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Hierarchical Analysis of Genetic Structure in Native Fire Ant Populations: Results From Three Classes of Molecular Markers

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Manuscript received January 30, 1997
Accepted for publication June 18, 1997

ABSTRACT

We describe genetic structure at various scales in native populations of the fire ant *Solenopsis invicta* using two classes of nuclear markers, allozymes and microsatellites, and markers of the mitochondrial genome. Strong structure was found at the nest level in both the monogyne (single queen) and polygyne (multiple queen) social forms using allozymes. Weak but significant microgeographic structure was detected above the nest level in polygyne populations but not in monogyne populations using both classes of nuclear markers. Pronounced mitochondrial DNA (mtDNA) differentiation was evident also at this level in the polygyne form only. These microgeographic patterns are expected because polygyny in ants is associated with restricted local gene flow due mainly to limited vagility of queens. Weak but significant nuclear differentiation was detected between sympatric social forms, and strong mtDNA differentiation also was found at this level. Thus, queens of each form seem unable to establish themselves in nests of the alternate type, and some degree of assortative mating by form may exist as well. Strong differentiation was found between the two study regions using all three sets of markers. Phylogeographic analyses of the mtDNA suggest that recent limitations on gene flow rather than longstanding barriers to dispersal are responsible for this large-scale structure.

THE nature of genetic structure in wild populations can reveal much about the contemporary influence of evolutionary forces such as selection, drift, and migration (BARTON and CLARK 1990). Also, properties of an organism's reproductive and dispersal biology, often difficult to discern through direct means, can be gleaned through the signatures left in the population genetic structure (AVISE 1994; MITTON 1994; SLATKIN 1994). Moreover, important historical features of populations, such as major changes in population size or the presence of earlier barriers to gene flow, may be detected if relevant data on genetic structure are obtained (AVISE *et al.* 1987; AVISE 1994; MEYER *et al.* 1996). Studies combining information from appropriate genetic markers with knowledge of an organism's natural history thus can reveal how current demographic traits and past events shape genetic structure, as well as predict how such structure may influence the subsequent course of evolution.

Empirical studies of genetic structure increasingly have employed multiple types of genetic markers (AVISE 1994; MITTON 1994). Each class of marker can differ with respect to its effective population size, mode of inheritance, level of polymorphism, and the nature of selection to which it is subjected, and thus each can give a somewhat different view of genetic structure and its determinants. Markers for which the evolutionary relationships of variants can be inferred, such as mitochondrial DNA (mtDNA) restriction site polymorphisms, add a further dimension to studies of genetic structure because an overtly historical perspective based on the gene phylogenies can be incorporated into the analyses (AVISE 1994).

Studies of genetic structure in eusocial insect populations attract special interest for several reasons. These insects form family-structured colonies that inhabit sedentary nests distributed unevenly throughout the environment. Depending on the specific social and reproductive systems, a complex hierarchical network of genetic affinities within and between nests may exist (ROSS and CARPENTER 1991; CROZIER and PAMILO 1996). Furthermore, eusocial Hymenoptera have a male-haploid genetic system that, because of the unorthodox transmission dynamics, can lead to patterns of structure different from those expected in male-diploid species with similar demographies (CROZIER and PAMILO 1996). Also, ants and other eusocial Hymenoptera display extreme reproductive altruism, the origin and maintenance of which is widely believed to entail kin selection in genetically structured populations (WADE 1985a; BARTON and CLARK 1990; CROZIER and PAMILO 1996). Finally, social insects provide examples of how changes in social behavior can alter local patterns of gene flow

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and perhaps even foster the development of reproductive isolation in sympatry (CROZIER 1977; WEST-EBERHARD 1986; BOURKE and FRANKS 1995; ROSS and KELLER 1995). Thus, knowledge of genetic structure in social insects can shed light on several fundamental micro- and macroevolutionary processes.

In this paper we describe genetic structure at various spatial scales in native populations of the fire ant *Solenopsis invicta* using three classes of genetic markers. Such data are important because a single comprehensive study of structure employing multiple types of nuclear markers as well as mtDNA markers has not been conducted previously in eusocial insects. Also, *S. invicta* is an introduced pest species that went through a population bottleneck and experienced significant changes in its population densities and colony social organization as it colonized the U.S. (PORTER *et al.* 1992; ROSS *et al.* 1993, 1996a). A comparison of native and introduced populations may reveal how such changes influence genetic structure, especially at microgeographic scales where altered colony social organization is most likely to affect structure via its association with dispersal and reproductive habits. A final reason for the importance of these data concerns the fact that fire ants display a prominent intraspecific polymorphism in social organization that may act to promote population differentiation or even create permanent barriers to intraspecific gene flow. Two different social forms often occur in sympatry in both native and introduced populations, the monogyne (M) form, in which colonies contain a single reproductive queen, and the polygyne (P) form, in which colonies contain multiple queens. Differences in the reproductive habits of the two forms appear to cause a cessation of interform gene flow via most routes in introduced populations (ROSS and SHOEMAKER 1993; SHOEMAKER and ROSS 1996), illustrating how divergence in social organization can constrain or even preclude particular types of gene flow. Comparative studies of native populations may shed further light on the question of whether social differentiation generally promotes genetic differentiation in these and other social insects.

MATERIALS AND METHODS

Sample collection: Specimens of *S. invicta,* identified on the basis of their morphology (TRAGER 1991), were collected from two localities in northeastern Argentina centered around the cities of Corrientes and Formosa. The Río Paraná, a major drainage basin that periodically floods extensively, separates the two collection areas, which are ~160 km apart. Both social forms occur sympatrically in both areas. Specimens were collected on liquid nitrogen and stored at −75° pending protein or DNA extraction. Twelve or more individuals per nest were genotyped for each of the polymorphic allozyme loci, whereas only a single individual per nest was used to determine genotypes at the microsatellite and mtDNA markers. Numbers of individuals used in various analyses are shown in the relevant tables reporting the results.

The social form of each sampled nest was determined initially in the field (GREENBERG *et al.* 1985) and confirmed subsequently by inspecting genotype arrays for at least 12 nestmates at eight or more variable allozyme loci. The genotypic data are definitive for determining social form because monogyne (M) nests contain simple progeny arrays consistent with a single monandrous mother queen, whereas polygyne (P) nests contain more complex arrays (*e.g.,* ROSS *et al.* 1993, 1996b). A total of 37 M nests and 44 P nests from Corrientes, and 35 M nests and 35 P nests from Formosa, were sampled.

Methods of generating the genetic data: Allozyme genotypes were determined by means of starch gel electrophoresis (protocols in SHOEMAKER *et al.* 1992). Mendelian inheritance of the products of most polymorphic loci was confirmed earlier (SHOEMAKER *et al.* 1992; ROSS *et al.* 1993) and is verified here for the remainder with data from monogyne nests. All genetic analyses involving P populations excluded the locus *Pgm-3,* which is affected by selection in this social form (ROSS *et al.* 1996b).

Microsatellite genotypes were determined by means of PCR amplification using one of seven pairs of primers, followed by separation of the PCR products in polyacrylamide sequencing gels and visualization using autoradiography. Details of the protocols for primer development and methods of PCR are reported in KRIEGER and KELLER (1997).

Haplotypes of the mtDNA genome were determined by means of PCR amplification of a 4-kb segment that includes the A+T-rich noncoding region, followed by restriction endonuclease digestion of this product with 16 enzymes. Digestion products were separated in agarose gels and visualized by staining with ethidium bromide. Composite mtDNA haplotypes were defined by the unique set of restriction sites present across all enzymes. Primers and methods used in determining mtDNA haplotypes are reported in ROSS and SHOEMAKER (1997).

Methods of analyzing the genetic data: Tests for conformity of the observed nuclear genotype frequencies to those expected under Hardy-Weinberg equilibrium (HWE) were conducted using chi-square tests or exact tests. Because multiple nonindependent allozyme genotypes were scored from each nest, a resampling procedure in which a single genotype per locus was drawn at random from each nest was used to estimate observed allele and genotype frequencies and test for HWE. The resampling procedure was conducted 1000 times for each deme and social form within a region (see below), and allele and genotype frequencies at these levels were estimated as the mean frequencies for the 1000 draws. The allozyme genotype frequencies for each form were tested for correspondence to HWE by applying chi-square tests to each of the resampled genotype distributions (LESSIOS 1992) and determining the proportions of tests that yielded values of chi square greater than the critical value for $\alpha = 0.05$. Genotype distributions were judged to deviate significantly from HWE when >5% of these tests yielded significant chi-square values. For those samples in which the frequencies of the pooled rare alleles resulted in expected genotype frequencies of less than one, exact tests were performed on the resampled genotype frequencies using the tables of VITHAYASAI (1973). Only a single genotype per nest was scored for the microsatellite loci, so conformity of the genotype frequencies observed in each form to HWE was tested by means of exact tests or, when more than four alleles were present at a locus, the Markov chain approximation of the exact test using the program GENEPOP (RAYMOND and ROUSSET 1995a).

Genetic structure in Argentina was investigated at several levels. Structure at the level of the nest was assessed by estimating average nestmate relatedness for workers and young nonreproductive queens from the allozyme genotype frequencies using the program RELATEDNESS (QUELLER and GOOD-

NIGHT 1989). For all relatedness estimates, nests (or groups at higher levels) were weighted equally, and all available individuals were used to estimate $\bar{p}$, the allele frequency in the reference population. Standard errors were obtained by jackknifing over nests (or other groups), and 95% confidence intervals (CIs) about the point estimates were constructed by assuming the t-distribution. Nestmate relatedness was corrected for significant relatedness (structure) at the level of the nest cluster (the next higher level analyzed) using the DEMES option in RELATEDNESS.

Structure at the level of the "nest cluster," a group of nests of the same form located within 10 m of one another and separated from other such groups by >10 m, was assessed by calculating relatedness between nonnestmate workers within such clusters from allozyme, microsatellite, and mtDNA data. Again, estimates were corrected for significant structure at the level of the deme (the next higher level analyzed).

Structure at the level of the "deme," a group of nests of the same form located within 5 km of one another and separated from other such groups by >5 km, was assessed for the allozymes by estimating single-level F_{ST} values using the approach of WEIR and COCKERHAM (1984). Estimates of values for all F-statistics using allozymes made use of allele and genotype frequencies derived from the resampling procedure conducted at the appropriate level. The corrected sample sizes employed for these estimates, which are based on the number of nests sampled and within-nest relatedness, were calculated as in SUNDSTRÖM (1993). Rare alleles present at a maximum frequency of 0.05, as well as one common allele per locus, were excluded when calculating values for all F-statistics, because low-frequency alleles are estimated with large error and estimates from alleles at a single locus are not independent (*e.g.*, NÜRNBERGER and HARRISON 1995). The 95% CIs about the point estimates were constructed by jackknifing over the single-locus values. Deme-level structure was assessed for the microsatellites and mtDNA by calculating single-level Φ_{ST} values using the AMOVA approach of EXCOFFIER *et al.* (1992), as implemented in the program WINAMOVA. These analyses were conducted both with and without divergence between pairs of alleles or haplotypes taken into account. Divergence was defined by a Euclidean metric equaling the square of the difference in repeat numbers for the microsatellite alleles (MICHALAKIS and EXCOFFIER 1996) or the square of the number of restriction site differences for the mtDNA haplotypes (EXCOFFIER *et al.* 1992). The 95% CIs for the microsatellite Φ_{ST} values were constructed by jackknifing over the single-locus values, and the significance probabilities for the mtDNA Φ_{ST} values were generated using permutation analysis on 1000 randomly permuted distance matrices.

Structure at the levels of social form (monogyne, polygyne) and region (Corrientes, Formosa) was studied by calculating hierarchical and single-level F-statistics for the allozymes and microsatellites (WEIR and COCKERHAM 1984) and Φ_{ST} values for the microsatellites and mtDNA. Structure at these levels was examined further by conducting exact tests of allele (haplotype) frequency differentiation (RAYMOND and ROUSSET 1995b) using the program GENEPOP. The joint probabilities of differentiation over all markers of each class were obtained using Fisher's combined probability test (SOKAL and ROHLF 1981). Exact tests for the allozymes were performed on 25 data subsets constructed from the original data sets by randomly sampling a single genotype per nest, with the mean chi-square values for the 25 subsets used to judge overall significance.

Percent sequence divergence between all pairs of mtDNA haplotypes (number of nucleotide substitutions per site; d) was estimated using the maximum likelihood method of NEI and TAJIMA (1983). Distributions of the numbers of restriction site differences for all pairs of individuals in each social form in each region were plotted as frequency histograms.

Relationships among the observed mtDNA haplotypes were inferred by two methods. First, a minimum spanning tree reflecting the most parsimonious (unrooted) genealogy of the observed mtDNA haplotypes was constructed using the program WINAMOVA. Observed haplotypes can occupy internal nodes of such a network and thus can be ancestral to other haplotypes. Second, a neighbor-joining tree in which haplotypes occupy terminal tips (and thus cannot be ancestral to one another) was constructed using the program MEGA (KUMAR *et al.* 1993), with the sequence divergence between haplotypes (d) employed as a distance metric. This tree was rooted at the midpoint of the longest distance between any two haplotypes.

Estimates of N_em, the genetically effective migration rate, were obtained using data from all three classes of genetic markers. For the allozymes and microsatellites, N_em was estimated from the single-level values of F_{ST} or Φ_{ST} by assuming an island model of structure (WRIGHT 1951; SLATKIN 1985; HUDSON *et al.* 1992). For the mtDNA, N_em was estimated in this way as well as by an independent method using the haplotype relationships portrayed in the neighbor-joining tree and gene coalescence theory (SLATKIN and MADDISON 1989). N_em represents the effective migration rate via both sexes when estimated from nuclear markers and the migration rate via females only when estimated from mtDNA data.

Trees illustrating genetic relationships of the social forms in each region were constructed based on pairwise distance measures between populations for the nuclear markers and nucleotide diversity within and between populations for the mtDNA. Nei's standard genetic distance (D_S, NEI 1972) was calculated for all pairs of populations based on allele frequencies at the electrophoretic loci (excluding *Pgm-3*), and the neighbor-joining method (implemented in MEGA) was used to construct a tree of relationships rooted at the midpoint. CAVALLI-SFORZA and EDWARDS' (1967) chord distance (D_C) was computed for all pairs of populations using the microsatellite allele frequencies, and neighbor-joining again was used to construct a midpoint-rooted tree (*e.g.*, TAKEZAKI and NEI 1996). Support for the nuclear trees was determined by bootstrapping over loci (100 replicates) using the program PHYLIP (FELSENSTEIN 1989). The mtDNA nucleotide diversity within populations and groups of populations was used to construct a tree of the population relationships using the procedure of HOLSINGER and MASON-GAMER (1996). Statistical support for each node of the mtDNA tree was determined by permutation analysis using 10,000 randomly permuted haplotype distributions.

RESULTS

Basic genetic data: Data were obtained for 11 variable allozyme loci and seven variable microsatellite loci. From two to 11 alleles were found at each allozyme locus in the entire collection of samples, yielding a total of 56 alleles of this class. From seven to 26 alleles were found at each microsatellite locus, yielding 118 alleles of this class.

No length variants were observed in the amplified mtDNA segment. Eleven restriction enzymes cleaved the PCR product at a total of 39 sites, six of which were present in all individuals. The presence or absence of the 33 variable sites defines 20 unique composite mtDNA haplotypes. From one to 22 restriction site dif-

ferences distinguish each haplotype, and the sequence divergence between pairs of haplotypes (d) ranges from 0.55% to 21.36%, with an unweighted mean of 7.73%. The mean sequence divergence in each social form within each region (hereafter referred to as "populations") ranges from 6.0% to 9.7%, values that are high relative to other animals (below) and suggest that many of the mtDNA variants in these fire ant populations are distantly related to one another.

Gene frequencies in the four study populations are available from K.G.R. upon request.

Tests for Hardy-Weinberg equilibrium: Genotype proportions at the allozyme loci are largely concordant with genotype proportions expected under Hardy-Weinberg equilibrium (HWE). Sixty-four of 67 individual tests (95.5%) showed no significant departure from HWE, with all three departures involving heterozygote deficiencies in the Formosa P population. Genotype proportions at the microsatellite loci also accord well with HWE. Twenty-six of 28 tests (92.9%) revealed no significant departure from HWE proportions. Combining probabilities over populations for the microsatellite loci (using Fisher's method) shows that only one locus displays a marginally significant departure from HWE due primarily to a deficiency of heterozygotes in the Formosa M population; combining probabilities over loci reveals no significant departures in any of the four populations. These data suggest a general lack of extensive inbreeding or pronounced population subdivision at the nuclear genome within the four study populations.

Population genetic structure: Estimates of within-nest relatedness are presented for each population in Table 1. Relatedness within M nests in each region is high and statistically indistinguishable from 0.75, the value expected for families composed of daughters of a single queen and single haploid male. Relatedness within P nests in each region is moderately high for both workers and nonreproductive queens, with all values significantly greater than zero but significantly less than the relatedness in M nests. The significantly positive values in Formosa P nests are preserved even when corrected for higher-level relatedness detected at the level of the nest cluster (see below).

Genetic structure at the level of nest clusters was assessed by calculating relatedness of non-nestmate workers in such clusters (Table 2). Using the allozyme data, the only significantly positive relatedness value was found for clusters of P nests in Formosa. Using the microsatellite data, the only significantly positive value was found for clusters of P nests in Corrientes; when corrected for significant structure at the level of the deme (see below) this value becomes nonsignificant. Using the mtDNA data, nest cluster relatedness values are significantly positive in both P populations but do not differ from zero in either M population. The former values remain significantly positive when corrected for structure at the deme level also found in the P populations. These results indicate slight nuclear structure and strong mtDNA structure at the level of nest clusters in the P form but not the M form in our study areas.

TABLE 1

Relatedness values for nestmates in the two social forms of *S. invicta* from two regions of Argentina estimated using allozyme markers

	Workers	Nonreproductive queens
Corrientes		
Monogyne (M) form	0.769 (0.723–0.814) $N = 37$, $n = 444$ 10 loci	0.712 (0.595–0.830) $N = 18$, $n = 130$ 7 loci
Polygyne (P) form	0.217 (0.162–0.272) $N = 44$, $n = 528$ 8 loci	0.460 (0.337–0.583) $N = 20$, $n = 181$ 5 loci
Formosa		
Monogyne (M) form	0.751 (0.714–0.788) $N = 35$, $n = 417$ 8 loci	0.728 (0.662–0.794) $N = 21$, $n = 145$ 8 loci
Polygyne (P) form	0.290 (0.228–0.352) $N = 35$, $n = 411$ 7 loci	0.287 (0.189–0.384) $N = 22$, $n = 171$ 7 loci
	0.272[a] (0.180–0.364) $N = 34$, $n = 399$ 7 loci	0.264[a] (0.149–0.379) $N = 21$, $n = 164$ 7 loci

The 95% CIs about the values are shown in parentheses. N indicates the number of nests and n the number of individuals for which genotypic data were obtained. The number of variable loci on which each estimate is based also is indicated.

[a] Value is corrected for significant higher-level relatedness observed at the level of the nest cluster.

Results of the analyses of structure at the deme level are presented in Table 3. Single-level F_{ST} estimates from the allozyme data reveal that demic structure is significant in the Corrientes P form and marginally so in the Formosa P form but is not detectable in either M population. Values of Φ_{ST} computed from the microsatellite data reveal significant differentiation among demes in the Formosa P form both when allelic divergence is considered (Euclidean metric) and ignored (equidistant metric) and in the Corrientes P form when it is ignored. Demic-level structure is not detectable in either M population using these markers. Values of Φ_{ST} computed from the mtDNA data reveal highly significant differentiation among demes in both P populations ($P < 0.001$) using either distance metric. Demes in the Formosa M population also exhibit significant

TABLE 2

Relatedness values for workers in nest clusters in the two social forms of *S. invicta* from two regions of Argentina estimated using three classes of markers

	Allozymes	Microsatellites	mtDNA
Corrientes			
Monogyne (M) form	−0.121 (−0.398–0.157) $N = 7$, $n = 228$ 10 loci	0.057 (−0.003–0.116) $N = 7$, $n = 19$	−0.231 (−0.479–0.017) $N = 7$, $n = 19$
Polygyne (P) form	0.055 (−0.048–0.158) $N = 10$, $n = 276$ 8 loci	0.193* (0.052–0.333) $N = 10$, $n = 23$	0.735* (0.388–1.081) $N = 10$, $n = 23$
		0.103[a] (−0.067–0.273) $N = 8$, $n = 19$	0.697*[a] (0.171–1.223) $N = 10$, $n = 23$
Formosa			
Monogyne (M) form	0.107 (−0.060–0.274) $N = 8$, $n = 264$ 8 loci	0.055 (−0.029–0.138) $N = 8$, $n = 23$	0.149 (−0.090–0.389) $N = 8$, $n = 23$
Polygyne (P) form	0.111* (0.034–0.188) $N = 7$, $n = 162$ 7 loci	0.137 (−0.006–0.280) $N = 7$, $n = 14$	1.000* (1.000–1.000) $N = 7$, $n = 14$
			1.000*[a] (1.000–1.000) $N = 6$, $n = 12$

The 95% CIs about the values are shown in parentheses (values with confidence intervals that do not overlap zero are indicated by an asterisk). N indicates the number of nest clusters and n the number of individuals for which genotypic data were obtained. The number of variable loci on which each allozyme estimate is based is indicated; all microsatellite estimates are based on seven loci.

[a] Value is corrected for significant higher-level relatedness observed at the level of the deme.

mtDNA differentiation, which is more pronounced when haplotype divergence is considered, while demes in the Corrientes M population show no such differentiation. Results at this level thus indicate weak nuclear differentiation among demes in both P populations but no such differentiation in either M population, with <5% of the total nuclear variation within each population residing between demes. In contrast, pronounced mtDNA differentiation occurs at this scale in both P populations (as well as the Formosa M population), with 20–40% of the total mitochondrial variation within each P population residing between demes.

Estimated values for the hierarchical *F*-statistics and Φ_{ST} at the levels of the social form and region obtained from all three classes of markers are presented in Table 4. Significantly positive values of F_{ST} and Φ_{ST} were obtained at both the form and region levels from both classes of nuclear loci (equidistant metric only for Φ_{ST} for the microsatellites). Values of F_{IS} are not significant at either level for either class of nuclear marker, whereas the value of F_{IT} is significantly positive for the allozymes but not the microsatellites. Thus, nuclear differentiation between the sympatric forms and regions apparently is sufficiently pronounced for the allozymes to cause a slight but significant overall Wahlund effect (deficiency of heterozygotes). The absence of significant F_{IS} values for either type of nuclear marker reflects the general agreement of the observed genotype proportions with those expected under HWE in each population. The Φ_{ST} values obtained from the mtDNA reveal pronounced differentiation at both the form and region levels when the Euclidean metric is used (and also when the equidistant metric is used at the form level). The hierarchical analyses show that ~12% of the total allozyme variation is partitioned between regions and ~11% is partitioned between sympatric social forms. Less of the total microsatellite variation is distributed between the regions and sympatric forms than is the case for the allozymes, with no more than 5% occurring at either level. More of the mtDNA variation is partitioned at these levels than is the case for either class of nuclear marker, with close to 20% of the total distributed between the regions and 15–20% distributed between sympatric social forms.

The significant differentiation discovered between sympatric social forms in the hierarchical analyses indicates the need for independent analyses within each region (Table 5). In Corrientes, extensive mtDNA differentiation between the sympatric forms is evident, and weak nuclear differentiation is suggested by the

TABLE 3

F_{ST} and Φ_{ST} values for differentiation among demes in the two social forms of *S. invicta* from two regions of Argentina estimated using three classes of markers

		Φ_{ST}			
		Microsatellites		mtDNA	
	F_{ST} Allozymes	Euclidean metric	Equidistant metric	Euclidean metric	Equidistant metric
Corrientes					
Monogyne (M) form	0.015	−0.021	0.010	0.052	0.035
	(−0.011–0.041)	(−0.049–0.007)	(−0.011–0.030)	(P = 0.262)	(P = 0.287)
	N = 6, n = 54	N = 6, n = 35	N = 6, n = 35	N = 6, n = 35	N = 6, n = 35
	7 loci				
Polygyne (P) form	0.017*	0.038	0.042*	0.422*	0.406*
	(0.009–0.025)	(−0.037–0.114)	(0.002–0.083)	(P < 0.001)	(P < 0.001)
	N = 5, n = 223	N = 5, n = 42	N = 5, n = 42	N = 5, n = 43	N = 5, n = 43
	8 loci				
Formosa					
Monogyne (M) form	0.039	0.033	0.029	0.391*	0.155*
	(−0.010–0.088)	(−0.030–0.097)	(−0.002–0.061)	(P < 0.001)	(P < 0.020)
	N = 3, n = 51	N = 3, n = 34	N = 3, n = 34	N = 3, n = 34	N = 3, n = 34
	8 loci				
Polygyne (P) form	0.029	0.048*	0.045*	0.319*	0.192*
	(0.000–0.059)	(0.017–0.079)	(0.008–0.083)	(P < 0.001)	(P < 0.001)
	N = 5, n = 132	N = 5, n = 34	N = 5, n = 34	N = 5, n = 33	N = 5, n = 33
	8 loci				

The 95% CIs about the values of F_{ST} for the allozymes and Φ_{ST} for the microsatellites, as well as the probabilities that the estimates of Φ_{ST} for the mtDNA do not differ from zero (no differentiation), are shown in parentheses. Values with CIs that do not overlap zero or permutation probabilities that are <0.05 are indicated by an asterisk. N indicates the number of demes and n the number of individuals for which data were obtained (values of n for F_{ST} represent corrected sample sizes that account for the numbers of nests sampled and the average relatedness within nests). The numbers of variable loci on which each allozyme estimate is based are indicated; all microsatellite estimates are based on seven loci.

small and marginally significant allozyme F_{ST} value. In Formosa also, mtDNA differentiation is apparent between the social forms, although it is much less extensive than in Corrientes and significant only when the equidistant metric is used. Again, weak nuclear differentiation between the forms is suggested by the small and marginally significant microsatellite F_{ST} and Φ_{ST} values. Exact tests of allele and haplotype frequency differences between the sympatric social forms largely substantiate these results. These tests reveal differentiation between the two social forms in Corrientes that is significant for all three types of markers (allozymes: $P = 0.045$; microsatellites: $P < 0.001$; mtDNA: $P < 0.001$) and differentiation in Formosa that is significant for the microsatellite and mtDNA markers (allozymes: $P > 0.280$; microsatellites: $P < 0.010$; mtDNA: $P = 0.020$).

Finally, single-level F_{ST} and/or Φ_{ST} values at the region level are 0.192 for the allozymes, 0.038–0.057 for the microsatellites, and 0.227–0.307 for the mtDNA, with all of the values statistically significant. Thus, as indicated by the hierarchical analyses, the mtDNA exhibits the greatest differentiation and the microsatellites the least at this highest level of structure. Exact tests also show pronounced structure at this level for all three genetic data sets (all $P < 0.001$).

Phylogeographic patterns of mtDNA variation: The minimum spanning tree depicting haplotype relationships (Figure 1, top) reveals that many of the haplotypes are highly divergent from other haplotypes in the same region or population. For instance, haplotypes C and L, present in both social forms in Corrientes, are separated by 46 inferred restriction site changes, and haplotypes P and B, present in both social forms in Formosa, are separated by 34 inferred restriction site changes. Nonetheless, some closely related haplotypes separated from others in the same population by as few as a single restriction site change also were found. The neighbor-joining tree (Figure 1, bottom) confirms that haplotypes within each population often are highly divergent (up to 19% sequence divergence in the Corrientes M form, 21% in the Corrientes P form, 11% in the Formosa M form, and 14% in the Formosa P form), but that closely related variants occur as well within single populations. Little phylogeographic structuring of the haplotypes is apparent, with the haplotypes present within any given population commonly occupying remote regions of the trees.

Frequency distributions of numbers of restriction site differences between all pairs of individuals are presented for each population in Figure 2. The distributions invariably are multimodal, reflecting the presence of some closely related haplotypes and many distantly

TABLE 4

Hierarchical values of *F*-statistics and Φ_{ST} for the levels of social form and region in *S. invicta* from Argentina estimated using three classes of markers

	Allozymes			Microsatellites					mtDNA	
				Φ_{ST}					Φ_{ST}	
	F_{ST}	F_{IS}	F_{IT}	Euclidean metric	Equidistant metric	F_{ST}	F_{IS}	F_{IT}	Euclidean metric	Equidistant metric
Social form	0.129*	0.001	—	0.009	0.007*	0.048*	−0.006	—	0.250*	0.173*
(within region)	(0.017–0.242)	(−0.025–0.026)		(−0.020–0.038)	(0.001–0.013)	(0.021–0.074)	(−0.045–0.034)		($P < 0.001$)	($P < 0.001$)
Region	0.117*	0.015	0.127*	0.052	0.035*	0.038*	0.004	0.042	0.210*	0.155
	(0.002–0.232)	(−0.011–0.041)	(0.007–0.247)	(−0.028–0.132)	(0.011–0.058)	(0.006–0.070)	(−0.036–0.045)	(−0.012–0.097)	($P < 0.001$)	($P = 0.167$)

The 95% CIs about the values of the *F*-statistics and of Φ_{ST} for the microsatellites, as well as the probabilities that the estimates of Φ_{ST} for the mtDNA do not differ from zero, are shown in parentheses. Values with CIs that do not overlap zero or permutation probabilities that are <0.05 are indicated by an asterisk. Sample sizes for the allozymes (numbers of individuals, corrected to account for numbers of nests sampled and average relatedness within nests) are as follows: Corrientes M population, 56; Corrientes P population, 228; Formosa M population, 53; Formosa P population, 136. Sample sizes for the microsatellites and mtDNA are as follows: Corrientes M population, 36; Corrientes P population, 43; Formosa M population, 35; Formosa P population, 35. The allozyme and microsatellite estimates are based on 10 and seven loci, respectively.

related haplotypes within each population. The patterns are not consistent with these being relatively isolated populations that experienced a recent flush of growth, such as would follow a founder event, because such events are expected to produce "starlike" haplotype phylogenies and distributions of restriction site differences approximating Poisson distributions (*e.g.*, ROGERS and HARPENDING 1992; LAVERY *et al.* 1996a).

Gene flow between populations: Estimates of N_em between sympatric social forms and between the regions are presented in Table 6. Both sets of nuclear markers suggest quite substantial gene flow between the social forms in each region but low or modest gene flow between the regions. Indeed, the allozyme data give an estimate of N_em between regions of ~1.0, the critical value for inferring whether gene flow is sufficient to prevent differentiation at neutral genes via drift (SLATKIN 1985, 1994). N_em values from the mtDNA generally are less than those from the nuclear markers and are lower when estimated from the haplotype phylogeny than from the Φ_{ST} values. Most of the N_em values from the mtDNA are close to or below 1.0, suggesting that female-mediated gene flow between sympatric social forms and regions may not be sufficient to prevent their continuing divergence at the mitochondrial genome.

Genetic relationships of populations: Trees depicting the genetic relationships of the four study populations are presented in Figure 3. Both classes of nuclear markers yield the same topology, with each social form most closely allied to the alternate sympatric social form (this topology is retrieved in 100% and 99% of the bootstrap replicates using the allozyme and microsatellite loci, respectively). The mtDNA tree, on the other hand, indicates a closer affinity of the Corrientes P population to the two Formosa populations than to the Corrientes M population (all nodes are significant at $P < 0.03$ according to the permutation analysis). Thus interform gene flow caused by the combined migration of both sexes is sufficient to cause sympatric populations to cluster when nuclear markers are employed, but female-mediated interform gene flow is not always sufficient to cause such clustering when mtDNA markers are employed.

DISCUSSION

This study describes hierarchical genetic structure in native fire ant populations determined by surveying variation at different classes of markers of the nuclear and mitochondrial genomes. The microgeographic structure revealed by these markers is predictable to a large extent from the known reproductive and social habits of these insects. Superimposed on this lower-level structure attributable to the contemporary social demography are macrogeographic and phylogeographic features that probably reflect the influence of historical patterns of gene flow and changes in range and population size.

TABLE 5

F_{ST} and Φ_{ST} values for differentiation between the two social forms of *S. invicta* from two regions of Argentina estimated using three classes of markers

	F_{ST}		Φ_{ST}		
			Microsatellites[a]	mtDNA	
	Allozymes	Microsatellites	Equidistant metric	Euclidean metric	Equidistant metric
Corrientes	0.009	0.006	0.005	0.356*	0.316*
	(0.000–0.018)	(−0.005–0.018)	(−0.003–0.014)	($P < 0.001$)	($P < 0.001$)
Formosa	0.007	0.014*	0.010	0.049	0.035*
	(−0.002–0.016)	(0.002–0.026)	(0.000–0.020)	($P = 0.063$)	($P = 0.026$)

The 95% CIs about the values of F_{ST} and of Φ_{ST} for the microsatellites, as well as the probabilities that the estimates of Φ_{ST} for the mtDNA do not differ from zero, are indicated in parentheses. Values with CIs that do not overlap zero or permutation probabilities that are <0.05 are indicated by an asterisk. Sample sizes are listed in the Table 4 footnote. The allozyme and microsatellite estimates are based on eight and seven loci, respectively.

[a] Φ_{ST} was not estimated using a Euclidean metric for the microsatellites because no significant structure was found at the level of social form in the hierarchical Φ_{ST} analyses using this metric (see Table 4).

In ants, colony social organization, especially the number of queens per nest, is thought to be a primary determinant of patterns of reproduction and local gene flow, and thus of microgeographic genetic structure (Ross and Keller 1995; Chapuisat *et al.* 1997; Ross and Shoemaker 1997). For instance, the family composition of nests, which is affected by the number of queens and their relatedness, influences within-nest genetic variation as well as differentiation between nests (Wade 1985b; Ross 1993). Moreover, because colony queen number strongly influences mating and dispersal habits (Keller 1991, 1993; Bourke and Franks 1995; Ross and Keller 1995), genetic relationships between adjacent nests may be affected by this element of social organization. Sexuals of monogyne ants commonly mate on the wing and disperse widely, and the dispersing queens are physiologically equipped to establish nests without the assistance of workers. In contrast, sexuals of polygyne ants often mate within or around their natal nests, and queens typically initiate reproduction in existing nests, often their natal or a neighboring nest. Colonies of such ants usually reproduce by budding, a process in which groups of workers and queens establish a new nest in proximity to the parent nest. Thus the reproductive habits of polygyne ants suggest limited dispersal and gene flow, at least via females, compared to closely related monogyne ants (Pamilo and Rosengren 1984; Sundström 1993; Bourke and Franks 1995; Ross and Keller 1995; Seppä and Pamilo 1995; Banschbach and Herbers 1996). Social organization can further influence genetic structure when contrasting breeding strategies of different social forms are incompatible, leading to substantial barriers to interbreeding where the alternate forms co-occur (Ross and Keller 1995).

The nest represents an important level of structure in both social forms of *S. invicta* in Argentina. In the monogyne (M) form, relatedness of female nestmates is as high as possible in the absence of inbreeding (0.75), reflecting the fact that the single queens heading each colony mate singly (Ross *et al.* 1993). Nestmate relatedness also is moderately high in the polygyne (P) form, reflecting the fact that such nests in the native range are headed by relatively few singly mated queens that are close relatives (Ross *et al.* 1996a). Two important conclusions stem from these results. The first concerns the nature of queen dispersal and recruitment in the P form. To explain the high relatedness of nestmate females, it is clear that queens of this form must not disperse far from their natal nest (if at all) before mating and beginning to lay eggs. This is an important line of evidence that native fire ants display the same sorts of dichotomies in mating and dispersal behaviors between social forms that are seen in other ants. The second conclusion is that the nest must represent an important level of selection in both social forms in the native range (*e.g.*, Crozier and Pamilo 1996). Selection is unlikely to be important at this level in introduced P populations because nests contain many distantly related queens and nestmate relatedness therefore is effectively zero (Ross *et al.* 1996a).

Reduced queen dispersal during mating, combined with budding as a mode of colony founding (Vargo and Porter 1989), is expected to generate microgeographic genetic structure above the level of the nest in native P populations of *S. invicta*. Significant structure indeed was found at the level of nest clusters and/or demes in both P populations studied using all three classes of markers. Structure at these levels consistently was more pronounced for the mtDNA markers than for the two classes of nuclear markers, which may be expected given the lower effective population size for the mitochondrial genome and its correspondingly greater susceptibility to drift compared to nuclear mark-

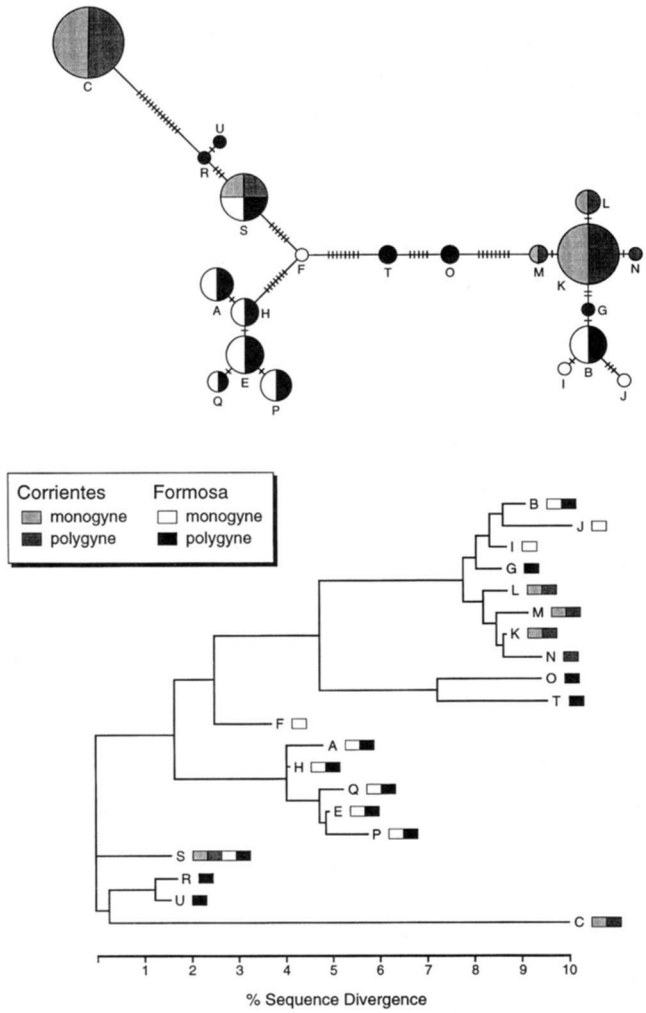

FIGURE 1.—Minimum spanning tree (top) and neighbor-joining tree (bottom) representing mtDNA haplotype relationships for *S. invicta* from Argentina. The frequency of each haplotype in the entire collection is proportional to the area of the circle representing it in the minimum spanning tree. Branch lengths are proportional to the inferred number of restriction site changes (indicated by ticks) in the minimum spanning tree or the percent sequence divergence in the neighbor-joining tree. The occurrence of each haplotype in each region and social form is indicated by the patterns of shading.

ers (AVISE 1994; LAVERY *et al.* 1996b). On the other hand, this result may also reflect real differences in dispersal patterns between the sexes, with queens being less vagile than males in P populations. This latter possibility has been postulated for other polygyne ants (PASSERA and KELLER 1994; SUNDSTRÖM 1995) and is consistent with the general lack of heterozygote deficiencies at nuclear markers in the two P populations we studied (which signals effective panmixis, such as would result from high male vagility).

Extensive dispersal by both sexes during mating flights, combined with founding of new nests by newly mated queens, suggests that minimal microgeographic structure should occur above the nest level in M populations. Indeed, nuclear and mtDNA structure was absent in this form at the level of the nest cluster (and generally the deme level as well), illustrating that differences in dispersal and reproductive behaviors associated with colony queen number have important consequences for local genetic structure in native fire ants. Similar results have been obtained for introduced *S. invicta,* where the P form exhibits strong mtDNA structure and very weak allozyme structure at microgeographic scales but the M form exhibits no detectable structure at such scales using either type of marker (ROSS and SHOEMAKER 1997). Thus, the substantial changes in colony queen number and mode of queen recruitment in the P form that followed colonization of the U.S. have not altered the fundamental effects that polygyny and its behavioral correlates exert on microgeographic structure above the level of the nest. The fact that nuclear structure at such scales is weak in both native and introduced P populations indicates that these are not "viscous" social insect populations in which higher-level selection significantly promotes or interferes with kin selection operating at the nest level (*e.g.,* KELLY 1994).

The question of whether divergence in social organization can constrain gene flow between sympatric social forms has become a major concern of population biologists (ROSS and KELLER 1995). Our data reveal significant mtDNA differentiation between the sympatric forms in each study region, with this differentiation especially pronounced in Corrientes. Strong mtDNA differentiation has been found as well in an area in the introduced range where the two forms co-occur (SHOEMAKER and ROSS 1996; ROSS and SHOEMAKER 1997). Such mtDNA differentiation between the forms was anticipated in the introduced range, where studies of the social biology suggest that queens of each form are unlikely to establish themselves commonly in nests of the alternate form (KELLER and ROSS 1993a,b; ROSS and SHOEMAKER 1993, 1997). Specifically, young P queens are not accepted into queenright M nests and generally do not possess sufficient nutrient reserves to found single-queen nests independently, whereas young M queens possess phenotypes inappropriate for being accepted by workers in P nests and do not cooperate to form persistent multiple-queen associations. The extensive interform mtDNA differentiation observed in Argentina suggests that similar social differences are likely to restrict queen-mediated gene flow between the two forms in native populations. Such restrictions apparently are not absolute or have not been absolute for long because all haplotypes found at appreciable frequencies in one form are present also in the alternate sympatric form (data not shown).

Nuclear gene differentiation has not been detected between sympatric social forms in the U.S. using allozyme markers (ROSS and SHOEMAKER 1993, 1997), but weak differentiation at this level is apparent in Argentina from both the allozymes and microsatellites. Sev-

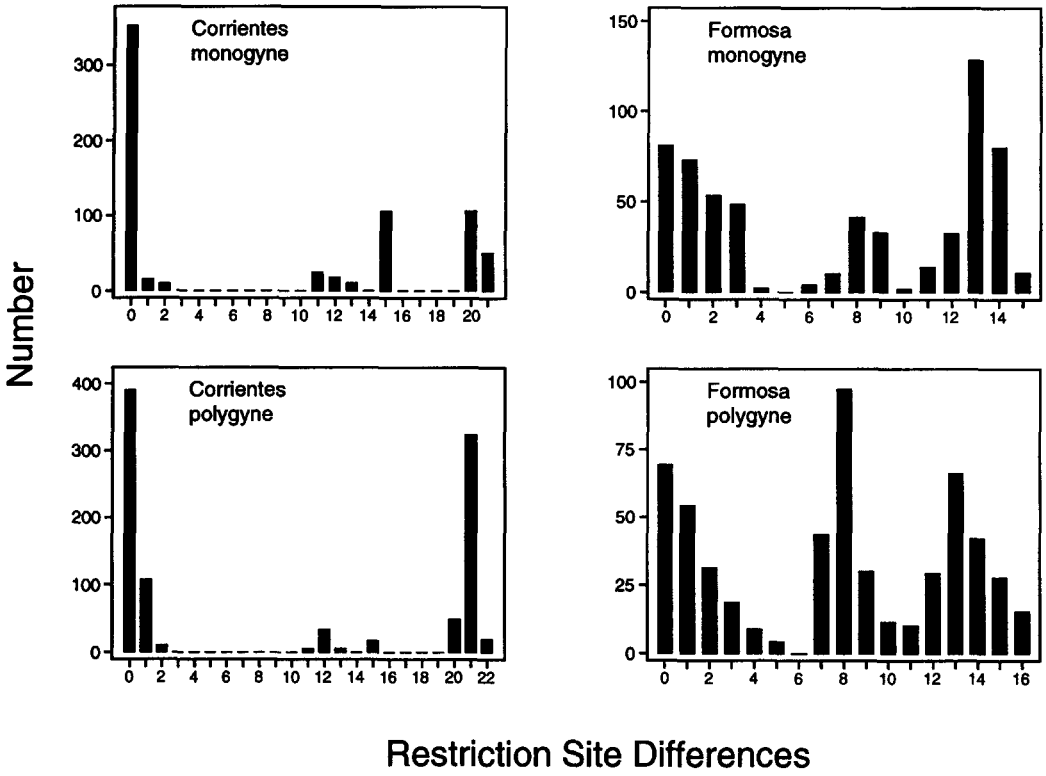

FIGURE 2.—Distributions of numbers of mtDNA restriction site differences between all pairs of individuals of *S. invicta* in four populations from Argentina.

eral factors may be involved in this difference between the native and introduced ants. Extensive nuclear gene flow mediated by P queens mating with M males is thought to homogenize allele frequencies at neutral loci in the U.S., and it is possible that gene flow via this route is not as extensive in the native range. Interform mating of this type apparently is promoted in the U.S. by the strong female bias in operational sex ratios in the P form (VARGO 1996), which is associated with a loss of allelic variation at the sex-determining locus and consequent sterility of most males produced in P nests (ROSS *et al.* 1993). Male sterility apparently is far less common in Argentina (ROSS *et al.* 1993), so that operational sex ratios may be more balanced and P males may be able to compete more effectively with M males for matings with P queens in the native than in the introduced range. Another reason that mating between P queens and M males may be more limited in Argentina than the U.S. is that assortative mating by social form may be better developed in the native populations. Finally, the inability to detect nuclear differentiation between the forms in the U.S. may simply result from there having been insufficient time for such differences to accumulate following the recent origins of P populations from local M populations.

Our analyses of mitochondrial and nuclear gene differentiation between sympatric social forms of *S. invicta* in Argentina bear on the issue of whether divergence in social organization in ants can lead to sympatric speciation by preventing gene flow between social variants with incompatible breeding systems (CROZIER 1977; WEST-EBERHARD 1986; ROSS and KELLER 1995; SHOEMAKER and ROSS 1996). Queen-mediated interform gene flow appears minimal in *S. invicta,* especially in Corrientes where all N_em estimates from the mtDNA are less than one. On the other hand, nuclear gene flow between the sympatric social forms appears substantial (all $N_em > 8$), which implies that strong disrup-

TABLE 6

Estimates of N_em between sympatric social forms and geographic regions for *S. invicta* from Argentina derived from three classes of markers

			mtDNA	
	Allozymes	Microsatellites	From Φ_{ST}	From haplotype phylogeny
Between social forms				
Corrientes	13.76	20.71–24.88	0.45–0.54	<0.10
Formosa	17.73	8.80–12.38	4.85–6.89	0.46
Between regions	1.05	4.14–6.33	1.13–1.70	0.57

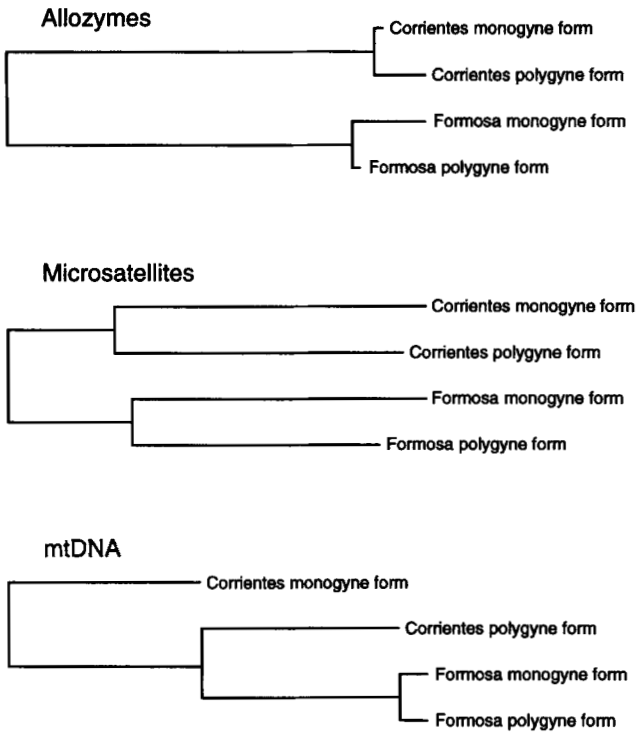

FIGURE 3.—Trees depicting genetic relationships of four study populations of *S. invicta* from Argentina based on three classes of genetic markers. Branch lengths reflect genetic distance (nuclear markers) or the proportion of genetic variation (mtDNA) between nodes.

tive selection would be required to promote the divergence of sympatric forms. Variation in colony queen number clearly affects patterns of local gene flow and generates a novel level of population genetic structure in native fire ants. Whether such constraints on interform gene flow ever develop into complete reproductive isolation, perhaps via the buildup of assortative mating, remains speculative. If polygyny arises repeatedly within local monogyne populations (*e.g.*, ROSS and KELLER 1995), then it is reasonable to expect that a range of development of assortative mating and genetic differentiation may exist in multiple sets of sympatric social forms sampled at various points in the process of divergence. Thus, genetic analyses of additional sympatric M and P populations of *S. invicta* and related species will be required to fully resolve this issue.

Differentiation between fire ants from the two regions studied in Argentina was discernible using all three classes of genetic markers and, judging from the estimates of N_em, populations from the two regions may be on largely independent evolutionary paths with respect to neutral genes. This assumes that the Río Paraná, which separates the two regions, currently serves as a major dispersal barrier, rather than an alternative scenario in which the two regions represent formerly fragmented parts of the range that have recently regained contact and begun free genetic exchange. The assumption that contemporary gene flow between the regions is minimal gains some credence from the fact that several allozyme and microsatellite alleles that are moderately common in one region are absent in the other, and that only one of the 20 mtDNA haplotypes discovered is shared between the regions (data not shown). A notable difference between the results from the two classes of nuclear markers at the regional level is that at least four times as much of the total allozyme variation is partitioned between the regions than is the case for the microsatellites. This is probably the result of substantial homoplasy at this level in the microsatellite data set that is associated with the high mutation rates and finite numbers of unique alleles generated at these loci (ESTOUP *et al.* 1995; JARNE and LAGODA 1996).

Additional insights into the evolutionary history of the study populations can be gleaned from the phylogeographic analyses of mtDNA variation. Little phylogeographic structure is evident from the inferred haplotype genealogies. That is, the major mtDNA clades are not confined to single regions or social forms, as would be expected if the populations were relatively closed to genetic exchange over evolutionary time periods and the distributions of variants resulted primarily from their gradual spread from endogenous points of origin (*e.g.*, NEIGEL and AVISE 1993). Rather, a history of repeated female-mediated gene flow between these and other populations would seem to be required to explain the phylogeographic distribution of the mtDNA variants. This conclusion reinforces the view that the nuclear and mtDNA structure observed at the form and regional levels is due to contemporary rather than long-standing restrictions on gene flow.

A distinctive feature of the fire ant mtDNA data is the large divergence between many of the haplotypes coexisting within single populations. Average sequence divergence between co-occurring fire ant haplotypes (6–10%) far surpasses the maximum sequence divergence typically reported within species of insects and other invertebrates (MARTIN and SIMON 1990; HALE and SINGH 1991; NÜRNBERGER and HARRISON 1995; BROWN *et al.* 1996; LAVERY *et al.* 1996b; ROSSI *et al.* 1996; FOLTZ 1997; GUILLEMAUD *et al.* 1997; WILCOX *et al.* 1997), including another group of eusocial hymenopterans, the honey bees (MORITZ 1994). Such comparisons are biased to some extent because about one-quarter of the mtDNA sequence we amplified in fire ants includes the noncoding A+T-rich region. Nonetheless, mean haplotype divergence in fire ants is more than six times greater than in either of two Drosophila species surveyed using PCR amplification and restriction enzyme digestion of a similar mtDNA fragment (D. D. SHOEMAKER, unpublished), and it is slightly greater than the divergence reported among six species in the lepidopteran genus Jalmenus surveyed by sequencing part of the A+T-rich region (TAYLOR *et al.* 1993). One possible explanation for the high mitochondrial divergence in fire ants is that the sampled populations are very old

and stable. The multimodal distributions of the pairwise restriction site differences are not typical of recently founded populations, and thus the study populations may well have been large and stable over long periods. Another possible explanation is persistent mitochondrial introgression from heterospecific populations. Although there presently is no evidence from nuclear markers or the morphology that hybridization occurs commonly between *S. invicta* and its sympatric congeners in the native range (Ross and Trager 1990; Trager 1991), examples of mtDNA introgression occurring in the face of barriers to nuclear gene flow are well known in other animals (Barton and Hewitt 1989).

To conclude, the three classes of markers we employed provide complementary information on genetic structure in native fire ants, with the nature of the information at different scales influenced by the mode of inheritance and level of variation characterizing each class. A consistently greater ability of the mtDNA to detect structure at all levels may be due to its lower effective population size relative to nuclear markers, as well as female vagility generally being more limited than male vagility in these ants. The largely compatible results from the two sets of nuclear markers at most scales may result from the superior resolving power of the highly polymorphic microsatellites being offset by the larger sample sizes obtained for the less variable allozyme markers. The range of spatial scales over which the markers are informative apparently is more limited for the microsatellites than for the allozymes or mtDNA, presumably because the particular mutation processes making the microsatellites particularly useful at microgeographic scales generate homoplasy at a regional scale. Finally, use of the equidistant metric appeared to detect structure more efficiently than use of the Euclidean metric for the microsatellites, a difference not apparent for the mtDNA. This may be because the particular distance metric employed for the microsatellites does not reflect the real evolutionary separation of variants as well as does the metric employed for the mtDNA (*e.g.*, Jarne and Lagoda 1996).

We thank J. Costa, C. DeHeer, J. Evans, and M. Goodisman for comments on the manuscript and R. Seay, J. Smith, and J. White for technical assistance. This work was funded in part by the National Geographic Society, the Georgia Agricultural Experiment Stations (University of Georgia), and the Swiss National Science Foundation.

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Communicating editor: M. SLATKIN