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Research article

Lack of patriline-specific differences in chemical composition of the metapleural gland secretion in *Acromyrmex octospinosus*

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Summary. Multiple queen-mating (polyandry) in social insects increases the genetic variability among worker offspring, which may enhance colony survival, social productivity and defence against parasites. The unique and complex symbiosis of leaf-cutting ants with a clonal mutualistic fungus makes this social system particularly vulnerable to contamination by pathogenic and unwanted saprophytic fungi and bacteria. Proper defence against such threats requires effective and flexible chemical defence mechanisms. A prime candidate for providing such defences is the metapleural gland secretion, which is known to have broad antibiotic properties. Here we use the leaf-cutting ant *Acromyrmex octospinosus* to specifically test the hypothesis that genetically more diverse worker-offspring produce a more variable spectrum of metapleural gland compounds. We used DNA microsatellite markers to assign workers from two colonies to the six most common patrilines in each colony, and have analysed the degree to which the observed variance in the quantitative chemical composition of the metapleural gland secretion can be explained by genetic differences among patrilines. We found a marginally significant patriline-effect on the overall variability of metapleural gland compounds in one colony, but could not detect such effect in the other colony. We discuss a number of possible reasons why the genetic variance component for quantitative variation in metapleural gland secretion may be low.

Key words: Acromyrmex octospinosus, polyandry, parasitism, metapleural gland.

Introduction

Theoretical studies have shown that single paternity or low frequencies of multiple paternity favour the evolution of eusociality in Hymenoptera, especially when combined with female-biased sex ratios (Hamilton, 1964; Trivers and Hare, 1976; Frank and Crespi, 1989) or worker produced males (Charnov, 1978). Consequently the evolution and maintenance of obligate multiple queen-mating is not expected until social evolution has advanced towards a stage where workers have lost their full reproductive potential and their ability to mate. As far as genetic mother-offspring data are available, this pattern seems to hold up (Boomsma and Ratnieks, 1996), as documented cases of obligate multiple queen-mating seem to be restricted to relatively few derived lineages such as *Apis* honeybees, *Vespula* wasps, *Atta* and *Acromyrmex* leafcutter ants, and *Pogonomyrmex* harvester ants (Estoup et al., 1994; Fjerdingstad et al., 1998; Boomsma et al., 1999; Cole and Wiernasz, 1999; Foster et al., 1999).

It is reasonable to assume that multiple queen-mating is costly and that multiple queen-mating can thus only be maintained if the costs of multiple copulation are somehow compensated by beneficial effects of colony genetic diversity, queen-optimal sex allocation and/or queen fecundity (Hamilton, 1978; Crozier and Page, 1985; Sherman et al., 1988; Ratnieks and Boomsma, 1995). In contrast to previous belief, extreme multiple mating in the honeybee (10–20 males) seems to incur very low costs to queens (Fletcher, 1978; Ratnieks, 1990), but costs are thought usually to outweigh the eventual benefits from additional matings in species with single or low queen-mating frequencies (Boomsma and Ratnieks, 1996).

Two sets of hypotheses for the eventual benefits of multiple mating have been proposed. Firstly, it has been suggested

that mating with multiple males enables queens to store more sperm and thereby achieve higher lifetime fitness (Hamilton, 1964; Cole, 1983). This idea has received some support for *Atta colombica* (Fjerdingstad and Boomsma, 1998), although a study on spermathecal content in this species showed that queens only store the equivalent of a single male worth of sperm (Fjerdingstad and Boomsma, 1997). The second set of hypotheses is based on the fact that multiple queen-mating leads to multiple paternity and therefore increases the genetic diversity among worker-offspring. Higher genetic diversity has been hypothesized to enhance colony fitness by increasing worker task efficiency (Crozier and Page, 1985; Page and Robinson, 1991), to improve colony resistance against infectious diseases (Hamilton, 1987; Sherman et al., 1988; Schmid-Hempel, 1994; 1998) and to reduce colony-level genetic load due to the production of sterile diploid males (Page, 1980; Ratnieks, 1990; Pamilo et al., 1994). Support for some of these genetic diversity hypotheses has recently increased, but general consensus on the relative importance of these alternative mechanisms of selection for multiple queen-mating is still far away. A positive effect of polyandry on parasite load and overall fitness has been reported for *Bombus terrestris* (Baer and Schmid-Hempel, 1999), but queens of this species are not known to mate multiply because males actively prevent multiple-queen mating by mating plugs (Baer et al., 2001). Honeybee colonies on the other hand are headed by a highly multiply-mated queen, but evidence for reduced parasite susceptibility in genetically heterogeneous colonies remains contradictory (Rinderer et al., 1975; Taber, 1982; Ratnieks, 1989; Woyciechowski et al., 1994). Finally, a recent study by Cole and Wiernasz (1999) showed that genetically variable colonies of *Pogonomyrmex* harvester ants survived better than less variable colonies, but had the disadvantage that multiple queen mating was estimated indirectly from offspring relatedness, so that the absolute number of queen-matings remained unknown.

After an earlier comparative tests of the "genetic variability versus disease resistance" hypothesis across ant species (Schmid-Hempel and Crozier, 1999) we here report the first empirical study testing this hypothesis in a single ant species. We have chosen the leafcutter ant *Acromyrmex octospinosus*, as this species was recently reported to have the highest queen-mating frequency known in ants so far (Boomsma et al., 1999). We sampled two colonies of *Acromyrmex octospinosus* to compare the qualitative and quantitative composition of the metapleural gland secretion of workers from different patrilines. The metapleural gland secretion of *Acromyrmex* leafcutter ants is known to be highly diverse in chemical composition (Ortius-Lechner et al., 2000a) and to contain many compounds with antibiotic properties (Bot et al., 2002; Poulsen et al., 2002). This justifies the assumption that the metapleural gland is important for disease resistance in leafcutter ants and that genetic variation in the expression of this defence may affect colony survival and performance. Patriline-level differences in metapleural gland secretion approximately reflect the additive genetic variance in the traits under investigation, as same-age workers of different patrilines share all environmental influences during development and all phenotypic maternal effects from having the same mother (cf. Falconer, 1981). We used advanced GC-MS techniques to quantify the chemical composition of metapleural gland secretions (Ortius-Lechner et al., 2000a) and DNA microsatellite typing (Ortius-Lechner et al., 2000b) to separate the patrilines.

Material and methods

Sampling methods

The colonies of *Acromyrmex octospinosus* were collected in 1994 in Gamboa, Panama. After their transfer to the laboratory in Aarhus they were housed in nestboxes in a rearing room with a constant temperature of 25 °C and a RH of ca. 70%. The ants were fed twice a week with leaves and flowers of mainly rosaceous woody perennials (during Danish summer) and bramble leaves, *Rubus fruticosus* (all year round) (Bot and Boomsma, 1996).

To avoid age or caste specific variation in the composition of the metapleural gland secretion (Ortius-Lechner pers. obs.) only large workers of approximately the same intermediate age class (we avoided both callows and old workers and sampled only workers of the same intermediate cuticular pigmentation) were collected from the two colonies. Workers were killed immediately by freezing and their bodies were partitioned with sterile scalpels into head, mesosoma, gaster and legs. Head, gaster and legs of each individual worker were subsequently used for microsatellite analysis, while the mesosomas were kept frozen at –70 °C until extraction of the metapleural gland secretion.

Genetic analysis

Paternity analysis was performed on 64 large workers from colony 19 and 92 large workers from colony 18. Patrilines were assigned after genotyping for three DNA microsatellite loci developed for the sympatric congener species *Acromyrmex echinatior* (Ech1390, Ech4126 and Ech3385) and segregating for 15, 14 and 13 alleles, respectively (Ortius-Lechner et al., 2000b). Total genomic DNA was extracted from individual ants by using the CTAB procedure by Doyle and Doyle (1987). One primer of each primer pair was end-labelled with Cy5 for an ALF express™ automatic DNA Sequencer.

Routine amplification of the *Acromyrmex octospinosus* samples was performed for all primer pairs with the following conditions for 6 µl reactions: 0.1 units Taq polymerase, 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.0), 0.2 mM dNTPs, 2 pmol of each primer and 1 µl of genomic DNA. PCR reactions were carried out on a Peltier Thermal Cycler 200 programmed to denature at 94 °C for 3 min followed by 30 s at 93 °C, 30 s at the respective annealing temperature (53 °C for Ech1390 and Ech3385, 59.5 °C for Ech4126), 40 s at 72 °C, 39 cycles of step 2 to step 4, 7 min at 72 °C and finally cooling down to 10 °C. The amplification products were diluted with 50 µl H_2O. Of this solution, 1.5 µl was mixed with 3 µl of formamide loading dye and denatured by heating to 95 °C before running it on the ALF express™ DNA Sequencer together with the internal standard ALf express™ sizers 100 and 300 and the external standard ALf express™ sizer 50–500. Allele sizes were scored by comparison with the internal and external size markers using the software Allele Links (Pharmacia). Genotypes of queens and their multiple mates were inferred from the multi-locus offspring genotypes. As the two colonies were kept alive for other experiments, the genotypes of mother-queens were reconstructed on the basis of worker and male offspring. Paternity of a few heterozygous workers could not be determined with certainty, because two inferred fathers had the same alleles as the heterozygous queen (Pamilo, 1982). These individuals were excluded from GC-MS analysis. All workers from these two colonies (156) were genotyped and analyzed a second time by an

independent person to avoid any errors of determination. We both give an estimate of the total pattern of paternity in the two colonies and a more detailed analysis of a subset of samples belonging to the six major patrilines in the two colonies (those represented with at least 3 workers), i.e. of 41 workers of colony 18 and 60 workers of colony 19. Only the thoraces of these ants were subsequently analysed for their chemical composition of the metapleural gland secretion.

Chemical analysis

For the extraction of the metapleural gland secretion, the posterolateral parts of the mesosoma containing the paired glands were dissected with a sterile scalpel. These entire pieces were then sealed in a soft glass capillary for further chemical analysis. This extraction method was chosen because it recovered a higher total amount of secretion compared to extracting secretion directly from the gland reservoir (details in Ortius-Lechner et al., 2000a).

The analytical separation was carried out with a Hewlett-Packard 5890 Gas Chromatograph directly coupled to a 5970B mass selective detector (quadrupole mass spectrometer using 70 eV electron impact ionisation). The system was controlled by a Hewlett-Packard series 300 computer with HP 5972/5971 MSD Chemstation on which data were accumulated. Mass spectra were scanned from m/z 35 to m/z 550 with a scanning time of about 2.4 s. Chromatography was performed on a 15 m × 0.32 mm ID column coated with a bonded polyethylene glycol stationary phase (0.25 µm thickness, Stabilwax, Restek) using quartz glass liners (produced in the lab in Keele) with Restek silanized glass wool. The samples were injected in splitless mode (injection temperature 250 °C) by crushing the capillary tubes immediately inside the injector port after insertion, as described by Maile et al. (1998). The oven temperature was programmed to increase from 40 °C (3 min) at 11 °C/min to a maximum of 200 °C. The split valve was closed before the sample was crushed and reopened 45 s later. Helium was used as carrier gas at a flow of 1 ml/min.

The compounds were identified by comparing their mass spectra with standard MS-data bases. Their identity was confirmed by co-injection of synthetic standards of the acids (Sigma-Aldrich Co. Ltd., Gillingham, UK). As the chromatograms were characterised by many co-eluting compounds, quantification was carried out using single ion monitoring appropriate to each individual compound. The amounts were quantified by acquiring calibration curves of six different concentrations for each compound under the same conditions.

Statistics: compositional data and their standardisation

The quantified amounts of all compounds in the individual metapleural gland secretions are absolute figures whose magnitude depends on the amount of secretion analysed. Standardisation adjusting for such total concentration effects normally involves dividing each amount by the sum of all compound-amounts measured for the focal ant, which results in proportional data adding up to one. However, this procedure poses problems for multivariate statistics (Aitchison, 1986), as the compositional nature of such data may induce spurious correlations. A more elaborate standardisation was therefore required, and in the present study we followed the one proposed by Aitchison (1986, p. 78–79):

$$Z_{ij} = ln\,(Y_{ij}/g\,(Y_j))$$

where Z_{ij} is the standardised total substance amount i for individual ant j, Y_{ij} is the observed total substance amount i for individual ant j, and $g(Y_j)$ is the geometric mean of all substances for ant j included in the analysis.

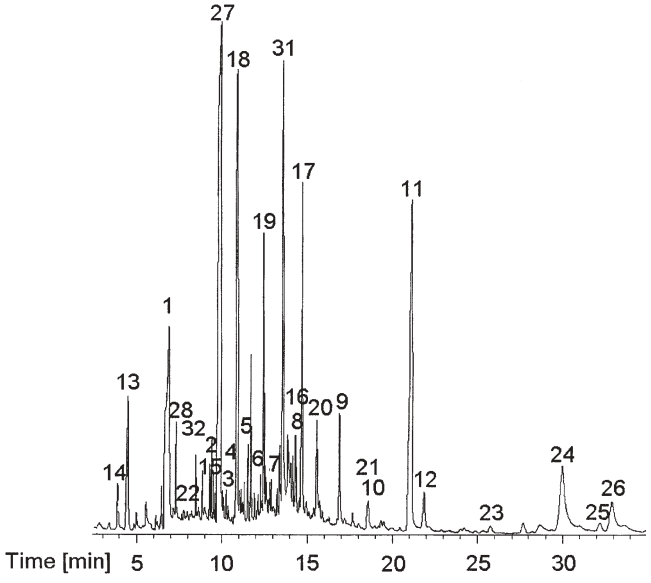

Figure 1. A typical chromatogram of an individual *Acromyrmex octospinosus* worker illustrating the chemical composition of the metapleural gland secretion. Only compounds that were detected in the metapleural gland secretion of all ant workers were given numbers. See Table 1 for details and for the grouping of compounds into the seven functional groups used in the multivariate analyses

The chemical composition of the metapleural gland secretion in *Acromyrmex octospinosus* is highly complex, with 21 major compounds identified (Fig. 1; for details see Ortius-Lechner et al., 2000a). Overall analysis of quantitative variation in specific compounds was therefore done by principal component analysis. Due to the very small amounts of some of the major compounds, only 17 of these were used. To ensure that the number of variables was about an order of magnitude less than the number of samples (a feature that generally makes multivariate analyses more reliable), these 17 compounds were combined into seven different functional groups: acetic acid, short chain acids, medium chain acids, long chain acids, indoleacetic acid, lactones and keto-acids (Table 1). These functional groups combine compounds of similar structure, which we assumed exert similar functional effects within groups. Across groups, functions were assumed to be different, an inference that was confirmed by a recent empirical study in which diverse classes of micro-organisms were shown to react differently to these classes of

Table 1. Chemical compounds detected in the metapleural gland secretion, their peak numbers (see Figure 1) and the classification into the seven functional groups used in the analysis

Functional Group	Acetic acid	Short chain acids	Medium chain acids	Long chain acids	Indoleacetic acids	Lactones	Ketoacids
Compound (peak number)	(1)	Propionic (22) Valeric (2) Hexanoic (3)	Octanoic (5) Nonanoic (6) Decanoic (7) Dodecanoic (8)	Myristic (9) Pentadecanoic (10) Palmitic (11)	(17)	γ-Butyrolactone (32) γ-Octalactone (18) γ-Decalactone (19)	4-Oxo-octanoic (20) 4-Oxo-decanoic (21)

compounds (Bot et al., 2002). This approach also avoids that differences among chemically very similar compounds would affect the analysis.

To investigate the distribution of variation in metapleural gland secretion at the colony and patriline level, the scores for each of the six principal components were subjected to ANOVAs in two steps: ANOVAs comparing colonies and ANOVAs comparing patrilines nested within colonies. We also performed discriminant analysis for comparing patrilines in each of the colonies, using the same standardized compound volumes. Finally we applied a power test (Sokal and Rohlf, 1981) on the patriline data nested within colonies.

Results

The total number of males that contributed to the offspring was 7 and 11 for colonies 18 and 19, respectively (Fig. 2). Paternal contributions were moderately skewed, with relatively many rare patrilines in colony 19. Taking this skew into account, the (sample size corrected – cf. Boomsma and Ratnieks, 1996) effective queen-mating frequencies were estimated to be 5.3 and 6.8 for colonies 18 and 19, respectively. The principal component analysis for the combined data set of all 101 ants from the two colonies gave eigenvalues and percentage of variation explained by each eigenvector as shown in Table 1. The number of principal components reflects the (maximally seven) classes of compounds for which cumulative peak-volumes were available. The last principal component is equal to zero by definition, because of the compositional nature of the data.

ANOVAs were run to compare workers from the two colonies with regard to their scores for the six non-zero principal components. A first analysis with patrilines nested within colonies gave a significant difference between colonies for PC1 and PC4, but no significant effect of patriline ($p > 0.06$ for all six components). In this analysis, the colony-level significance for PC1 and PC4 disappeared when using Bonferroni adjustments (Rice, 1989). However, when we excluded the non-significant patrilines as a nested factor, we obtained a significant result at the colony-level for the first, second and fourth principal component (PC1: $F_{1,99} = 14.85$, $p < 0.0005$; PC2: $F_{1,99} = 4.24$, $p = 0.042$; PC4: $F_{1,99} = 12.54$, $p = 0.001$), where only the significance of PC2 disappeared after a Bonferroni correction. A power test on the total data set of 101 workers produced a power of 52.33%. To achieve a power of at least 90% the sample size would have had to be twice as large, i.e. 201 workers, divided equally over the two colonies. This power indicates that we cannot accept a null hypothesis of no difference with a high probability after rejecting an alternative hypothesis.

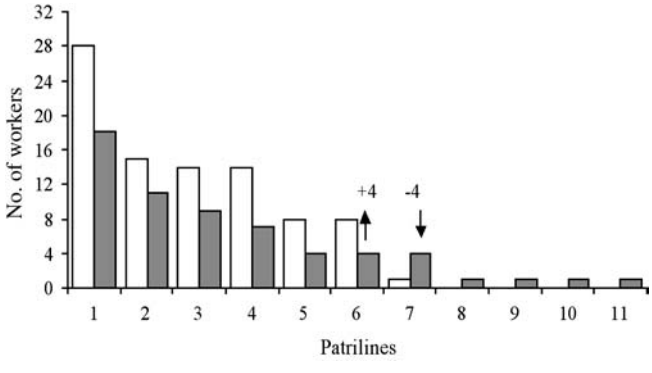

Figure 2. Paternity profiles of *Acromyrmex octospinosus* colony 18 (white bars) and 19 (dark grey bars), indicating the total number of fathers detected in samples of 88 and 64 workers, respectively, and the relative differences in representation of these patrilines (paternity skew). Arrows, whose length corresponds to the possible error made, indicate the few cases in which paternity assessments could not discriminate between two of the fathers, which means that either patriline 7 does not exist or both 6 and 7 had 4 workers assigned

Colony-wise discriminant analyses of the quantitative variation in the standardized compound-class volumes per patriline produced the patterns shown in Figure 3. No significant overall patriline effect on the quantitative variation in the seven groups of chemicals could be detected in colony 18 ($\chi^2 = 31.257$; df = 30; $p = 0.403$), whereas a marginally significant effect was found for colony 19 ($\chi^2 = 43.567$; df = 30; $p = 0.052$). Using a cross-validation procedure for assigning individual ants on the basis of their metapleural gland profiles, 17.1% and 23.3% were correctly assigned to their patrilines in colony 18 and 19, respectively. These percentages are very close to the 16.7% expected if assignment would have been completely random.

Discussion

Using three highly variable microsatellite markers, we detected 7 and 11 patrilines in the samples of 88 and 64 workers from colony 18 and 19, respectively. A previous study, using three allozyme markers and a 4-allele microsatellite marker detected 6 and 10 fathers for these same two colonies from samples of 79 and 68 workers (Boomsma et al., 1999). The only marginally better detection efficiency in the present study indicates that: 1. The combined detection efficiency of four markers with only 2–4 alleles each is reasonably good, as was inferred already in Boomsma et al. (1999). 2. The present analysis with three highly variable markers easily detects all patrilines in a sample. This is illustrated by the fact that our third microsatellite marker almost never detected a patriline that had not already been identified by the other two markers (never in colony 18 and only for five individuals in colony 19). The present estimates of effective queen-mating frequency were higher than the ones obtained in the previous study: 5.3 versus 3.6 for colony 18, and 6.8 versus 6.5 for colony 19. The new estimates confirm that little effective paternity may be gained by additional matings beyond the population-specific average, a trend that was noted in the data of Boomsma et al. (1999), although it was not statistically significant.

Our results show that the quantitative composition of the metapleural gland secretion may differ significantly among colonies, but that this composition is unlikely to show similar differences among patrilines in the same colony. Although we only sampled two colonies the differences between them were significant despite the fact that they had been kept under identical laboratory conditions for several years before

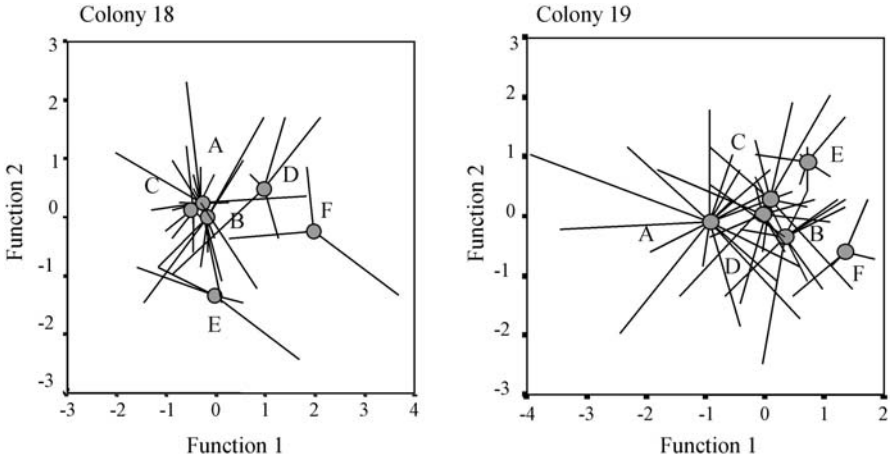

Figure 3. Within and among patriline variation in the chemical composition of the metapleural gland secretion in workers from colony 18 and 19 of the leafcutter ant *Acromyrmex octospinosus*. The plots illustrate the variation at the first and second discriminant axis, which together explain >75% of the total variation (PC1: 2.53 eigenvalue/47.0% variance; PC2: 1.53/28.5%). The six patrilines are marked A-F for both colonies and contained the following number of workers analysed (colony 18: A = 18; B = 7; C = 4; D = 5; E = 4; F = 3, colony 19: A = 18; B = 11; C = 10; D = 9; E = 7; F = 5). Group centroids (filled circles) indicate the mean score for each of the patrilines. The scores of individual ants are the tips of the lines emanating from these centroids

these analyses. These colony-level differences may well be even more substantial in the field, where nesting habitats and type and quality of forage are less constant than in the lab. These differences across colonies may be partly genetic, but they are also affected by environmental differences (although we tried to standardize these by analysing laboratory colonies) and phenotypic maternal effects (differences in queen quality) and can therefore not be used to infer the heritable component of traits under study. Differences among patrilines nested within colonies do allow such inferences as variance components at this level are directly proportional to the additive genetic variation, except that genetic dominance effects may still induce overestimations of heritability (Falconer, 1981). However, the patriline-specific differences in our study are apparently minor. In the current samples patriline assignment on the basis of the chemical characteristics of the metapleural gland secretion were only 0.4–4.2% better than random assignments. Although it cannot be excluded that these differences might become statistically significant with much larger samples (see the power-test results), it seems doubtful that such possible differences would be biologically very meaningful. As outlined in the Methods section, the across-patriline variance component is directly proportional to the narrow-sense heritability of a trait (cf. Falconer, 1981), because workers of different patrilines only differ in their genes and have all environmental variation in common. The present results thus imply that the heritable component for chemical variation in the metapleural gland secretion of *Acromyrmex octospinosus* is low.

Low heritability of the chemical composition of metapleural gland secretion means that selection for the spectrum of compounds analysed is weak and that worker-daughters of queens mating with more males are not or hardly more variable in their expression of these spectra than workers whose mother mated with fewer males. It is possible that our patriline samples were not maximally homogeneous because we could only assess age-cohorts approximately by cuticular pigmentation and such sample-heterogeneity may have obscured some of the genetic differences between patrilines. However, it should be kept in mind that colonies of *Acromyrmex* leafcutter ants always have overlapping age-cohorts of workers, so that patrilines in reality probably overlap even more in metapleural gland chemistry than the ants that we sampled. We therefore conclude that, given the labour-intensiveness of the present study, doubling the sample size to obtain a power of 90% and possibly a significant variance component among patrilines seems hardly worthwhile. The reason is that substantial benefits from multiple queen mating are needed to compensate for the increased mortality costs of this queen-behaviour. The present data show that the variation between patrilines in the spectrum of metapleural gland compounds investigated is unlikely to select for multiple mating of queens in *Acromyrmex* leafcutter ants. However, this does not preclude that other compounds in the metapleural gland secretion may show more genetic variation. Although we find no clear patriline differences in the volatile part of the metapleural gland secretion, we must remember that the metapleural gland secretion also includes some proteins (do Nascimento et al., 1996). Quantitative analysis of these proteins, which would require completely different techniques than the ones used in the present study, would therefore be highly interesting. In addition this secretion is not the only known defense mechanism that leafcutting ants have at their disposal and mandibular gland secretions (North et al., 1997) and/or cuticular *Streptomyces* bacteria (Currie et al., 1999) may add further, and possibly genetically variable, perspectives to the overall robustness of leaf-cutting ants towards disease.

The results of our study show that the "genetic variability against disease" hypothesis for the evolution and maintenance of multiple queen-mating is hard to test and even harder to prove. Although we have far from exhausted the exploration of traits that could potentially give worker-collectives of leafcutter ants important colony-level fitness advantages from multiple queen-mating, the present exclusion of the seemingly relevant "antibiotic spectrum" variable is a marked case in point. It adds to the controversial results from tests of the same hypothesis in multiply mated honeybees, *Apis mellifera*. While Rothenbuhler and Thompson (1956) and Laidlaw and Page (1984) found weak support for the

"genetic variability against disease" hypothesis, Ratnieks (1989) and Woychiechowski et al. (1994) found no evidence for higher levels of infection in low versus high genetic diversity colonies of honeybees.

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