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DYNAMICS AND GENETIC STRUCTURE OF ARGENTINE ANT SUPERCOLONIES IN THEIR NATIVE RANGE

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Some introduced ant populations have an extraordinary social organization, called unicoloniality, whereby individuals mix freely within large supercolonies. We investigated whether this mode of social organization also exists in native populations of the Argentine ant *Linepithema humile*. Behavioral analyses revealed the presence of 11 supercolonies (width 1 to 515 m) over a 3-km transect. As in the introduced range, there was always strong aggression between but never within supercolonies. The genetic data were in perfect agreement with the behavioral tests, all nests being assigned to identical supercolonies with the different methods. There was strong genetic differentiation between supercolonies but no genetic differentiation among nests within supercolonies. We never found more than a single mitochondrial haplotype per supercolony, further supporting the view that supercolonies are closed breeding units. Genetic and chemical distances between supercolonies were positively correlated, but there were no other significant associations between geographic, genetic, chemical, and behavioral distances. A comparison of supercolonies sampled in 1999 and 2005 revealed a very high turnover, with about one-third of the supercolonies being replaced yearly. This dynamic is likely to involve strong competition between supercolonies and thus act as a potent selective force maintaining unicoloniality over evolutionary time.

KEY WORDS: **Biological invasions, *Linepithema humile*, social evolution, social insects, supercolonies, unicoloniality.**

The evolution of animal societies in which some individuals forego their own reproductive opportunities to help others to reproduce poses an evolutionary paradox that can be traced to Darwin (1859). Altruism may evolve through kin selection when the donor and recipient of altruistic acts are related to each other (Hamilton 1963, 1964), as is generally the case in social birds and mammals (Riedman 1982; Emlen 1987; Cockburn 1998; Covas and Griesser 2007). Similarly, social insect workers are generally highly related to the brood they rear when colonies are headed by a single queen (Bourke and Franks 1995). However, insect colonies frequently contain several queens, which decreases the relatedness among colony members (Keller 1995).

The evolution of multiple-queen colonies can be explained under some ecological conditions, in particular when there is high dispersal risk, high queen mortality and a low success of independent colony founding (Nonacs 1988; Pamilo 1991; Keller 1995). In some species, however, colonies contain a very large number of queens (Hölldobler and Wilson 1977; Keller 1995) which

greatly reduces relatedness among colony members (Bourke and Franks 1995; Crozier and Pamilo 1996). At the extreme, unicolonial species form networks of interconnected nests that are so large that workers of distant nests are too far away to physically interact with each other (Hölldobler and Wilson 1977; Passera 1994; Chapuisat and Keller 1999; Chapman and Bourke 2001; Holzer et al. 2006; Pedersen et al. 2006; Debout et al. 2007).

Unicoloniality confers important ecological advantages in terms of colonization ability (Holway 1999; Tsutsui et al. 2000; Giraud et al. 2002), resource exploitation (Hölldobler and Lumsden 1980; Holway and Case 2001) and interspecific competition (Passera 1994; Human and Gordon 1996; Holway 1999). Indeed, of the 17 land invertebrates species listed among the world's worst invaders (http://www.issg.org), five are ant species with documented or inferred unicolonial structures. However, if unicoloniality is a key attribute responsible for the ecological dominance of some ants, it is also an evolutionary paradox and a potential problem for kin selection theory because this mode of social organization leads to an extremely low relatedness between nestmates (Hamilton 1964; Crozier 1979; Bourke and Franks 1995; Queller and Strassmann 1998; Queller 2000; Helanterä et al. 2009). Thus, selfish traits, such as larvae developing into queens rather than workers should be selected for, so that the worker caste would gradually vanish, disrupting colony function. Also, worker traits should no longer evolve adaptively given that workers do not share genes by descent with the reproductive individuals they raise. Rather, they would evolve by genetic drift and mutation, so that accumulation of deleterious traits leads to decline in fitness over time.

Until very recently, it was thought that unicolonial species never exhibited within-species aggression. However, this dogma has been challenged by recent findings showing that several unicolonial species are organized into mutually aggressive supercolonies (i.e., large networks of nests exhibiting no within- but strong between-supercolony aggression). In the little fire ant *Wasmannia auropunctata*, several supercolonies were detected in the introduced range (Mikheyev and Mueller 2007). Similarly, distinct supercolonies of the yellow crazy ant *Anoplolepis gracilipes* were discovered within the pacific islands (Abbott et al. 2007) and in Malaysian Borneo (Drescher et al. 2007). Finally, aggression between colonies has been observed both in native (Suarez et al. 1999; Tsutsui et al. 2000; Pedersen et al. 2006) and introduced populations (Suarez et al. 1999; Tsutsui et al. 2000; Giraud et al. 2002; Thomas et al. 2006; Sunamura et al. 2007) of the Argentine ant *Linepithema humile*.

The observation that unicolonial species may form mutually aggressive supercolonies and that such supercolonies may even coexist in the same locality has important implications for our understanding of the origin and maintenance of unicoloniality (Pedersen et al. 2006), because the relatedness relevant for predictions from kin selection theory should be measured at the scale at which intraspecific competition takes place (Taylor 1992; Kelly 1994; Queller 1994; Griffin and West 2002; Lehmann et al. 2008). Thus, if supercolonies exist in the same habitat and compete with each other for access to territory and resources, relatedness should not be measured between individuals of different nests of the same supercolony, as typically is the case, but among cooperative individuals using as a reference all individuals that may eventually compete. It is therefore critical to know the spatial scale of intraspecific competition.

Most of the work on unicolonial species has been conducted in their introduced ranges. A notable exception is the Argentine ant, which has been studied both in the native range and introduced populations (mostly Europe and California). Although it was first thought that introduced colonies had a different social and genetic structure than native colonies (Tsutsui et al. 2000; Giraud et al. 2002; Tsutsui and Suarez 2003), a recent genetic study revealed that this might not be true. On the basis of indirect measures of relatedness obtained at the population level Pedersen et al. (2006) concluded that native populations in Argentina are composed of genetically distinct supercolonies also containing genetically undifferentiated nests (i.e., workers are not more related to nestmates than to workers from distant nests of the same supercolony). The first aim of the present study was to confirm this claim by conducting behavioral tests to identify groups of nests among which there is no aggression and determine to what extent the clustering of nests into supercolonies correlates with genetic differentiation. The demonstration that the Argentine ant is also unicolonial in the native range is important because explanations for the evolution of unicoloniality have to be found in the native range of the species and not as a consequence of introduction into new habitats, as has been frequently done. The second and related aim was to estimate the degree of genetic differentiation between putative supercolonies using both nuclear and mitochondrial regions, and to determine whether, as in the introduced range, there is no genetic differentiation between nests within supercolonies. Such information is important to quantify the amount of gene flow within and between supercolonies. The third aim was to determine whether there was a correlation between the genetic differentiation between supercolonies, their spatial distance, their aggression level, and the worker variation in chemical recognition cues (the cuticular hydrocarbon profile) to have a better understanding of the mechanisms underlying nestmate discrimination. Finally, we compared the size, distribution, and genetic composition of supercolonies sampled from the same locality in 1999 and 2005. These data were used to quantify the supercolony turnover over time, an important parameter affecting the scale of competition and the selective forces at work in the native habitat.

Materials and Methods

STUDY AREA

The study area is situated in the Natural Reserve of Otamendi, 70 km north of Buenos Aires, Argentina. The population of *L. humile* in Otamendi described by Pedersen et al. (2006) and originally sampled in October 1999 was reinvestigated during October and November 2005. This population runs alongside a straight road of 6 km that joins the railway station of the village of Otamendi to the Parana River. A canal runs parallel to its NW side, separated from the road by an approximately 2-m-wide strip of vegetation. No ants were found on the NW side of the canal and aggression tests indicated that ants on the opposite (SE) side of the road belong to other supercolonies. Although Pedersen in 1999 sampled ants approximately every 200 m on both sides of the road along the 6 km, we concentrated our study on the first 3 km and on the 2-m-wide strip of vegetation forming a natural transect between the road and the canal.

BEHAVIORAL TESTS

To determine the number of distinct supercolonies, we sampled workers from nests (defined here as aggregations of workers and queens) approximately every 50 m to conduct standard aggression tests (Holway et al. 1998; Giraud et al. 2002) between adjacent nests. We randomly selected a single worker from each of two adjacent nests and placed them together for 10 min in a 5.5-cm-diameter vial with fluon-coated walls. Interactions were scored as follows: 0 = ignore, physical contact in which neither ant showed any interest; 1 = antennation, repeated tapping the antennae on the other ant; 2 = avoidance, one or both ants retreating in opposite directions after contact; 3 = dorsal flexion, gaster raised to vertical position as escalation to chemical defense; 4 = aggression, biting, or pulling extremities or head, or deposition of venom; 5 = fight, prolonged aggression often involving locking the mandibles onto a body part of the other and/or carrying it. Aggression levels 0 and 1 were considered as neutral, nonaggressive behaviors, and levels 2–5 as antagonistic. This procedure allowed us to group contiguous nests into putative supercolonies. When two adjacent nests were aggressive, we performed a more detailed search within this 50 m to locate the exact limits of the two supercolonies. With this approach we identified 11 putative supercolonies along the NW side of the road (see Results). To verify if nests within putative supercolonies were nonaggressive toward each other, we sampled whenever possible, at least five nests within each of the 11 putative supercolonies including the two nests at the limits of the supercolony, and we performed aggression tests between all pairs of nests sampled within the supercolony. For each pair of nests we did 10 trials, using different workers for each trial. Finally, one nest per supercolony (the focal nest) was chosen to perform between-supercolony aggression tests to confirm that the 11 supercolonies were really distinct from one another by consistently showing mutual aggression. Again 10 trials were performed per pair of supercolonies. For all aggression tests, the maximum level of aggressiveness during the 10 min of each test was used to calculate a mean value over the 10 replicates. All tests were conducted blindly.

DISTRIBUTION AND DENSITY OF SUPERCOLONIES

To assess the distribution of supercolonies along the road and estimate their density, we recorded the presence (value = 1) or absence (value = 0) of nests each meter on the transect and calculated an index of density with a sliding window method. The index was estimated as follows: for every point of the transect, we summed the number of presences of ants at each meter for the 10 m before and after that point and divided the sum by 20, resulting in an index between 0 and 1.

GENETIC ANALYSES

All nests sampled for aggression tests ($n = 53$) were used for genetic analyses. DNA was extracted from 10 workers per nest (total 530) using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and analyzed at 11 microsatellites loci: *Lhum*-3, *Lhum*-11, *Lhum*-13, *Lhum*-14, *Lhum*-19, *Lhum*-28, *Lhum*-33, *Lhum*-35, *Lhum*-39, *Lhum*-52, and *Lhum*-62 (Krieger and Keller 1999). One worker per nest was also amplified for two mitochondrial regions. For the three supercolonies in which less than five nests were sampled the number of individuals per nest was increased to have at least five individuals ($n = 5$–11) per supercolony. In total, 63 individuals were amplified for the mitochondrial regions. We amplified 524 bp of the cytochrome *b* gene (Cyt *b*) according to Pedersen et al. (2006) and 803 bp of the cytochrome oxidase *c* subunit-I gene (COI) using the primers L-LhCOI (5′-TAATATGGCAGATAAGTGCA-3′) and R-LhCOI (5′-TCATATCTTCAATATCATTG-3′) which were developed for this study. The amplification conditions for COI were identical to Cyt *b*, except for annealing temperature (45°C) and extension time (1.5 min). We sequenced COI for the individuals of the population of Otamendi studied at Cyt *b* by Pedersen et al. (2006).

Tests for linkage disequilibrium over the entire dataset and for deviations from Hardy–Weinberg genotype proportions (HW) among individuals within nests were performed with FSTAT 2.9.3 (Goudet 1995; http://www2.unil.ch/popgen/softwares/fstat.htm) applying 10,000 randomizations. There was no significant ($P > 0.05$) linkage disequilibrium between any pair of loci. A significant deviation from HW was detected for *Lhum*-11, *Lhum*-13, *Lhum*-28, and *Lhum*-35 ($P < 0.01$ for all) suggesting a potential occurrence of null alleles. Consequently, all analyses were carried out both with and without these four loci. As all results remained unchanged, we only present the analyses based on the entire set of 11 loci.

COMPARISON BETWEEN BEHAVIOR AND GENETIC STRUCTURE

To check the concordance between behavior and genetic structure, we used the Bayesian clustering method implemented in BAPS 4.14 (Corander et al. 2003, 2004). This method clusters, without any prior assumption, groups of individuals who are likely to come from the same mating subpopulation. The analysis was performed at the "group of individuals level" and each nest constituted a group. The maximum number of genetically diverged groups (K) tested was set to 53, which corresponds to the number of nests sampled. The analysis was repeated 10 times to test whether the results were robust and consistent between runs.

GENETIC STRUCTURE WITHIN AND BETWEEN SUPERCOLONIES

To compare the level of structure between and within supercolonies, a hierarchical analysis was performed with HIERFSTAT (Goudet 2005; http://www2.unil.ch/popgen/softwares/hierfstat.htm). The different levels considered were the individuals (Ind) within the nests (Nest), the nests within the supercolonies (Sc), and the supercolonies within the entire population (Pop). Significance of $F_{\mathrm{Nest/Sc}}$ was tested in HIERFSTAT (de Meeus and Goudet 2007) with 10,000 permutations of individuals between nests within supercolonies and $F_{\mathrm{Sc/Pop}}$ with 10,000 permutations of nests between supercolonies.

The relatedness between workers was determined at two levels. First, we calculated the mean relatedness between nestmate workers relative to their supercolony. Second, we calculated the mean relatedness among workers in a supercolony relative to the entire population. Both calculations were performed with the method of Queller and Goodnight (1989) as implemented in the computer program Relatedness 5.0.4 (http://www.gsoftnet.us/GSoft.html). The standard errors of the estimated relatedness values were obtained by jackknifing over nests and over supercolonies for the first and second calculation, respectively.

CUTICULAR HYDROCARBONS

To determine if cuticular hydrocarbons correlated with the level of aggression or the genetic and geographic distances between nests, we collected five workers from each of the nests used for the between-supercolony aggression tests (focal nests) and analyzed their cuticular profiles. Workers were placed in 2 mL glass vials (Sigma-Aldrich, Brøndby, Denmark) and stored at −20°C until extraction. For four of the 11 supercolonies, the focal nest could not be sampled because it disappeared during the 6 weeks of fieldwork; therefore only supercolonies S1, S3, S4, S8, S9, and S10 were used for chemical analysis. To extract cuticular hydrocarbons, 50 μL of solvent (pentane) was added to the vial containing the five nestmate workers. After 10 min, the extract was transferred into glass inserts (200 μL) and the solvent let evaporate. The dry extracts were kept frozen (−20°C) until analysis by gas chromatography-mass spectrometry (GC-MS). Each extract was rediluted in 8 μL of pentane and 2 μL of this were injected into an Agilent Technologies (Palo Alto, CA) 6890N GC equipped with a capillary column (HP5MS 30 m × 250 μm × 0.25 μm). The injector was a split-splitless type with helium carrying gas at 1 mL/min. The initial temperature was 70°C, which was held for 1 min and then increased at 30°C/min to 180°C, then to 320°C at 5°C/min, and finally held for 15 min. The GC was coupled with a 5375 Agilent Technologies Mass Spectrometer, using 70 eV electron impact ionization. Compounds were identified on the basis of their mass spectra, and comparison with standards and published data (Liang et al. 2001; de Biseau et al. 2004; Buczkowski et al. 2005).

A principal component analysis (PCA) was performed on peak area data, the relative proportions of which were log-ratio transformed according to Aitchison (1986). As some of the compounds were not detected in some supercolonies we replaced these zero values for the log-ratio transformations by half the minimum values obtained for the peak. The first four principal components, which explained a minimum of 95% of the variation, were used for calculating the intersupercolony chemical (Euclidian) distances as a measure of their relative differences.

ASSOCIATION OF FACTORS

To test for an association between the genetic distance (pairwise F_{ST}), geographic distance (minimum distance between supercolonies), chemical distances (Euclidian distance based on cuticular hydrocarbons), and mean level of aggression between supercolonies we performed Mantel tests applying FSTAT 2.9.3. We first determined whether the genetic distance increased with spatial distance. Then, we tested if the chemical distances between supercolonies are explained either by the geographic or genetic distances. We finally examined the variation in the level of aggression as a function of geographical distance, genetic differentiation and chemical distances. Because only supercolonies S1, S3, S4, S8, S9, and S10 were sampled for the chemical analysis, the Mantel tests were performed on these six supercolonies when the chemical distance was involved in the correlation tested. In addition, genetic isolation by distance was tested within all supercolonies for which more than two nests were sampled. In all cases, we determined statistical significance by performing 10,000 random permutations.

GENETIC DIVERSITY AND BOTTLENECKS

We tested if supercolonies had gone through recent genetic bottlenecks using the method described by Cornuet and Luikart (1996), which is based on differences between the observed and expected heterozygosity given the number of alleles in the

population assuming mutation-drift equilibrium. When a population has been subjected to a recent bottleneck, allele numbers are reduced faster than gene diversity (expected heterozygosity), and a significant number of loci with excess heterozygosity may be observed. This was tested with the program BOTTLENECK 1.2.02 (Piry et al. 1999; http://www.montpellier.inra.fr/URLB/bottleneck/bottleneck.html), according to the two-phased model of mutation (TPM) with 10,000 permutations. Significant heterozygosity excess was determined with a Wilcoxon signed rank test (Cornuet and Luikart 1996). To test for a global bottleneck effect in the locality of Otamendi, we considered the average of standardized differences between the expected and observed heterozygosity on the 11 supercolonies and tested with a *t*-test whether it was significantly different from zero.

TURNOVER OF SUPERCOLONIES

The population dynamics in the locality was assessed by comparing the genetic composition of supercolonies found in 1999 and 2005, the aim being to determine whether supercolonies sampled in 1999 were still present in 2005 and, if so, whether they were localized at the same places. The two mitochondrial regions (Cyt *b* and COI) and the five microsatellites common to both studies (*Lhum*-11, *Lhum*-13, *Lhum*-19, *Lhum*-28, *Lhum*-35) were used for this comparison analyzing all supercolonies sampled in both years ($n = 30$).

First, to test whether the entire gene pools were similar in 1999 and 2005 we performed a hierarchical analysis as implemented in HIERFSTAT (Goudet 2005). The different levels considered were the nests (Nest) within supercolonies (Sc) and the supercolonies within sampling year (Year). Significance of $F_{\mathrm{Nest/Sc}}$ was tested in HIERFSTAT according to de Meeus and Goudet (2007) with 10,000 permutations of individuals between nests within supercolonies, $F_{\mathrm{Sc/Year}}$ with 10,000 permutations of nests between supercolonies within years and $F_{\mathrm{Year/Tot}}$ with 10,000 permutations of supercolonies between years.

Second, to test whether supercolonies discovered in 2005 were also present in 1999 we used the software BAPS 4.14 (Corander et al. 2003, 2004). The analysis was performed at the "group of individuals level" and each supercolony constituted a group ($n = 30$). The maximum number of genetically diverged groups (K) tested was set to 30, which corresponds to the number of supercolonies detected with both samplings. The analysis was repeated 10 times to tests whether the results were consistent between the different runs.

Finally, we developed an analytical model to rule out the hypothesis that high levels of genetic differentiation between workers of the same supercolony sampled in 1999 and 2005 could stem from genetic drift caused by a limited number of breeding queens per supercolony (which would erroneously be taken as evidence of supercolony replacement instead of within-supercolony change in allele frequencies). For this purpose, we defined a space–time measure of population differentiation at a single neutral locus (e.g., Epperson 1993) using classical approaches of spatial pairwise measures of population differentiation (Rousset 2004). The genetic differentiation between individuals sampled from the same supercolony at different points in time is given by

$$F_{\mathrm{SS}}(t) = \frac{Q(0) - Q(t)}{1 - Q(t)}, \tag{1}$$

where $Q(t)$ is the probability that a gene randomly sampled in a queen in a focal supercolony in a focal generation is identical-by-descent with a homologous gene randomly sampled from a queen living in the focal colony t generations previous to the focal generation and $Q(0)$ is the probability of identity between pairs of individuals sampled in the same generation. At $t = 0$ we have $F_{\mathrm{SS}}(0) = 0$ and the genetic differentiation $F_{\mathrm{SS}}(t)$ then increases with time t as a result of migration between supercolonies and drift (including queen turnover) within supercolonies, both changing supercolony allele frequencies. To evaluate $F_{\mathrm{SS}}(t)$ explicitly, we constructed a model on the basis of the known life cycle of the Argentine ant. In this species, queens typically have a life span of 1 year (Keller et al. 1989) and mate with a single male (Krieger and Keller 2000). Genetic data and field collection revealed that the number of queens per supercolony is generally very high (more than 1000 queens). To be conservative we considered in the model lower values of queens per supercolony (i.e., $N = 10$, 100, and 1000). These queens were assumed to die every year after reproduction and their reproductive offspring to disperse independently of each other with probability m to another supercolony, where males compete for mating (both males and females can mate only once). Density-dependent competition among females then brings each supercolony back to size N. The equilibrium value of $Q(t)$ for this model is then given for $t > 1$ by

$$Q(t) = \frac{(1-m)^t \left(2m(1-m)\left(-\frac{1}{2}\right)^t + (3-m)(1+m)\right)}{3\,(1+2m(1-m)\,\{2m(1-m)(N-1)+3N\})}, \tag{2}$$

which was obtained by solving explicitly equation (21) of Lehmann (2007) and using the probability of identity between pairs of females. We also have

$$Q(0) = \frac{(1-m)^2\,(m(1-m)/2 + (3-m)(1+m))}{3\,(1+2m(1-m)\,\{2m(1-m)(N-1)+3N\})}. \tag{3}$$

Substituting into the left-hand side of this equation the value of population structure estimated directly in the field (i.e., the F_{ST} value between supercolonies) allows us to estimate the migration rate between supercolonies for $N = 10$, 100, and 1000 queens per supercolony and the corresponding increase in genetic differentiation over time (0–6 years).

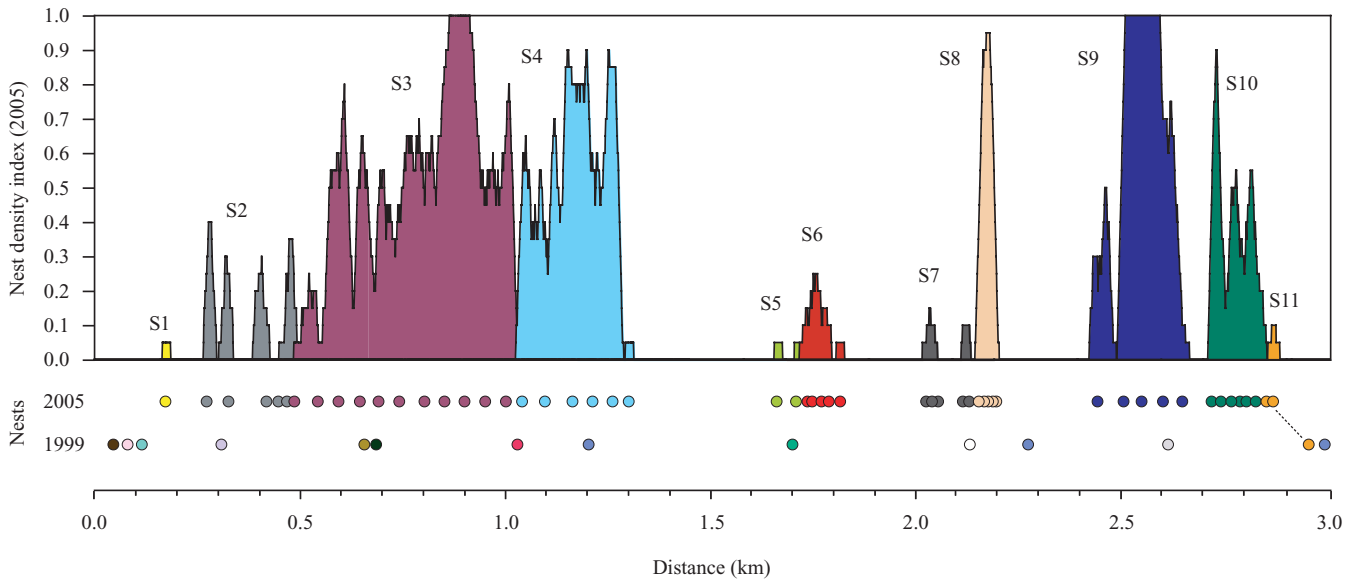

Figure 1. Localization of the *Linepithema humile* nests sampled in 1999 and 2005 and nest density in 2005 along the 3-km transect. The circles and their colors indicate nest location and their assignment to supercolonies on the basis of the genetic and behavioral data. The dotted line between two of the 1999 and 2005 nests indicates those that were assigned to the same supercolony.

Results

CHARACTERISTICS OF THE SUPERCOLONIES

All nests sampled contained queens. The aggression tests performed between adjacent nests along the 3 km revealed the presence of 11 putative supercolonies (Fig. 1). The level of aggression between workers from nests belonging to the same supercolony was always very low, with the mean aggression value being only 1.10 ± 0.02 (mean ± SE). By contrast, the aggression level was high (3.02 ± 0.07) between pairs of supercolonies (Fig. 2).

The genetic data were in perfect agreement with the behavioral tests. The clustering method implemented in BAPS also identified 11 groups in nine of the 10 runs. These groups consisted of exactly the same nests that were assigned to each of the 11 supercolonies by the behavioral tests. This partition of nests into the 11 groups was strongly supported by the analyses with a posterior probability $P = 1$ to have that partition for each of the nine runs. The remaining run was somewhat more ambiguous with a probability $P = 0.68$ of having 11 groups (with the same assignment of nests as found by the behavioral tests and nine other BAPS runs) and a probability $P = 0.32$ of having 12 groups. A total of five distinct mitochondrial haplotypes were identified in the 11 supercolonies (GenBank accession numbers for Cyt *b* and COI, respectively; L1: FJ466647 and FJ466666, L2: FJ466654 and FJ466673, L3: FJ466655 and FJ466674, L4: FJ466662 and FJ466681, L5: FJ466665 and FJ466684), but we never found more than a single haplotype per supercolony (Table 1).

The characteristics of each of the 11 supercolonies are given in Figure 1 and Table 1. Their length varied greatly (mean ± SD = 145 ± 151 m), with the smallest supercolony being only 1 m and the largest 515 m long (Table 1). The distance between supercolonies was also variable with a mean (± SD) value of 105 ± 120 m and a range from 11 to 356 m. The nest density (mean ± SD = 0.29 ± 0.23 nests/m) was also quite variable both within and among supercolonies (Fig. 1).

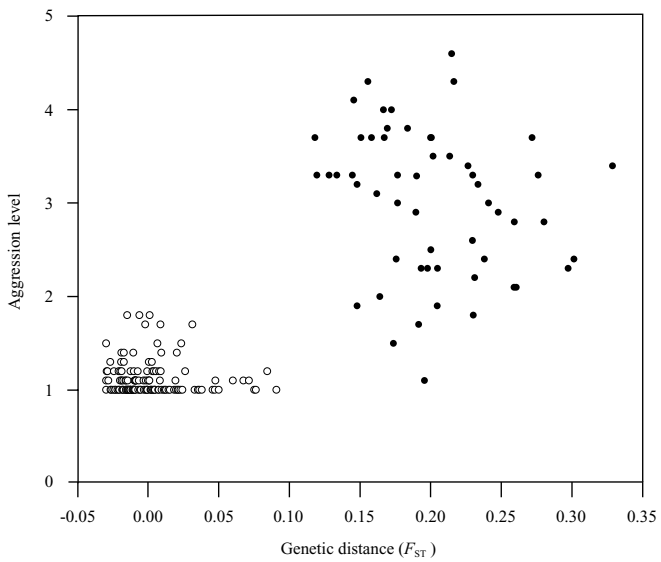

Figure 2. The association of pairwise genetic differentiation and aggression level within (open circles) and between (closed circles) supercolonies ($n = 11$) of *Linepithema humile*.

Table 1. Characteristics of *Linepithema humile* supercolonies. Number of nests refers to the number of nests sampled for aggression tests and genetic analyses; *k′* is the total allelic richness over all loci; *DH*/SD is the standardized differences between the expected and observed heterozygosities with positive values suggesting the occurrence of a genetic bottleneck (bold figures show tests for which $P < 0.05$, and asterisks the level of significance after sequential Bonferroni correction for multiple tests: $*P < 0.05$, $**P < 0.01$.

Supercolony ID	Number of nests	Supercolony length (m)	Nest density (No. nests/m)	Haplotypes found	Relatedness within supercolony (r±SE)	k'	DH/SD±SE
S1	1[1]	1	0.05	L5	N/A	50.0	0.59±0.35
S2	5	205	0.18	L2	0.018±0.027	39.1	**1.13±0.19***
S3	11	515	0.52	L1	−0.007±0.010	45.5	0.79±0.24
S4	6	262	0.54	L5	−0.028±0.009	33.9	**1.20±0.17****
S5	2[1]	48	0.05	L2	0.001±0.015	36.6	**1.05±0.17****
S6	5	88	0.12	L2	0.008±0.018	50.0	0.87±0.21
S7	5	86	0.10	L2	−0.022±0.005	45.6	**1.04±0.19***
S8	5	38	0.59	L4	0.070±0.043	44.7	**1.00±0.18***
S9	5	220	0.60	L2	0.045±0.063	55.9	0.74±0.18
S10	6	121	0.39	L1	0.000±0.022	46.0	**0.98±0.22**
S11	2[1]	9	0.07	L3	0.093±0.027	52.6	**0.84±0.22**
Overall	53	145±151 (SD)	0.29±0.23 (SD)	5	0.018±0.012	70.1	**0.93±0.05**

[1]For these supercolonies we did not find additional nests than those sampled.

GENETIC STRUCTURE WITHIN AND BETWEEN SUPERCOLONIES

The hierarchical analysis revealed that, although statistically significant, the genetic differentiation among nests within supercolonies was very low ($F_{\text{Nest/Sc}} = 0.006$, $P < 0.001$). Accordingly, the relatedness among workers from the same nest was statistically indistinguishable from zero when nests of the same supercolony were taken as the reference population (Table 1). The mean relatedness among nestmate workers within each of the 11 supercolonies was also not significantly different from zero (one-tailed t-test, $P = 0.09$).

By contrast, there was strong and significant genetic differentiation between supercolonies ($F_{\text{Sc/Pop}} = 0.203$, $P < 0.001$). This differentiation resulted in a significant and positive relatedness among nestmate workers (mean $r = 0.304 \pm 0.016$ SE) when all nests of the 11 supercolonies were used as a reference population. As expected, estimating the relatedness among nonnestmate workers of the same supercolony gave an identical value.

CUTICULAR HYDROCARBONS

GC-MS analysis of cuticular compounds revealed the presence of 24 different hydrocarbons, which were a mixture of linear, mono- and dimethyl-branched alkanes with 20 to 37 carbons. The quantity of the 24 hydrocarbons varied between supercolonies and three of them (peaks no. 3, 9, and 19) were not detected in some of the supercolonies (Fig. 3).

ASSOCIATION OF FACTORS

The association of pairwise genetic differentiation and aggression level showed a bimodal pattern (Fig. 2) with all the within-supercolony combinations having F_{ST} values below 0.1 and aggression levels below 2 (corresponding to avoidance) whereas almost all combinations between supercolonies had F_{ST} values higher than 0.15 and aggression levels higher than 2.

The correlation analyses between geographic, genetic, chemical, and behavioral distances (Fig. 4) showed that the geographic and genetic distance between supercolonies were not significantly associated (Mantel test, $r = -0.07$, $P = 0.65$). The chemical distance between supercolonies was significantly correlated with the genetic distance ($r = 0.51$, $P = 0.042$) but not the geographical distance ($r = -0.27$, $P = 0.33$). Finally, the level of aggression was neither correlated to the geographic ($r = 0.06$, $P = 0.69$), genetic ($r = -0.22$, $P = 0.96$), or chemical distances ($r = 0.008$, $P = 0.51$). When applying a Bonferroni correction for multiple tests there was no significant genetic isolation by distance within any of the supercolonies (without correction only significant in S8; $P = 0.036$).

GENETIC DIVERSITY AND BOTTLENECKS

The comparison between the observed and expected heterozygosities provided evidence of a genetic bottleneck in seven of the 11 supercolonies (Table 1). The mean standardized difference between the heterozygosity expected under mutation-drift equilibrium and the observed heterozygosity estimated over the 11 supercolonies was also significantly different from zero (two-tailed t-test, $P < 0.001$) indicating a general bottleneck effect in the population studied. The microsatellite allelic richness of each supercolony is presented in Table 1. There was no significant

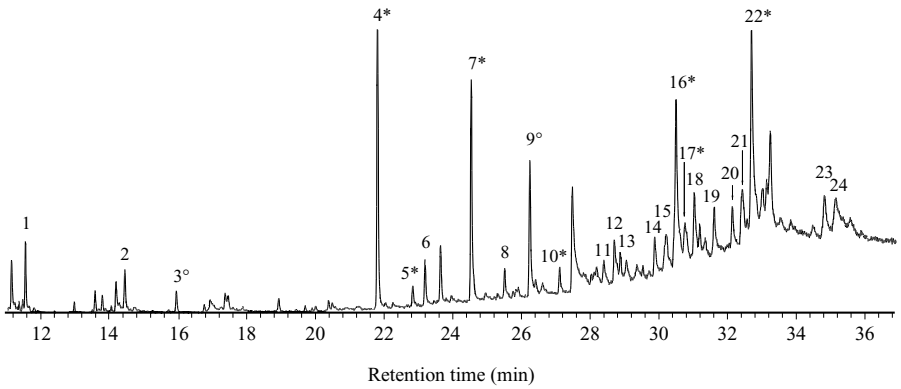

Figure 3. Gas-chromatogram of the typical cuticular hydrocarbon profiles of *Linepithema humile* workers. The numbers on the peaks denote the identified hydrocarbons: (1) C20, (2) C22, (3) C23, (4) C27, (5) 3-MeC27, (6) C28, (7) C29, (8) 3-MeC29, (9) 12,14diMeC30, (10) C31, (11) unknown, (12), unknown (13) x,y-diMeC31, (14) 13- + 15-MeC33, (15) unknown, (16) 11,15,21-triMeC33, (17) 5,15-diMeC33, (18) x,y-diMeC33, (19) unknown, (20) 13- + 15- + 17-MeC35, (21) 5,15- + 5,17-diMeC35, (22) 13,17,21-triMeC35, (23) 13,17-diMeC37, (24) x,y-diMeC37. Circles indicate hydrocarbons that were not detected in all supercolonies, and asterisks denote the hydrocarbons that explain most of the variance in the overall principal component analysis, that is, factor loadings >0.7.

correlation between the size of supercolonies and their genetic diversity measured as allelic richness ($r = 0.083$, $P = 0.82$).

TURNOVER OF SUPERCOLONIES

There was no significant genetic differentiation between the gene pool of individuals sampled in 1999 and 2005 ($F_{\text{Year/Tot}} = 0.003$, $P = 0.32$). On the basis of a Bayesian analysis, we identified 19 supercolonies on the 6-km transect investigated in 1999 (Pedersen et al. 2006), 12 of them were found on the 3-km we investigated in this study. To determine whether some of these 19 supercolonies were the same as the 11 sampled in 2005 we conducted new Bayesian analysis including all nests sampled in 1999 and 2005, and with no information on year of collection. This analysis revealed the existence of 29 genetically divergent groups. In each of

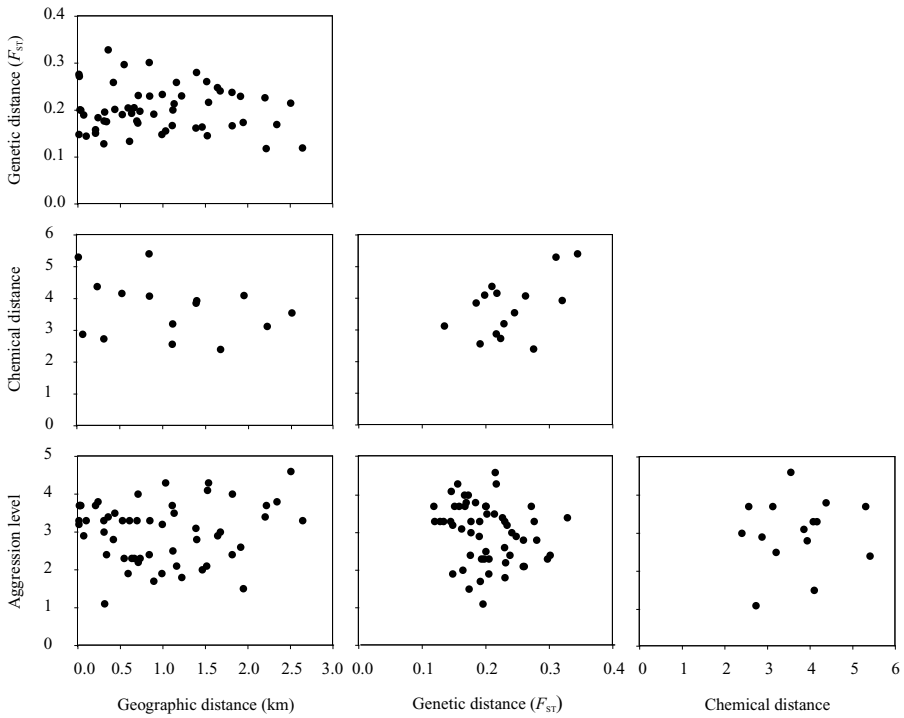

Figure 4. The associations of pairwise geographic (km), genetic (F_{ST}), chemical (Euclidian) and behavioral (aggression level) distances between supercolonies of *Linepithema humile*. Only the correlation between genetic and chemical distances is significant ($P = 0.042$; not corrected for multiple tests).

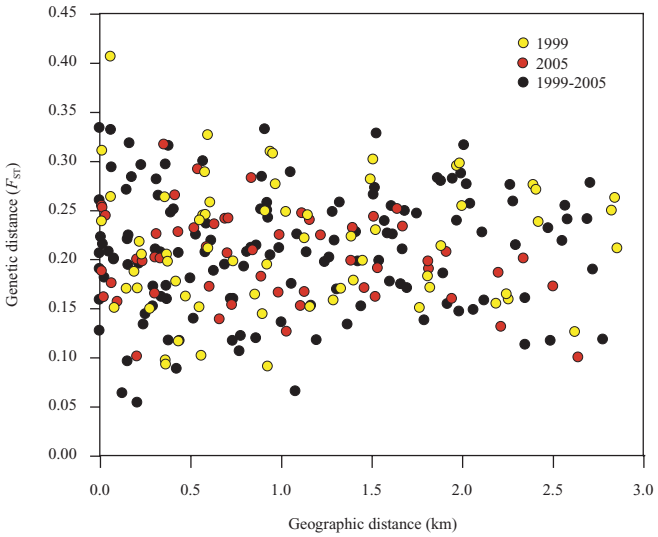

Figure 5. **The pairwise geographic and genetic distance between *Linepithema humile* supercolonies sampled in 1999 (yellow circles; $n = 66$ pairs) and 2005 (red circles; $n = 55$), and between supercolonies sampled at the same place in different years (black circles; $n = 132$).**

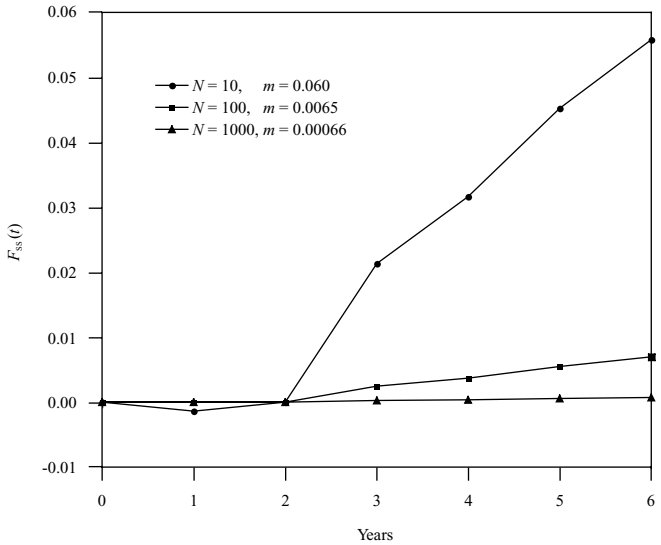

Figure 6. **The theoretically expected $F_{SS}(t)$ values between individuals of the same supercolony as a function of the number of years (t) separating the samples. The differentiation is shown for three parameter values of queen number per colony (N). See text for explanation.**

the 10 runs, two supercolonies, one sampled in 1999 and the other in 2005, were indistinguishable (Bayesian posterior probability, $P = 1$ for all runs; Fig. 1, S11 orange marker). Two more lines of evidence further support the view that these two supercolonies are actually the same supercolony sampled at 6 years of interval. First, they were sampled at almost the same location in 1999 and 2005. Second, they had the same mitochondrial haplotype (L3).

With this single exception, there was no indication that any of the 2005 supercolonies were derived from the 1999 supercolonies. The supercolonies localized at the same place in 1999 and 2005 were not genetically more similar than two supercolonies sampled at random within the first 3 km between the 2 years (Fig. 5; $r = -0.004$; partial Mantel test on geographic and genetic distance, $P = 0.94$). There was also no evidence that new supercolonies originate from nearby extant ones. In both 1999 and 2005, there was no genetic isolation by distance between supercolonies (Fig. 5; 1999, 5 loci: $r = 0.06$, $P = 0.66$; 2005, same 5 loci: $r = -0.25$, $P = 0.65$; 2005, all 11 loci: $r = -0.07$, $P = 0.62$). Moreover, for the total sample in 1999 and 2005, supercolonies having an identical haplotype did not exhibit greater genetic similarity at microsatellite loci than those with different haplotypes (same haplotype: $F_{ST} = 0.227 \pm 0.057$ SD, $n = 24$ pairs; different haplotypes: $F_{ST} = 0.209 \pm 0.056$, $n = 169$; two-tailed t-test, $P = 0.19$).

Finally, our analytical model also showed that genetic drift over 6 years cannot generate high genetic differentiation between individuals collected from the same supercolony in 1999 and 2005 (cf. observed estimates of F_{ST} above). On the basis of the observed differentiation between supercolonies, the estimated migration rates were $m = 0.060$, 0.0065, and 0.00066 for values of queen number per supercolony of $N = 10$, 100, and 1000, respectively. Figure 6 reveals that there was an increase in genetic differentiation over time only under the unrealistic assumption of supercolonies containing only 10 queens. With values of 100 and 1000 queens there was almost no change in the genetic composition of supercolonies over 6 years. If one assumes a larger number of queens, as is likely to be the case given the large size of supercolonies (Table 1), the ratio of about 1 queen per 100 workers (Markin 1970; Keller et al. 1989) and the large number of queens that can be found in nests in native populations (Heller 2004, V. Vogel, pers. obs.), the expected differentiation would be even lower, ruling out the possibility that the presence of the same supercolony sampled in 1999 and 2005 would remain undetected.

Altogether these data suggest that only one of the 12 supercolonies found along the 3-km transect in 1999 still existed 6 years later. This implies a rapid turnover with about one-third of the supercolonies disappearing and being replaced every year (estimated annual turnover $= 1–12^{-1/6} = 34\%$).

Discussion

CHARACTERISTICS OF NATIVE SUPERCOLONIES

The behavioral tests revealed the presence of 11 supercolonies over the 3-km transect. Although there was never any aggression between workers from nests of the same supercolony, the

aggression between workers from different supercolonies was always very high. The microsatellite genetic data were in perfect agreement with the behavioral tests, the Bayesian clustering method assigned all the nests to the same 11 groups. Furthermore, we never found more than a single mtDNA haplotype per supercolony.

Within supercolonies, the genetic differentiation between nests was extremely low. Thus, when measured at the level of the supercolony, the workers were not significantly more related to nestmates than workers from other nests of the same supercolony. By contrast, there was high genetic differentiation between supercolonies, and accordingly, workers were significantly related to nestmates when all 11 supercolonies were taken as the population of reference.

The contrasting patterns of aggressiveness and genetic differentiation observed within and between supercolony highlight the importance of considering the within versus between supercolonies levels when investigating the social structure of Argentine ants. Failing to do so may lead to the conclusion that the social organization of introduced colonies is different from that of colonies in native populations. Because supercolonies are usually very large in the introduced range (Giraud et al. 2002; Suarez and Tsutsui 2008), genetic and behavioral analyses have largely been done within supercolonies (Krieger and Keller 2000; Giraud et al. 2002; Corin et al. 2007), even in studies in which several supercolonies were investigated (Tsutsui et al. 2000; Tsutsui and Case 2001). By contrast, because supercolonies are smaller in Argentina (Suarez and Tsutsui 2008), studies have typically included a larger number of supercolonies (Tsutsui et al. 2000; Tsutsui and Case 2001). As both studies in the native and introduced range did not clearly distinguish between patterns occurring within or between supercolonies, this has lead to the erroneous conclusion that, in contrast to the introduced range, relatedness within nests and colonies in the native range is relatively high (Tsutsui and Case 2001). However, once the supercolony level is considered, the same pattern is found in native and introduced populations with a complete lack of genetic differentiation (i.e., zero relatedness among nestmates) and a complete lack of aggression between nests within supercolonies. Similarly, the conclusion that native populations are characterized by a higher intraspecific aggression than introduced populations (Tsutsui et al. 2000; Tsutsui and Case 2001) results from a higher proportion of aggression tests being conducted between nests from distinct supercolonies in the native range than in the introduced range. Once the within versus between supercolonies levels are appropriately considered there is again no difference between the native and introduced range. In both cases, there is never any aggression within supercolonies and always a strong aggression between supercolonies.

Several other findings from previous studies need also to be considered with caution because the within- and between-supercolony effects were not considered in the analyses. For example, a significant correlation between genetic differentiation (or genetic similarity), chemical differentiation and aggression level was reported in three studies (Tsutsui et al. 2000; Suarez et al. 2002; Thomas et al. 2006). However, an artificial relationship between the level of aggressiveness, genetic differentiation, and chemical distances may appear when several nests originate from the same supercolony (which is probably the case in these three studies). If the association is only due to nests from the same supercolony being more similar than nests from different supercolonies, this should result in a binomial distribution of observations, with no clear difference in genetic and chemical distance between closely located nests (i.e., those belonging to the same supercolony) and a marked difference between more distant nests (i.e., those belonging to distinct supercolonies). This is indeed the pattern we observed for the level of aggressiveness as function of the genetic differentiation (Fig. 2). We also found no evidence for a correlation between the level of aggression and the geographic distance or the chemical differentiation between supercolonies. There was only a significant association (when not being corrected for multiple tests) between chemical distance and genetic differentiation, in line with the observation that there is a genetic component underlying the production of different amounts of chemical cues used in nestmate discrimination (Giraud et al. 2002; Suarez et al. 2002).

SUPERCOLONY TURNOVER AND POPULATION DYNAMICS

The comparison of the genetic composition of colonies sampled in 1999 and 2005 revealed a very high turnover with only one of the 12 supercolonies detected on the 3-km transect in 1999 still being present in 2005. This means that about one-third of the supercolonies disappear and are replaced yearly, raising the questions of why do supercolonies disappear and where do new supercolonies come from.

We have currently almost no information of why colonies disappear at such a high rate. Many authors (Crozier 1979; Bourke and Franks 1995; Queller and Strassmann 1998) predicted that supercolonies should not be stable over time because they should be prone to the invasion of selfish mutants. However, this explanation cannot account for the observed turnover because these processes take place over a much longer evolutionary time scale. Therefore, the disappearance of supercolonies is more likely to occur from intra- and interspecific interactions as well as abiotic perturbations. In the native habitat competition among supercolonies at territory borders (Thomas et al. 2006) and competition with other species such as the fire ant *Solenopsis invicta* commonly exist and are likely to affect supercolony growth and survival (LeBrun et al. 2007). Although there are almost no studies on *L. humile* pathogens (but see Tsutsui et al. 2003; Reuter et al. 2005) they

are probably common in the native range, as appears to be the case for fire ants (Porter et al. 1997). Finally, it is also likely that flooding is an important factor leading to supercolony extinction, in particular because flooding is common in the regions inhabited by Argentine ants (LeBrun et al. 2007) and because Argentine ants are extremely vulnerable to such environmental disturbance.

As for the origin of new supercolonies our study provides some clues. First, and most importantly, the lack of isolation by distance between supercolonies suggests that new supercolonies do not frequently arise from the splitting of extant supercolonies into closely located entities. This view is in line with the finding that supercolonies sharing the same mitochondrial haplotype are not more similar at nuclear loci than colonies with different haplotypes. Finally, the overall lack of similarity between supercolonies sampled at the same location in 1999 and 2005 (except for the pair of supercolonies that were found to cluster together with the BAPS analysis) also suggests that new supercolonies do not originate from nearby colonies but that they instead come from other localities, for example, by rafting on the water during flooding (Newell and Barber 1913; Wild 2004) or inadvertent human transportation. This implies that new supercolonies get established from relatively few founding queens which is consistent with the overall strong signature of likely recent genetic bottlenecks (Table 1, Williamson-Natesan 2005). Remarkably, once established, the native supercolonies are able to grow to very high densities of individuals and nests (Fig. 1, see also Heller 2004) and to expand their spatial size by several tens of meters per year, and are thus also for these characteristics comparable to colonies in the introduced range (Suarez et al. 2001).

Conclusions

Our study reveals that, contrary to previous suggestions, the social organization and kin structure of Argentine ant colonies is very similar in the native and introduced ranges. Similar to introduced populations, native populations consist of supercolonies in which there is almost no genetic differentiation between nests, so that workers within a nest are not significantly more related to their nestmates than to workers in other nests of the same supercolony. Moreover, there is also high genetic differentiation between supercolonies indicating that, as in the introduced range, gene flow between supercolonies is very limited or even absent. Thus, both native and introduced supercolonies form closed breeding units, which have the potential to expand rapidly and reach high densities. The only difference between native and introduced populations lies in the sizes of supercolonies that are much smaller in the native range. Thus, the success of the Argentine ant as an invasive species does not result from a shift in social organization associated with introduction into new habitats (Tsutsui et al. 2000; Giraud et al. 2002; Tsutsui and Suarez 2003) but is most probably explained by the characteristics they develop in their native range (Passera 1994; McGlynn 1999; Aron 2001) in association with the ecological release from predators, parasites, and competitors that follows the introduction into a new habitat (Cremer et al. 2008). The findings that supercolonies are closed breeding units, that they are much smaller, and that they are subject to a very high turnover in the native range have important implications for our understanding of the evolution of unicoloniality. The relatively small size of native supercolonies together with the strong genetic differentiation between supercolonies and the evidence of between-supercolony competition (Thomas et al. 2006) and high supercolony extinction rate makes it a less daunting task to explain the evolution and stability of unicoloniality (compared to the introduced range) because it sets the necessary conditions for kin selection to operate between supercolonies (Sturtevant 1938; Crozier 1979; Pedersen et al. 2006; Helanterä et al. 2009). If more competitive supercolonies are more likely to survive or replace other supercolonies, a subtle dynamical process between the spread of detrimental traits within supercolonies and the selective elimination of colonies with such traits may allow a stable equilibrium and the persistence of unicoloniality over considerable stretches of evolutionary time.

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