

# Identifying the transition between single and multiple mating of queens in fungus-growing ants

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Obligate mating of females (queens) with multiple males has evolved only rarely in social Hymenoptera (ants, social bees, social wasps) and for reasons that are fundamentally different from those underlying multiple mating in other animals. The monophyletic tribe of ('attine') fungus-growing ants is known to include evolutionarily derived genera with obligate multiple mating (the *Acromyrmex* and *Atta* leafcutter ants) as well as phylogenetically basal genera with exclusively single mating (e.g. *Apterostigma*, *Cyphomyrmex*, *Myrmicocrypta*). All attine genera share the unique characteristic of obligate dependence on symbiotic fungus gardens for food, but the sophistication of this symbiosis differs considerably across genera. The lower attine genera generally have small, short-lived colonies and relatively non-specialized fungal symbionts (capable of living independently of their ant hosts), whereas the four evolutionarily derived higher attine genera have highly specialized, long-term clonal symbionts. In this paper, we investigate whether the transition from single to multiple mating occurred relatively recently in the evolution of the attine ants, in conjunction with the novel herbivorous 'leafcutter' niche acquired by the common ancestor of *Acromyrmex* and *Atta*, or earlier, at the transition to rearing specialized long-term clonal fungi in the common ancestor of the larger group of higher attines that also includes the genera *Trachymyrmex* and *Sericomyrmex*. We use DNA microsatellite analysis to provide unambiguous evidence for a single, late and abrupt evolutionary transition from exclusively single to obligatory multiple mating. This transition is historically correlated with other evolutionary innovations, including the extensive use of fresh vegetation as substrate for the fungus garden, a massive increase in mature colony size and morphological differentiation of the worker caste.

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

## 1. INTRODUCTION

Multiple mating of queens in social Hymenoptera is a derived trait, occurring only in relatively few evolutionarily derived taxa of ants, social bees and social wasps (Boomsma & Ratnieks 1996). A possible reason for this remarkable pattern is that kin selection on helping behaviour is less effective when workers are mostly half sisters than when they are full sisters (Hamilton 1964). Multiple queen mating is therefore only evolutionarily stable after helpers (workers) have irreversibly lost their ability to mate and when the behaviour significantly enhances queen fitness. The possible fitness benefits to queens of social Hymenoptera are fundamentally different from those applying to females of non-social animals (Boomsma & Ratnieks 1996; Strassmann 2001), because queens of social insects choose the prospective father(s) of their offspring only once and do not subsequently re-mate. Current hypotheses to explain multiple mating in social insects have therefore concentrated on sperm limitation and on

the advantages of genetically diverse offspring, rather than on female choice of superior mates and sperm displacement (Boomsma & Ratnieks 1996; Schmid-Hempel 1998; Strassmann 2001). Multiple mating in social insects is thus predicted to be associated with high longevity and fecundity of queens, traits that are typical for the evolutionarily more advanced species of ants, social bees and social wasps, and, indeed, it is in some of these taxa that obligate multiple mating has been found (Boomsma & Ratnieks 1996).

The 12 genera of fungus-growing ants (Formicidae: Attini) contain ca. 210 described species (Chapela *et al.* 1994; Schultz & Meier 1995; Wetterer *et al.* 1998), all of which obligately cultivate mutualistic fungi upon which they are completely dependent (Weber 1972). Based on their fungal associations, attine ants can be divided into two distinct groups. On the one hand the lower attines are characterized by small colony sizes, a relatively heterogeneous collection of symbionts with free-living counterparts, and rather generalized behavioural interactions with these fungi (Chapela *et al.* 1994; Mueller *et al.* 1998). Higher attines, on the other hand, cultivate a highly derived, monophyletic group of fungi lacking known

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free-living relatives and possess unique adaptations for interacting with these fungi (Chapela *et al.* 1994; Mueller *et al.* 2001). Within the four genera of higher attine ants, there is another major, but more gradual evolutionary transition. The basal genera *Trachymyrmex* and *Sericomyrmex* have approximately monomorphic worker castes, as in the lower attines. Colony sizes of *Trachymyrmex* spp. are hardly larger than the largest colonies of lower attines and they exploit green leaves and flowers only marginally better than do the lower attines. However, the colonies of some *Sericomyrmex* spp. may be an order of magnitude larger. A significant part of the diet of *Sericomyrmex* spp. consists of flowers and fruit, but they have not been observed to process green leaves to any significant degree in the field. The two most derived genera *Acromyrmex* and *Atta* are often referred to as the leafcutter ants. They have large to very large colonies, rely exclusively on fresh vegetation (mostly leaves), and have highly polymorphic worker castes (Wilson 1971; Weber 1972; Hölldobler & Wilson 1990).

Previous studies with microsatellite DNA markers have shown that lower attines have singly mated queens (Villesen *et al.* 1999; Murakami *et al.* 2000), whereas the most derived higher attines, the *Atta* and *Acromyrmex* leaf-cutting ants, are invariably multiply mated (Fjerdingstad *et al.* 1998; Bekkevold *et al.* 1999; Boomsma *et al.* 1999; Fjerdingstad & Boomsma 2000). Because queen mating frequencies of leafcutter ants are the highest so far documented for ants, these comparative data imply that fundamental changes in selection on the mating system have taken place during the evolutionary history of the attine ants. The objectives of the present study are: (i) to assess whether a single transition or multiple transitions from single to multiple queen mating occurred; (ii) to assess whether such transition(s) occurred in the common ancestor of all higher attine ants and/or in the common ancestor of the leafcutter ants, i.e. to assess whether they occurred in conjunction with the shift to rearing specialized long-term clonal fungi, or in conjunction with entering the herbivorous 'leafcutter' niche; (iii) to provide a first estimate of the distinctness of the transition(s) by inferring the likelihood that facultative multiple mating occurs in at least some of the extant genera; (iv) to re-evaluate the earlier data on queen mating frequency in attine ants with novel statistical methods, allowing the estimation of confidence limits around the means obtained; and (v) to make a more precise inference of the selection forces that may have promoted the evolution and maintenance of multiple queen mating. To fulfil these objectives, we analysed queen mating-frequency in five species of the key genera *Trachymyrmex* and *Sericomyrmex*, by using a series of highly variable DNA microsatellite markers (Villesen *et al.* 1999, 2002).

#### (a) *Natural history and systematic position of Trachymyrmex and Sericomyrmex*

The genus *Trachymyrmex* currently consists of 41 described species. These are geographically widespread in the Americas, ranging from the northern United States to Argentina. Worker size is strictly monomorphic in some species and slightly polymorphic in others (Hölldobler & Wilson 1990; Beshers & Traniello 1994). *Trachymyrmex* spp. seem to prefer insect faeces, dead vegetable matter

and flower parts as primary substrates for their fungus gardens. Worker number in mature colonies of the investigated species varies from *ca.* 100–500 (Weber 1966, 1972; Hölldobler & Wilson 1990; J. J. Boomsma, personal observation), which implies that colony sizes are generally larger than those typical for the lower attines but orders of magnitude smaller than those of the leafcutter ants. Based on available data, *Trachymyrmex* colonies have a single queen (Murakami 1998; J. J. Boomsma, unpublished data).

The genus *Sericomyrmex* currently consists of 20 described species, which are easily distinguished from other attines by the abundant flexuous hairs on their cuticle. The genus is distributed from Mexico to southern Brazil. In the field, *Sericomyrmex* spp. use fruit, flowers, some leaves and possibly dead vegetable matter as fungal substrates (Urich 1895; Weber 1945, 1972). Experimental laboratory colonies readily accept, cut and use fresh leaves of bramble (*Rubus* sp.; not occurring in the native range of the ants), showing that these ants can handle fresh leaves when available in or very close to the nest (P. Villesen, personal observation). Colony sizes range from less than 100 to *ca.* 3000 workers (Weber 1972; Murakami 1998; T. R. Schultz, personal observation). The mature colonies of the *Sericomyrmex* species investigated here had a few thousand workers and several garden chambers. Workers were monomorphic, but a few species with a slight polymorphism in the worker caste are known (Hölldobler & Wilson 1990). A recent study by Murakami *et al.* (2000), based on a CA-repeated primed-PCR DNA fingerprinting technique, has suggested that some *Trachymyrmex* species are singly mated, but that multiple mating occurs in *Sericomyrmex amabilis*. This technique allows the estimation of colony kin-structure based on band-sharing statistics, but does not produce accurate assessments of the effective queen mating frequency and of possible confounding effects of facultative polygyny or occasional unrelated workers due to raids on or merging with neighbouring colonies (Adams *et al.* 2000). As multiple queens are occasionally found in Panamanian *Sericomyrmex* colonies (J. J. Boomsma, personal observation), we therefore reanalysed the *S. amabilis* samples of Murakami *et al.* (2000) with our DNA microsatellite markers in order to distinguish between multiple queen mating and polygyny.

The higher attines as a whole (*Trachymyrmex*, *Sericomyrmex*, *Acromyrmex* and *Atta*) are a monophyletic group (Chapela *et al.* 1994; Schultz & Meier 1995; Wetterer *et al.* 1998). Within the higher attines, the leafcutting genera *Atta* and *Acromyrmex* comprise a derived, monophyletic group, whereas *Sericomyrmex* is the basal monophyletic clade of the higher attines (Schultz & Meier 1995; Schultz 2000). The genus *Trachymyrmex* as currently defined is probably paraphyletic, both with respect to *Sericomyrmex* and with respect to (*Acromyrmex* plus *Atta*). Thus, some *Trachymyrmex* species will ultimately have to be transferred to the genus *Sericomyrmex*, whereas one or a few others (e.g. *T. diversus*, which does not occur in Panama) may be transferred to the genus *Acromyrmex* (Schultz & Meier 1995; Schultz 2000; T. R. Schultz, unpublished data). Natural history data for *Acromyrmex* and *Atta* have been summarized by various authors (e.g. Weber 1972, 1979; Fowler *et al.* 1986; Hölldobler & Wilson 1990).

## 2. METHODS

### (a) Population sampling

Field collections were made in and around Gamboa, Fort Sherman and El Llano in the Republic of Panama. Colonies of *Trachymyrmex* cf. *zeteki* ( $n = 8$ ), *T. cornetzi* sp. 1 ( $n = 6$ ) and *T. cornetzi* sp. 2 ( $n = 10$ ) were collected in May–June 1998, whereas colonies of *S. amabilis* ( $n = 5$ ) and *S. cf. amabilis* ( $n = 5$ ) were collected in spring 1996 and May–June 1998. The colonies of *S. amabilis* ( $n = 4$ ) previously analysed by Murakami *et al.* (2000) were collected in 1996–1997 at Barro Colorado Island, ca. 18 km from Gamboa. Mother queens and offspring workers were genotyped on an ALF express automatic sequencer for three to four microsatellite loci per species (see also below).

The genera *Trachymyrmex* and *Sericomyrmex* have not been revised taxonomically, and many new and cryptic species are likely to exist. The names used in this study are the most accurate that are currently available. In spite of the lack of described species, the molecular data indicate that all species identified are proper taxonomic entities with homogeneous non-overlapping gene pools for the marker loci. Voucher specimens have been deposited at the National Museum of Natural History (USNM), Smithsonian Institution, Washington, DC, under species names used in the present paper: *S. amabilis*, *S. cf. amabilis*, *T. cf. zeteki*, *T. cornetzi* sp. 1 and *T. cornetzi* sp. 2.

### (b) DNA extractions and PCR

DNA for genotyping individual ants was extracted using a modified Chelex 100 extraction protocol (Walsh *et al.* 1991). After homogenization, samples were incubated for 1 h at 55 °C in 200 µl 5% Chelex solution (5% Chelex in 50 mM Tris pH 8.0), followed by vortexing for 30 s and boiling for 12 min. The DNA was then centrifuged at 13 000 rpm for 2 min and stored at 20 °C until use.

PCR reactions were carried out in 6 µl volumes containing 1 µl of extracted template DNA, 2 pmol of each PCR primer (one primer was 5' Cy end-labelled for specific use on the ALF express), 0.2 mM dATP, dCTP, dGTP and dTTP, one unit of *Taq* polymerase buffer and 0.375 units of *Taq* polymerase (Pharmacia).

After testing a total of 32 microsatellite loci (Fjerdingstad *et al.* 1998; Villesen *et al.* 1999, 2002; Ortius-Lechner *et al.* 2000) for amplification and variability, we decided to use six of these loci in the present analysis of queen mating frequency in *Trachymyrmex* and *Sericomyrmex*. The loci used had  $4.9 \pm 2.4$  alleles, with expected heterozygosities of  $0.697 \pm 0.159$  (mean  $\pm$  s.d.). Two of the markers (Cypho9\_10, Cypho15B\_16B) were originally developed for *Cyphomyrmex longiscapus* (Villesen *et al.* 2002) and one of these, Cypho15B\_16B, was highly variable in all five species. We further used three markers (Trachy 3\_4, Trachy 5\_6, Trachy 11\_12) developed for *T. cf. zeteki* (Villesen *et al.* 2002) and one locus (Ech3385) developed for *Acromyrmex echinator* (Ortius-Lechner *et al.* 2000).

### (c) Phylogeny

Bootstrap values are based on 100 bootstrap pseudoreplicates (Felsenstein 1985) obtained with maximum-likelihood phylogenetic analyses of an 804 bp fragment of the nuclear EF-1 $\alpha$  gene and an 807 bp fragment of the mitochondrial COI gene using the computer program PAUP 4.0b4a (Swofford 2001). Likelihood analyses employed the most complex model available (general time-reversible with gamma-distributed rates and a proportion of nucleotide sites assumed to be invariant; Rodriguez

*et al.* 1990). Due to the model and methods employed and the bootstrap values obtained (all greater than or equal to 85%), the accuracy of the topology relating the higher attine genera and species is expected to be robust at greater than 95% confidence (Hillis & Bull 1993).

### (d) Data analysis

The number of haploid fathers per colony could easily be derived by comparing the mother–offspring genotypes. We normally genotyped the mother queen and 10–15 workers ( $12.89 \pm 2.98$ ) from each colony with three microsatellite loci. In one case (*T. cf. zeteki*), a fourth locus was applied to see how it affected the quantitative analysis and conclusions. The results showed no difference in the accuracy of effective paternity estimates between the four-locus analysis in this species and the three-locus analyses in the four other species.

Two sources of error are possible in the estimation of queen mating frequencies from genotypic pedigree data. Both a non-detection error, due to limited genetic variation at marker loci, and an error of non-sampling of paternal genotypes when the number of offspring analysed is relatively small, may lead to an underestimation of queen mating frequency (Boomsma & Ratnieks 1996). 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) by using a beta version of the MATESOFT program (Moilanen & Pedersen 2001). In some colonies ( $n = 9$ ) the queen was not found, so her genotype was inferred by the computer program (Moilanen & Pedersen 2001), which takes limited sample size and limited resolution of genetic markers into account. If more than one combination of queen-mate(s) is possible, the program assigns probabilities to different combinations and uses these in the subsequent estimations of errors and corrected frequencies of possible double matings (see table 1 for details).

We calculated the 95% confidence intervals (CI) of the observed fraction of multiply mated queens  $F$  (where  $F$  can be equal to zero) according to a method by Schmid-Hempel & Schmid-Hempel (2000). The upper 95% CI of an estimate in which no cases of multiple mating were observed ( $D_{\text{obs}} = 0$ ) is

$$L_2 = 1 - \left(\frac{\alpha}{2}\right)^{1/n},$$

where  $n$  is the number of colonies analysed and  $\alpha$  is the desired significance level (Zar 1999). After correcting for the sampling and detection errors estimated by the MATESOFT program (Moilanen & Pedersen 2001), the 95% CI is obtained as

$$D_{0.95} = \left[0; \frac{L_2}{1-f}\right],$$

where  $f$  is the combined detection and sampling error according to Pedersen & Boomsma (1999). As the observed number of multiple matings in the present dataset is zero, we had to use a hypothetical value for paternity skew. As an estimate, we have used the average uncorrected value of 19 ant species,  $c = 0.77$  (corrected value  $c = 0.753$ ) (Boomsma & Ratnieks 1996). Higher hypothetical values of  $c$  will result in larger errors and thus higher estimates of  $D_{\text{est}}$ , the inferred fraction of multiply mated queens in the population. However, high values of paternity skew make inferred cases of multiple mating ineffective for increasing genetic variation among offspring, so that relatedness estimates are hardly affected by different values of

Table 1. Paternity and offspring relatedness in five species of *Trachymyrmex* and *Sericomyrmex* fungus-growing ants.

	<i>Trachymyrmex</i> cf. <i>zeteki</i>	<i>Trachymyrmex</i> <i>cornetzi</i> sp. 1	<i>Trachymyrmex</i> <i>cornetzi</i> sp. 2	<i>Sericomyrmex</i> <i>amabilis</i>	<i>Sericomyrmex</i> cf. <i>amabilis</i>
observed frequency of double matings ( $D_{\text{obs}}$ )	0	0	0	0	0
number of microsatellite loci used	4	3	3	3	3
number of colonies analysed	8	6	10	11	5
number of offspring workers genotyped (mean $\pm$ s.d.)	13.0 $\pm$ 4.6	12.0 $\pm$ 0.0	13.5 $\pm$ 3.7	10.9 $\pm$ 3.8	12.2 $\pm$ 1.8
upper 95% CI for frequency of double queen mating (uncorrected)	0.369	0.459	0.308	0.285	0.522
combined error for identification of double mating ( $f$ ) <sup>a</sup>	0.075	0.066	0.090	0.107	0.059
upper 95% CI for frequency of double queen mating (corrected) <sup>b</sup>	0.399	0.492	0.339	0.319	0.555
maximal effective paternity of nest-mate workers <sup>c</sup>	1.165	1.211	1.136	1.127	1.244
minimal inferred relatedness among nest-mate workers <sup>d</sup>	0.679	0.663	0.690	0.694	0.652

<sup>a</sup> Estimated by the MATESOFT program (Moilanen & Pedersen 2001), based on error estimates and corrections described in Pedersen & Boomsma (1999).

<sup>b</sup> The uncorrected upper 95% confidence limit divided by  $(1 - f)$ ; see also figure 1.

<sup>c</sup> Calculated as  $1/m_e = (1 - D_{\text{est}}) \times 1.0 + D_{\text{est}} \times \sum p_i^2$ , where  $\sum p_i^2 = 0.646$  based on  $p_1 = 0.77$ ,  $p_2 = 0.23$  from Boomsma & Ratnieks (1996).

<sup>d</sup> Calculated as  $r = 0.25 + (0.5/m_e)$  (Pamilo 1993).

paternity skew (Pedersen & Boomsma 1999; Villesen *et al.* 1999).

### 3. RESULTS

Not one doubly mated queen was detected in the 38 colonies analysed. Of special interest were the genotypes in the four *S. amabilis* colonies that were previously analysed by Murakami *et al.* (2000) and inferred to have had multiply mated queens. Our analysis allowed an unambiguous rejection of this inference, as we could show that the two colonies that had elevated numbers of band differences in the analysis by Murakami *et al.* (2000) each consisted of two genetically distinct groups of workers that originated from different queens, each mated to a single male. This was obtained by visual inspection of the genotype distribution and was later confirmed by the MATE-SOFT program, which indicated that the genotypic patterns in these two colonies were in accordance with two unrelated groups of offspring. These two polygynous colonies were therefore counted as four cases of single queen mating in the re-analysis. Following the statistical procedures outlined above, we obtained maximum effective paternities ( $m_e$ ) ranging from 1.12 to 1.24 (table 1) and estimates of minimum intracolony relatedness of 0.65 to 0.69, corresponding to effective single mating in most if not all of the cases.

The statistical analysis of the estimates of queen mating frequency is summarized in figure 1. The middle part of figure 1a shows the upper 95% confidence limits for the expected proportion of multiply mated queens in the five species analysed in the present study. The estimate varies somewhat due to differences in sample size, but shows that the upper confidence limit is usually less than 50% double mating. For comparison, we have plotted a series

of similar analyses for three species of lower attine ants (the left side of figure 1a) and four species of leafcutter ants (the right side of figure 1a). These data were taken from published papers (Fjerdingstad *et al.* 1998; Fjerdingstad & Boomsma 2000; Bekkevold *et al.* 1999; Boomsma *et al.* 1999; Villesen *et al.* 1999), but had not yet been analysed for statistical confidence limits. When we calculated the 95% confidence limits after pooling the data (assuming that each colony is a representative, random and independent sample of a distinct group of attine ants) into three overall categories (lower attines, higher non-leafcutting attines, leafcutter ants; figure 1b), the differences become even more convincing, because the larger sample sizes result in narrower confidence limits.

### 4. DISCUSSION

Our analysis shows that five species of the higher attine genera *Trachymyrmex* and *Sericomyrmex* have singly mated queens. A much larger sample of colonies would be required to establish whether multiple mating is completely absent or just rare, but the current analysis is sufficient to demonstrate that queen mating frequency is effectively very close to 1.0 in all species investigated. Although all species were collected in Panama, there is no reason to assume that they are not representative of their genera, as typical nesting habitats were variable across species and similar to the variation that occurs in South America (Weber 1966; Hölldobler & Wilson 1990; T. R. Schultz, personal observation). The result for *S. amabilis* is especially interesting because this species was inferred to have multiple queen mating by Murakami *et al.* (2000). Our current analysis of material from the same five nests showed that three colonies were headed by a lone, singly mated queen, whereas two colonies had two genetically

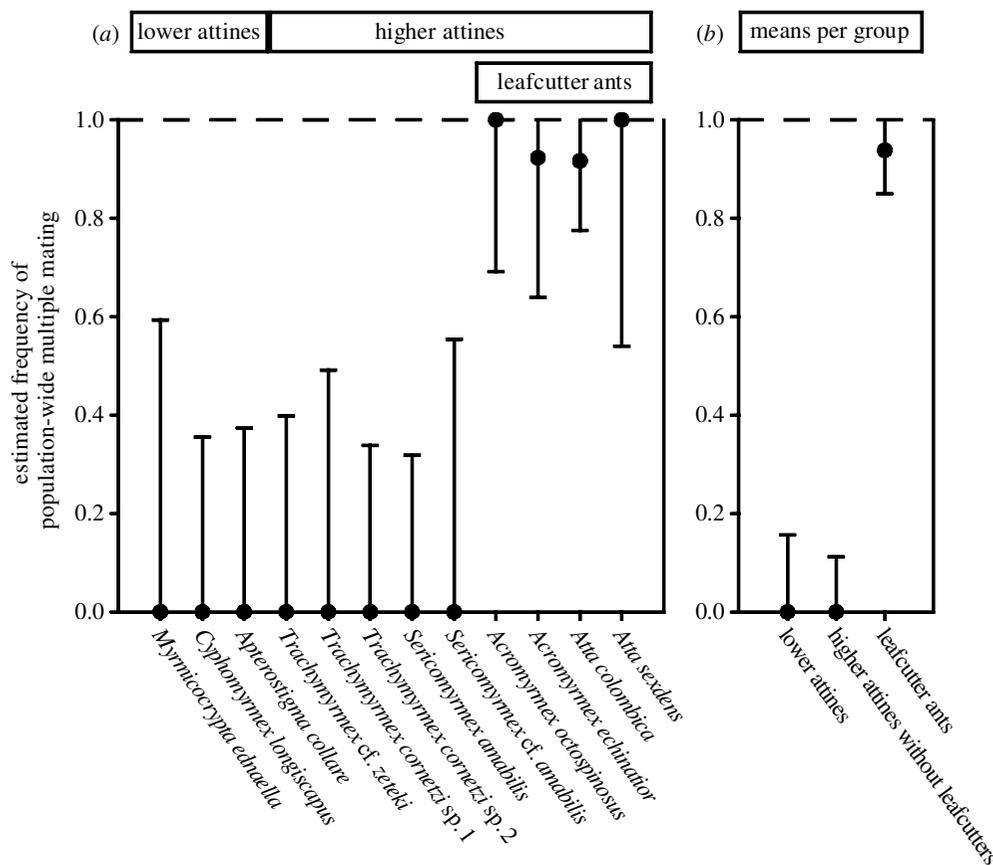


Figure 1. An overview of the best available estimates of queen mating frequency throughout the attine ants, giving the observed frequencies of multiple mating (dots) and their 95% CI (bars), corrected for combined errors (' $f$ ' in table 1). (a) The data per species, and (b) the pooled data per group. The central part of (a) includes both the data for the five species of non-leafcutting higher attines obtained in this study, and a series of similar data for three species of lower attine ants (at the left; from Villesen *et al.* 1999) and four species of *Atta* and *Acromyrmex* leafcutter ants (at the right; from Fjerdingstad *et al.* 1998; Bekkevold *et al.* 1999; Boomsma *et al.* 1999; Fjerdingstad & Boomsma 2000). (b) The same data pooled for the three major categories of fungus-growing ants (the lower attines, the higher non-leafcutting attines (this study) and the leafcutter ants).

distinct groups of workers that originated from different queens, each mated to a single male. This indicates that facultative polygyny occurs in *S. amabilis*, perhaps similarly to the pattern observed in *A. echinator* (Bekkevold *et al.* 1999), although occasional raiding of broods from a neighbouring colony could also explain the genotype distributions observed. Further studies combining complete colony excavation and microsatellite genotyping will be needed to clarify this issue.

Our results demonstrate that it is highly unlikely that the transition from single to multiple mating occurred before or coincident with the origin of the entire higher attine group (the four terminal genera in figure 2). To be supported, such a hypothesis would require the improbable scenario that we have by chance sampled five species in two different genera, which have all secondarily lost multiple mating. It thus appears that multiple queen mating originated in the common ancestor of *Atta* and *Acromyrmex* (a group that may, due to the paraphyly of *Trachymyrmex* discussed above, ultimately include one or a few additional *Trachymyrmex* species, none of which were examined in this study). These novel insights concerning attine phylogeny (cf. figure 2) have made a third hypothetical transition (Villesen *et al.* 1999), intermediate between the two scenarios addressed here, obsolete. A

relatively recent mating system transition in the higher attines implies that the origin of multiple mating in fungus-growing ants coincided with the specialization for leafcutting behaviour and not with the shift from non-specialized fungal symbionts (utilized by all lower attines) to long term clonal symbionts (utilized by all higher attines) (see also final paragraph of § 4). The occupation of the new herbivorous 'leafcutter' niche produced larger colonies with longer lived queens and more complex forms of social organization, and a higher 'visibility' to parasites, but the present data do not allow us to infer whether the mating system transition was causal or consequential in this rapid sequence of evolutionary developments. While it remains possible that future studies will reveal occasional double matings in some of the non-leafcutter attine genera, the phylogenetic correlation between the queen mating system and particular social and ecological grades documented here (figure 2) indicates that it is unlikely that similar major transitions to multiply mated queens have occurred in other branches of the attine tree.

The fact that multiple queen mating is restricted to the socially most derived clade of the attine ants has interesting parallels with phylogenetic patterns of multiple mating found in bees and wasps (Boomsma & Ratnieks 1996; Strassmann 2001). In the vespine wasps, multiple queen

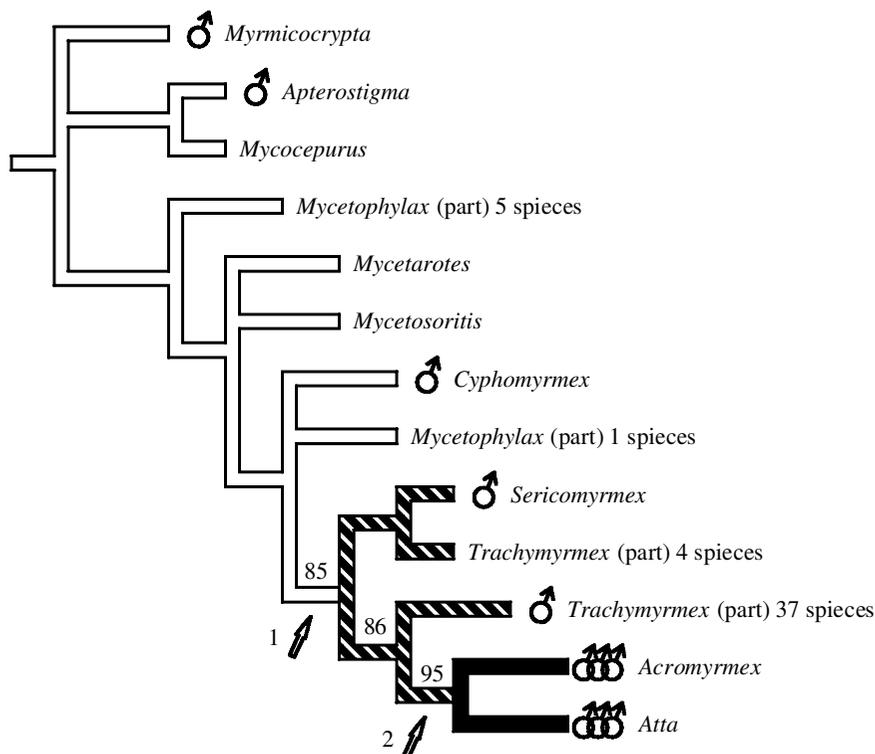


Figure 2. A simplified attine phylogeny based on Schultz (2000) and T. R. Schultz (unpublished data); see also Schultz & Meier (1995) and Wetterer *et al.* (1998). Numbers above branches are bootstrap values from maximum-likelihood analyses of DNA sequences from CO1 and EF-1 $\alpha$  using the model GTR + I + gamma (see text). (White branches, lower attines; shaded branches, higher attines; black branches, leafcutter ants; single male sign, single mating; three male signs, multiple mating; arrow, possible origin of multiple mating.) The two mutually exclusive hypotheses for the transition from single to multiple queen mating tested in the present study are indicated by the arrows: (i) a hypothetical transition that occurred prior to, or coincident with, the origin of the higher attines; and (ii) a hypothetical transition that coincided with the origin of the leafcutter clade, which may include one or a few species currently still assigned to the paraphyletic genus *Trachymyrmex*. The results of the present study confirm the second hypothesis. See text for further details.

mating is also restricted to the most derived genera, although there may have been two independent transitions, one at the base of the genus *Vespula* and one within the *Dolichovespula norwegica* group (Foster *et al.* 1999). Interestingly, however, the latter (less derived) transition only includes partial multiple mating rather than the obligatory multiple mating present in the genus *Vespula*. Within the family Apidae, all species of honeybees (*Apis*) so far investigated have extremely high queen mating frequencies (Estoup *et al.* 1994; Moritz *et al.* 1995; Palmer & Oldroyd 2000), but outside this derived clade, evolution towards multiple queen mating has been rare. One case of partial double mating has been documented in bumble-bees (Estoup *et al.* 1995; Schmid-Hempel & Schmid-Hempel 2000) and another isolated case exists in stingless bees (Paxton *et al.* 1999; Peters *et al.* 1999). It thus seems that evolutionary transitions from exclusively single mating to some combination of single–double or single–multiple mating have originated a number of times in bees and wasps, but that the species occupying these grades have not necessarily undergone further evolutionary modification towards obligatory multiple mating with considerably higher queen mating frequencies. This overall pattern of rarity of multiple mating was previously documented for ants by Boomsma & Ratnieks (1996). Since that review, a new case of multiple queen mating has been discovered in *Pogonomyrmex* harvester ants (Cole & Wiernasz 1999), confirming earlier observations by

Hölldobler (1976). Although there is some controversy over the interpretation of these results (Fjerdingstad & Keller 2000) because estimates of absolute and effective queen mating frequencies are lacking, it seems that the *Pogonomyrmex* case is similar to the leafcutter ants in that all queens mate with multiple males. Other recent studies on ants have shown exclusive single mating (*Pheidole pallidula*; Aron *et al.* 1999) or some combination of single and multiple mating: weaver ants *Oecophylla smaragdina* (Fraser & Crozier 1998) and three species of *Formica* ants (Walsh *et al.* 1998). These studies all showed average effective queen mating frequencies close to unity (less than 1.04 for *P. pallidula*, 1.10 for *O. smaragdina* and 1.35–1.47 for the *Formica* species). The data thus remain consistent with the hypothesis that multiple mating in ants is relatively rare, and that obligatory multiple mating is restricted to very few derived taxa, as inferred by Boomsma & Ratnieks (1996).

Queens of monogynous large colonies could be sperm limited (Cole 1983), a factor sufficient to generate selection for multiple mating. However, this mechanism seems unlikely to be very strong or general, because examples of species with large colonies and singly mated queens are common (Boomsma & Ratnieks 1996). In *Atta colombica* there is a significant increase in the amount of sperm stored by the queen and the number of males with which she mates (Fjerdingstad & Boomsma 1998). However, this effect might have arisen because, after the origin of

multiple mating by queens, males started to mate multiply as well and were more discriminatory in allocating their sperm, forcing queens to obtain matings from an even greater number of males. Another argument against sperm limitation as a general explanation for the evolution of multiple queen mating is that queens of *Acromyrmex octospinosus* (Boomsma *et al.* 1999) and possibly *Pogonomyrmex occidentalis* (Hölldobler 1976; Cole & Wiernasz 1999) have high numbers of mates, but colony sizes at least an order of magnitude less than *A. colombica* (Wilson 1971; Wiernasz & Cole 1995).

The leading hypothesis for the evolution of multiple queen mating has been that genetically diverse colonies suffer less from omnipresent parasites than genetically homogeneous colonies (Schmid-Hempel 1998). This hypothesis is supported by correlative comparative evidence for ants (Schmid-Hempel & Crozier 1999), and convincing specific evidence was recently obtained for bumble-bees (Baer & Schmid-Hempel 1999, 2001). The disease argument carries most force when addressing specialized and well-adapted parasites. Of this category only one is known in the attine ants, a newly discovered fungal parasite of the genus *Escovopsis*, which attacks gardens of attine ants (Currie *et al.* 1999). This parasitic fungus is most prevalent in the higher attines, which may be due to a long evolutionary history of clonal propagation of the fungus (Chapela *et al.* 1994). The lower attines by contrast have been shown to be able to acquire novel cultivar fungi from free-living sexual populations (Mueller *et al.* 1998) and lower attine colonies are perhaps therefore more robust against *Escovopsis* infection. If increased within-colony genetic variation in attine ants results in better defence against a specialized pathogen like *Escovopsis*, targeted at an increasingly vulnerable symbiont, multiple queen mating might be selected for. This 'evolutionary cost of long-term clonality' hypothesis is consistent with the fact that *Escovopsis* is present in all attine genera (Currie *et al.* 1999). However, all fungi cultivated by the higher attines (including *Trachymyrmex* and *Sericomyrmex* as well as the leafcutter ants) seem to share a similar and equally long history of clonal propagation, as they all belong to the monophyletic G1 group of cultivars (Chapela *et al.* 1994). All other things being equal, there should thus be no reason for queen mating frequency to differ between *Trachymyrmex* and *Sericomyrmex* on the one hand, and *Atta* and *Acromyrmex* on the other, unless the previously mentioned differences in colony size, social complexity and longevity are also important. We conclude, therefore, that we now have very good correlational evidence for a single major transition from single to multiple queen mating in attine ants, but that additional research is required to identify the decisive causal factors that drove attine societies across this evolutionary boundary.

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