Multiple paternity, relatedness and genetic diversity in *Acromyrmex* leaf-cutter ants

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Multiple queen-mating occurs in many social insects, but high degrees of multiple paternity have only been found in honeybees and some yellowjacket wasps. Here we report the first case of an ant species where multiple mating reduces relatedness among female offspring to values significantly lower than 0.5. Genetic analysis of a Panamanian population of the leaf-cutter ant *Acromyrmex octospinosus* showed that queens mate with at least 4–10 males. The detected (minimum) genetically effective paternity of nestmate females was 3.9 and estimates of mean relatedness among nestmate females were *ca.* 0.33. This implies that multiple queen-mating in *Acromyrmex octospinosus* reduces relatedness to 44% of the value in full-sib colonies (0.75), realizing 84% of the maximum reduction (to 0.25) that would be obtained with an infinite number of matings. Queens of Panamanian *Acromyrmex octospinosus* mate with more males than sympatric queens of *Atta colombica*, which is contrary to the positive relationship between queen-mating frequency and colony size found across more distantly related ant species. Possible selective forces that maintain high queen-mating frequencies in leaf-cutter ants are discussed.

Keywords: *Acromyrmex*; leaf-cutter ants; multiple mating; paternity; relatedness; social evolution

1. INTRODUCTION

The evolution and maintenance of multiple queen-mating in the eusocial Hymenoptera (ants, some bees and some wasps) is an enigmatic phenomenon. The behaviour is common, although it is likely to be costly for queens who often expose themselves to significant predation pressure when seeking additional matings. Reviews by Page & Metcalf (1982), Cole (1983), Starr (1984), Page (1986), Keller & Reeve (1994), Bourke & Franks (1995), Boomsma & Ratnieks (1996) and Crozier & Pamilo (1996) have addressed various not mutually exclusive fitness benefits that could potentially compensate for the costs associated with multiple mating, but explicit tests have been few and analyses of comparative data have so far failed to produce unambiguous general explanations.

Comparative data (Boomsma & Ratnieks 1996) suggest that queen-mating and offspring paternity are much more variable among genera than within genera, exemplified by the existence of genera with exclusively single mating (*Solenopsis* fire ants; Ross 1993) and exclusively high (*Vespula* yellowjacket wasps; Ross 1986; Ross & Carpenter 1991) or very high queen-mating frequencies (*Apis* honeybees; Estoup *et al.* 1994; Moritz *et al.* 1995; Oldroyd *et al.* 1995). This pattern has been corroborated by studies on other honeybee species (Oldroyd *et al.* 1996, 1997) and by a comparative analysis on *Formica* wood ants, where eight species were shown to have mostly low but somewhat variable queen-mating frequencies (Boomsma & Sundström 1998). Substantial degrees of multiple paternity among nestmate workers have also

been documented for leaf-cutter ants: *ca.* three fathers per colony in *Acromyrmex versicolor* (Reichardt & Wheeler 1996), 1–5 fathers per colony in *Atta colombica* (Fjerdingstad *et al.* 1998), but averages for ants have so far remained far below the levels of multiple paternity found in honeybees and yellowjacket wasps.

Here we present a genetic marker study on multiple paternity and relatedness in a Panamanian population of the leaf-cutter ant *Acromyrmex octospinosus*. The typical form of this species is one of the most widespread of all leafcutter ants, ranging from Mexico to Brazil. An account of the taxonomic status of Panamanian *Acromyrmex octospinosus* and some of its close relatives is given by Schultz *et al.* (1998). We show that *Acromyrmex octospinosus* has higher queen-mating frequencies than any ant species studied with genetic markers so far and that multiple queen-mating significantly reduces relatedness among (female) colony offspring. Evolutionary causes and consequences of these exceptional patterns of multiple paternity are discussed.

2. METHODS

Twenty-two mature colonies of *Acromyrmex octospinosus* were excavated in the so-called 'pond-area' of Gamboa, Panama at the beginning of the rainy season (April–May 1993 and 1994) and in January 1996. Given the modest size of the total collection area (<0.5 km²), the colonies are highly likely to belong to a single breeding population. Ten of these colonies were collected with a queen and fungus garden and set up as laboratory colonies. For the other 12 colonies no queen was available because: the queen was in an additional nest chamber that could not be reached; she was missed during excavation; the colony was queenless; or the queen died shortly after collection.

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For all 22 colonies, samples of workers and (if present) gynes (winged daughter queens) and males were stored on dry ice and afterwards in an ultra freezer at -70°C .

The queenright colonies were transported to the laboratory in Aarhus, Denmark, and reared at 25°C and 60–70% relative humidity (RH) for 2–4 years. Ants were provided with a variety of leaves and flower petals and given access to water so that they could keep their fungus gardens in good condition at close to 100% RH. Upside-down transparent plastic beakers containing the fungus garden were covered with non-transparent plastic flower pots to keep them as dark as possible. Workers from these colonies were sampled and frozen at intervals of 6–12 months.

Samples were analysed by horizontal starch-gel electrophoresis for the allozyme loci *Got* and *Tpi*, segregating for two and three alleles, respectively. Buffers (Aarhus lab code: S5 for *Got* and S4 for *Tpi*) are described in Schultz *et al.* (1998). Normally heads were used to stain for *Tpi* and gasters for *Got*. A third allozyme locus (*Mdh*) segregating for two alleles was scored from separate slices of the S5 gels, but had such low heterozygosity that it was excluded from the relatedness calculations (see below), although it did reveal one extra rare patriline in colony 41. The remaining mesosomas or parts of gasters were used to also analyse genetic variation at a microsatellite-DNA locus, segregating for six alleles in *Acromyrmex octospinosus*. For the latter analyses, total genomic DNA was isolated from individual ants using the CTAB procedure of Doyle & Doyle (1987). Microsatellite primers *Etta-5* (5'-CAGCTCTCGTAGAAGAGTAGCATG-3') and *Etta-6* (5'-CTGAACTTCGCCAGCG-3') were used to amplify the microsatellite locus *Etta5-6TF* (Fjerdingstad *et al.* 1998). The *Etta-6* primer was end-labelled with Cy5 for an ALF expressTM DNA Sequencer. PCR reactions were run in 6 ml volumes in a Peltier Thermal Cycler 200 using 2 pmol of each primer per reaction. The PCR programme was: one denaturation at 94°C for 3 min; 40 cycles of 94°C for 30 s; 58°C for 30 s; 72°C for 40 s; and finally 72°C for 10 min. PCR products were diluted three times and 1 ml of this solution was run in an ALF expressTM DNA Sequencer together with the internal standard ALF expressTM size 100 and external standard ALF expressTM size 50–500.

Between 10 and 40 (mean 29.7) female offspring per colony were analysed from the field collected samples and 40–80 (mean 59.3) additional offspring for the colonies that were reared in the laboratory after 12–30 months had passed since the colonies were collected. This time-lag was observed to excluded as much as possible any offspring that might have originated from other queens or colonies in the field (see §4). Genotypes of queens and their multiple mates were inferred from the multi-locus offspring genotypes produced in the lab and where possible checked when a recently dead queen or offspring males were available. All such checks confirmed the queen genotype inferred from the worker offspring genotypes. Because of the large offspring samples taken from the laboratory colonies, inferences of parental genotypes were normally unambiguous. Even if alternative interpretations were possible, these could be excluded because they were less parsimonious or statistically unlikely (assuming Mendelian segregation).

The inferred paternal genotypes per colony gave a minimum estimate of the number of queen-matings in *Acromyrmex octospinosus*. Two sources of error induce underestimations of the real number of patrilines present in a colony. The first is a non-detection error due to limited genetic variation at the available marker loci. Although $2 \times 3 \times 2 \times 6 = 72$ multi-locus paternal

genotypes were possible in the investigated population, allele frequencies were sufficiently uneven to make it likely that some patrilines were missed because the respective fathers had the same multi-locus genotype. An overall estimate of the magnitude of this genetic non-detection error was obtained by using the population-wide sum of squared male genotype frequencies (Pamilo 1993; see also Boomsma & Ratnieks 1996). The second source of error is due to limited (offspring) sample size, making it likely that especially rare patrilines are missed (Boomsma & Ratnieks 1996). This non-sampling error can be corrected by using a general expression developed by Pamilo (1993), $\Sigma p_i^2 = (n \Sigma y_i^2 - 1)/(n - 1)$, where y is the proportional contribution of each father and n is the number of offspring sampled. In terms of sampling, this expression yields an unbiased estimation of the genetically effective queen-mating frequency (m_e), which is equal to the reciprocal of Σp_i^2 (Starr 1984). Note, however, that this estimate still underestimates multiple paternity to some extent because genetic non-detection errors and reduced effectiveness of assigning paternity to offspring from heterozygous queens (Pamilo 1982; Pedersen & Boomsma 1999) have not been taken into account.

One way to circumvent some of these estimation errors is to calculate the effective queen-mating frequency directly from estimates of relatedness among colony offspring. Such relatedness estimates are especially accurate when large samples of offspring individuals are available so that the effect of under-sampling on relatedness estimations is negligible (in this study the number of individuals genotyped per colony was approximately an order of magnitude larger than the number of patrilines per colony). For estimations of regression relatedness (b) we used the algorithm of Queller & Goodnight (1989) as implemented in the Relatedness 4.2b program (Goodnight & Queller 1994), which provides jackknife estimates of the standard errors. This program also gives estimates of inbreeding coefficients (F_{IS}) and their (jackknife) standard errors. Regression relatednesses were subsequently compared with pedigree relatednesses (g), which were calculated from the relationship $g = 0.25 + 0.5 \Sigma p_i^2$ (Page 1986; Pamilo 1993). The distribution of the pedigree-based estimate of effective paternity across colonies was normal (Shapiro–Wilk test: $W = 0.822$; $p > 0.50$), but the corresponding distribution of Σp_i^2 deviated from normality ($W = 0.822$; $p < 0.05$). We therefore estimated the 95% confidence limits (c.l.) of the mean pedigree relatedness (g) via transformation from the 95% c.l. of effective paternity.

3. RESULTS

Queens of *Acromyrmex octospinosus* mate with 4–10 males, with an arithmetic mean of 6.1 (figure 1). Paternity contributions were moderately skewed so that the average genetically effective paternity, estimated from the reciprocals of colony-level Σp_i^2 , was 3.93 (95% c.l. 3.08–4.77) and significantly lower ($t = 3.233$; one-tailed $p < 0.01$) than the harmonic mean of the absolute number of fathers observed per colony (5.79; 95% c.l. 5.08–6.74). The observed mean genetically effective paternity (3.93) corresponds to a mean pedigree relatedness among female nestmates of 0.377 (95% c.l. across colonies 0.355–0.412). Based on the population-wide allele frequencies in the 61 inferred colony fathers there was an average probability of 0.11 that two paternal males shared the same multi-locus genotypes (Σp_i^2 taken over all multi-locus haplotypes; Pamilo 1993). This would indicate that

Figure 1. Absolute number of fathers of workers in ten colonies of *Acromyrmex octospinosus* (white bars) and the corresponding genetically effective paternities (black bars).

roughly six males were missed in the entire analysis of the ten laboratory colonies. However, the true underestimation of the absolute number of fathers must have been higher, as especially fathers with a small share in the colony offspring are likely to have been missed due to non-sampling. However, even if we assume that several rare fathers per colony may have been missed, this will not have induced a large bias because the non-sampling of fathers with small proportional contributions to offspring hardly affects estimates of relatedness and genetically effective paternity.

Direct estimates of mean relatedness and inbreeding coefficients of nestmates across colonies are given in table 1. Relatedness among female offspring (all workers except for two sets of 20 gynes in the respective field samples of colonies 19 and 25) was around 0.33 and varied little among means based on the 22 field colonies, the field samples from the ten colonies set up in the laboratory or the same ten colonies after having lived in the laboratory for at least 12–30 months. A combined estimation of relatedness among offspring females of the ten colonies

sampled in the laboratory and in the field gave virtually the same result (0.339) (table 1). Estimates of inbreeding were not significantly different from zero and were thus assumed not to have inflated the relatedness estimates (Pamilo 1985). Using the standard errors over loci, all three average relatednesses among female nestmates of the same cohort were significantly lower than 0.75, the value expected for single queen-mating ($7.31 < t < 8.18$; $p < 0.001$), and also significantly lower than 0.50, the value expected for a non-variable effective queen-mating frequency of two ($3.00 < t < 3.28$; $p \leq 0.01$). However, there was no significant difference between the three average regression relatednesses ($\pm$ s.e. loci) and the observed pedigree relatedness of 0.377 ($0.73 < t < 1.08$; $p > 0.20$). Relatedness estimates among inferred males mating with the same queen were consistent with the hypothesis that males mix randomly in mating swarms, i.e. have zero relatedness. The average relatedness between queens and their inferred multiple mates was also not significantly different from zero.

Colony-specific effective paternity, calculated from offspring relatedness, was significantly correlated ($r = 0.819$; $t = 4.044$; $p = 0.006$) with the absolute number of patriline observed per queen (figure 2). The slope of the regression plotted in figure 2 was less than one suggesting that paternity skew increases with increasing mate number, but this effect was not statistically significant (95% c.i. of the regression slope 0.31–1.13; $t = 1.558$; two-tailed $p = 0.162$).

4. DISCUSSION

The absolute and genetically effective queen-mating frequencies in the Gamboa population of *Acromyrmex octospinosus* are the highest so far reported for ants and rank among the highest reported in the eusocial Hymenoptera. Our estimates for *Acromyrmex octospinosus* are similar to those reported for *Vespula* yellowjacket wasps (Ross 1986; Ross & Carpenter 1991), and only in *Apis* honeybees with their very derived male-swarming mating system have higher queen-mating frequencies been detected (Estoup *et al.* 1994; Moritz *et al.* 1995; Oldroyd *et al.* 1995, 1996, 1997). Until the present study, the largest reported reduction of relatedness through multiple queen-mating in ants was found in a population of the leaf-cutter ant *Atta colombica* in Gamboa, Panama (average relatedness 0.52; s.e. = 0.010 over two microsatellite loci

Table 1. *Relatedness and inbreeding coefficients of female nestmates and parents in Acromyrmex octospinosus*

		relatedness		inbreeding	
	n_{colonies} ($n_{\text{individuals}}$)	mean (b)	s.e. colonies (s.e. loci)	F_{IS}	s.e. colonies (s.e. loci)
female offspring:					
queenright colonies (lab samples)	10 (593)	0.315	0.072 (0.057)	0.040	0.047 (0.031)
queenright colonies (field samples)	10 (297)	0.333	0.093 (0.051)	0.002	0.064 (0.052)
queenright colonies (lab + field samples)	10 (890)	0.339	0.089 (0.052)	0.022	0.068 (0.067)
all field colonies	22 (569)	0.326	0.044 (0.058)	0.033	0.036 (0.052)
males mating with the same queen:					
queenright colonies (lab samples)	10 (61)	0.026	0.054 (0.024)	—	—
queens and mates:					
queenright colonies (lab samples)	10 (10 + 61)	−0.024	0.113 (0.088)	—	—

Figure 2. Effective paternity as a function of the absolute number of fathers detected. Regression equation: $y = 0.722x - 0.463$ ($r^2 = 0.671$). The expected relationship when all fathers contribute equally is plotted for comparison (45° diagonal).

and 0.023 over 33 colonies; Fjerdingstad *et al.* 1998). However, our current estimate of mean nestmate regression relatedness in the ten *Acromyrmex octospinosus* laboratory colonies (the sample where multiple maternity of offspring could be completely excluded; see §2 and below) was significantly lower than the estimate in *Atta colombica* (from s.e. colonies: $t = 2.712$, $p_{\text{two-tailed}} < 0.01$).

The reduction in nestmate relatedness in *Acromyrmex octospinosus* (from the maximum of 0.75 in hymenopteran full-sib families to 0.33) amounts to 84% of the maximum possible reduction (from 0.75 to 0.25) that can theoretically be achieved through multiple mating with an infinite number of males. This implies that queens of *Acromyrmex octospinosus* realize a very substantial increase in the mean genetic diversity of their colonies through their multiple mating behaviour. However, the maximal relatedness-reducing effect of multiple mating was not achieved as there was significant skew in the paternity contributions of males mating with the same queen: all dots in figure 2 are below the diagonal and the difference between the effective paternity of (at least) 3.93 and the harmonic mean of the absolute number of fathers of (at least) 5.79 was significant. Paternity skew tends to increase with the number of fathers per colony (figure 2), but further analyses will be needed to confirm this result.

As in *Atta colombica*, multiple mating is apparently the only way by which an increase in genetic diversity among *Acromyrmex octospinosus* nestmates can be achieved. More than one wingless queen (2–3) are occasionally found when colonies of *Acromyrmex octospinosus* are excavated during the season that winged reproductives are present. It seems, however, that these additional queens are newly dealated but unmated nestmates, because they are ignored by the workers, in contrast to the mother queen who is covered by workers when disturbed and exposed to light. No such additional queens were collected in 1996, when

sampling took place outside the reproductive season. The *Acromyrmex octospinosus* population in Gamboa thus seems to be exclusively monogynous, in contrast to another sympatric *Acromyrmex* species which is facultatively polygynous (Bekkevold *et al.* 1999). The inference that *Acromyrmex octospinosus* is monogynous is corroborated by comparison of the offspring relatedness estimates from single-queen laboratory colonies and field samples from these same colonies. If additional mother queens had significantly contributed to the worker offspring sampled in the field, the latter relatedness estimates should have been lower (table 1). However, it was also clear that not all nestmate genotypes observed in the ten laboratory colonies were found in the field samples from the same colonies and vice versa. Such rare deviations may reflect sampling error involving rare patriline (perhaps unlikely because $b_{\text{field+lab}} > b_{\text{field}}, b_{\text{lab}}$; see table 1), a low frequency of brood raiding from neighbouring colonies (possible in the field but not in the laboratory), a remnant of colony founding by foundress association (Rissing & Pollock 1988), or some degree of sperm clumping making lifetime queen-mating frequencies even higher than the ones reported in figures 1 and 2.

The high queen-mating frequencies documented for *Acromyrmex octospinosus* and (to a lesser degree) for *Atta colombica* raise two kinds of questions. The first concerns the extent to which these patterns are likely to be more generally valid among other species of leaf-cutter ants. The second concerns the selective factors that could possibly explain why leaf-cutter ants have exceptionally high queen-mating frequencies.

Several other studies in leaf-cutter ants have also indicated high degrees of multiple queen-mating. Reichardt & Wheeler (1996), carrying out controlled mating experiments, found that queens of *Acromyrmex versicolor* mated with 3–4 males. Subsequent parent–offspring analysis with randomly amplified polymorphic DNA (RAPD) markers showed that these males contributed rather equally to the colony offspring. Although this study did not allow a direct estimate of relatedness among offspring of naturally mated queens, it suggests that colony-level genetic diversity through multiple queen-mating may be comparable to the values obtained for *Acromyrmex octospinosus*. A recent genetical analysis of multiple paternity in another Panamanian *Acromyrmex* species (Bekkevold *et al.* 1999) also showed a high degree of genetically effective multiple queen-mating (regression relatedness among offspring of monogynous queens = 0.40). Earlier non-genetic studies on the amounts of sperm stored by queens and males in Brazilian populations of *Atta sexdens* and *Atta leavigata* (Kerr 1961; Corso & Serzedello 1981) have suggested slightly higher absolute queen-mating frequencies (3–4) than could be genetically documented in the Panamanian *Atta* species (2.6 for *Atta colombica*, Fjerdingstad *et al.* 1998; 2–3 on average for Panamanian *Atta sexdens*, E. J. Fjerdingstad and J. J. Boomsma, unpublished data). These genetic paternity data thus confirm that multiple queen-mating is likely to be a general trait in both *Atta* and *Acromyrmex* leaf-cutter ants, which fits the earlier observed pattern that paternity is generally not highly variable among species of the same ant genus (Boomsma & Ratnieks 1996).

A variety of selection regimes could potentially explain the evolution and maintenance of multiple queen-mating

in eusocial Hymenoptera (Crozier & Page 1985). The currently most likely hypotheses are that multiple mating allows: (i) the storage of more sperm so that queens can realise a longer reproductive life span (Hamilton 1964; Cole 1983); (ii) a reduction of colony-level genetic load due to the production of non-viable diploid males (Page 1980; Page & Metcalf 1982; Pamilo *et al.* 1994); (iii) a better colony performance and more efficient defence against diseases because of increased genetic diversity among workers (Hamilton 1987; Sherman *et al.* 1988); (iv) active mating strategies of males precluding the transmission of their total sperm store while mating with a single queen (Boomsma 1996).

The Cole (1983) hypothesis predicts that multiple queen-mating is more likely to occur in species with large mature colony sizes. This trend has recently been confirmed by comparative data analysis across ants (Boomsma & Ratnieks 1996). In a more direct test on the Gamboa population of *Atta colombica*, it was further shown that there is a positive correlation between queen-mating frequency and the amount of sperm stored, suggesting that queens may indeed need to mate multiply to realize maximal lifetime fitness (Fjerdingstad & Boomsma 1998). However, data on sperm storage in both *Atta colombica* (Fjerdingstad & Boomsma 1997) and *Acromyrmex versicolor* (Reichardt & Wheeler 1996) have shown that queens store only a single male's sperm, which cannot be explained by the Cole hypothesis. In addition, *Atta colombica* has much larger and probably more long-lived colonies than *Acromyrmex octospinosus*, which is incompatible with the higher number of matings found in the latter species compared to the former.

The diploid male hypothesis (Page 1980; Page & Metcalf 1982; Pamilo *et al.* 1994) predicts selection in favour of multiple mating when the average fitness penalty for queens receiving a single matched mating at the sex determining locus is less than the reciprocal of the number of males mated with. In the current study on *Acromyrmex octospinosus*, two matched matings were detected in the four colonies for which a total of 122 males were run. This suggests that at least 7% of the matings were matched. However, both cases concerned fathers with a very low paternity share (one and two offspring, respectively), which may imply that most diploid males are removed as eggs or larvae, so that the fitness load due to matched matings may remain rather limited.

The genetic diversity hypothesis (Crozier & Page 1985; Hamilton 1987; Sherman *et al.* 1988; Keller & Reeve 1994; Schmid-Hempel 1994) has not yet been tested in leaf-cutter ants, although this group holds great potential to do so. Parasites are likely to impose a particular challenge to the higher attine ants (such as *Atta* and *Acromyrmex*), because they are completely dependent on rearing clonal fungi in their underground nest chambers (Chapela *et al.* 1994). These ants therefore not only have to control their own parasites, but also the parasites of their mutualistic fungus, which has very limited potential to evolve novel resistance genes as recombination is probably absent. The leaf-cutter ants are therefore particularly suitable to test whether genetically diverse colonies perform better under disease-stress than genetically more homogeneous colonies, but such work has not yet been done.

Finally, it has recently been suggested that ant males may be selected to mate with more than one queen (Boomsma 1996; Fjerdingstad & Boomsma 1997; additional references of multiple mating males in Boomsma & Ratnieks (1996, p. 953)). This possibility has not been taken into account in earlier considerations and would imply that some of the critique by Crozier & Page (1985) on the Cole (1983) hypothesis would need reconsideration. Crozier & Page's main point was that if storing more sperm would be the main reason for queens to pursue multiple matings, it was hard to understand why natural selection had not produced males with larger sperm stores, so that queens could avoid costly additional matings. This argument implicitly assumes that males have no fitness agenda of their own and that additional matings for males are always difficult to obtain, conjectures which at closer inspection may prove to be not universally true.

We thank Kirsten Petersen and Camilla Håkansson for running many of the gels necessary for this study, the Smithsonian Tropical Research Institute for providing logistic help and facilities to work in Gamboa and the Instituto Nacional de Recursos Naturales Renovables (INRENARE) for permission to sample ants in Panama. Jeanette Bot, Rebekka Gadeberg and Mark Goldsmith helped maintain the lab colonies and Dorte Bekkevold, Jeanette Bot, David Nash and Jes Pedersen gave constructive comments on the manuscript. This work has been supported by several grants from the Carlsberg Foundation and the Danish Natural Science Research Council.

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