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Evolutionary transition from single to multiple mating in fungus-growing ants

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Abstract

Queens of leafcutter ants exhibit the highest known levels of multiple mating (up to 10 mates per queen) among ants. Multiple mating may have been selected to increase genetic diversity among nestmate workers, which is hypothesized to be critical in social systems with large, long-lived colonies under severe pressure of pathogens. Advanced fungus-growing (leafcutter) ants have large numbers (10^4–10^6 workers) and long-lived colonies, whereas basal genera in the attine tribe have small (< 200 workers) colonies with probably substantially shorter lifespans. Basal attines are therefore expected to have lower queen mating frequencies, similar to those found in most other ants. We tested this prediction by analysing queen mating frequency and colony kin structure in three basal attine species: *Myrmicocrypta ednaella*, *Apterostigma collare* and *Cyphomyrmex longiscapus*. Microsatellite marker analyses revealed that queens in all three species were single mated, and that worker-to-worker relatedness in these basal attine species is very close to 0.75, the value expected under exclusively single mating. Fungus growing *per se* has therefore not selected for multiple queen mating. Instead, the advanced and highly productive social structure of the higher attine ants, which is fully dependent on the rearing of an ancient clonal fungus, may have necessitated high genetic diversity among nestmate workers. This is not the case in the lower attines, which rear fungi that were more recently derived from free-living fungal populations.

Keywords: attine ants, fungus-growing ants, microsatellite markers, multiple mating, paternity, relatedness

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Introduction

Fungus-growing ants are fascinating products of evolution through natural selection. The ancestors of what was to become the attine tribe of fungus-growing ants started to cultivate lepiotaceous fungi ≈ 50 million years ago (Ma) (Wilson 1971; Mueller *et al*. 1998). Since then, a spectacular radiation has occurred, resulting in 12 extant genera with ≈ 200 described species, all obligately dependent on farming mutualistic fungi in (usually) underground nest cavities (Schultz & Meier 1995; Wetterer *et al*. 1998). Current life histories of attine ants are remarkably variable. At one extreme, some of the lower attines have a single queen and up to 50 monomorphic workers cultivating a walnut-sized fungus garden on a substrate of fragmented organic debris. At the other extreme are the highly specialized *Atta* leafcutter ants that have mature colonies with a (usually single) giant queen, a total of up to five million workers subdivided into multiple, physically different worker castes and hundreds of football-sized underground fungus gardens (Hölldobler & Wilson 1990).

Adaptations of attine ants and their symbiont fungi reflect the progressive evolution to larger and more complex mutualistic societies that has occurred across the

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attine tribe. The lower attines appear to occasionally acquire new fungal symbionts (Mueller *et al*. 1998), which suggests that these ants have retained relatively unspecialized adaptations towards fungus growing. The higher attines, however, have a long history of cultivating clonal fungal lineages and show much closer co-evolutionary connections between ant and fungal lineages (Chapela *et al*. 1994).

Low genetic variation under clonal reproduction is thought to reduce the ability of populations to adapt to changing environments and to increase the relative evolutionary potential of associated pests and diseases. In human societies the rearing of clonal crops is a high-risk enterprise: the yield may be superior in the short run and under optimal conditions, but the chance that stands without genetic variation are swept away by some virulent pathogen are substantial (Fisher 1930; Beardmore 1983; Hamilton 1987). A number of large, long-lived ant societies are known to have multiple queens and/or multiple matings. This complex pedigree structure gives a high genetic diversity among colony nestmates (Keller & Reeve 1994), compared with the low genetic diversity of the full-sib family structures found in many other ant societies (Boomsma & Ratnieks 1996). Societies such as the higher attine leafcutter ants have been hypothesized to be in particular need of genetic diversity (Boomsma *et al*. 1999), to optimize both colony performance (Crozier & Page 1985) and defence against infectious diseases of the ants themselves (Hamilton 1987; Sherman *et al*. 1988; Schmid-Hempel 1994) or their fungal gardens (Boomsma *et al*. 1999; Currie *et al*. 1999).

Most attine species have a single queen per colony (Weber 1972; Weber 1979). This implies that within-colony genetic diversity can only be increased by selection on queens to mate with multiple males, a behaviour that is likely to incur fitness costs through the prolonged exposure to predators during the mating flight (Weber 1972; Fowler *et al*. 1986). Recent studies have shown that *Atta* and *Acromyrmex* leafcutter ants have the highest queen mating frequencies reported so far for ants (effective queen mating frequencies of ≈ two and four, respectively) (Fjerdingstad *et al*. 1998; Boomsma *et al*. 1999; Bekkevold *et al*. 1999). This suggests that queen mating frequency and colony kin structure may have evolved in concert with the increase in colony size, caste complexity and degree of cultivar specialization that typifies the various clades in the attine tribe (Chapela *et al*. 1994; Mueller *et al*. 1998). However, this hypothesis remains to be tested because mating frequencies in the lower attines are unknown. Here we report the first study on queen mating frequency and colony kin structure in representatives of three genera of lower attine ants. DNA microsatellite markers indicate that single queen mating prevails in all cases.

Materials and methods

Population sampling

Field collections were made in the vicinity of Gamboa in the Republic of Panama. Colonies of *Cyphomyrmex longiscapus* ($n = 11$) and *Apterostigma collare* ($n = 10$) were collected in May and June 1998, and colonies of *Myrmicocrypta ednaella* ($n = 8$) in the spring of 1996. Mother queens and eight to 15 offspring per nest (except for four colonies of *M. ednaella* and one of *C. longiscapus* where only three to six and four workers were available, respectively) were genotyped on an ALF express™ automatic sequencer for two microsatellite loci for each species.

Cloning, DNA extractions and microsatellite analysis

DNA for constructing genomic libraries for each species was extracted from single worker ants (except for *C. longiscapus*, where it was necessary to pool three individuals) using a high-salt purification procedure. Two to six micrograms of high-molecular-weight DNA was digested with *Sau*3AI, and fragments of 200–500 bp were purified using Chromaspin 100 columns. The fragments were then ligated into the *Bam*HI site of the dephosphorylated plasmid vector pBluescript II-SK+. The ligation products were transformed into competent *Escherichia coli* cells, followed by recombinant screening using selective agar plates containing X-Gal, IPTG and ampicillin. Two-thousand recombinant clones were then screened for each species with synthetic oligonucleotides $(GT)_{10}$ $(CT)_{10}$ and $(AAT)_{10}$, end-labelled with DIG-dUTP. The positive clones were sequenced using an ALF express™ automatic sequencer. Six primer sets were designed and polymerase chain reaction (PCR) conditions were optimized (Table 1).

DNA extractions used for genotyping individual ants were performed using a standard CTAB extraction protocol (Doyle & Doyle 1987). After homogenization, the samples were incubated for 1 h with 1 μL of proteinase K (10 mg/mL) at 55 °C in 350 μL of CTAB buffer (1% hexadecyltrimetyl ammonium bromide, 0.75 M NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA). DNA was then purified using an isoamylalcohol–chloroform extraction, followed by ethanol precipitation. The DNA was suspended in 50 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and stored at –20 °C.

PCR reactions were carried out in 6-μL volumes containing 1 μL of template DNA, 2 pmol of each primer (one primer was end-labelled for specific use on the ALF express™), 0.2 mM of dATP dCTP, dGTP and dTTP, 1× *Taq* polymerase buffer and 0.375 units of *Taq* polymerase (Pharmacia).

Table 1 Primer sequences, number of alleles observed (n_a), expected heterozygosity (H_E) and optimal annealing temperatures (T_m; °C) for six polymorphic microsatellite loci from three species of fungus-growing ants

Locus	Species	Core sequence	Primer sequences (5′ → 3′)	n_a	H_E	T_m
Crypta1–2	*Myrmicocrypta ednaella*	$(GA)_{16}(GT)_9$	F: GTATCACGTTCTTGCACAATCC R: CGCGCGTTTATTTACATGAC	3	0.65	56.0
Crypta3–4	*Myrmicocrypta ednaella*	$(AG)_{12}$	F: CGTTCAAGGCGTATATATTGC R: GCTCTGCCTGGATAAGATGC	3	0.48	56.0
Cypho3–4	*Cyphomyrmex longiscapus*	$(TC)_{22}$	F: ATAACAGCGAATGGGTGCC R: GCAACGTGAACACAGGGC	8	0.84	59.7
Cypho5–6	*Cyphomyrmex longiscapus*	$(TG)_{18}$	F: CTTTAATCTTCTGCCGCC R: AAAAAATCCCTACATACTCGTG	9	0.82	58.5
Aptero1–2	*Apterostigma collare*	$(AC)_{20}$	F: TCGCACAAATAACAAATACAC R: CACCACGGACAAAACTCAC	5	0.65	53.5
Aptero3–4	*Apterostigma collare*	$(TG)_{18}$	F: ACTGCACTGAGATTGGAAACC R: TATCGACACGCTTAGAAATCC	3	0.52	55.0

Data analysis

The number of haploid fathers per colony could easily be derived by comparing the mother–offspring genotypes. In some of the nests, the queen was not found, so that her genotype had to be inferred from the multilocus genotypes of the worker offspring, under the assumption of Mendelian segregation. Because of the high heterozygosity of the microsatellite markers, such inferences were not problematic and allowed the unambiguous assessment of the paternal haplotypes in each colony. Estimates of relatedness of nestmate workers (r) were derived from the inferred effective paternities (m_e) by using the relationship (Pamilo 1993):

$$r = 0.25 + 0.5/m_e$$

Two sources of error are possible in the estimation of queen mating frequencies from genotypic pedigree data and both may lead to an underestimate of queen mating frequency. The first is a nondetection error caused by limited genetic variation at marker loci, while the second is a result of nonsampling of paternal genotypes when the number of offspring analysed is relatively small (Boomsma & Ratnieks 1996). Accurate correction procedures for both sources of error exist (Pamilo 1982, 1993; Boomsma & Ratnieks 1996; Pedersen & Boomsma 1999), and we have followed the procedure of Pedersen & Boomsma (1999). Such corrections require a known proportion of double-mated queens and an empirical estimate of the mean and variance of paternity skew among workers in colonies with double-mated queens. When double-mated queens are not present in the sample of colonies investigated, one can assume that the next colony genotyped would have revealed a double-mated queen and thus evaluate the detection efficiency of double mating based on a range of possible paternity skews (Boomsma & Ratnieks 1996). This produces an upper limit for the frequency of double queen mating when no such double matings have been observed. This procedure is very conservative, especially when the number of colonies analysed is limited and paternity skew is high, as is often the case when multiple queen mating is rare (Boomsma & Sundström 1998; Boomsma & Van der Have 1998).

Results

Primer sequences, expected heterozygosity (H_E) and optimal annealing temperatures (T_m) for the six microsatellite loci are given in Table 1. Genetic analysis of the 29 colonies of the three species, *Myrmicocrypta ednaella*, *Apterostigma collare* and *Cyphomyrmex longiscapus*, showed only single queen mating (Table 2). Following the procedure outlined above, we estimated the upper limit of double queen mating in these populations by: (i) assuming that the next colony sampled would exhibit a double-mated queen; and (ii) deriving nondetection and nonsampling errors for a range of paternity-skew values of 0.6–0.9. This yielded nonidentification errors for double mating of 0.06–0.60 and frequencies of double queen mating of maximally 28% (Table 2). In particular, a hypothetical paternity skew of 0.9 yields a large nonsampling error, and thus a relatively high estimate of the expected frequency of double matings. However, the genetic contribution of second fathers with a paternity share of 10% is very low, so that the effective paternity and relatedness estimates are only minimally affected. For the three species studied, the genetically effective queen mating frequency was estimated to be, at most, 1.07, and the corresponding minimum relatedness among nestmate workers was 0.715 (Table 2).

Table 2 Paternity and offspring relatedness in three basal species of attine fungus-growing ants

	Myrmicocrypta ednaella	*Cyphomyrmex longiscapus*	*Apterostigma collare*
Observed frequency of double mating	0	0	0
No. of colonies analysed	8	11	10
No. of offspring workers genotyped	51	88	130
Maximal frequency of 'observed' double mating*	0.111	0.083	0.091
Nondetection error*	0.18	0.03	0.11
Nonsampling error†	0.06–0.52	0.03–0.44	0.00–0.26
Total error range for identification of double mating†	0.23–0.60	0.06–0.46	0.17–0.38
Corrected maximal frequency of double queen mating†	0.14–0.28	0.09–0.15	0.11–0.15
Effective paternity of nestmate workers*	1.05–1.07	1.03–1.04	1.03–1.06
Inferred relatedness among nestmate workers*	0.715–0.725	0.728–0.736	0.723–0.737

*Estimated according to Pamilo (1993) and Boomsma & Ratnieks (1996), assuming that the next colony would have had a double-mated queen.
†Estimated according to Pedersen & Boomsma (1999).

Discussion

Our analysis shows that three species of lower attine ants have predominantly, if not exclusively, single queen mating. A larger sample of colonies will be required to establish whether double queen mating never occurs, or to produce a precise estimate of the frequency of this behaviour. Even if double queen mating did occur, there is no doubt that it happens at such a low frequency that the average relatedness of nestmate workers is affected only minimally. This implies that the mating systems of the lower attine ants are not different from those of other ants with small colonies, which generally exhibit a low frequency of double mating or are exclusively single mated (Boomsma & Ratnieks 1996).

The mating systems of the three investigated lower attines are in sharp contrast to the record high queen-mating frequencies that have recently been documented for higher attine ants. These earlier studies showed that relatedness among nestmate workers was reduced to 0.50 in *Atta colombica* (minimum range of mates per queen: 1–5; Fjerdingstad *et al*. 1998), to 0.40 in *Acromyrmex echinatior* (minimum range of mates per queen: one to four; Bekkevold *et al*. 1999) and to 0.33 in *Ac. octospinosus* (minimum range of mates per queen: four to 10; Boomsma *et al*. 1999). Statistical comparison of the reported relatedness estimates for these higher attines with the lower limits of the relatednesses (equivalent to the highly conservative upper limits of m_e) found for the lower attines (Table 2), showed that the differences between these groups of species were highly significant ($5.29 < t < 8.92$, $P < 0.001$ in all *t*-tests comparing the relatedness distribution of means across colonies in the higher attines with each single minimum value for the lower attine species).

The phylogeny of the attine tribe is known from studies by Schultz & Meier (1995) and Wetterer *et al*. (1998). Although the number of attine lineages examined for mating frequency is too low for a formal phylogenetic analysis of attine queen mating frequency (cf. Grafen 1989; Harvey & Pagel 1991), some definite conclusions are possible. Table 3 summarizes the genetically effective number of matings (m_e) and the observed distribution of matings in five species of attine ants, for which accurate genetic mother–offspring data are available from this study and from previous studies by Fjerdingstad *et al*. (1998) and Boomsma *et al*. (1999). Non-detection errors for the higher attines were 0.016 for *At. colombica* (Fjerdingstad *et al*. 1998) and 0.11 for *Ac. octospinosus* (Boomsma *et al*. 1999). A third study on *Ac. echinatior* (Bekkevold *et al*. 1999) confirmed multiple queen mating, but here the nondetection error was much larger (0.26–0.41) so that the absolute number of queen mates per colony could not be estimated accurately. After pooling the data on absolute paternity into a lower attine group (the three species analysed here) and a higher attine group (the previously reported data for *Ac. octospinosus and At. colombica*; Fjerdingstad *et al*. 1998; Boomsma *et al*. 1999), the difference in queen mating patterns between the groups was also found to be highly significant when applying a contingency table test ($G = 68.20$, $P < 0.001$). The test on the differences in the observed (minimal) number of queen matings given in Table 3 thus confirms the analysis based on relatedness among nestmate workers presented in the Results. As reported by Boomsma *et al*. (1999), the difference in queen mating frequency between Panamanian *At. colombica* and *Ac. octospinosus* was also significant ($P < 0.01$), based on the relatedness estimates reported in that study and in Fjerdingstad *et al*. (1998).

Table 3 Comparative data on paternity and colony size in attine ants

Species*	m_e†	Observed distribution of the number of fathers per colony				Colony size‡
		1	2	3	≥ 4	
Atta colombica	2.31	3	13	15	5	10^6–10^7
Acromyrmex octospinosus	3.43	0	0	0	10	10^4–10^5
Cyphomyrmex longiscapus	≤ 1.04	11	0	0	0	10^1–10^2
Apterostigma collare	≤ 1.07	10	0	0	0	10^1–10^2
Myrmicocrypta ednaella	≤ 1.06	8	0	0	0	10^1–10^2

*The five species are shown in the order in which they appear in the attine phylogeny of Schultz & Meier (1995). The genera *Atta* and *Acromyrmex* are the most advanced genera, *Cyphomyrmex* is placed in the middle of the tree and *Apterostigma* and *Myrmicocrypta* are considered to be basal attine genera (Schultz & Meier 1995).

**$P < 0.01$; ***$P < 0.001$.

†m_e: effective paternity (Pamilo 1993).

‡Approximate sizes of mature colonies for the lower attines were obtained from the colonies collected for this study (see also Mueller & Wcislo 1998). Colony sizes typical for *Acromyrmex* and *Atta* were obtained from the literature (Lewis 1975; Fowler *et al*. 1986).

The results of Table 3 indicated that there are three possible scenarios for the evolutionary transition from single to multiple queen mating. First, it occurred prior to or coincident with the origin of the higher attines. This would imply that the higher attine genera *Trachymyrmex* and *Sericomyrmex* also have multiple queen mating. Second, it occurred during the evolution of the *Trachymyrmex* lineage (which is paraphyletic, Schultz & Meier 1995) and the *Sericomyrmex* lineage, so that some primitive *Trachymyrmex* have low mating frequencies, whereas some derived *Trachymyrmex* (those closer to the leafcutters) have elevated mating frequencies. Finally, it occurred at the origin (coincident with) of the leafcutter clade (including only *Acromyrmex* and *Atta*). To discriminate between these possible scenarios, we are currently performing detailed genetic analyses of several species of the *Trachymyrmex/Sericomyrmex* group. The results reported here indicate that fungus growing itself has not triggered a concurrent transition of queen mating behaviour and colony kin structure in attine ants. Apparently, the adaptive radiation and further specialization after the origin of fungus farming led to correlated responses in a suite of traits, one of them being multiple queen mating and the resulting increased genetic diversity of colonies.

Other factors that may have driven the evolution towards high queen mating frequency are: (i) selective pressures related to large colony size and physical caste differentiation; (ii) physiological innovations required for processing fresh leaves; and/or (iii) the mutualistic interaction with highly specialized fungi. The first explanation is supported by some correlative evidence across and within ant species (Cole 1983; Boomsma & Ratnieks 1996; Fjerdingstad & Boomsma 1998), but cannot explain why other ants (e.g. *Formica, Lasius, Solenopsis*; reviewed in Boomsma & Ratnieks 1996) with large colonies did not evolve mating systems similar to the higher attine ants. It can also not explain why queens of *Ac. octospinosus* (colony sizes of 10–100 000; Weber 1972) mate significantly more often than queens of *At. colombica* (colony sizes of 1–5 million; Weber 1972; Fjerdingstad *et al*. 1998; Boomsma *et al*. 1999). The second and third hypotheses are more plausible because the processing of fresh leaves and the rearing of clonal fungi are unique traits for the higher attine ants (Hölldobler & Wilson 1990). The lower attines appear to acquire new fungal symbionts frequently over evolutionary and even ecological time (Mueller *et al*. 1998), whereas the higher attines have a much longer history of clonal fungus transmission and thus tight coevolution between ant lineages and a limited number of fungal clones (Chapela *et al*. 1994). Ancient clones accumulate deleterious mutations and have low genetic variation on which natural selection can take place (Kondrashov 1988). In addition, their constant and slowly evolving genotypes make it easy for parasites to evolve precise adaptations for evading host defences (Seger & Hamilton 1988). Compensation for these disadvantages can only come from increased genetic diversity in the mutualistic partner of the fungal clones. It is therefore not unlikely that the evolution of multiple queen mating in attine ants is driven by a 'red queen' process, where increased genetic diversity of host ants became necessary in attine lineages that had become highly specialized on a derived and vulnerable group of clonally propagated fungal symbionts. This hypothesis is akin to the so-called 'disease hypothesis' or 'genetic diversity hypothesis' for the evolution of multiple queen mating in social Hymenoptera

(Hamilton 1987; Sherman *et al*. 1988; Schmid-Hempel 1994). However, it adds an interesting dimension, related to the cost of long-term clonality in an associated mutualist, whose first group of specialized, sexual and horizontally transmitted virulent fungal parasites has just been identified (Currie *et al*. 1999).

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