Queen execution increases relatedness among workers of the invasive Argentine ant, Linepithema humile

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Abstract
Polygyny in social insects can greatly reduce within-nest genetic relatedness. In polygynous ant species, potential rival queens in colonies with multiple queens are often executed by other queens, workers, or both. The Argentine ant, Linepithema humile, native to South America, forms a “supercolony” that is composed of a large number of nests and is considered to contribute to the ant’s invasion success. Currently, four mutually antagonistic supercolonies are contiguously distributed within a small area of Japan. Here, we analyzed the genetic structure and relatedness within and among the four supercolonies using microsatellite markers to clarify how L. humile maintains its supercoloniality. The results of AMOVA and BASP, the F_{ST} values, and the existence of several private alleles indicated that the L. humile population in the Kobe area had a characteristic genetic structure. Within a given supercolony, there was significant genetic differentiation (F_{ST}) among workers collected in May and those collected in September. The significant deviation from Hardy–Weinberg equilibrium increased, and the relatedness among workers significantly increased from May to September in all supercolonies. This result suggested that the supercolonies replaced old queens with new ones during the reproductive season, thus supporting the plausibility of queen execution. From the perspective of kin selection, workers collectively eliminate queens, thereby increasing their own inclusive fitness. Restricted gene flow among supercolonies, together with mating with sib and queen execution, could help to maintain the unique social structure of L. humile, the distribution of which is expanding worldwide.

Introduction
Eusocial Hymenoptera (ants, bees, and wasps) vary greatly in their social structure. The single queen structure (monogyny), which maximizes relatedness among offspring, is ancestral in the major lineages of eusocial insects (Boomsma 2007; Hughes et al. 2008), and the multiple queen structure (polygyny) has evolved secondarily. Workers in monogynous species help raise their full sibs and so gain inclusive fitness returns from their helping behavior. On the other hand, polygyny can greatly reduce within-nest genetic relatedness, thus diluting the benefits of helping behavior. An increase in queen numbers is associated with a decrease in each queen’s reproductive output (Sommer and Holldobler 1995). These queens compete for reproductive shares within a colony with limited resources. To increase their inclusive fitness, stingless bee workers of Melipona execute emerging virgin queens when the queen’ numbers are very high (Jarau et al. 2009), being up to 16% (Wenseleers et al. 2004).

Among ant species, polygyny is a common social structure in which queens usually reduce their dispersal abilities and mate in, or close to, their natal nests (Bourke and Franks 1995). This is probably because mating among sibs increases relatedness between workers and the brood they rear within a colony (Crozier and Pamilo 1996). In polygynous ant species, potential rival queens in colonies newly established by multiple queens (i.e., colonies with pleometrosis) are often executed by other queens (Sommer and Holldobler 1995; Balas and Adams 1996a; Aron et al. 2009), the workers (Balas and Adams 1996b), or
both (Helms et al. 2013). Queen execution could benefit not only queens themselves by eliminating rivals but also workers by killing extra queens and thus increasing exclusive fitness.

In places where it is introduced, the Argentine ant, *Linepithema humile* (Mayr), which is native to South America, forms a “supercolony” composed of a large number of nests and ranging over several thousand kilometers (Giraud et al. 2002). This social structure is considered to contribute to the ant’s invasion success, because a supercolony, by reducing the costs associated with territoriality, may have high worker densities, thus promoting ecological dominance (Holway et al. 1998). On the other hand, a supercolony is also an evolutionary paradox for kin selection theory because supercolonies are genetically homogeneous entities, with the majority of workers having low relatedness to each other within colonies (Bourke and Franks 1995; Crozier and Pamilo 1996; Tsutsui and Case 2001; Helantera et al. 2009). *Linepithema humile* lacks nuptial flights (Passera and Keller 1990), and queens mate with related males within their natal nests (Markin 1970). At the beginning of the reproductive season, up to 90% of queens are usually killed by the workers (Keller et al. 1989; Reuter et al. 2001), and this queen execution may increase average worker relatedness among nestmates (Keller et al. 1989).

*Linepithema humile* was first reported in Japan in 1993 (Sugiyama 2000), and four mutually antagonistic supercolonies (*Kobe A*, *Kobe B*, *Kobe C*, and *Japanese main*), each of which is associated with one of four different mitochondrial haplotypes (Inoue et al. 2013), have been found in the city of Kobe, in western Japan (Murakami 2002; Okaue et al. 2007; Sunamura et al. 2009). Here, we used microsatellite markers to analyze genetic structure and relatedness within and among the four supercolonies to elucidate how *L. humile* maintains its supercoloniality.

### Materials and Methods

Our studies were conducted in 2009 in the four *L. humile* populations established in the Kobe area: the KA (Kobe A) and KB (Kobe B) supercolonies at Port Island, and the KC (Kobe C) and JM (Japanese main) supercolonies at the Maya Wharf (Fig. 1). We collected 11–20 workers from each of 19 nests in May and September 2009 for genetic analysis. Sexual production of *L. humile* generally occurs in the spring and early summer (M. N. Inoue et al. unpubl. data): May is just before reproduction begins and September is when the first workers are produced by the new queens. We also collected workers from 17 nests (excluding KA4 and KB5) to perform aggression tests between workers from different nests. The distance between the nests ranged from 30 to 380 m (mean ± SE, 177.50 ± 69.6 m) at the Maya Wharf and 40–1270 m (506.00 ± 29.5 m) at the Port Island site.

### Aggression tests

Intercolony aggression tests between workers were performed in May and September to confirm that workers sampled from each nest belonged to the expected supercolony. We randomly selected one worker from one of the nests from each supercolony and placed it in a plastic dish (diameter: 4 cm). One worker from a nest from the adjacent supercolony was then added and the two were observed for 5 min. To quantify the workers’ behavior, we scored each contact using a 0–4 scale (modified from the work of Suarez et al. 1999; see also Inoue et al. 2013), as follows: 0 = ignoring (physical contact between individuals in which neither ant showed any interest in the other ant); 1 = avoidance (one or both ants retreated after contact) or antennation (repeated tapping of the antennae somewhere on the other ant); 2 = dorsal flexion (abdomen pointed toward the other ant in a threatening

![Figure 1](image_url). Map of the study populations of *Linepithema humile* in the Kobe area. Population codes: KA, Kobe A; KB, Kobe B; KC, Kobe C; and JM, Japanese main.
posture); 3 = aggression (biting the other ant); and 4 = fighting (prolonged aggression, resulting in severe injury or death). Six tests were conducted for each pair of nests using different individuals each time.

**DNA extraction and PCR amplification**

We assessed the genotypes of the ants in each nest using microsatellite loci. DNA was extracted from individual workers according to the method described by Goka et al. (2000). After the application of 60 μL of lysis buffer (1 mg/mK proteinase K, 0.01 mol/L NaCl, 0.1 mol/L EDTA, 0.01 mol/L Tris–HCl [pH 8.0], 0.5% Nonidet P-40), each worker was homogenized at 50°C for 60 min and then at 94°C for 10 min. We then diluted 30 μL of the homogenate with 270 μL of TE buffer (0.001 mol/L EDTA, 0.001 mol/L Tris–HCl [pH 8.0]) and stored it at −20°C. Amplification reactions were performed in 25 μL volumes consisting of 0.5 μL of template DNA, 0.2 mmol/L dNTP, 4 mmol/L MgCl₂, 0.5 units of Taq DNA polymerase (Amplitaq Gold; Applied Biosystems, Foster City, CA), and 0.4 μmol/L of each primer. Samples were amplified using PCR under the following conditions: initial denaturing at 95°C for 10 min followed by 30 cycles of denaturing at 94°C for 10 sec, annealing at 55°C for 20 sec, and extension at 72°C for 20 sec, with a final extension step at 72°C for 7 min. Seven microsatellite loci were analyzed: Lhum-3, Lhum-18, Lhum-19, Lhum-28, and Lhum-52 (Krieger and Keller 1999); and Lihu-M1 and Lihu-S3 (Tsutsui et al. 2000). Primers were labeled with fluorescent dyes, and PCR products were visualized with an ABI 3770 sequencer (Applied Biosystems).

**Statistical analysis**

Data are expressed as means ± SD. To compare the levels of aggression between workers at each site, we employed a generalized linear mixed-effect model (GLMM; Venables and Ripley 2002) with the assumption of a normal distribution of errors with “pair” (i.e., workers of KA and KB and workers of KC and JM) as the fixed effect and “nest” as the random effect, using R version 3.0.1 (R Development Core Team 2013) and the nlme libraries (Pinheiro and Bates 2000).

Random mating (i.e., agreement with the conditions for Hardy–Weinberg equilibrium, HWE) within each supercolony was tested using Arlequin version 3.5.1.3 (Excoffier and Lischer 2010). An unbiased estimate of the exact P value for each locus was computed using the MCMC (Markov chain Monte Carlo) method with a forecast chain length of 100,000 steps and 10,000 dememorization steps. Linkage disequilibrium between loci in each population was conducted with MCMC with 10,000
tion history of a species; BAPS performs better in clustering together populations with recent gene flow (Díez-del-Molino et al. 2013).

**Results**

**Aggression**

Aggressive behavior was observed between workers from adjacent supercolony pairs. The average levels of aggression between the KA and KB supercolonies (3.65 ± 0.33 in both May and September) were significantly larger than those of the KC and JM supercolonies (GLMM, estimate = −0.67, SE = 0.16, t = −4.19, P < 0.0001 in May; GLMM, estimate = −0.50, SE = 0.17, t = −2.81, P = 0.0081 in September).

**Genetic diversity and structure**

At seven microsatellite loci, we assessed the genotypes of 717 individuals collected from the four supercolonies in May and September (for a total of eight population—

| Month   | Supercolony | n  | H_s | H_O | A_O | A_R | P_HW | Mean | SD   |
|---------|-------------|----|-----|-----|-----|-----|------|------|------|
| May     | KA          | 120| 0.76| 0.66| 6   | 4   | 6.00 | 0.51 | 0.20 |
|         | KB          | 117| 0.83| 0.72| 2   | 1   | 8.43 | 0.70 | 0.04 |
|         | KC          | 80 | 0.69| 0.68| 5   | 3   | 4.73 | 0.44 | 0.13 |
|         | JM          | 60 | 0.67| 0.69| 5   | 2   | 4.00 | 0.44 | 0.05 |
| September | KA         | 120| 0.54| 0.66| 6   | 3   | 5.21 | 0.39 | 0.05 |
|         | KB          | 89 | 0.70| 0.72| 6   | 1   | 5.61 | 0.44 | 0.05 |
|         | KC          | 71 | 0.62| 0.69| 7   | 4   | 4.00 | 0.67 | 0.05 |
|         | JM          | 60 | 0.39| 0.66| 4   | 1   | 3.93 | 0.37 | 0.05 |

*Table 1.* The expected heterozygosity (H_s), observed number of alleles per locus (A_O), allelic richness (A_R), and P value for Hardy–Weinberg equilibrium (P_HW) for the four supercolonies in the Kobe area, as determined from individuals collected in May and September 2009. See Fig. 1 for colony abbreviations.

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Table 2. Mean and standard deviation (SD) of relatedness (rxy) values for nestmate workers collected from the four supercolonies in May and September 2009. See Fig. 1 for colony abbreviations.

| Month | Supercolony | Mean rxy | SD  |
|-------|-------------|----------|-----|
| May   | KA          | 0.208    | 0.260 |
|       | KB          | 0.395    | 0.208 |
|       | KC          | 0.290    | 0.202 |
|       | JM          | 0.407    | 0.253 |
| September | KA       | 0.318    | 0.261 |
|         | KB          | 0.449    | 0.209 |
|         | KC          | 0.307    | 0.190 |
|         | JM          | 0.584    | 0.200 |

month combinations) (Table 1). We detected 59 alleles in total. Of the 56 tests, 14 (25%) showed significant deviation from HWE (P < 0.05) in L. humile: The number of tests that deviated significantly from the expectation increased in the supercolonies was greater in September (nine) than in May (five). Significant linkage disequilibrium was found between Lhum18 and LihM1 (P < 0.05) after Bonferroni correction. The presence of null alleles was suggested at five loci: Lhum3, Lhum18, Lhum19, Lhum28, and LihM1. The mean estimated null allele frequency ranged from 0.004 (Lhum-52) to 0.180 (LihuM1); across all seven loci, it was 0.079.

Across loci, the expected heterozygosity (H_e) ranged from 0.00 to 0.83 (mean = 0.51 ± 0.25), and the observed heterozygosity (H_o) ranged from 0.00 to 0.82 (mean = 0.42 ± 0.24). The number of alleles per locus (A_o) for all samples ranged from 1 to 9, and the mean allelic richness (A_r) ranged from 2.99 to 6.12. Mean A_r was highest in the KC supercolony and lowest in the JM supercolony in both months and decreased in all supercolonies between May and September. In all, 24 private alleles were detected across the seven loci, with at least one private allele per locus: Lhum-3 produced three; Lhum-18, seven; Lhum-19, four; Lhum-28, three; Lhum-52, one; Lihu-M1, four; and Lihu-S3, two. All supercolonies had two or more private alleles: Five were detected in KA; four in KB; 13 in KC; and two in JM.

The mean relatedness values (rxy) of worker nestmates within each supercolony ranged from 0.208 to 0.407 in May and from 0.307 to 0.584 in September (Table 2); the rxy values within each supercolony were significantly lower in May than in September (t-test, P < 0.0001).

The FST values ranged from 0.172 (KB vs. JM) to 0.370 (KC vs. JM) in May and from 0.191 (KA vs. KB) to 0.416 (KC vs. JM) in September among the four supercolonies; all values were significant (P < 0.0001, Table 3). The FST values also differed significantly among workers collected in May and those collected in September within each given supercolony, ranging from 0.027 (KB vs. KB) to 0.068 (JM vs. JM) (P < 0.0001, Table 3). AMOVA detected a significant population structure for the microsatellite data, with small but significant components of variance within (FSC) and between (FST) supercolonies (FSC = 1.78, %V = 73.80, P < 0.0001; FST = 0.63, % V = 26.20, P < 0.0001). The relationship between the pairwise FST and the geographic distance between nests was not significant within each supercolony: KA, r^2 = 0.057, P = 0.316 in May and r^2 = 0.093, P = 0.142 in September (Fig. 2A and B); KB, r^2 = 0.060, P = 0.193 in May and r^2 = −0.001, P = 0.339 in September (Fig. 2A and B); KC, r^2 = −0.245, P = 0.912 in May and r^2 = −0.569, P = 0.051 in September (Fig. 2C and D); and JM, r^2 = 0.949, P = 0.900 in May and r^2 = −0.786, P = 0.788 in September (Fig. 2C and D).

Figure 3 shows the relationship between lnP(D) and K. The lnP(D) values increased with increasing K until they reached a plateau at about K = 9. If the supercolonies had formed a significant genetic barrier to the flow of nuclear genes, we would have expected four population clusters to be revealed in this analysis; therefore, the lnP(D) values would have approached a maximum at K = 4. However, the curve failed to produce a clear peak at any

Table 3. Pairwise FST comparisons between samples from the four supercolonies in the Kobe area, collected in May and September 2009. All values were significant (P < 0.0001). Values for the comparison of a supercolony in May with the same supercolony in September are shown in the bottom half of the table. See Fig. 1 for colony abbreviations.

| Month | Supercolony | KA   | KB   | KC   | JM   | KA   | KB   | KC   | JM   |
|-------|-------------|------|------|------|------|------|------|------|------|
| May   | KA          | 0.188|      |      |      |      |      |      |      |
|       | KB          | 0.293| 0.393|      |      |      |      |      |      |
|       | KC          | 0.203| 0.172| 0.370|      |      |      |      |      |
|       | JM          | 0.040| 0.224| 0.329| 0.234|      |      |      |      |
| September | KA       | 0.191| 0.027| 0.403| 0.191| 0.215|      |      |      |
|         | KB          | 0.292| 0.388| 0.051| 0.382| 0.306| 0.393|      |      |
|         | KC          | 0.241| 0.222| 0.414| 0.068| 0.244| 0.195| 0.416|      |
|         | JM          |      |      |      |      |      |      |      |      |
value of $K$ and the STRUCTURE analysis did not detect any signs of population structure in *L. humile*. The characteristics of the curve were not altered by extending the burn-in period or by increasing the number of MCMC iterations. The log-likelihood curve was further tested using the $\Delta K$ statistic, but this statistic also did not produce a distinct peak that would have been correlated with the most likely value of $K$. All individuals were assigned to one of the respective clusters with a probability of $>82\%$ at $K = 4$ according to the number of supercolonies using STRUCTURE (Fig. 4A). In contrast, BAPS identified eight homogenous units within the study area, indicating that each supercolony represented genetic differentiation between the seasons (Fig. 4B).

**Discussion**

In our analysis using microsatellite genotypes, the results of AMOVA and BAPS, the $F_{ST}$ values, and the existence of several private alleles indicated that the *L. humile* population in the Kobe area was characterized by a pronounced genetic structure. As in previous studies conducted in Europe (Giraud et al. 2002; Jaquiery et al. 2005), North America (Tsutsui and Case 2001; Thomas et al. 2006), and Japan (Sunamura et al. 2009), our findings support the notion that gene flow among supercolonies is largely restricted and that mutually antagonistic supercolonies remain genetically differentiated. On the other hand, our STRUCTURE results did not detect clear genetic units; instead, they demonstrated that some workers of the KA supercolony were assigned to the adjacent KB supercolony with low probability (Fig. 4A). This suggested that gene flow occurred from the KB supercolony to the KA supercolony. In *L. humile*, only the males disperse, whereas the queens mate and stay within or close to their natal nests (Passera and Keller 1990). During the early reproductive period, under laboratory conditions, foreign males are not attacked by workers (M.N. Inoue, pers. obs.); instead, they are adopted by, and successfully mate with, resident queens (Passera and Keller 1994). However, at the end of reproduction, males are aggressively attacked by the workers (Sunamura et al. 2011). Therefore, male-mediated gene flow might occur between the supercolonies, but only during the early reproductive period.

Within each supercolony, the genetic differentiation ($F_{ST}$) between nests was low; this is also consistent with previous studies both in native populations in Argentina (Pedersen et al. 2006) and in areas where the ant has been introduced (Giraud et al. 2002). In addition, there was no evidence for a correlation between genetic differentiation and the geographic distance among the nests of the four supercolonies, suggesting that isolation by distance does not appear to be occurring at a small spatial...
scale; this supports the findings of Tsutsui and Case (2001). On the other hand, we demonstrated that significant deviation from HWE was greater in September than in May in all supercolonies. This result suggested the occurrence of nonrandom mating, probably because queens mate with their sibs within a nest, despite the above-mentioned possibility of gene flow among supercolonies. The significant genetic differentiation ($F_{ST}$) between workers collected in May and those collected in September within a given supercolony suggested that the supercolonies replaced old queens with new ones after the reproductive season, supporting the plausibility of queen execution. Queen execution has been reported in two introduced populations – in the United States (Markin 1970) and in France (Keller et al. 1989) – but not in native populations. Most executed queens were <1 year

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**Fig. 3.** Plot of log-likelihood values [lnP(D)] as a function of K indicating the number of distinct groups for *Linepithema humile* sampled from 19 nests in the Kobe area in both May and September 2009, for a total of 38 nest–month combinations. All data were obtained from analysis of the microsatellite DNA loci using 20 runs with STRUCTURE. K values tested ranged from 1 to 20, with a burn-in of 100,000 and with 100,000 Markov chain Monte Carlo iterations per value of K.

**Fig. 4.** Bayesian analyses of population structure. Analyses were performed with (A) STRUCTURE and (B) BAPS among the *Linepithema humile* supercolonies: (A) Each individual is represented as a vertical bar partitioned into segments of different color according to the proportion of the genome belonging to each of the four identified clusters ($K = 4$); each sampling is a different color according to the cluster to which it belongs.
old, suggesting that queen age, like weight and fecundity (Keller et al. 1989), does not play an important role in the choice of queen for execution. From the perspective of kin selection, one hypothesis proposed by Keller et al. (1989) is that workers collectively eliminate queens, to which they are less related, thereby increasing their own inclusive fitness. Reuter et al. (2001), however, reported that the workers in a nest were, on average, not significantly less related to executed queens than to surviving ones. We revealed here that relatedness among workers increased significantly from May to September in all supercolonies, probably as a result of queen execution, supporting the hypothesis of Keller et al. (1989). Our results also showed that genetic diversity (allelic richness) decreased significantly from May to September, indicating that the occurrence of genetic drift due to queen execution may contribute to genetic differentiation by changing the gene frequencies within a supercolony.

The genetic differentiation among supercolonies and the genetic homogeneity within each supercolony in this recently established Japanese population suggest that each introduced supercolony reflects the genetic constitution of the founder colony rather than the mixing of several mutually antagonistic supercolonies. Thus, the colony structure does not differ between its area of origin and area of introduction. Among the four supercolonies, the JM and KB supercolonies were characterized by lower genetic diversity and higher relatedness among workers than were the KA and KC supercolonies (see Tables 2, 3). First, these differences among supercolonies may arise from differences in invasion history. The JM supercolony represents the dominates across Europe, North America, Australasia, and Japan on each continent or island (Tsutsui et al. 2000; Corin et al. 2007; Giraud et al. 2002; Inoue et al. 2013; Suhr et al. 2009). The secondary large KB supercolony has also been found in some populations in Japan and the United States, whereas the minor KA and KC supercolonies are locally distributed within their introduced ranges (Inoue et al. 2013). The two dominant supercolonies are likely to have been transferred stepwise from their native range to a new region and then on to other new regions and to have originated from established populations. In the areas to which they are introduced, they may have little opportunity for gene flow with other supercolonies because of their high levels of dominance. Therefore, low genetic diversity as well as high relatedness due to mating with sibs and queen execution can be found in these two dominant supercolonies. On the other hand, the two minor supercolonies may not have had repeated invasion histories and thus maintain relatively high genetic diversity but low relatedness despite queen execution. Second, the differences in genetic structure among the supercolonies may reflect those of the source populations in the native ranges, although variations in relatedness among nestmates have not been found in the native supercolonies (Vogel et al. 2009).

In conclusion, restricted gene flow among supercolonies, mating with sibs, and queen execution could help to maintain the unique social structure of L. humile, the distribution of which is expanding worldwide.

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Conflict of Interest

None declared.

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