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## Patterns of male parentage in the fungus-growing ants

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**Abstract** Ant queens from eight species, covering three genera of lower and two genera of higher attine ants, have exclusively or predominantly single mating. The ensuing full-sib colonies thus have a strong potential reproductive conflict between the queen and the workers over male production. This is because, all other things being equal, relatedness incentives should favour traits expressed in both workers and the queen to monopolise the production of the colony's male offspring. Microsatellite genotyping of males from these attine species shows that workers in queenless colonies are able to produce males, but that no worker-produced males were found in queenright colonies. Our results suggest that worker reproduction is rare or even absent in colonies with a fertile queen. This indicates that either the queen directly prevents the workers from raising their own sons, or that worker reproduction is absent in the presence of a fertile queen due to high ergonomic costs of this behaviour.

**Keywords** Fungus-growing ants · Worker reproduction · Worker-queen conflict · Attini · Relatedness

### Introduction

Social insects often live in large colonies where harmony seems to prevail despite the potential for reproductive conflicts. Such conflicts may concern sex allocation or production of males and have been well documented in recent years, both theoretically and empirically (see

Sundström and Boomsma 2001 for a recent review). The eusocial Hymenoptera are particularly suitable as model systems for the study of reproductive conflict, owing to their haplo-diploid genetics (Hamilton 1964a, 1964b) and to the highly variable numbers of female and male breeders that contribute to colony offspring. Both factors induce variation in patterns of relatedness, i.e. variation in colony kin structure, which leads to a considerable diversity in the expression and regulation of reproductive conflicts (Ratnieks and Reeve 1992).

In colonies with a single, once-mated queen, the potential conflict over male production between the queen and her workers is maximal. Based on relatedness differences, the workers should prefer to rear worker-produced sons, to whom they are more related ( $r=0.375$ ) than to their brothers, the queen-produced males ( $r=0.25$ ). The queen, however, is more related to her own sons ( $r=0.5$ ) than to worker-produced sons ( $r=0.25$ ) and will thus prefer her own sons over grandsons (Trivers and Hare 1976; Woyciechowski and Lomnicki 1987; Ratnieks 1988). When a colony-queen is mated to two or more males, the conflict over male production is reduced (Ratnieks 1988), because the relatedness of workers to half-sister sons ( $r=0.125$ ) is lower than the relatedness of workers to brothers. In the absence of ergonomic costs of worker reproduction, the break-even point for the expression of conflict over male production is reached when each worker interacts with equal amounts of full-sisters and half-sisters, i.e. when the effective queen-mating frequency is two (Ratnieks 1988; Boomsma and Ratnieks 1996).

Several mechanisms for restricting worker fertility have been proposed. When queens mate with multiple males, relatedness incentives by themselves are sufficient and worker policing or self policing are invariably expected to evolve (Ratnieks 1988). When queens mate exclusively or predominantly with single males, effective queen control or additional costs of worker reproduction are required to enforce worker sterility. Although worker reproduction has been regularly reported (e.g. Bourke 1988) and, indeed, seems to be restricted to species with

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low queen-mating frequency (Ratnieks 1990), the pattern is far from universal, as illustrated by several recent studies showing that workers in full-sister colonies do not produce their own sons (Heinze et al. 1997; Arevalo et al. 1998; Walin et al. 1998; Foster et al. 2000). This suggests that queen control is more effective than has previously been assumed or that there are substantial recognition constraints and/or hidden costs at the colony level, which prevent the expression of reproductive traits in workers.

To understand the selection forces that govern the expression and resolution of conflict over male production, it is most informative to compare related species, which differ in queen-mating frequency. One such comparative analysis was recently completed for the vespine wasps (Foster et al. 1999, 2000). Here we present a similar analysis for the attine fungus-growing ants, another tribe of social insects for which the phylogeny is known (Schultz and Meier 1995; Wetterer et al. 1998) and where multiple queen-mating is restricted to the most derived terminal genera, the *Acromyrmex* and *Atta* leafcutter ants (Villessen et al. 1999, 2002b). All species of leafcutter ants so far investigated have effective queen-mating frequencies of two or above (Fjerdingstad et al. 1998; Bekkevold et al. 1999; Boomsma et al. 1999; Fjerdingstad and Boomsma 2000), so that worker reproduction is not expected to occur in queenright colonies (Ratnieks 1988). However, representatives of the lower attines (Villessen et al. 1999; Murakami et al. 2000) and the basal genera of the higher attines, *Trachymyrmex* and *Sericomyrmex* (Villessen et al. 2002b), are exclusively or predominantly singly mated, so that the conflict over male production may be expressed and may at least be partly won by the workers. To test this hypothesis, we have determined the maternity of males in a sample of colonies from eight different species of non-leafcutting fungus-growing ants.

## Methods

### Species sampled

Colonies were collected in Panama in spring 1996, 1998 and 2000. Samples were preserved in either 96% ethanol or kept frozen at  $-80^{\circ}\text{C}$  until genotyping. We analysed a total of 211 males from 27 colonies belonging to 8 different species (see Table 1 for sample sizes): *Apterostigma collare*, *Cyphomyrmex costatus*, *C. longiscapus*, *Sericomyrmex amabilis*, *S. cf. amabilis*, *Trachymyrmex cf. zeteki*, *T. cornetzi* sp. 1, and *T. cornetzi* sp. 2. Voucher specimens have been deposited at the Museum of Natural History, Smithsonian Institution, Washington, DC for all colonies of all species. Colonies of *T. cornetzi* were found to be an assembly of two different species (showing some morphological differences as well), which could easily be separated on the basis of amplification differences and allele sizes (Villessen et al. 2002a, 2002b). Hence, these species are referred to as *T. cornetzi* sp.1 and *T. cornetzi* sp. 2. *C. longiscapus* (Mueller and Weislo 1998) has also been found to consist of two species (Schultz et al. 2002), but only the nominal species is included here.

### DNA extractions and analysis

Ant DNA was extracted using either a modified CTAB method (Doyle and Doyle 1987) or a modified chelex extraction (Walsh et al. 1991). After homogenisation, the samples were incubated for 1 h with 1  $\mu\text{l}$  of proteinase K (10 mg/ml) at  $55^{\circ}\text{C}$  in 350  $\mu\text{l}$  CTAB buffer (1% hexadecyltrimethyl ammonium bromide, 0.75 M NaCl, 50 mM Tris pH 8.0, 10 mM EDTA). DNA was then purified using an iso-amyl-alcohol/chloroform extraction, followed by ethanol precipitation. The DNA was suspended in 50  $\mu\text{l}$  TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and stored at  $-20^{\circ}\text{C}$ . Later extractions were done using a modified chelex extraction (Walsh et al. 1991), where part of the ant (head, hind leg or whole ant, dependent on size) was dried at  $60^{\circ}\text{C}$  for 20 min, homogenised in liquid  $\text{N}_2$ , followed by the addition of 400  $\mu\text{l}$  20% chelex solution (Chelex 100 in 100 mM Tris-HCl, pH 8.0). Samples were subsequently vortexed and boiled for 12 min and stored at  $-20^{\circ}\text{C}$  until genotyping. PCR reactions were carried out in 6- $\mu\text{l}$  volumes containing 1  $\mu\text{l}$  of template DNA, 2 pmol of each primer (one primer was end labelled for specific use on the ALF express), 0.2 mM dATP, dCTP, dGTP and dTTP, 1x *Taq* polymerase buffer and 0.375 units of *Taq* polymerase (Pharmacia). Details on primers and Genbank accession numbers are given in Table 2 and in Ortius-Lechner et al. (2000), Villessen et al. (1999) and Villessen et al. (2002a).

### Hypothesis testing and analysis

Adult male offspring of both queenright and queenless colonies were genotyped. Males from queenless colonies could be queen-produced or worker-produced, depending on whether the brood was queen-produced or not. If the queen was not present, we used the presence of gynes (winged prospective queens) to infer whether the mother-queen had died recently or had been missed during excavation (in which case diploid offspring are expected), or whether the colony had been queenless for some time. The former type of colony was categorised as having a "functional queen" and was included in the queenright class, whereas the latter type formed the real queenless class of colonies.

The following alternative hypotheses were tested: (1) all males are queen-sons, both in queenright and queenless colonies. This implies that the worker-queen conflict over male production is not expressed. Workers are thus effectively sterile, possibly because of self-restraint or self-policing. Self-restraint would follow from workers being unable to discriminate eggs and brood of different sex or maternity (Nonacs and Carlin 1990; Nonacs 1993). Self-policing would result from substantial costs of worker reproduction relative to the potential relatedness advantages both under queenright and queenless conditions (Ratnieks 1988; Foster and Ratnieks 2001). (2) Males are queen-produced in queenright colonies and worker-produced in queenless colonies. This implies that workers under some conditions lay eggs, but that either queen-policing (the queen removes worker-laid brood) or worker-policing operates, despite the high relatedness of workers to worker-produced males (Woyciechowski and Lomnicki 1987; Ratnieks 1988). (3) Some males are worker-sons, both in queenright and queenless colonies. This implies that workers are effective in adding to or replacing some queen-laid haploid eggs and in raising worker-sons.

Given that single mating is the rule in all non-leafcutting attine species studied so far (Villessen et al. 1999, 2002b), offspring males with an allele exclusively found in the father can be unambiguously assessed as being worker-produced. However, maternal alleles in male offspring may be derived both from the queen and from her worker daughters, so that indirect maximum likelihood methods have to be used to arrive at unbiased estimations of the population-wide proportion of worker-produced males. Such analyses require estimates of the allele frequencies in queens and workers, which we obtained by genotyping 4–17 workers from each colony (mean  $10.1 \pm 3.8$  SD). We reconstructed missing queen genotypes based on worker genotypes, which were never in conflict with an assumption of a single once-mated queen. In queenless colonies, loci for which

**Table 1** Male parentage across species and colony classes (queen present or absent) of fungus-growing ants

| Species                            | Queen present      |                       |                                 | Queen absent       |                       |                                 | Total              |                       |                                 |
|------------------------------------|--------------------|-----------------------|---------------------------------|--------------------|-----------------------|---------------------------------|--------------------|-----------------------|---------------------------------|
|                                    | Number of colonies | Total number of males | Number of worker-produced males | Number of colonies | Total number of males | Number of worker-produced males | Number of colonies | Total number of males | Number of worker-produced males |
| Worker reproduction detected       |                    |                       |                                 |                    |                       |                                 |                    |                       |                                 |
| <i>Apterostigma collare</i>        |                    |                       |                                 | 1                  | 8                     | 4                               | 1                  | 8                     | 4                               |
| <i>Sericomyrmex cf. amabilis</i>   |                    |                       |                                 | 2                  | 28                    | 16                              | 2                  | 28                    | 16                              |
| <i>Trachymyrmex cf. zeteki</i>     |                    |                       |                                 | 1                  | 8                     | 3                               | 1                  | 8                     | 3                               |
| Subtotal                           |                    |                       |                                 | 4                  | 44                    | 23                              | 4                  | 44                    | 23                              |
| Worker reproduction not detected   |                    |                       |                                 |                    |                       |                                 |                    |                       |                                 |
| <i>Apterostigma collare</i>        | 1                  | 14                    | 0                               | 1                  | 7                     | 0                               | 2                  | 21                    | 0                               |
| <i>Sericomyrmex cf. amabilis</i>   |                    |                       |                                 | 1                  | 12                    | 0                               | 1                  | 12                    | 0                               |
| <i>Trachymyrmex cf. zeteki</i>     | 4                  | 30                    | 0                               | 2                  | 17                    | 0                               | 6                  | 47                    | 0                               |
| <i>Cyphomyrmex costatus</i>        | 3                  | 17                    | 0                               |                    |                       |                                 | 3                  | 17                    | 0                               |
| <i>Cyphomyrmex longiscapus</i>     | 3                  | 18                    | 0                               |                    |                       |                                 | 3                  | 18                    | 0                               |
| <i>Sericomyrmex amabilis</i>       | 2                  | 19                    | 0                               |                    |                       |                                 | 2                  | 19                    | 0                               |
| <i>Trachymyrmex cornetzi</i> sp. 1 | 3                  | 20                    | 0                               |                    |                       |                                 | 3                  | 20                    | 0                               |
| <i>Trachymyrmex cornetzi</i> sp. 2 | 3                  | 13                    | 0                               |                    |                       |                                 | 3                  | 13                    | 0                               |
| Subtotal                           | 19                 | 131                   | 0                               | 4                  | 36                    | 0                               | 23                 | 167                   | 0                               |
| Total                              | 19                 | 131                   | 0                               | 8                  | 80                    | 23                              | 27                 | 211                   | 23                              |

**Table 2** Loci, Genbank accession numbers, number of alleles and observed heterozygosities (*in parentheses*) for the microsatellite loci used in this study. The locus cypho15b-16b is highly variable in all species (Villessen et al. 2002a)

| Locus        | Genbank accession no. | <i>A. collare</i> | <i>C. costatus</i> | <i>C. longiscapus</i> | <i>S. amabilis</i> | <i>S. cf. amabilis</i> | <i>T. cf. zeteki</i> | <i>T. cornetzi</i> sp. 1 | <i>T. cornetzi</i> sp. 2 |
|--------------|-----------------------|-------------------|--------------------|-----------------------|--------------------|------------------------|----------------------|--------------------------|--------------------------|
| Aptero1-2    | AF521215              | 3 (0.478)         |                    |                       |                    |                        |                      |                          |                          |
| Aptero3-4    | AF521216              | 2 (0.167)         |                    |                       |                    |                        |                      |                          |                          |
| Cypho3-4     | AF521213              |                   | 5 (0.889)          |                       |                    |                        |                      |                          |                          |
| Cypho5-6     | AF521214              |                   | 2 (0.25)           |                       |                    |                        |                      |                          |                          |
| Cypho9-10    | AF453707              |                   |                    | 3 (0.792)             | 4 (1.0)            |                        | 3 (0.542)            |                          | 2 (0.459)                |
| Cypho15b-16b | AF453708              | 5 (1.0)           | 4 (0.375)          | 6 (0.75)              |                    |                        | 6 (1.0)              |                          | 3 (1.0)                  |
| Trachy3-4    | AF453709              |                   |                    |                       |                    | 4 (0.758)              |                      |                          |                          |
| Trachy5-6    | AF453710              |                   |                    | 4 (1.0)               | 8 (0.889)          | 3 (0.750)              |                      |                          |                          |
| Trachy11-12  | AF453712              |                   |                    | 3 (0.5)               | 3 (1.0)            | 4 (0.877)              |                      |                          |                          |
| Ech3385      | AF160987              |                   |                    |                       |                    | 5 (0.858)              |                      |                          | 2 (0.25)                 |

all workers were heterozygous were discarded, because they do not allow the queen genotype to be determined.

The maximum likelihood approach has been pioneered by Arevalo et al. (1998) and estimates the likelihood  $L(Q)$  of males being produced by the queen as:

$$L(Q) = K \prod_{loci} \prod_{alleles} ((Q \times f_q + (1 - Q) \times f_w))^n$$

where  $f_q$  is the frequency of a specific allele in the queen,  $f_w$  is the frequency of the same allele in the workers,  $Q$  is the fraction of males produced by the queen [ $0 \leq Q \leq 1$ ; note, however, that  $L(Q)$  was sampled at short intervals],  $n$  is the number of males with the focal allele, and  $K$  is a multinomial constant. To arrive at population-wide estimates across colonies, we used the product of the likelihoods of single colonies. The maximal likelihood value ( $\hat{Q}$ ) was normalised so that  $L(\hat{Q}) = 1.00$ , allowing the difference between this value and an observed likelihood value to be evaluated with the approximation:

$$-2 \ln \left( \frac{L_{Q=Q_0}(Q)}{L(\hat{Q})} \right) \approx \chi^2$$

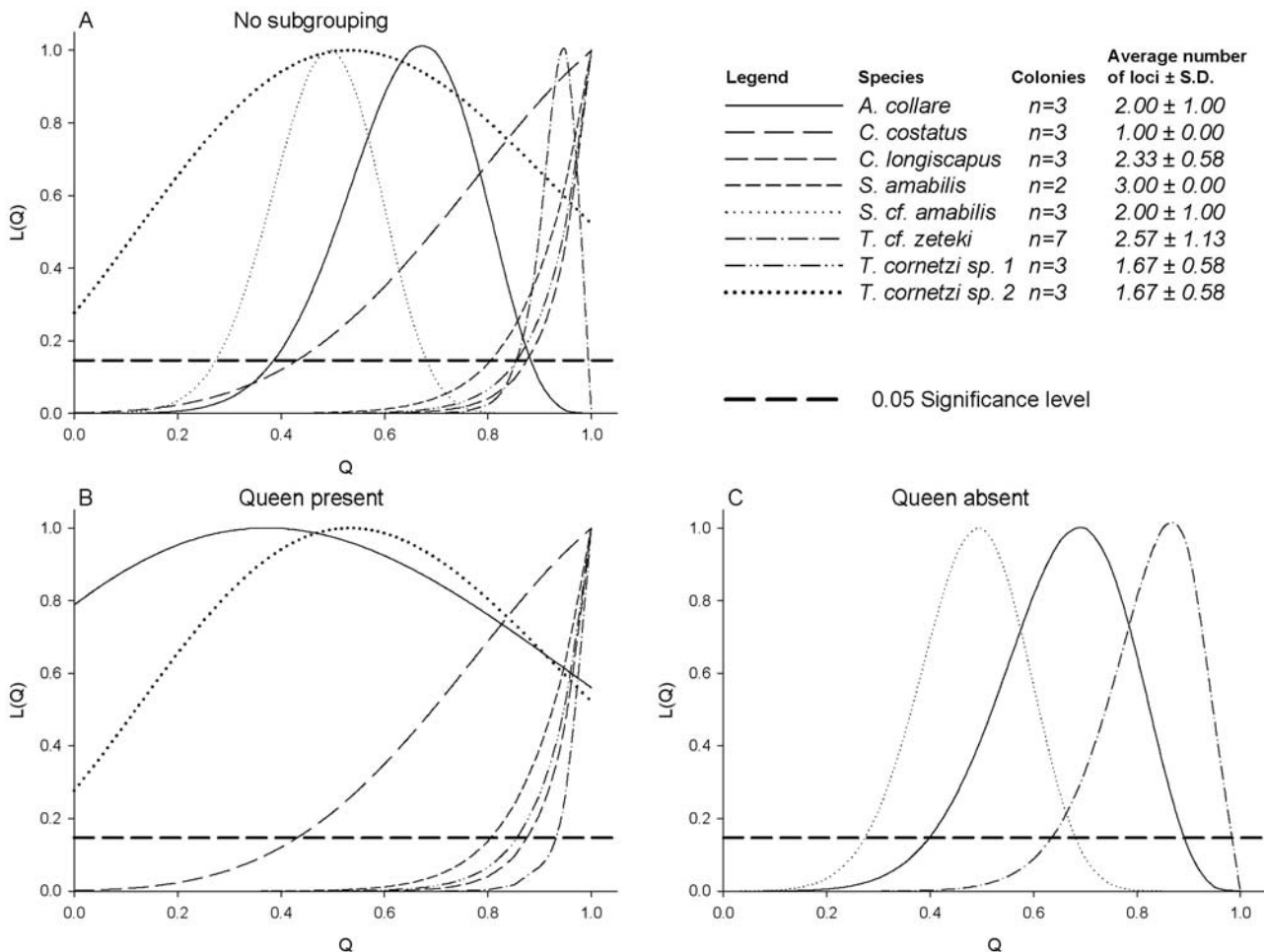
To be significantly different ( $P \leq 0.05$ ) from the normalised maximum likelihood estimate [ $L(\hat{Q}) = 1.00$ ],  $\chi^2$  should equal or exceed 3.84, corresponding to the critical value of  $L_{Q=Q_0}(Q) = 0.1466$  with 1 *df*. Values of  $Q$  resulting in right-hand side likelihood values [ $L(Q)$ ] below this critical value imply that the null hypothesis ( $Q=Q_0$ ) can be rejected in favour of the alternative hypothesis ( $Q \in [0;1]$ ) at the 5% level. In the plotted likelihood curves, significant deviations from the null hypothesis thus depend on two characteristics: (1) the top of the curve should be reached at  $Q$ -values below 1.00, indicating that some worker reproduction may have occurred; (2) the right-hand side of the curve should drop below the significance threshold, indicating that the deviation from the null model is significant.

## Results

### Genotype distributions

Using only informative loci (i.e. with non-overlapping maternal and paternal alleles), worker-produced males were detected in four queenless colonies belonging to three species (upper part of Table 1), whereas all male genotypes were compatible with being queen-produced in another four queenless colonies of the same three species (lower part of Table 1). In the remaining 19 queenright colonies, belonging to 7 species of 4 genera, not one of the 131 analysed males could be positively assessed as worker-produced. Assuming  $Q=0.95$  (i.e. 5% worker reproduction), the chance of neither sampling nor detecting any worker-produced males was 0.036, indicating that  $Q$  is indeed likely to be close to 1.00 (i.e. worker reproduction rare or absent).

As only 50% of worker-laid males can be detected when a single locus is examined, the data corrected for this bias indicated that an overall average of 98% of the males in queenless colonies (ranging from 75% to 114%) was worker-produced. As the other four queenless colonies of the same three species most likely produced queen-sons, there seems to be a clear trend that any remaining sexual brood of the queen is apparently raised to adulthood before worker-sons are raised. Five species were only sampled as queenright colonies and showed no



**Fig. 1A–C** Likelihood plots of  $Q$ , the fraction of males produced by the queen for the total data set and for the two classes of colonies. The dashed horizontal line indicates the 5% significance level, so all values of  $Q$  for which  $L(Q)$  falls below this line are significantly different from the null-model maximum likelihood estimate of  $Q$  [ $L(Q) = 1.00$ ]. **A** Data for all eight species: three

species show significant ( $P < 0.05$ ) worker reproduction, whereas the  $L(Q)$  values of five species are consistent with no worker reproduction. **B** Queenright colonies only: in no cases is there evidence for significant worker reproduction. **C** Queenless colonies only: all three cases show significant deviations from exclusive male production by the queen. See text for further details

male genotypes that were positively compatible with worker reproduction. However, we cannot exclude the possibility that the workers of these species are able to lay and raise some male eggs under queenright conditions and perhaps even substantial amounts under queenless conditions. A larger data set will be needed to settle this.

#### Maximum likelihood analysis

The maximum likelihood analysis evaluates allele frequency differences between the queen and the workers to estimate the proportion of queen-produced males. Analysis of the full data set indicated that worker reproduction does take place in at least three species (*A. collare*, *S. cf. amabilis* and *T. cf. zeteki*; right-hand tail of distribution significant), and perhaps also in a fourth species (*T. cornetzi* sp. 2; only peak of curve shifted to the left) (Fig. 1A). When analysing the data per colony class, it

appeared that worker reproduction in the three former species was restricted to the queenless colonies, whereas the latter species (*T. cornetzi* sp. 2) gave ambiguous results for queenright colonies, due to small sample size (Fig. 1B, C). In five species, *C. costatus*, *C. longiscapus*, *S. amabilis*, *T. cf. zeteki* and *T. cornetzi* sp. 1, the maximum likelihood estimate of  $Q$  in queenright colonies was 1.0 (all males queen-produced). In two species, *A. collare* and *T. cornetzi* sp. 2, the maximum likelihood estimate of  $Q$  in queenright colonies was below 1.0, indicating that some males might have been worker-produced, but the deviation from the null hypothesis of no worker reproduction ( $Q=1.0$ ) was not significant (Fig. 1B). For one species, *S. cf. amabilis*, we did not sample any colonies with a queen present, so that we only had three colonies in the “queen absent” category for this species. Of these three colonies, two showed unambiguous evidence of worker reproduction, whereas the third remained inconclusive, resulting in an overall species

estimate of  $Q < 1.0$  (Fig. 1A, C). The two remaining species, *A. collare* and *T. cf. zeteki*, which were sampled in the “queen absent” category, also showed significant evidence of worker reproduction (Fig. 1C).

In summary, we found that males are partly (but not exclusively) worker-produced in queenless colonies of three species (*A. collare*, *S. cf. amabilis* and *T. cf. zeteki*), but mostly queen-produced in queenright colonies of five species (*C. costatus*, *C. longiscapus*, *S. amabilis*, *T. cf. zeteki* and *T. cornetzi* sp. 1). However, for two species (*A. collare* and *T. cornetzi* sp. 2), the results for queenright colonies remained ambiguous, so that larger samples will be required to assess whether most of the males in queenright colonies are queen- or worker-produced (Fig. 1B).

## Discussion

This study shows that workers of three species of non-leafcutting attine ants (one species each of the genera *Apterostigma*, *Trachymyrmex* and *Sericomyrmex*) are capable of laying functional male eggs and of raising these to adulthood under queenless conditions. Not a single worker-produced male was found in the queenright colonies of these three species or in any of the other five species (of the same genera and *Cyphomyrmex*). However, we have not investigated queenless colonies from these last five species, so we cannot exclude the possibility that these workers are capable of laying and raising male eggs under these conditions. Although we cannot completely rule out worker reproduction in queenright colonies, recent studies on workers of the same non-leafcutting attine ants from Panama have so far confirmed that: (1) worker ovary development in queenright colonies is rare in all species except *Apterostigma*, and (2) workers tend to have active ovaries in queenless colonies of *Apterostigma*, *Trachymyrmex* and *Sericomyrmex* (T. Murakami, personal communication; M.B. Dijkstra, unpublished work). This implies that patterns of worker ovary development seem to match the patterns of male-parentage reported here.

Our data provide most support to hypothesis 2, i.e. that worker-produced males are not produced in queenright colonies, whereas male eggs are laid and raised by workers in queenless colonies. This implies either queen-policing or worker-policing, or that workers do not lay eggs in queenright colonies. The occurrence of alternative policing behaviours deserves further explicit study in attine ants, because both different colony sizes and variable degrees of polydomy occur along the attine phylogenetic tree. We hypothesise that in the lower attines (represented in our study by *Cyphomyrmex* and *Apterostigma*), queen-policing is perhaps most likely, because the number of workers per colony is below 100 (Villesen et al. 1999), so that the queen has easy access to all the eggs and brood in the colony, facilitating queen-policing (Ratnieks and Reeve 1992). Although still small compared to the leafcutting ants, colonies of *Tra-*

*chymyrmex* and *Sericomyrmex* (the basal genera of the higher attines) are often larger and more complex than those of the lower attines. Panamanian *Sericomyrmex* species have several thousands of workers per colony and multiple fungus chambers (Weber 1966; Wilson 1971; Hölldobler and Wilson 1990; M.B. Dijkstra, personal observation). Also Panamanian *T. cornetzi* and *T. zeteki* frequently have multiple nest chambers (Hölldobler and Wilson 1990; J.J. Boomsma, personal observation), in contrast to earlier records (Weber 1941). Large, complex colony structures could limit access of the queen to all brood, especially if the workers have reasons to prevent her free movement. Further comparative studies of worker ovary development and egg laying across the lower and basal higher attines could thus potentially reveal different degrees of worker sterility and different mechanisms of policing or self restraint.

If costs of expressed worker-queen conflict over male production are significant, it will be in the interest of all parties to avoid these costs by having all eggs laid by the queen. Empirical examples of these costs are scarce, but include aggression and reduced brood care (Cole 1986). Theoretical explorations have further emphasised that recognition errors make culling of haploid queen-eggs by workers less profitable and that competition between workers for egg laying may decrease the foraging efficiency of a colony (Ratnieks and Reeve 1992). Such costly conflicts may even result in arbitrary conventions to avoid the expression of conflict (Pollock 1996). Factors like this could result in the abandonment of worker reproduction for ergonomic reasons, even when the relatedness incentive for this behaviour is present (Queller and Strassman 1998). A number of recent studies have indeed shown that workers fail to reproduce in colonies with a high relatedness among workers (Arevalo et al. 1998; Foster et al. 1999, 2000). An additional hypothesis for the evolution of worker-policing in high relatedness colonies has recently been proposed by Foster and Ratnieks (2001). Assuming that workers control sex allocation by killing excess male larvae at a considerable cost to the colony, this model links sex allocation and worker reproduction. By policing worker-laid (male) eggs instead of killing male larvae, this total cost is reduced so that the evolution of worker-policing may be favoured in spite of high relatedness. The model assumes that workers are able to discriminate between worker-laid eggs and queen-laid eggs, but not between female and male eggs.

Sexual deception models by Nonacs and Carlin (1990) and Nonacs (1993) have shown that non-distinctness of queen-produced female and male larvae may add a considerable extra cost to attempts to replace queen eggs by worker eggs, which may in itself, and independent of any policing behaviour, also explain why workers do not attempt to reproduce in queenright nests. Detailed behavioural observations will be needed to test the validity of egg-policing versus sexual deception in the attine ants, to see whether workers actively lay eggs that are subsequently policed, or whether workers refrain from laying altogether until the queen has been removed.

However, the latter would not necessarily prove that sexual deception is operating, as other ergonomic costs of worker reproduction may tip the balance towards worker sterility. If worker-laid eggs are allowed to hatch, but are removed later, the timing of this removal would be crucial to discriminate between policing and sexual deception. Culling of worker-sons as young larvae would be compatible with worker-policing, whereas their removal after they are halfway developed would – all other things being equal – support the sexual deception hypothesis (Nonacs and Carlin 1990; Nonacs 1993).

Only the evolutionarily most derived genera of the higher attine ants, the *Atta* and *Acromyrmex* leaf-cutting ants, have high queen-mating frequencies (Fjerdingstad et al. 1998; Bekkevold et al. 1999; Boomsma et al. 1999; Fjerdingstad and Boomsma 2000). Recent evidence indicates that the transition from single to multiple queen-mating has occurred only once in the common ancestor of these two genera (Villesen et al. 1999, 2002b). Colony sizes of leaf-cutting ants are generally in the order of  $10^4$ – $10^5$  (*Acromyrmex*) and  $10^5$ – $10^6$  (*Atta*), which makes queen control by direct physical dominance virtually impossible. However, with multiple mating and the ensuing genetically variable colony kin structure, theory predicts no conflict over male production (Woyciechowski and Lomnicki 1987; Ratnieks 1988). Therefore, worker-policing is expected to have evolved in the leafcutter ants, as in the honeybee (Ratnieks and Visscher 1989) and in vespine wasps with multiple queen-mating (Foster et al. 1999; Foster and Ratnieks 2000).

At least three key questions on costs, benefits and constraints of workers reproduction in attine ants remain to be investigated. First, our data suggest that worker-policing may have evolved in the basal lineages of the higher attine ants, i.e. before multiple queen-mating arose, but the reasons for this are as yet unclear. Second, the possibility that queen-policing or sexual deception operate in the lower attines cannot be excluded. If sexual deception proves to be a valid mechanism preventing worker reproduction in the lower attines, this phenomenon would also deserve explicit investigation in the higher attines (the opposite scenario of later evolution of sexual deception seems unlikely in light of the increasing sexual dimorphism in the higher attines). Third, the mechanism of worker-policing or self-policing in leafcutting ants deserves explicit study because fungus culturing implies a very specific nesting biology and an unusual distribution of eggs. In contrast to bees and wasps, ants do not have brood cells, but tend to sort their eggs and larvae in one or several piles (Hölldobler and Wilson 1990), which means that the identification of worker-laid eggs to be culled is possibly less efficient. However, this is not so in the fungus-growing ants, where eggs are deposited relatively scattered throughout the fungus garden, implying that they are perhaps harder to find but easier to recognise because their chemical signature is more distinct.

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