed and stored at –20 °C for microsatellite mother–offspring analyses. Effective paternity (M_E) was estimated from the mean relatedness within female brood of a single queen (Pamilo 1993): $M_E = 1/(2r - 0.5)$, where r is the regression relatedness calculated using Relatedness 5.0.7.

Mother–offspring combinations can leave multiple mating undetected because of nonsampling errors (i.e. limited sample size of offspring) and/or nondetection errors (i.e. limited variation of genetic markers). The latter can cause underestimation of mate frequencies because of (i) males having identical genotypes and/or (ii) heterozygous queens exhibiting one allele identical to that of one of the males contributing sperm. We combined nonsampling and nondetection errors into a 'nonidentification' probability following Pedersen & Boomsma (1999b; equ. 11, page 582).

Number of queens

The minimum number of queen(s) in each colony, Q, was inferred from the observed genotypes of workers at all loci. For the overall population, the 'pedigree effective queen number' per colony $N_{E,P}$ was estimated as the harmonic mean of the number of queens across colonies (Ross 1993): $N_{E,P} = N/\sum_i 1/Q_i$, where N is the total number of colonies and Q_i is the minimum number of queens in colony i. An estimator of the effective mean number of reproductive queens per colony, weighted by the respective contribution of each queen to the production of workers, was also inferred from relatedness indices among workers (Ross 1993; Seppä 1994): $N_E = (4r_{fs} - r_q - 2r_m)/(4r_w - r_q - 2r_m)$, where r_{fs} is the average relatedness among workers sharing the same mother ($r_{fs} = 0.75$ if they also share the same father), r_q is the average relatedness among queens within a single colony, r_m is the average relatedness among the males inseminating the same reproductive queen, and r_w is the average relatedness among worker nestmates in the population. Because reproductive queens were not available from our summer field collection (see above), we did not determine r_q. The lowest and highest values of N_E were estimated assuming that queens were unrelated ($r_q = 0$) or that the average relatedness among queens from a single colony was equal to the mean worker relatedness ($r_q = r_w$). Standard errors for N_E and $N_{E,P}$ were estimated by bootstrapping (1000 replicates). Statistical comparisons between the two estimators were performed using Wilcoxon signed ranks tests.

Results

Isolation and characterization of microsatellite sequences

Among the 160 positive clones, 158 effectively contained at least one microsatellite sequence (of four repeats or more). In total (many of the clones contained several microsatellites), we identified 348, 30, 11 and two microsatellite sequences corresponding to di-, tri-, tetra-, and hexa-nucleotide repeats, respectively. Twenty-two of these loci were tested on 12 winged females and 15 loci (see Table 1 for the sequence of the flanking primers) were selected on the basis of two parameters: level of polymorphism and readability of the amplification products. The number of alleles and observed heterozygosity at these loci ranged from 3 to 12 and from 0.333 to 1.000, respectively.

Population structure

F-statistics and genetic relatedness coefficients were estimated from worker genotypes at four microsatellite loci: *Ppal-03*, *Ppal-12*, *Ppal-83* and *Ppal-19T* (Table 1). No significant

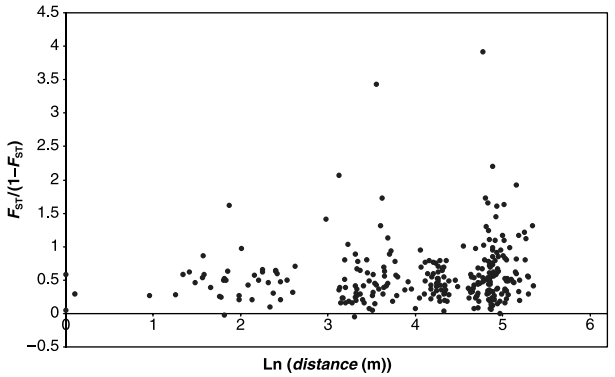

Fig. 1 Relationship between genetic differentiation and geographical distance between pairs of nests in the whole population investigated.

difference of allele frequencies was observed between minor and major workers in 25 out of 26 colonies (the difference remained significant after Bonferroni adjustments). Worker genotypes showed no significant departure from Hardy–Weinberg equilibrium over the whole population. Neither the population-average inbreeding coefficient (F_{IS} = 0.054, jackknife SE = 0.056), nor the four individual inbreeding coefficients (i.e. for each locus) were significantly different from zero (all $P > 0.34$).

The correlation between genetic differentiation between pairs of colonies and the geographical distance indicated a low but significant isolation-by-distance pattern (matrices correlation = 0.118, $P = 0.043$; Fig. 1). Consistent with this result, groups of adjacent nests showed a significant nonzero relatedness to each other ($r = 0.26 \pm 0.02$; two-tailed *t*-test, $t = 14.379$, $n = 26$, $P < 0.001$).

The average within-colony genetic relatedness among nestmate workers (10 workers analysed per colony) was $r = 0.65$ ($SE_{jackknife} = 0.05$). This value is significantly different from that expected (0.75) in colonies headed by a single, once-mated queen (Table 2), indicating that some level of polygyny and/or polyandry influences kin structure in the investigated population.

Mating frequency

Queen mating frequencies were estimated on the basis of four microsatellite loci: *Ppal-12*, *Ppal-84*, *Ppal-01T* and *Ppal-16T*. In each of the 20 samples of seminal fluid that could be genotyped (one spermatheca was lost during the dissection), only one allele was detectable at each of the four microsatellite loci. As the probability of two males sharing the same allele is as low as 0.0003 or 0.0085 (when estimated from population allele frequencies or male allele frequencies, respectively), this result indicates that the investigated queens of *Pheidole pallidula* were singly mated. No contamination by female DNA was detected. The estimates of the 'upper limit of double mating frequency' (D_{est} and D^*_{est}, cf. Materials and methods) ranged from 0.0476 to 0.0480, indicating that, in *P. pallidula*, a very low proportion of queens, if any, mate with two or more males. Comparison between the genotypes of females and that of the sperm cells stored in their spermathecae indicated that mating occurred between unrelated ($r = -0.091 \pm 0.038$) individuals.

Distributions of genotypes in mother–offspring combinations (280 callow workers from 12 queens were investigated; mean number of workers analysed per queen = 23.33 ± 9.85) were consistent with single mating. This result is also corroborated by the high mean relatedness ($r = 0.756 \pm 0.028$; $M_E = 1$) among laboratory-reared offspring. The probability of nonidentification of a second mate (Pedersen & Boomsma 1999b; equ. 11, page 582), was 0.0040 (range: 0.0000–0.0131), and 0.0127 (range: 0.0000–0.0391) assuming paternity skews (Boomsma & Ratnieks 1996) of 0.5 and 0.7, respectively.

Table 2 Regression relatedness of workers, female (sexual) progeny and male progeny in *Pheidole pallidula* colonies

	No. individuals workers; females; males	Relatedness ± SE			
		workers–workers	females–females	males–males	females–males
Overall colonies ($n = 26$)	260; 165; 194	0.651 ± 0.051*	0.576 ± 0.070**	0.461 ± 0.035	0.352 ± 0.081*
Monogynous colonies ($n = 16$)	160; 48; 160	0.733 ± 0.031	0.671 ± 0.075	0.473 ± 0.016	0.484 ± 0.081
Polygynous colonies ($n = 10$)	100; 117; 34	0.552 ± 0.087*	0.538 ± 0.093*	0.430 ± 0.044	0.259 ± 0.128*

The observed relatedness values were tested (one-tailed *t*-tests) for significant differences from various expected relatedness values (0.75 if workers or females were full sisters, 0.5 if males were full brothers or if females and males were full siblings). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Number of queens

Observed genetic relatedness among workers ($r = 0.65$) in the study population was significantly lower than that expected ($r = 0.75$) under combined monandry and monogyny. As the analyses above indicated that each queen mated with a single male, polygyny was very probably responsible for this pattern. This hypothesis is also supported by the fact that the average relatedness between females and males over all colonies (0.352 ± 0.081, Table 2) was significantly lower than 0.5.

For the overall population, the mean number of queens per colony ($N_{E,P}$) = 1.30 ± 0.10. The weighted estimator of mean number of queens per colony (N_E, see Materials and methods) = 1.18 ± 0.07 or 1.23 ± 0.09, depending on whether the queens were assumed unrelated ($r_q = 0$) or related ($r_q = r_w$). Determination of the effective queen number on the basis of workers' pedigree showed that the level of polygyny varied among colonies: 10 out of the 26 colonies (38%) contained more than one matriline of full-sisters (six colonies with two matrilines, two with three matrilines, and two with four matrilines). Average within-colony genetic relatedness (among workers) of single-matriline colonies (0.733 ± 0.031; Table 2) did not differ from the expected value of 0.75. On the other hand, the genetic relatedness among workers in multiple-matriline colonies (0.552 ± 0.087; Table 2) was significantly lower than 0.75, but significantly higher than 0.375, i.e. the theoretical value expected when colonies are headed by two unrelated queens (*t*-test, $n = 10$, $P = 0.035$). When considering polygynous colonies only, the mean number of queens per colony ($N_{E,P}$) was 2.41 ± 0.21, and the weighted estimator of the number of queens per colony (N_E) was 1.41 ± 0.21 ($r_q = 0$) or 1.55 ± 0.29 ($r_q = r_w$).

Discussion

Our analyses of microsatellite data indicate both monandry and facultative polygyny in the ant *Pheidole pallidula*. No multiple mating of queens was detected through the analysis of mother–offspring combinations and direct typing of seminal fluid. This result is consistent with previous analyses indicating that effective queen mating frequencies are close to 1 in the Formicidae (Boomsma & Ratnieks 1996; Strassmann 2001). However, in contrast with most ant species in which a significant proportion of queens mate multiply, our data show that *P. pallidula* queens are strictly monandrous. Estimates of the inbreeding coefficient *F* and of the relatedness between mates were not significantly different from zero, indicating that mating occurs between unrelated individuals.

Thirty-eight per cent of the investigated colonies were found to be polygynous with two to four functional queens (average 2.41). These numbers are substantially higher than those previously observed through excavation censuses (6% of polygynous colonies, with a maximum of three queens per colony; L. Passera and S. Aron, personal observation).

Facultative polygyny, as reported here for *P. pallidula*, has been documented in two other species belonging to the genus *Pheidole*: *P. morrisi* (Hölldobler & Wilson 1990) and *P. dentata* (Helms 1999). In ants, polygyny may arise from various factors such as primary polygyny through pleometrosis (i.e. foundresses association; e.g. Mintzer 1987; Rissing *et al*. 1989; Hölldobler & Wilson 1990; Heinze *et al*. 2001a), intranidal mating (e.g. Passera *et al*. 1988; Chapuisat & Keller 1999a), re-adoption of related queens after the mating flight (e.g. Keller 1995; Gadau *et al*. 1998), and adoption of unrelated queens (Hölldobler & Wilson 1990). Pleometrosis in *P. pallidula* seems unlikely: foundresses associations were never observed under natural conditions. Furthermore, laboratory experiments showed that the association of young mated queens is possible but becomes unstable when the first eggs are laid: foundresses fight each other until a single queen survives (Emery 1911). Intranidal mating is equally unlikely: reproductives of *P. pallidula* engage in large mating flights and laboratory experiments showed that queens never mate close to or in their colony (Bontpart 1964). The most likely explanation for the origin of facultative polygyny in our sample might be the adoption of unrelated queens into established colonies. Our analyses show that the average within-colony relatedness in multiple queen colonies ranges from 0.138 ± 0.123 to 0.736 ± 0.111. The lowest values of this range demonstrate that at least some coexisting queens are not close relatives while the highest values do not rule out the possibility of secondary adoption of related queens after the mating flight.

Aside from variation in the number and/or relatedness among breeders, colony kin structure is also directly affected by other breeding properties (Ross 2001), such as queen replacement (Heinze & Keller 2000) and variation in reproductive skew (Keller & Reeve 1994). Queen replacement in polygynous ants is generally associated with a short lifespan of queens (Keller & Genoud 1997) and leads to transient coexistence of different matrilines in a colony, resulting in a lower nestmate relatedness than expected from the number of queens observed in the colony at a given time. A short lifespan of queens in *P. pallidula* is unlikely as natural colonies of this species extend deeply underground (such that queen mortality in winter is probably low), and queens of this species can live for more than three years in laboratory rearings (L. Passera, G. Sempo, personal communication).

Contrary to queen replacement, unequal respective contribution of queens to reproduction (reproductive skew) leads to a higher relatedness than expected from the number of queens in the colony (Heinze & Keller 2000). Several molecular genetic studies have found evidence of

reproductive skew in polygynous ants and showed that the magnitude of the skew may vary greatly not only at the species and population levels, but also among colonies within populations (Ross 1988; Bourke & Heinze 1994; Heinze 1995; Bourke *et al*. 1997; Fournier & Keller 2001; Heinze *et al*. 2001b; Reeve & Keller 2001). Although the relatively high within-colony relatedness we observe in polygynous colonies (mean = 0.552 ± 0.087) can be due to either a close kinship among queens or to reproductive skew, the latter hypothesis is supported by our data. First, some of the polygynous colonies exhibit relatedness values (range = 0.138 ± 0.123 to 0.736 ± 0.111) higher than that theoretically expected (0.469) if colonies are headed by two full-sister queens. Second, the mean queen number per colony ($N_{E,P} = 2.41 \pm 0.21$), i.e. the estimation of the absolute number of queens contributing to the brood, is significantly higher ($P < 0.0001$) than the weighted mean queen number ($N_E = 1.41 \pm 0.21$ and 1.55 ± 0.29 for $r_q = 0$ and $r_q = r_w$, respectively), i.e. the estimation of the number of queens weighted by their respective contribution to the brood. The two estimators ($N_{E,P}$ and N_E) should be equal if reproductive skew was absent. Third, the relatedness among workers estimated from our genotype frequency data is larger than the relatedness deduced from the effective pedigree number of queens. Indeed, Queller *et al*. (1988) showed that mean nestmate worker relatedness in polygynous colonies is equal to: $r_w = (3/4N) + \{[(N-1)/N] \times (r_q/4)\}$, where r_q and N are the relatedness among and the number of resident colony queens, respectively. This expression is equal to $0.75/N$ when queens are not relatives ($r_q = 0$). Using our estimate of the pedigree effective number of queens per colony ($N_{E,P} = 2.41$), this equation gives a minimum worker relatedness of 0.311 ($r_q = 0$) and a maximum relatedness of 0.364 ($r_q = r_w$) (note that if queens were full sisters $r_w = 0.421$). These estimates are largely below the value of $r_w = 0.552$ calculated from the worker genotypes. In short, the three above-mentioned points suggest the existence of reproductive skew in *P. pallidula*. However, this hypothesis is very tentative and warrants further study in this species.

While monogynous colonies are usually characterized by widely dispersing sexuals, polygynous colonies often exhibit a mixed dispersal strategy, with some females mating near the colony (and being eventually re-adopted), whereas others leave the colony and participate in mating flights. Young mated queens from polygynous colonies may (i) found colonies independently after dispersal, (ii) seek adoption in their own or an unrelated colony, or (iii) found new colonies by budding (i.e. queens leave the mother colony with workers and establish new colonies near the parental site) (Keller 1991). Previous studies reported that sexuals of *P. pallidula* engage in extensive mating flights, leading to solitary founding (Bontpart 1964). Our analyses show a significant isolation-by-distance in the investigated population of *P. pallidula*, suggesting the occurrence of some level of colony budding in this species.

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