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Mating frequency and genetic structure of the Argentine ant *Linepithema humile*

Abstract

The nest and population genetic structures of the Argentine ant, *Linepithema humile* were investigated using eight microsatellite loci. Genotypes of the sperm from spermathecae of 87 queens were consistent with all queens being singly inseminated. The probability of a double mating remaining undetected was low (0.012) suggesting that no queens or only a very low proportion mate multiply. The relatedness between the queens and their mates was negative ($R = -0.164 \pm 0.044$) and significantly different to zero ($P = 0.020$). However, the high negative relatedness value was caused by a significant allele frequency difference between the sexes at a single locus (*Lhum-28*). When this locus was removed from the analyses, the relatedness was not significantly different from zero ($R = 0.013 \pm 0.050$, $P = 0.812$). Analysis of 10 nests revealed that the genetic differentiation among nests was weak ($F_{ST} = 0.003$) and not distinguishable from zero ($P = 0.468$). Similarly, the overall relatedness among nestmate females was not significantly different from zero ($R = 0.007 \pm 0.018$, $P = 0.706$). These results are consistent with the lack of distinct nest boundaries and the large number of queens per nest in the population studied. Although mating takes place inside the nest, the inbreeding coefficient was close to zero ($F = 0.007 \pm 0.025$, $P = 0.786$). Overall, these data indicate substantial local gene flow mediated by movement of reproductives among colonies.

Keywords: *Linepithema humile*, mating frequency, microsatellites, population structure, social insects

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Introduction

Hamilton (1964) showed that the evolution and maintenance of reproductive altruism in insect societies can be explained by kin selection when workers favour the reproduction of related individuals, and by so doing indirectly transmit copies of their own genes to the next generation. Indeed social insect workers are highly related to the brood they rear when colonies are headed by one queen. However, insect colonies frequently contain several queens (polygyny), and these queens can mate with more than one male, with the effect of decreasing relatedness among colony members (Hamilton 1964; Nonacs 1988; Ross 1993; Keller 1995; Boomsma & Ratnieks 1996; Pamilo *et al*. 1997).

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The presence of multiple queens is not challenging for kin selection as long as the brood is significantly related to the workers (Bourke & Franks 1995). However, in some species the average relatedness among nestmate queens and workers is indistinguishable from zero (Fletcher & Ross 1985; Herbers 1993; Rosengren *et al*. 1993; Keller 1995) and this is considered as a potential problem for kin selection theory (Fletcher & Ross 1985; Herbers 1993; Nonacs 1993; Bourke & Franks 1995).

An important step toward gaining a better understanding of the evolution and maintenance of high levels of polygyny in ants requires information on the genetic structure of nests and populations as well as data on dispersal patterns of sexuals in highly polygyne ants (Chapuisat *et al*. 1997). The aim of the present study was to document the genetic structure and breeding system of such a species, the Argentine ant *Linepithema humile* (previously *Iridomyrmex humilis*). This species has been accidentally

introduced from South America to many areas with Mediterranean-like climate such as South Africa, California (USA), southern Europe and Australia (e.g. Newell & Barber 1913; Passera 1994; Visser *et al*. 1996). It is frequently a significant pest either because it tends herbivorous aphids, invades human habitations or displaces indigenous species (Visser *et al*. 1996; Human & Gordon 1997). *L. humile* queens generally mate in the nest a few days after eclosion from the pupae (Newell & Barber 1913; Keller & Passera 1992). Males have been frequently observed in mating flights and/or captured in aerial traps (Newell & Barber 1913; Markin 1970; Benois 1973) and can mate with queens from foreign colonies (Passera & Keller 1994). By contrast, females do not participate in a mating flight (Newell & Barber 1913; Passera & Keller 1990a). A genetic study with two allozyme markers (average $H_E = 0.279$) showed that nestmate individuals are not significantly related (Kaufmann *et al*. 1992). To obtain more precise data we made use of the recently developed polymorphic microsatellite markers (Krieger & Keller 1999) to study the genetic structure of *L. humile* populations. The higher numbers of microsatellite loci screened are expected to provide more accurate estimates of the population genetic structure.

Another objective of this study was to obtain genetic data on mating frequencies. Mate-choice experiments suggested that *L. humile* females effectively mate only once (Keller & Passera 1993). However, copulation frequencies are not always a good measure of the effective mating frequency because not all copulations necessarily translate into insemination. Furthermore, the mating frequencies observed in artificial experiments might differ from the true mating frequencies in natural populations. Hence, effective mating frequencies are best studied using genetic data only (Boomsma & Ratnieks 1996). The use of microsatellites also allowed us to estimate the relatedness between the queens and their mates by being able to genotype sperm.

Materials and methods

Sample collection

Linepithema humile were collected in Port Leucate, near Perpignan, Southern France. All individuals were collected along a road inside the town of Port Leucate. Ten workers were sampled from each of 10 locations, 20–30 m apart (total distance of sampling = 200 m). Worker samples were stored immediately in 100% ethanol. Queens were collected from the same area (total distance of sampling = 200 m) from 20 different locations. We collected and brought the soil back to the laboratory where the queens were slowly drawn out of the soil with a 100 W incandescent lamp. A total of 111 queens were recovered from the excavated soil (2–13 queens per location).

Microsatellite analysis of sperm, queens and workers

All workers were genotyped at eight microsatellite loci (*Lhum-11*, *Lhum-13*, *Lhum-19*, *Lhum-28*, *Lhum-35*, *Lhum-39*, *Lhum-52*, *Lhum-62*). The queens and their stored sperm were only genotyped at the five most polymorphic loci (*Lhum-11*, *Lhum-13*, *Lhum-19*, *Lhum-28*, *Lhum-35*) which possess 7, 5, 7, 4, and 10 alleles, respectively (Krieger & Keller 1999). Primer sequences and detailed methods of PCR amplifications are described in Krieger & Keller (1999).

DNA was extracted from whole individual workers or the heads and thoraxes of queens. The specimens were homogenized in 1.5-mL reaction tubes with a small plastic pestle. One hundred and fifty μL of extraction buffer (100 mM NaCl, 50 mM Tris-HCl pH 8, 1 mM EDTA, 0.5% SDS and 200 mg/mL proteinase K) was added and the tissue was digested for 2 h at 55 °C. After a phenol–chloroform extraction the DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 vols of ethanol (100%). The pellets were rinsed with 70% ethanol, dried at 37 °C and resuspended in 200 μL of distilled water.

For the isolation of sperm DNA, a queen's abdomen was dissected in distilled water, and intact spermathecae were removed and placed in a receptacle with distilled water. The spermatheca was then ruptured with forceps and transferred to a 1.5-mL reaction tube. We did not separate the sperm from the spermatheca because the amount of DNA from the spermathecal wall is likely to be small in comparison to the amount of DNA from the sperm. As the PCR is a competitive and exponential amplifying process the sperm DNA is expected to outcompete the maternal tissue (spermatheca wall). Further DNA isolation of sperm DNA followed the methods described above with the exception that the volume of distilled water used for final resuspension was reduced to 100 μL.

Statistical analyses

Deviation from Hardy–Weinberg genotype proportions at each locus was tested by means of exact tests, or a Markov chain approximation of the unbiased exact *P*-values (Guo & Thompson 1992) when more than four alleles were present at a locus. A global test of deviation from Hardy–Weinberg proportions across all loci was performed using Fisher's method. Genetic differentiation among nests was calculated for each locus using the fixation index (F_{ST}) of Wright (1951). Multilocus estimates were computed as in Weir & Cockerham (1984). To test for significant genetic differentiation at each locus, exact

genotypic *G*-tests were computed (Goudet *et al*. 1996). A global test across all loci was carried out using Fisher's method. Differences in allelic distributions between queens and their mates were calculated as described by Raymond & Rousset (1995a). A global test across all loci was carried out using Fisher's method. All calculations were performed using the program GENEPOP (version 2 and 3.1; Raymond & Rousset 1995b).

Genetic relatedness was measured with the method of Queller & Goodnight (1989). For all relatedness (R) and inbreeding (F) estimates, individuals were weighted equally. Standard errors were obtained by jackknifing over groups or nests. Relatedness and inbreeding coefficients were tested for significant departures from zero with two-tailed t-tests. Calculations were performed using the program RELATEDNESS 4.2b (Goodnight 1994).

Queen mating frequencies were estimated from the sperm in the queens' spermatheca by counting the maximum number of alleles detected at any locus. As fertile Hymenopteran males are almost always haploid the number of alleles detected provides a direct estimate of the mating frequency. However, assessing queen mating frequencies from amplified sperm is prone to potential errors (Gertsch & Fjerdingstad 1997; Chapuisat 1998) that can lead either to an over- or underestimation of the actual mating frequencies. First, extracted sperm can be a mixture of sperm and queen's DNA. This is only a problem if the relative amount of the queen DNA is sufficient to be amplified. Successful amplification of queen DNA results in an overestimation of the true mating frequency. Second, multiple insemination can remain undetected if unequal sperm contributions by males result in nonamplification of rarer DNA haplotypes (Gertsch & Fjerdingstad 1997). Finally, multiple matings can remain undetected because males have identical genotypes. Nonamplification of the rarer haplotype and nondetection of multiple mating partners with identical genotypes both lead to an underestimation of the true mating frequency (Gertsch & Fjerdingstad 1997).

Contamination with the female's DNA can be classified in two categories depending on whether the extract contains only maternal tissue or also sperm. In the former case contamination results in the same allelic pattern at all loci as the female from which the sperm was extracted. The chance that this pattern emerges from a genuine double mating can be calculated (assuming random mating) and is given by the product over all loci of $2p_iq_j$ for the heterozygous and p_i^2 for the homozygous loci, where p_i and q_j represent the population frequencies of the corresponding alleles. Even with only a few moderately polymorphic loci this probability is very small and all sperm athecal amplified showing this pattern can be safely regarded as being contaminated with maternal tissue. If the extract contains also sperm the allelic pattern corresponds to the genotype of the queen plus additional alleles at some or all of the loci depending on the queen's mating frequency and on the queen and male genotypes. If the queen has mated only once, at most three different alleles are present per locus, two corresponding to her own genotype and one corresponding to the male's genotype. The probability that this pattern emerges from a genuine triple mating is even smaller than in the case of a genuine double mating.

Undetected double matings caused by nonamplification of the rarer haplotype are likely to occur when the difference in individual sperm contributions is large. Males that contribute relatively few sperm have a lower detection probability because the PCR is a competitive and exponential amplifying process. However, sperm can be unambiguously detected until a ratio of about 1:10 (Gertsch & Fjerdingstad 1997; Chapuisat 1998) depending on the PCR characteristics of the loci used and on the relative sizes of the alleles involved.

Finally, the proportion of undetected double matings due to identical genotypes of the queen's mating partners can also be estimated. The proportion of undetected double matings among doubly mated queens is given by

$$\prod_j \sum_i p_{ij}^2$$

where p_{ij} stands for the population frequency of allele i at locus j (Pamilo 1993; see also Boomsma & Ratnieks 1996). The expected total proportion of doubly mated queens (D_{est}) in the data set is then calculated from the sum of undetected and observed number of doubly mated queens and is given by

$$D_{\text{est}} = \frac{D_{\text{obs}}}{1 - \prod_j \sum_i p_{ij}^2} \quad (1)$$

where D_{obs} is the proportion of observed double matings and i, j and p are as noted above. If no double mating is detected the upper limit of the proportion of doubly mated queens can be estimated by assuming that the $N + 1$th sampled queen would have been detected as a doubly mated queen (N is the sample size). This estimate is given by

$$D_{\text{est}} < \frac{1}{(N+1)\left(1 - \prod_j \sum_i p_{ij}^2\right)} \quad (2)$$

However, if the loci are not in linkage equilibrium the observed haplotype combinations of the different loci will not reflect what is expected under the hypothesis of random segregation, i.e. some allelic combinations will occur more and some less frequently than expected. This will lead to an over- or underestimation of the proportion of doubly mated queens. Even if the loci are unlinked

but sample sizes are small, the observed haplotype combinations can also differ from expected haplotype frequencies due to sampling error and hence influence the estimate. We describe below a second estimation procedure (D^*_{est}) which overcomes these limitations by integrating the haplotype combination of each sperm contributor into the calculation of the estimate. Another difference between D_{est} and D^*_{est} is that the former yields a better estimation of the source population, i.e. the expected proportion of double-mated queens in the total population, whereas the latter gives a more accurate estimate of a given data set. However, in the absence of linkage disequilibrium the two estimates are expected to converge with large sample sizes. D_{est} should be preferred with large samples because of its simpler calculation.

The second estimation procedure (D^*_{est}) that uses the information of the haplotype combination of each sperm contributor is calculated as follows (see also Pedersen & Boomsma 1999b). The probability that queen l, which has mated with male k, has also mated with a second male of identical haplotype is m, where m is the male allele frequency at the marker locus. This probability over all loci is then given by

$$\prod_j m_j$$

where m_j is the male allele frequency of allele m at locus j. This probability is calculated for all queens where only one mate has been identified. These probabilities are averaged and represent the average proportion of undetected doubly mated queens among all doubly mated queens. Again, the expected total proportion of doubly mated queens (D^*_{est}) in the data set, based on individual haplotype combinations of the sperm contributors, is then calculated from the sum of observed and undetected number of doubly mated queens and is given by

$$D^*_{est} = \frac{S \cdot D_{obs}}{S - \sum_k^s \prod_j m_{jk}} \quad (3)$$

where m_{jk} is the male allele frequency of allele m at locus j of male k, the sample size and S the number of queens with only one mate detected. It has to be noted, however, that the male allele frequencies used in the calculation might be inaccurate if they were solely assessed from the sperm extracts. Alleles at high frequencies will be underestimated. This is because, on average, an allele at a high frequency will be masked more often by a same allele in doubly inseminated queens as compared with alleles at low frequencies. This causes the estimate of the proportion of doubly mated queens to be slightly smaller than the true proportion (for a mathematical procedure to correct for this bias see Pedersen & Boomsma 1999b). Again, if no double mating is detected, the upper limit of the proportion of doubly mated queens in a sample of N queens can be estimated by

$$D^*_{est} < \frac{N}{(N+1)\left(N - \sum_k^N \prod_j m_{jk}\right)} \quad (4)$$

To estimate the proportion of doubly mated queens we used both estimates, D_{est} and D^*_{est}. However, in the calculation of D_{est} we replaced the population allele frequencies of females (workers and queens) by the male allele frequencies because males and females did not share the same allele frequencies at locus *Lhum-28*. Using the population allele frequencies instead would have led to an underestimation of the proportion of doubly mated queens.

Results

Hardy–Weinberg proportions

Results of the Hardy–Weinberg tests with the eight microsatellite markers in the worker sample showed no significant ($P < 0.05$) departure from Hardy–Weinberg proportions (HWP) with a combined probability over all loci of $P = 0.945$. Expected heterozygosity (H_E) for the eight polymorphic loci varied between 0.107 and 0.638 (Table 1).

Queen mating frequency

In 14 cases (12.6%) all amplified alleles from the spermathecal sperm corresponded to the queen's genotype, indicating that the extract contained only maternal tissue or a mixture of maternal tissue and sperm from a single male with an allele at any one locus identical to one of

Table 1 Relatedness estimates (R) and standard errors (SE) of 87 queens and the males they mated with, separated by locus. P-values indicate the alpha level of the relatedness estimates being different from zero. Standard errors were obtained by jackknifing

	Queens and their mating partner ($N = 87$)	
Locus	$R \pm$ SE	P
Lhum-11	−0.079 ± 0.108	0.505
Lhum-13	−0.009 ± 0.099	0.932
Lhum-19	0.063 ± 0.088	0.514
Lhum-28	−0.676 ± 0.081	0.001
Lhum-35	0.075 ± 0.093	0.465
All loci	−0.164 ± 0.044	0.020
All without Lhum-28	0.013 ± 0.050	0.812

Table 2 Relatedness estimates (R), inbreeding coefficients (F) and fixation indexes (F_{ST}) of 10 workers from 10 different nests, separated by locus. P-values of R and F-values indicate the alpha level of being different from zero, whereas the P-values of the F_{ST} values indicate the significance level of a genotypic structure (G-test). Standard errors were obtained by jackknifing across nests

Locus	H_E	$R \pm$ SE	P	$F \pm$ SE	P	F_{ST}	P
Lhum-11	0.486	−0.045 ± 0.018	0.034	0.093 ± 0.101	0.381	−0.025	0.606
Lhum-13	0.492	0.005 ± 0.041	0.906	0.004 ± 0.107	0.971	0.003	0.447
Lhum-19	0.638	0.010 ± 0.037	0.793	−0.003 ± 0.080	0.971	0.005	0.640
Lhum-28	0.304	0.111 ± 0.152	0.484	−0.013 ± 0.094	0.893	0.053	0.057
Lhum-35	0.376	0.025 ± 0.028	0.395	0.044 ± 0.056	0.452	0.013	0.442
Lhum-39	0.107	−0.013 ± 0.036	0.726	−0.032 ± 0.007	0.001	−0.006	0.512
Lhum-52	0.422	0.030 ± 0.068	0.670	−0.089 ± 0.061	0.179	0.014	0.234
Lhum-62	0.496	−0.035 ± 0.040	0.404	0.011 ± 0.092	0.908	−0.017	0.705
All loci	0.418	0.007 ± 0.018	0.706	0.007 ± 0.025	0.786	0.003	0.468

the queen's alleles. The probability that this pattern emerged from genuine double matings was, on average, 0.0007 and ranged from 0.009 to 0.00003, depending on the queen's individual genotype. Thus, these extracts probably contained only maternal DNA. In 10 cases (9%) the amplified alleles were a mixture of maternal alleles plus an additional allele at least at one locus. This suggests that the extract contained maternal tissue as well as sperm. In all 10 cases there was never more than one allele per locus which did not correspond to the queen's genotype. This is consistent with the queens being single-mated. The alternative explanations, that the amplified DNA came from three different males were very unlikely because the probability of obtaining this allelic pattern from genuine triple matings was very low ($P < 0.0001$). These 24 samples (21.6%) were excluded from further analyses. The sperm amplified from the remaining 87 queens (78.4%) was consistent with all queens being singly mated. At all loci the amplified sperm DNA resulted in a single electrophoretic band. The upper estimates of the proportion of doubly mated queens were low and almost identical ($D_{est} < 0.0122$, $D^*_{est} < 0.0123$). This suggests that none or only a very low proportion of queens are multiply inseminated.

Mating pattern

The relatedness of the queens and their mates over all loci was negative ($R = -0.164 \pm 0.044$) and significantly different from zero ($P = 0.020$). However, there was a marked difference in relatedness values among loci (Table 1) with only one of them (*Lhum-28*) showing a relatedness value significantly different from zero ($R = -0.676 \pm 0.081$, $P = 0.001$). The high negative relatedness value at locus *Lhum-28* was caused by different frequencies of the two most common alleles. Allele *Lhum-28*^{185} had a higher frequency among males (males: 0.862; queens: 0.178), whereas the allele *Lhum-28*^{187} was more common among the females (males: 0.115; queens: 0.782). This difference in frequencies of the two alleles between queens and their mates was significant (contingency test with Williams' correction, $G_{adj} = 120.276$, d.f. = 1, $P < 0.0001$). This difference remained highly significant when all alleles at locus *Lhum-28* were included in the analysis (contingency test with Williams' correction, $G_{adj} = 100.089$, d.f. = 3, $P < 0.0001$). Allele frequencies of these two most common alleles in workers at this locus (*Lhum-28*^{185}: 0.175; *Lhum-28*^{187}: 0.800) were similar and not significantly different (contingency test with Williams' correction, $G_{adj} = 0.023$, d.f. = 1, $P = 0.880$) from the allele frequencies of the queens. The difference between queens and workers was also not significant when all alleles were included (contingency test with Williams' correction, $G_{adj} = 1.089$, d.f. = 3, $P = 0.669$). When locus *Lhum-28* is removed from the analyses, the relatedness of the queens and their mates was close and not significantly different from zero ($R = 0.013 \pm 0.050$, $P = 0.812$).

Nest and population structure

The genetic differentiation among nests was assessed by calculating the relatedness value within nestmate workers. The relatedness value over all loci was low ($R = 0.007 \pm 0.018$) and not significantly different from zero ($P = 0.706$) suggesting that nestmate workers are as equally related to each other as workers from different nests. A detailed analysis separated by loci (Table 2) revealed that one of the eight loci (*Lhum-11*) had a relatedness value significantly different from zero ($R = -0.045 \pm 0.018$, $P = 0.034$). Removing locus *Lhum-28* (because of the different allelic frequencies among males and females) had little effect on the relatedness of females. Although the relatedness value decreased, it remained indistinguishable from zero ($R = -0.004 \pm 0.011$, $P = 0.725$).

The inbreeding coefficient assessed over all loci was also close and not significantly different from zero ($F = 0.007 \pm 0.025$, $P = 0.786$). When separated by loci (Table 2) one (*Lhum-39*) had an inbreeding coefficient significantly different from zero ($F = -0.032 \pm 0.007$, $P = 0.001$). Removing locus *Lhum-28* from the analysis again had little impact on the estimate of the inbreeding coefficient ($F = 0.009 \pm 0.033$, $P = 0.791$). Genetic differentiation among nests assessed over all loci was weak ($F_{ST} = 0.003$) and showed no significant structure ($\chi^2 = 15.794$, d.f. = 16, $P = 0.468$). Again, removing locus *Lhum-28* had little effect on the overall F_{ST} value ($F_{ST} = -0.002$, $\chi^2 = 9.810$, d.f. = 14, $P = 0.776$). None of the eight loci showed a significant differentiation (< 0.05%, Table 2).

Discussion

Results of the sperm extracts from the queens' spermathecae show clearly that queens were all singly mated. Because no doubly mated queen was detected, the maximal proportion of doubly mated queens was estimated by assuming that the hypothetical 88th queen (the next queen sampled) was doubly mated. Such an estimate represents the upper limit of the proportion of doubly mated queens in a data set where no double matings are detected. This estimated proportion was small (0.012), indicating that all or almost all queens mate once in the studied populations. In all cases, the largest number of alleles per loci which could not be attributed to the queen was only one, which is consistent with these queens being singly mated (see below). The conclusion that all, or almost all queens are inseminated by a single male support findings from behavioural studies which showed that although some of the queens mated with more than one male, only one of them effectively transferred sperm (Keller & Passera 1992).

The results of the two methods to estimate the proportion of multiple mating were almost identical ($D_{est} < 0.0122$, $D^*_{est} < 0.0123$). This partly results from the fact that no doubly mated queen was detected. This can be shown by comparing the estimated proportion of undetected doubly mated queens among all doubly mated queens, a variable used in both methods to calculate the estimate (but calculated differently). This proportion calculated in D_{est} (using only male allele frequencies) was 0.067 whereas the same proportion in D^*_{est} (using the additional information of the haplotype combinations) was 0.075. This difference indicates that in our sample some of the rarer haplotype combinations occurred less frequently than expected, increasing the proportion of undetected doubly mated queens. Yet, this difference had little impact on the total estimate because no doubly mated queens were detected. However, with a sufficient large number of detected double matings, this difference would have been large enough to generate substantial differences between the two estimates.

The relatedness of the queens and their mates was negative and significantly different from zero. However, the high negative relatedness value was caused by allele frequency differences between the sexes at a single locus (*Lhum-28*). When this locus was excluded from the analyses the relatedness was close to and not significantly different from zero. This result is expected because some of the males successfully disperse, enter foreign nests and mate with resident female sexuals whereas others stay and mate within the natal nest (Passera & Keller 1994). The different allele frequencies between the sexes at locus *Lhum-28* is more difficult to account for. In males allele *Lhum-28*^{185} occurred with a high frequency (0.862) but was rarer in females (0.178). The frequencies of the other common allele (*Lhum-28*^{187}) was reversed; it occurred at a high frequency in females (0.782) but was uncommon in males (0.115). Differences in allele frequency between the sexes have been reported in other ants (Boomsma *et al*. 1993; Pamilo 1993), yet its origin remains speculative (Pamilo 1993).

For *Linepithema humile*, there are at least two possible explanations for the allele frequency differences between the sexes in this population. First, males may originate from another population with different allele frequencies. Although males in *L. humile* are able to fly and enter foreign nests (Passera & Keller 1994), Port Leucate is isolated between the Mediterranean sea and salt water lakes, which makes it unlikely that males from a population outside Port Leucate reach this locality in high frequencies. Furthermore, the numerical sex ratio in this population is strongly male biased (Keller & Passera 1992) which should make it even harder for putative immigrant males to successfully mate with resident females. If males originate from another population the allele frequencies are expected to be dissimilar at most or all loci. However, a test for allelic differentiation between the queens and their mates revealed that *Lhum-28* was the sole cause for the significant differentiation across all loci ($\chi^2 = \infty$, d.f. = 10, $P < 0.0001$). Excluding *Lhum-28* from the analyses rendered the difference nonsignificant ($\chi^2 = 10.985$, d.f. = 8, $P = 0.203$). The other explanation is that a dominant factor which eliminates one set of chromosomes in diploid offspring is in linkage disequilibrium with *Lhum-28*^{185} (the most common allele in males at locus *Lhum-28*). Under this hypothesis females would only be produced in the absence of this allele. If one assumes that linkage disequilibrium is such that the proportion of the allele *Lhum-28*^{185} is 0.178, this yields an estimate of 73% diploid eggs ultimately giving rise to males.

Genetic factors that influence the sex ratio are widespread in animals (reviewed in Hurst *et al*. 1997). Sex-ratio distortion is frequently caused by maternally inherited

microorganisms that bias the sex ratio towards females. The best known genetic element that causes a sex ratio bias towards males is a supernumerary or B chromosome found in some natural populations of the parasitoid wasp *Nasonia vitripennis* (Werren *et al*. 1987; Nur *et al*. 1988). This B chromosome induces in fertilized eggs a condensation of the paternal chromosomes which are subsequently lost, causing the zygote to develop into a haploid male. This same mechanism cannot be invoked to explain the allele frequency differences between the sexes in *L. humile*, because under the hypothesis made above the paternal set of chromosomes is eliminated. However, it is conceivable that a similar genetic process is causing the elimination of the maternal chromosomes in the Argentine ant. It is currently impossible to test this hypothesis, but several lines of evidence suggest that it is not implausible.

First, allele frequencies in workers at locus *Lhum-28* did not correspond to the allele frequencies expected from matings between males and queens, they were almost identical to the allele frequencies of the queens. This consistent difference in allele frequency between the sexes points to a systematic deviation of allele frequencies at this locus. Second, a large proportion of eggs (30–50%) produced in this population (Aron *et al*. 1994) are haploid. Haploid eggs are found throughout the year, indicating that their production is decoupled from the short reproductive season in early summer. The high proportion of haploid eggs cannot be attributed solely to unmated queens because only 2.1% of the queens are unmated (Keller & Passera 1992). Furthermore, the proportion of haploid eggs might even be higher than 30–50% in this population. Aron *et al*. (1994) collected eggs that were kept between 48 and 96 h with workers before determining their sex. It is therefore conceivable that workers had already eliminated a proportion of male eggs in the first 48–96 h. Because the proportion of 48–96-h-old haploid eggs is the same as the proportion of males produced in queenless colonies it has been suggested that workers do not discriminate against male eggs (Passera & Aron 1996). However, because the available data do not allow us to determine whether workers eliminate males at the egg stage in queenless colonies, the question of whether workers may discriminate against male eggs, and what is the actual proportion of haploid eggs laid by queens, remains open.

Genetic differentiation among nests was low ($F_{ST} = 0.003$) and not distinguishable from zero. Similarly, the overall relatedness of nestmate workers was low ($R = 0.007$) and also not significantly different from zero. This shows that there is no significant structure among nests and that this population of *L. humile* can be regarded as truly unicolonial. However, it is possible that the distance among the sampled nest was too close to detect any significant population structure. The inbreeding coefficient (F) assessed over all loci was also not significantly different from zero. Although mating takes place within the nest, the dispersal of males is one important component of gene flow between colonies and appears to be an effective means of preventing inbreeding and population substructure (Passera & Keller 1994). A further possible mechanism decreasing relatedness and inbreeding is brood exchange between neighbouring nests. To some extent gene flow may also occur by female sexuals or mated queens dispersing from one nest to another, but the dispersal range of queens is limited because they have never been observed to fly (Passera & Keller 1990b). At present these hypotheses cannot be discriminated. More work on the genetic structure of native populations is required in order to achieve a better understanding of the evolution and maintenance of unicoloniality in the Argentine ant and other ants.

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