Population Genetics of the Invasive Fire Ant *Solenopsis invicta* (Hymenoptera: Formicidae) in the United States

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ABSTRACT Analyses of population genetic variation in invasive species can provide information on the history of the invasions, breeding systems, and gene flow patterns. We surveyed genetic variation in both social forms of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), throughout the species' introduced range in the United States, to learn how the unique breeding biology of each form shapes genetic structure at various scales, to discern genetic footprints of the invasion process, and to reconstruct the origin and spread of each form. Consistent with more limited earlier studies, our analyses revealed significant local mitochondrial DNA (mtDNA) differentiation in the polygyne (multiple colony queens) but not in the monogyne (single colony queen) social form as well as pronounced mtDNA differentiation coupled with weaker nuclear differentiation between sympatric populations of the two forms. At a larger scale, we found no mtDNA but significant nuclear regional differentiation. In general, populations were most similar to other populations of the same social form at their mtDNA genomes. These higher level patterns of structure are consistent with the spread of the ant by long-distance, human-mediated dispersal, with subfounder populations of each form typically established by queens of the same form. Bayesian analyses showed that study populations most distant from the claimed site of entry, Mobile, AL, have diverged most from the hypothetical founder population, consistent with an invasion scenario in which the ants spread outward from Mobile through repeated subfounder events. Several lines of evidence raise the possibility of secondary introductions of *S. invicta* into the United States.

KEY WORDS fire ants, genetic structure, invasion history, *Solenopsis invicta*

Analyses of population genetic variation can provide much relevant information on important issues in evolutionary biology such as patterns of dispersal and gene flow, characteristics of breeding systems, the existence of local adaptation and divergence, the form of host–parasite coevolution, and the history of biological invasions (Reilly 1987, Gillespie and Oxford 1998, Nason et al. 1998, Storfer and Sih 1998, Martinez et al. 1999, Clegg et al. 2002, Sved et al. 2003). In social organisms, such analyses take on an added dimension because social habits can affect patterns of gene flow and population structure and the nature of this structure at various scales is in turn expected to influence trajectories of social evolution (Hamilton 1964, West-Eberhard 1975, Queller 1992, Ross and Shoemaker 1997, Reeve and Keller 2001, Sundström and Boomsma 2001). Consequently, numerous studies characterizing genetic structure of social organisms have been conducted, especially on ants. Many ant studies have focused on genetic structure at localized scales to assess how properties of the breeding system influence relatedness within and between social groups (for review, see Ross 2001), how selection acts within colonies (Banschbach and Herbers 1996, Ross 1997, Keller and Ross 1998), the nature of queen–worker conflict (Queller 1993, Sundström et al. 1996), and rates of social parasitism (Foitzik and Herbers 2001). Others have evaluated structure at more expansive scales to determine patterns of dispersal and gene flow (Ross and Shoemaker 1993, Shoemaker and Ross 1996, Chapuisat et al. 1997, DeHeer et al. 1999, Goropashnaya et al. 2001, Sanetra and Crozier 2003, DeHeer and Herbers 2004), reveal historical demographic events (Goropashnaya et al. 2004, Ahrens et al. 2005), document the results of interspecific hybridization (Shoemaker et al. 1996, Cahan et al. 2002, Julian et al. 2002, Cahan and Keller 2003, Cahan and Vinson 2003), infer the genetic consequences of invasions (Ross et al. 1993, Tsutsui and Case 2001, Giraud et al. 2002, Jaqui-

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éry et al. 2005), and reveal relationships between native and introduced populations of invasive species (Tsutsui et al. 2001, Henshaw et al. 2005).

One ant for which population genetic tools have been applied extensively is the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). This species was inadvertently introduced into the United States from South America ≈75 yr ago (Lofgren 1986a). Since its initial introduction, *S. invicta* has spread throughout most of the southeastern United States, with the core area of its invasive range presently extending from central Texas to southern Virginia (Culpepper 1953, Lofgren 1986a, Callcott and Collins 1996). The species is considered a significant ecological, agricultural, and public health pest throughout this invasive range (Lofgren 1986a,b; Jouvenaz 1990; Porter and Savignano 1990; Patterson 1994; Vinson 1997; Carroll and Hoffman 2000; Gotelli and Arnett 2000; Kemp et al. 2000; Eubanks 2001; Wojcik et al. 2001; Williams and deShazo 2004). Consequently, an extensive literature on the biology of *S. invicta* has been generated, which in turn has led to its emergence as an important model system for ecological and evolutionary studies (see Tschinkel 2006, and references therein). An important element of its social behavior that has motivated many such studies is the existence of two distinct types of colony social organization within the species (Glancey et al. 1973). Colonies of the monogyne (M) form possess a single egg-laying queen, whereas colonies of the polygyne (P) form possess several to hundreds of these queens. Introduced populations were long assumed to consist solely of M colonies (Vinson and Greenberg 1986), but populations of P colonies have been documented with increasing frequency since the early 1970s (Glancey et al. 1973, 1975; Hung et al. 1974; Fletcher et al. 1980) and are now known to be widespread throughout the range in the United States. (Porter et al. 1991, Porter 1992, Mescher et al. 2003). The two social forms of *S. invicta* differ not only in colony queen number but also in important features of their reproductive and dispersal behaviors, which are expected to have a number of important effects on the distribution of genetic variation at various spatial scales (see below).

Previous studies have analyzed patterns of genetic structure in introduced *S. invicta* in the United States (Ross and Fletcher 1985b; Ross et al. 1987, 1996, 1999; Ross and Shoemaker 1993, 1997; Shoemaker and Ross 1996; Chen et al. 2003). However, no study to date has examined structure at multiple scales in both social forms over a broad geographic area by using numerous highly informative markers. The resulting lack of a detailed picture of population genetic structure in the United States has hindered progress on several fronts, including our ability to discern predicted genetic signatures of recently founded populations, to reconstruct the origin and spread of each social form, and to fully understand the role of social polymorphism in driving population divergence. The application of new analytical methods for interpreting population genetic data (Lessa 1990, Pritchard et al. 2000, Mank and Avise 2004), coupled with the availability of extensive population samples scored for numerous informative markers, now offers the promise of sustaining progress on these issues. In particular, newly developed Bayesian methods (Pritchard et al. 2000, Corander et al. 2003, Falush et al. 2003, Evanno et al. 2005) offer unprecedented power in extracting useful information concerning the form and causes of genetic differentiation from large, complex data sets.

In the current study, we make use of traditional as well as newer analytical methods to explore patterns of variation within *S. invicta* across its introduced range in the United States. The study has several specific objectives. First, we wanted to learn whether observed patterns of variation shed light on the invasion history of this ant. Second, we wanted to gain additional insight into the origin and spread of each social form. Early observers speculated that the polygyne form may have arisen "spontaneously" from sympatric monogyne populations due to environmental causes (Ross et al. 1987, Ross and Keller 1995b), but more recent genetic analyses implicate a strong heritable component to the expression of polygyny (see below). These two proximate mechanisms make different predictions that can be tested by comparing the genetic structure inferred from nuclear and mitochondrial markers. Third, we wanted to further test the long-standing idea that changes in social behavior can alter patterns of gene flow, eventually culminating in loss of some gene flow routes or even complete reproductive isolation between sympatric forms (West-Eberhard 1986, Ross and Keller 1995b, Shoemaker and Ross 1996). Finally, elucidation of the large-scale genetic structure of introduced *S. invicta* provides a necessary framework for future efforts aimed at pinpointing the native source population(s), a task crucial to focusing biological research aimed at developing new methods of control of this invasive pest.

Invasion Biology of Introduced *S. invicta*. A primary aim of our study is to use genetic information in conjunction with data on the invasion history to refine our knowledge of the processes that have led to the current distribution of *S. invicta* in the United States. Historical collections indicate that this ant was introduced inadvertently into the United States between the 1930s and early 1940s, with Mobile Bay, AL, the most likely point of entry (Lofgren 1986a). The ensuing spread of the ant was characterized by a gradual wave of colonization outward from Mobile, coupled with frequent long distance dispersal events through unintentional human transport. Federal quarantine measures put in place in 1958 apparently reduced the frequency of anthropogenic dispersal, so that subsequent spread likely occurred mostly via natural dispersal, although clear exceptions are documented (e.g., Fletcher 1983). The invasive range nonetheless continued to expand rapidly through the mid-1970s to encompass much of the southern United States (Culpepper 1953, Callcott and Collins 1996, Lofgren 1986a). The current range, which has not increased substantially over the past 25 yr (Callcott and Collins 1996), spans a core area from central Texas to southern

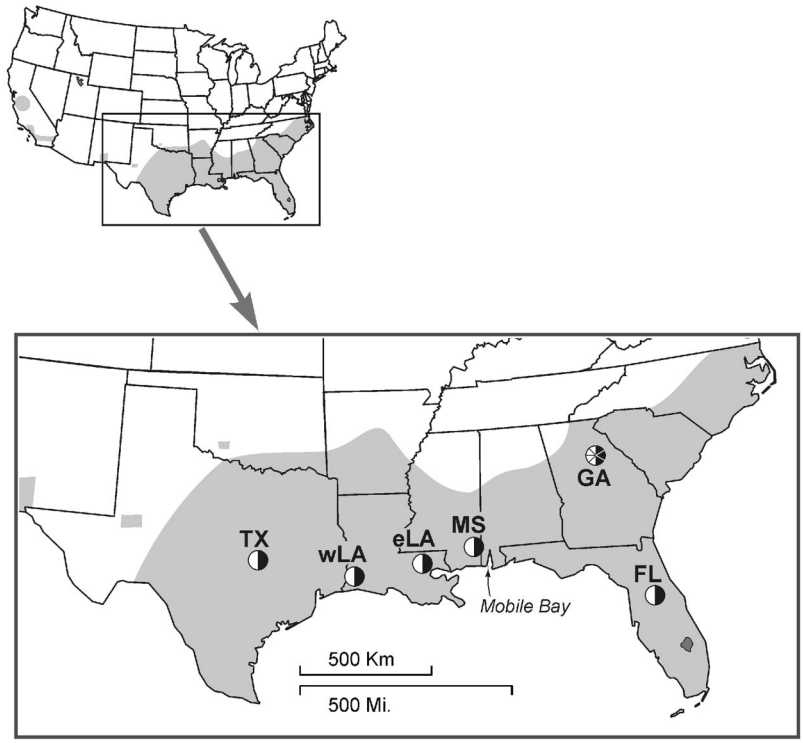

Fig. 1. Collection sites for *S. invicta* in the southern United States. Nests of both social forms (monogyne [M] and polygyne [P]) were sampled at each of six regional localities (TX, Texas; wLA, western Louisiana; eLA, eastern Louisiana; MS, Mississippi; GA, Georgia; and FL, Florida). Nests of each form were sampled at multiple sites in Georgia (three M and four P sites). The range of *S. invicta* in the United States at the time of collection of the samples (1995) is indicated by gray shading (source: www.aphis.usda.gov/ppq/maps/fireant.pdf). Mobile Bay is the presumed point of entry of *S. invicta* into the United States (Lofgren 1986b).

Virginia, with several isolated infestations in outlying states (Fig. 1).

Until the early 1970s, it was assumed that all *S. invicta* colonies in the introduced range were monogyne. This assumption changed with the discovery of colonies containing multiple mated queens in several widely separated areas (Glancey et al. 1973, 1975; Hung et al. 1974; Fletcher et al. 1980). Given that social form has a strong genetic basis (see below), a major question raised by the relatively late discovery of polygyny is whether its appearance reflects the occurrence of a secondary introduction involving this social form.

Natural dispersal occurs predominantly during mating flights in both social forms of *S. invicta.* In the M form, sexuals ascend to elevations of 100 m or more for pairing, and they can be transported several kilometers or more by wind currents during these flights (Markin et al. 1971). Mating swarms in the P form occur at lower elevations (often at head-height), and the vagility of queens of this form seems correspondingly more restricted than in the M form (DeHeer et al. 1999, Goodisman et al. 2000). An important additional means of natural spread of the P form is through colony budding or fissioning, a process in which workers and queens from a parent colony travel on foot to establish a new colony (Vargo and Porter 1989). Movement during this mode of colony reproduction is necessarily very limited. The differences in reproductive behavior between the two social forms of *S. invicta* are expected to yield different patterns of naturally occurring gene flow and genetic structure, with gene flow occurring over much longer distances in the M form than the P form (Shoemaker and Ross 1996; also see Goodisman and Ross 1998, 1999; Ross et al. 1999).

Genetic Basis of Colony Social Organization in *S. invicta*. Colonies of the M form of *S. invicta* are headed by a single reproductive queen, whereas those of the P form contain multiple reproductive queens (Vargo and Fletcher 1987, Ross and Keller 1995b, Ross et al. 1996). Association studies have demonstrated conclusively that this fundamental social attribute in this and other fire ant species depends on the presence within a colony of specific coding region variants of the gene *Gp-9*. Two such variants, designated as alleles *B* and *b*, occur in *S. invicta* in the United States. M colonies contain only the *B* allele, whereas P colonies invariably contain the *b* allele as well as the *B* allele (Ross 1997, Ross and Keller 1998, Krieger and Ross 2002). Because the P form harbors both *Gp-9* alleles, it is hypothetically possible for M populations to arise from previously existing P populations (via *BB* founder

queens that have mated with *b* males). However, it seems that it is not possible for P populations to arise from preexisting M populations, because the requisite variant at *Gp-9* for expression of polygyny (the *b* allele) is lacking.

Materials and Methods

Samples. Ants were collected at six localities distributed throughout the range of introduced *S. invicta* in the southern United States (Fig. 1), with nests of each social form sampled at each locality. In total, 1,060 nests were sampled, 135 from Austin, TX (referred to hereafter as Texas; 72 monogyne [M], 63 polygyne [P]), 135 from De Quincy, LA (western Louisiana; 75 M, 60 P), 130 from Hammond, LA (eastern Louisiana; 70 M, 60 P), 133 from Hurley, MS (Mississippi; 69 M, 64 P), 402 from Monroe, GA (Georgia; 152 M [40, 42, and 70 from three sites], 250 P [55, 65, 65, and 65 from four sites]), and 125 from Gainesville, FL (Florida; 65 M, 60 P). Sampled nests were located within 40 km of all other nests from the same locality, with the several sampling sites in Georgia located 10–30 km from other sites containing the same social form. A subset of the samples in this study (from western Louisiana and Georgia) was used in an earlier study of population genetic structure that used additional nuclear genetic markers (Ross et al. 1999, also see Mank and Avise 2004).

We determined the social form of each colony by using several lines of evidence, as described in Ross and Shoemaker (1997). Nest density, worker size, and nest brood composition were used to make initial identifications of social form in the field. Subsequently, polygyny was confirmed in a nest by discovering two or more inseminated wingless (reproductive) queens, by detecting multiple families represented among eight or more nestmate offspring females surveyed at six polymorphic allozyme loci and/or by detecting the presence of the *b* allele of the gene *Gp-9* among the female inhabitants (Ross 1997, see below; also see Krieger and Ross 2002). Monogyny was confirmed by detecting a single family represented among eight or more nestmate females surveyed at the six allozyme loci and by failing to find the $Gp\text{-}9^b$ allele among these females. Designation of social form was not entirely consistent across all criteria for 34 of the 1,060 sampled nests (3.2%). Four colonies (0.4%) were classified as monogyne in the field and lacked the $Gp\text{-}9^b$ allele, yet allozyme genotype distributions indicated that nestmate females made up multiple families. These are assumed to represent M colonies in which queen turnover had recently occurred (DeHeer and Tschinkel 1998). Another five colonies (0.5%) were classified as monogyne in the field but possessed multiple families as well as the $Gp\text{-}9^b$ allele, indicating that they were really polygyne. Finally, 25 colonies (2.3%) identified as either form in the field contained only one detectable matriline, but some females in each bore the $Gp\text{-}9^b$ allele. Given the apparent invariant association of this allele with polygyny in *S. invicta* (Ross 1997, Ross and Keller 1998, Krieger and Ross 2002), these are presumed to be P colonies with low effective queen numbers. We note that any misclassification of nests in this study is a conservative error in the sense that it reduces our ability to perceive real genetic differentiation between the social forms.

Genetic Markers. We scored genotypes of winged (nonreproductive) and/or wingless (reproductive) queens at 10 polymorphic allozyme or protein-coding loci by using horizontal starch gel electrophoresis coupled with specific staining (for procedures, see Shoemaker et al. 1992, DeHeer et al. 1999). For presumed M nests and for presumed P nests for which we failed to obtain two or more mated queens, seven of these loci (*Aat-2; Acoh-1,5; Est-4; Gp-9; G3pdh-1;* and *Pgm-1*) were scored from 8 to 12 winged queens per nest to determine family structure and look for the $Gp\text{-}9^b$ allele (this information was used to assist in correct classification of social form of each colony). Genotypes at three additional allozyme loci (*Acy*1, *Ddh-1*, and *Pgm-3*) were determined from a single individual per nest. Genotypic information from *Gp-9* was not used apart from assigning colonies to social form. Genotypic information from *Pgm-3,* which is tightly linked to *Gp-9* (Ross 1997), was used only for M nests in all of the analyses, because the effects of selection on *Gp-9* have been shown to distort *Pgm-3* genotype frequencies in the P form (Ross 1997, Ross et al. 1999).

Total DNA was extracted from the head of one of the queens in each nest used for allozyme electrophoresis by means of the Puregene Kit (Gentra Systems, Inc., Minneapolis, MN). We used this template DNA to score genotypes at seven polymorphic microsatellite loci. Primers used to amplify the seven loci were as described by Krieger and Keller (1997), except for the redesigned forward primer of *Sol-20* (*Sol-20.F2,* 5′-GACTTCCCTACTTTGTCTCTCTCC-3′) and redesigned forward and reverse primers of *Sol-55* (*Sol-55.3 F,* 5′-CAGTTTGCGAATATCCGGTC-3′ and *Sol-55.3R,* 5′-GCGTATTGCGACAATGAATG-3′). One primer of each pair was labeled at the 5′ end with 6FAM, HEX, or NED (Applied Biosystems, Foster City, CA), with the dyes chosen so that the products of all seven loci could be separated and detected simultaneously on single gels. The following primers were labeled: *Sol-6.rev* (6FAM), *Sol-11.rev* (6FAM), *Sol-18.for* (6FAM), *Sol-20.F2* (HEX), *Sol-42.for* (NED), *Sol-49.rev* (HEX), and *Sol-55.3 F* (NED). The seven microsatellite loci were amplified in two separate 20-μl multiplex polymerase chain reaction (PCR) reactions. The first reaction mixture contained 1.6× PCR buffer (2.4 mM $MgCl_2$ and 80 mM KCl), 1× Q-Solution (QIAGEN, Valencia, CA), 300 μM dNTPs, 0.63 μM *Sol-42* primers, 0.41 μM *Sol-49* primers, 0.47 μM *Sol-55* primers, 1.5 U of *Taq* polymerase, and 2 μl of hydrated template DNA. The second reaction mixture contained 2× PCR buffer (3 mM $MgCl_2$ and 100 mM KCl), 0.725× Q-Solution, 400 μM dNTPs, 0.38 μM *Sol-6, Sol-11,* and *Sol-18* primers, 0.5 μM *Sol-20* primers, 1.5 U of *Taq* polymerase, and 4 μl of template DNA. We conducted reactions in 0.5 ml thin-walled PCR tubes on a PTC-100 thermal cycler (MJ Research,

Watertown, MA) with the heated lid enabled; the cycling profile of Krieger and Keller (1997) was used.

After PCR, 1.0 μl of product from each of the two multiplex reactions was combined with 2.0 μl of deionized formamide, 0.5 μl of GeneScan-400 [ROX] size standard (Applied Biosystems), and 0.5 μl loading dye. The mixture was denatured for 3 min at 95°C and then 1.3 μl was loaded onto a polyacrylamide gel (4.8% acryl-bisacrylamide and 6 M urea). In addition to the GeneScan-400 [ROX] size standard, we included a sample of known repeat length for each of the loci on each gel to ensure accurate size assessments. The samples were run on an ABI 370 DNA Sequencer (Applied Biosystems), and the microsatellite genotypes were scored using GENESCAN 3.1.2 software (Applied Biosystems).

We used the same template DNA also to score haplotypes of the mitochondrial DNA (mtDNA) by PCR amplifying a 4-kb segment (including the control region) and then digesting the PCR product with 13 enzymes used individually (Ross and Shoemaker 1997). Composite haplotypes were defined by possession of unique sets of restriction sites across the battery of enzymes. Because *S. invicta* colonies comprise one or more families, we used only a single multilocus genotype from each nest in all of the genetic analyses reported in this article.

Data Analyses. We estimated allele and genotype frequencies at each of the nuclear loci and haplotype frequencies at the mtDNA for each of the 17 study populations by using the program GENEPOP on the Web (Raymond and Rousset 1995b). Genetic diversity in each population was measured in a number of ways. We obtained allele and haplotype counts for each nuclear marker and the mtDNA, respectively, and calculated the mean numbers of alleles per nuclear locus (allelic richness). Estimates of allelic richness were not corrected for sample size differences (Leberg 2002), because of the large sample sizes for most populations. Multilocus expected heterozygosity (gene diversity) and haplotype diversity were calculated according to Nei (1987) by using the program ARLEQUIN (Schneider et al. 2000).

Genotype proportions at the nuclear loci were tested for conformity to Hardy–Weinberg expectations (HWE) by using the exact tests implemented in GENEPOP, with Fisher's method of combining test results (Manly 1985) used to evaluate the overall significance of departures from HWE across subsets of these loci in each population. The α levels for each specific test were adjusted using the Bonferroni correction. Values of F_{IS} estimated by GENEPOP with the method of Weir and Cockerham (1984) were used to determine whether significant departures were due to excesses or deficiencies of heterozygotes.

Several different measures of genetic differentiation between pairs of populations were estimated. None of these estimates used information on the presumed evolutionary distance between variants of the microsatellite loci and the mtDNA, because the stochastic loss of genetic variation and rapid population expansion in introduced *S. invicta* in the United States violates the assumption of mutation-drift equilibrium underlying the use of such information in phylogeographic analyses. Values of F_{ST} were estimated using GENEPOP (method of Weir and Cockerham 1984), and values of the genetic distance of Nei (1972) and chord distance of Cavalli-Sforza and Edwards (1967) were estimated using the program PHYLIP. We estimated the values for different subsets of the markers such as the allozyme loci only, the microsatellite loci only, and all nuclear loci combined. Significance of the genetic differentiation measured between pairs of populations was determined by means of exact tests implemented in GENEPOP (Raymond and Rousset 1995a). Fisher's method of combining results was used to assess the significance of differentiation across the different subsets of nuclear markers. Because of the large numbers of comparisons, a conservative level of $\alpha = 0.005$ was used to evaluate significance.

We also estimated values of F_{ST} simultaneously at two levels (social form and geographic region) by using the hierarchical analysis of molecular variance procedure in ARLEQUIN to partition the total genetic variation (Excoffier et al. 1992). The multiple populations of each social form in Georgia were pooled for these analyses, which were conducted separately for the different subsets of the nuclear data as well as for the mtDNA data. Statistical significance of the differentiation at each level was established by means of 20,000 data permutations.

We undertook isolation-by-distance analyses to learn whether genetic differentiation between populations increased with their geographic separation. These analyses of the relationships of F_{ST} or $F_{ST}/(1 - F_{ST})$ with geographic distance or the natural logarithm of geographic distance (Slatkin 1993) were conducted separately on the nuclear markers and the mtDNA by using GENEPOP. The sites were pooled for each social form in Georgia. Significance of the relationships was determined by means of Mantel tests based on 10,000 data permutations coupled with estimation of Spearman rank correlation coefficients.

We further examined geographic genetic structure by using two methods that do not assume hierarchical relationships among the study populations (e.g., de Queiroz and Good 1997). First, the ordination technique known as nonmetric multidimensional scaling (NMDS) analysis was used in an exploratory manner to uncover patterns of genetic variation in the data. This technique visually portrays the genetic relationships among populations represented in a matrix of pairwise distances by reducing the multidimensional, multilocus allele frequency relationships to a few dimensions that explain most of the original distance data (Lessa 1990, Guiller et al. 1998). We used the program VISTA (Young 1996) to conduct NMDS analyses on the combined nuclear data or the mtDNA data by using pairwise values of F_{ST}, Nei's genetic distance, and Cavalli-Sforza and Edwards' chord distance in the original dissimilarity matrices. The best dimensionality for each analysis was determined by generating scree plots and looking for an elbow in the curve showing the total variance in the data explained with

each added dimension; any dimensions past the elbow explained relatively little additional variance and thus were not retained. A stress statistic, which measures the discrepancy between the matrix of model distances in *n*-dimensional space and the original distance matrix, was calculated (Kruskal 1964), and a method of iterative approximations was applied repeatedly until values of this statistic declined to an asymptote, at which point the model was accepted. We graphed projections of the model output in the first three dimensions, which always jointly accounted for 70–95% of the total data variance, to distinguish clusters of genetically similar populations.

Next, we used an individual-based Bayesian method to statistically recognize distinct genetic clusters of individuals and to infer levels of population admixture from individual multilocus genotypic data (Pritchard et al. 2000, Mank and Avise 2004). The method assumes a model with *K* populations, each with characteristic allele frequencies that are estimated while individuals are probabilistically assigned to each population. Prior information such as the geographic location of samples or colony social form also can be incorporated into the model to assist in clustering. The program STRUCTURE (Pritchard et al. 2000; http://pritch.bsd.uchicago.edu) was used to explore the parameter space of each model using Markov chain Monte Carlo (MCMC) algorithms, with competing models evaluated on the basis of the posterior probabilities given the data, model, and prior information. All simulations used 100,000 MCMC iterations in the burnin phase and 300,000 iterations in the data collection phase, with four independent runs conducted on each set of data and parameter values. We ensured accurate estimates of the simulation values by checking that model parameters equilibrated before the end of the burnin phase and that the posterior probabilities were consistent across the four runs for each data and parameter set.

We used two different models of individual ancestry for these Bayesian analyses, one that used only genetic information to infer clusters and degree of admixture, and one that used information on the geographic sample locality (and, in some cases, colony social form) for each sample as well. Both models assume some level of population admixture as well as correlations of allele frequencies among populations, as expected if there is recent shared ancestry or ongoing gene flow among populations (Falush et al. 2003). These model assumptions are appropriate given that *S. invicta* rapidly colonized the southern United States over the past 75 yr and that there likely has been considerable human-mediated and natural dispersal over large portions of this range during this period. All other model parameter values were the defaults for the program.

Differentiation between the social forms at each locality was explored with STRUCTURE by using only data for that locality to determine whether models assuming a single genetic cluster or two clusters gave the highest posterior probability. We then inferred the most probable number of distinct clusters represented in the entire data set by running STRUCTURE for different numbers of assumed populations ranging from 1 to 12. The estimated membership coefficients (degree of admixture) of individuals, and their averages for each of the regions, were obtained from the simulations yielding the preferred estimates of numbers of distinct clusters, as determined by the method of Evanno et al. (2005). Finally, we explored the utility of incorporating the geographic and social form information as priors in the models. In one set of simulations, each social form at each locality was considered a separate population, whereas in a second set the two forms at each locality were pooled into a single population. For the simulations incorporating prior information, values of F_K were estimated for each predefined population; these can be interpreted as F_{ST} values that indicate the divergence of each population from a single hypothetical ancestral population (Pritchard et al. 2000, Falush et al. 2003). All of the analyses were conducted on several different subsets of the data, such as all of the genes, all of the nuclear genes, and subsets of the nuclear genes. Graphs of individual and population membership coefficients were produced for select analyses using the program DISTRUCT (Rosenberg 2002).

Results

Genetic Diversity and Tests for Conformity to HWE. Frequencies of the variants at each marker in each study population are reported in *Appendix* 1. Over the entire collection of samples, two alleles were detected at each allozyme locus (except for three alleles detected at *Pgm-1*), and from 3 (*Sol-18*) to 14 (*Sol-42*) alleles were detected at each of the microsatellite loci. Eight unique restriction haplotypes were detected for the mtDNA.

Allozyme and microsatellite gene diversities within populations were found to be significantly correlated (Spearman rank correlation test; $P = 0.02$), suggesting that these two classes of nuclear genes provide complementary information for inferring population genetic variation and structure. However, no association between nuclear and mtDNA variation within populations was observed using estimates of allelic richness, haplotype number, or gene/haplotype diversity as measures, nor by considering allozymes and microsatellites either separately or together (Spearman rank correlation tests; all $P > 0.2$).

No consistent differences in levels of genetic diversity were apparent between the two social forms of *S. invicta* in the United States. Nuclear gene diversity was higher in the P form in five of the six comparisons between geographically paired forms, whereas haplotype diversity was higher in the M form in four of the six comparisons (mean values were used for the multiple sites for each social form in Georgia). Nuclear allelic richness was higher in each of the social forms in three of the six comparisons, and the number of haplotypes was higher in the M form in four of the six comparisons.

There is no evidence that the more peripheral localities in the introduced range harbor reduced ge-

netic variation compared with the area where the invasive population originally was established. Allelic richness, number of haplotypes, multilocus nuclear gene diversity, and haplotype diversity estimates were found not to be correlated with distance from Mobile, AL, regardless of whether the social forms at each locality were pooled as single populations or were considered separately (Spearman rank correlation tests, all $P > 0.16$). Moreover, the Mississippi samples, which are located <100 km from Mobile, exhibit unremarkable values for all these statistics. Thus, the distribution of genetic diversity in the study populations did not follow the geographic pattern expected under a simple scenario of a single introduction at Mobile followed by gradual range expansion through repeated peripheral subfounder events (Ramachandran et al. 2005).

Tests for conformity to HWE using the combined allozyme data revealed no significant departures in any study population. Tests using the combined microsatellite data revealed significant departures in 8 of the 17 study populations, including populations of each social form from diverse localities. Inspection of the single-locus results indicated that these departures are attributable to just two loci, *Sol-20* and *Sol-42*, each of which displays significant heterozygote deficiencies in virtually every population. Indeed, when these loci are excluded from consideration, all populations conform to HWE using either the remaining microsatellite data or the remaining combined nuclear data. These latter results accord with previous findings from diverse data sets showing that *S. invicta* populations in the United States generally conform to HWE (Ross et al. 1987, 1999; Ross 1993; Ross and Shoemaker 1997). The atypical results for *Sol-20* and *Sol-42*, also reported earlier for a subset of the samples presented here (Ross et al. 1999), may be due to a low frequency of nonamplifying (null) alleles or to scoring artifacts at these loci. For the remaining analyses, exclusion of these two loci generally did not alter the patterns in or significance of the results presented (detailed below).

Population Genetic Differentiation. The extent of genetic differentiation between pairs of populations, as assessed by F_{ST} values, was highly correlated between the two classes of nuclear markers, regardless of whether *Sol-20* and *Sol-42* were included in the comparison (Mantel tests of association with 10,000 permutations; both $P < 0.001$). This result again shows that the two classes of nuclear genes provide concordant information for inferring population structure. For the microsatellites, values of F_{ST} derived from all seven loci were highly correlated with the values derived from the five loci other than *Sol-20* and *Sol-42* (Mantel test; $P < 0.0001$), suggesting that inclusion of the latter two loci does not unduly bias analyses of population genetic structure. Finally, no association was found between paired population F_{ST} values derived from the nuclear markers and those from the mtDNA, regardless of whether all 16 nuclear loci, only the allozyme loci, or only the microsatellite loci were considered (Mantel tests; all $P > 0.38$). Thus, the mitochondrial and nuclear genomes do not register parallel patterns of population differentiation.

We divided the paired population F_{ST} values into three categories—those for sympatric populations of the alternate social forms, for allopatric populations of the same form, and for allopatric populations of the alternate forms. For the combined nuclear data, values for sympatric populations of the different forms tend to be considerably lower than values for allopatric populations of the same or different form (Fig. 2), with a conspicuous absence of relatively large F_{ST} values between sympatric populations. No such patterns are observed for the mtDNA-based F_{ST} values, the ranges of which are similar across the three categories. These results suggest that nuclear gene differentiation between social forms at a given locality generally is low compared with that among regions but that mtDNA differentiation is comparable at the two levels. Essentially identical patterns were detected using Nei's genetic distance and Cavalli-Sforza and Edwards' chord distance.

Exact tests based on all 16 nuclear loci revealed significant differentiation between virtually all pairs of populations from different regions. Exceptions were M populations from Mississippi and one Georgia site, P populations from Texas and eastern Louisiana, and M populations from Texas and Mississippi. Most comparisons between social forms within a region also revealed significant nuclear differentiation (sites for each form in Georgia were combined for this comparison), with the exceptions being the two populations closest to the point of introduction, eastern Louisiana and Mississippi. Within Georgia, none of the comparisons between sites containing the same social form were significant. Examination of the allozyme and microsatellite data separately revealed similar patterns for the two classes of markers at both the regional and local levels but with fewer examples of significant differentiation at each level for each class. Exclusion of the loci *Sol-20* and *Sol-42* did not appreciably alter the general conclusions based on all nuclear markers.

Exact tests based on the mtDNA revealed a pattern different from that found for the nuclear markers. Virtually all pairwise population comparisons at any scale were significant, with no evident general pattern to the few nonsignificant comparisons (except that the three Georgia M sites are not significantly differentiated; also see Shoemaker and Ross 1996, Ross et al. 1999). It is noteworthy that every population is significantly differentiated from its sympatric population of the alternate form at the mtDNA, a trend upheld across the multiple sampling sites in Georgia.

To further assess the relative magnitude of differentiation between sympatric forms and among regions, we partitioned the nuclear and mtDNA variation by estimating F_{ST} simultaneously at both levels by using the analysis of molecular variance approach (Excoffier et al. 1992). For the combined nuclear markers, the between-form variance component is slightly negative (though insignificant), whereas the among-region variance is small but significantly positive (Fig. 3). Qualitatively similar results are obtained when the

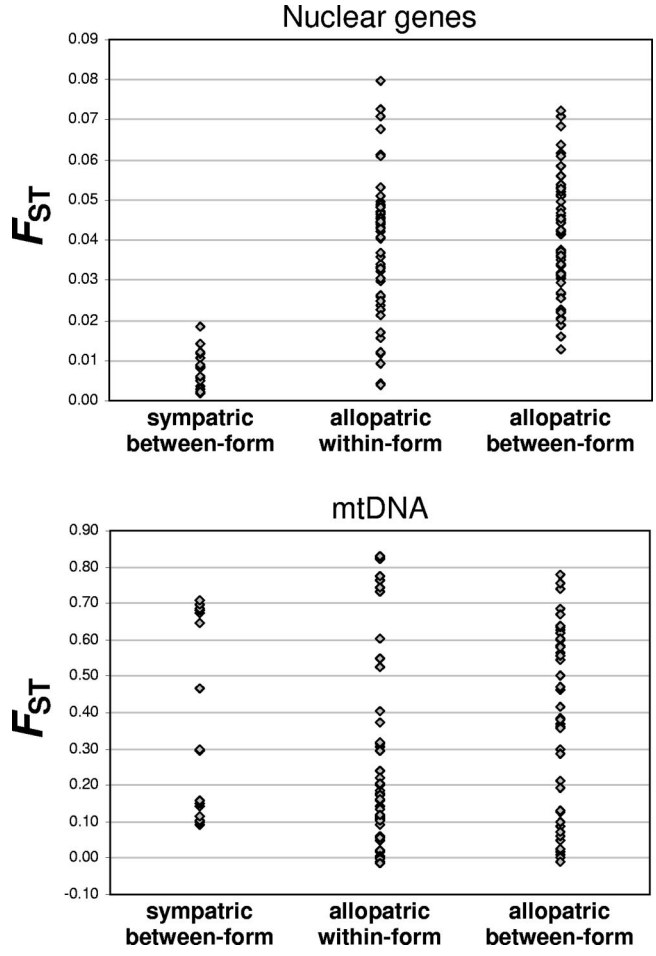

Fig. 2. Distributions of F_{ST} values between populations of *S. invicta* in the southern United States calculated using data from 16 nuclear genes and the mtDNA. Values are divided into three categories: values for sympatric populations of the alternate social forms (sympatric between-form), values for allopatric populations of the same form (allopatric within-form), and values for allopatric populations of the alternate forms (allopatric between-form).

allozymes and microsatellites are analyzed separately, although differentiation at both levels is insignificant for the former class but significant for the latter class of markers. An entirely different pattern again is observed for the mtDNA. In this case, the among-region variance is slightly negative (though insignificant) and the between-form variance is large and highly significant (Fig. 3). These results reinforce the conclusions from the previous analyses that variation at the two genomes is partitioned very differently over our different scales of sampling.

Analyses of isolation by distance using the combined nuclear markers indicate that genetic divergence tends to increase with geographical distance between populations. This result was obtained regardless of the measures of geographic and genetic distance employed and was stable to exclusion of the zero-distance class (sympatric social forms) from the analyses (all $P \leq 0.012$). However, no significant relationships were found when the sympatric social forms were pooled or when the forms were considered in separate analyses (all $P > 0.28$), perhaps because of the low power associated with the small sample sizes in these cases ($n = 6$). No analyses employing the mtDNA haplotype frequencies showed a signal of isolation by distance, regardless of whether the populations were considered simultaneously or separated by social form (all $P > 0.14$). We conclude that there is a weak signal of isolation by distance from the nuclear markers but no such signal from the mtDNA.

Results of the NMDS analyses using Nei's genetic distances are plotted in Fig. 4. For the combined nuclear genes, five dimensions were found to be optimal, although the first three dimensions (plotted in Fig. 4) explain >70% of the data variance. Several distinct clusters of study populations are apparent, four of which separate clearly on the first two dimensions (which together explain 60% of the variance). These individual clusters comprise all Georgia sites; both sites from Florida; both sites from western Louisiana; and a fourth, more diffuse cluster comprising the sites from Texas, eastern Louisiana, and Mississippi. Some

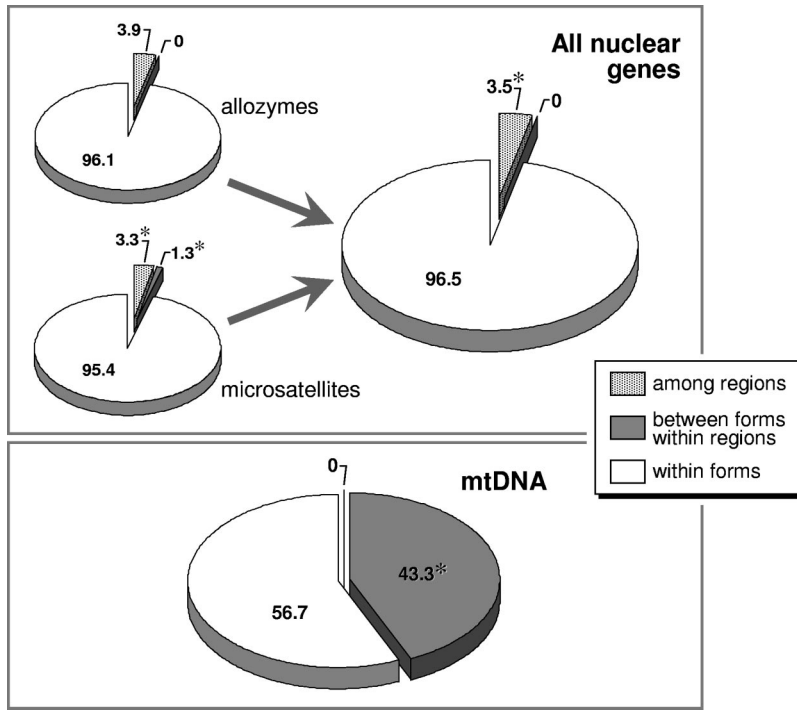

Fig. 3. Partitioning of genetic variance in *S. invicta* in the southern United States at 16 nuclear genes and the mtDNA estimated using analysis of molecular variance. This hierarchical approach partitioned the total variance among the six regions, between the two social forms within each region, and within the social forms in each region. The percentage of the total variance partitioned at each level is shown, with negative estimates indicated as zero. Asterisks indicate statistically significant differentiation at a given level.

separation is evident even in this fourth cluster, with the Texas populations evidently distinct from the others along the second and third dimensions. With the possible exceptions of eastern Louisiana and Mississippi, sympatric social forms clearly cluster together, as expected if geography rather than social organization is the primary determinant of nuclear genetic affinities. There is little evidence from the NMDS analyses of regional populations grouping together on the basis of geographic proximity, again with the exception of the eastern Louisiana and Mississippi populations. Thus, for instance, the two easternmost populations, Georgia and Florida, are relatively divergent from one another, whereas Texas and Mississippi, which are geographically distant, are relatively similar at the nuclear markers.

For the mtDNA, three dimensions were found to be optimal in the NMDS analyses, together explaining >91% of the variance. In contrast to the results from the nuclear data, well defined clusters of populations are not particularly conspicuous in the mtDNA data (Fig. 4). One exception is the three Georgia M sites, the clustering of which accords with the results from the exact tests (see above) and stands in contrast to the dispersion of the Georgia P sites. Significantly, sympatric populations differing in social form tend not to cluster together with regard to their haplotype composition. Rather, populations tend to cluster by social form along the first dimension, a dimension that explains almost three-fourths of the total variance (see Fig. 4, inset; segregation by form along the first dimension is significant based on a Kruskal–Wallis test and using single examplars for each form in Georgia [$P = 0.01$]). For both genomes, very similar results were obtained from the NMDS analysis when F_{ST} values or Cavalli-Sforza and Edwards' chord distances were used to assess pairwise population differentiation.

Simulations using the Bayesian clustering technique on the nuclear data were run separately for each region and without prior information on colony social form to infer whether sympatric social forms represent distinct genetic clusters. The results of these analyses uniformly failed to provide evidence of such differentiation. In five of the six regions, the simulations gave higher posterior probabilities, as calculated from Bayes' Rule, for single genetic clusters ($\Pr[1] \approx 1.0$) than for two clusters ($\Pr[2] \approx 0$). In western Louisiana, the posterior probability was higher for two clusters ($\Pr[2] > 0.999$) than for one cluster ($\Pr[1] < 0.001$), but, surprisingly, the two inferred clusters do not correspond to the different forms (average membership assigned to the first inferred cluster was 0.51 for the M form and 0.65 for the P form). This discovery of two distinct nuclear clusters in western Louisiana prompted us to rerun the NMDS analyses for the entire set of samples but to use the genetic distances between the two inferred clusters (calculated from the allele frequencies output by STRUCTURE) rather

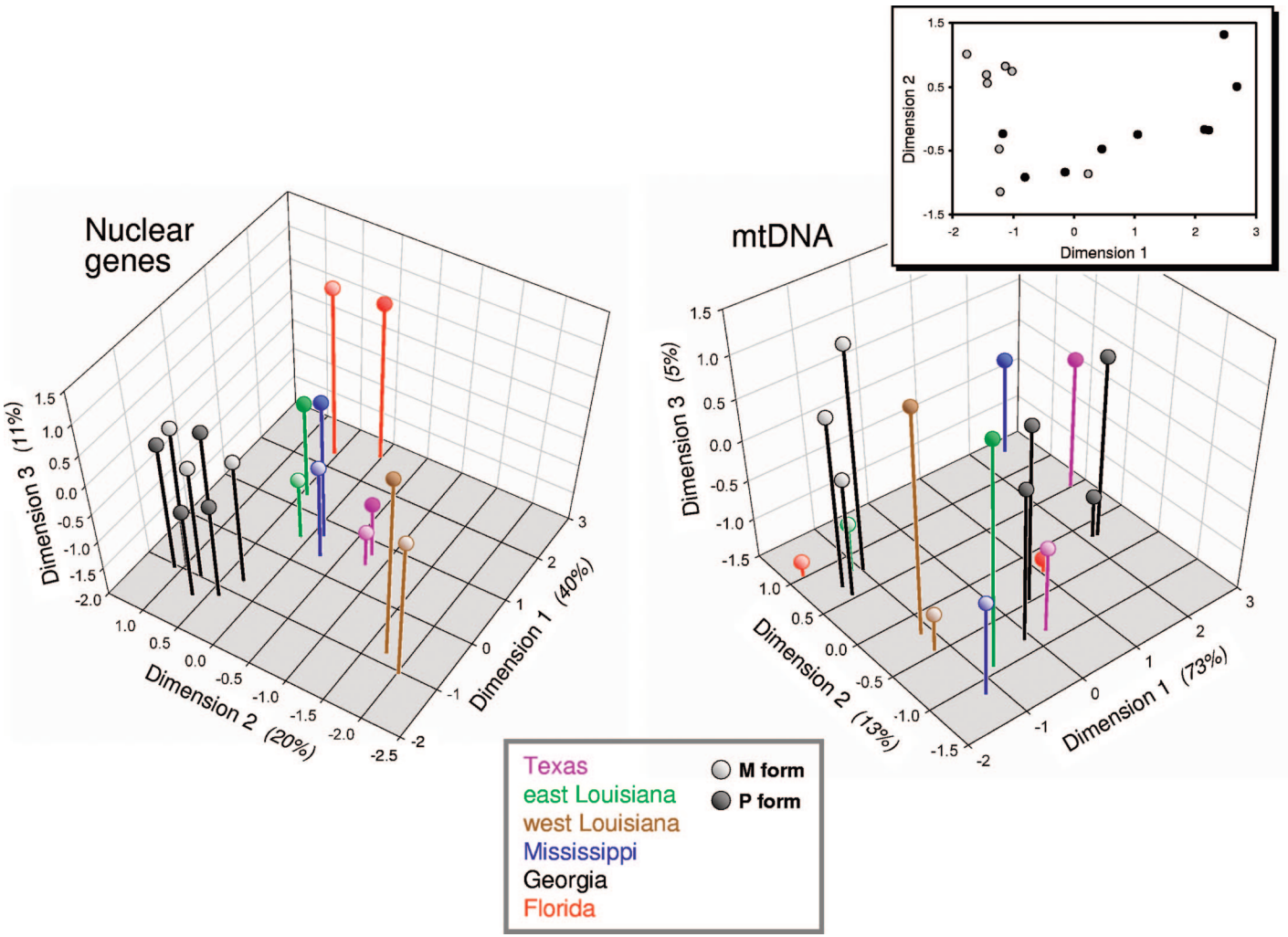

Fig. 4. Distributions of points in the three primary dimensions resulting from NMDS analyses based on Nei's genetic distance between pairs of *S. invicta* populations in the southern United States. Analyses were conducted separately for 16 nuclear genes and for the mtDNA. The percentage of the variance in the original genetic distance data explained by each dimension is shown on the appropriate axis. Inset, distributions of points for the mtDNA in the first two dimensions. Dark points represent P populations, and light points represent M populations.

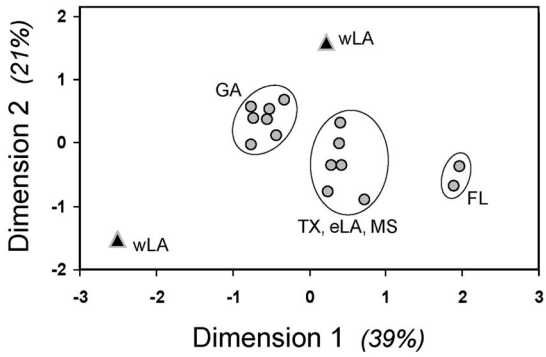

Fig. 5. Distributions of points in the two primary dimensions resulting from NMDS analyses based on Nei's genetic distances between *S. invicta* populations in the southern United States. Distances were based on data from 16 nuclear genes. Gray circles represent the different social forms (or sample sites in Georgia) within all regions except western Louisiana. Dark triangles represent the two clusters inferred by STRUCTURE in western Louisiana. The percentage of the variance in the original genetic distance data explained by each dimension is shown on the appropriate axis. See Fig. 1 caption for abbreviations.

than the genetic distances between the two social forms to define subpopulations in western Louisiana. The results were rather similar to those shown in Fig. 4, except that the two western Louisiana clusters were strongly divergent from each other. One was strongly divergent from all other populations, whereas the second grouped with the Texas, eastern Louisiana, and Mississippi populations along the first dimension (which explains 39% of the variance; Fig. 5).

Bayesian simulations run using the nuclear and mtDNA data combined, but without prior information on colony locality or social form, gave posterior probabilities that strongly support the existence of four distinct genetic clusters for our samples (based on calculation of the ΔK statistic of Evanno et al., 2005). Essentially identical results were obtained when data from just the 16 nuclear genes were used. Membership coefficients for each individual in the six regions based on all of the genetic information, as well as the average membership coefficient in each region, are shown in Fig. 6. (Sympatric populations were pooled because of the lack of evidence of consistent nuclear genetic differentiation between sympatric social forms.) The four clusters hypothesized by the Bayesian method correspond roughly to what may be called a "Texas cluster," a "western Louisiana cluster," a "Georgia cluster," and a "Florida cluster," based on average membership coefficients >0.5 for one of the four posterior clusters in each of these regions. The eastern Louisiana and Mississippi populations average <0.4 estimated membership in any single hypothetical cluster, suggesting that these are the least genetically distinctive (most strongly admixed) of the six regional samples. In general, each regional population bears evidence of considerable shared ancestry with several of the hypothetical genetic clusters detected by the Bayesian technique. Western Louisiana stands out in this regard as containing the genetically most unique ants (average membership coefficient >0.73).

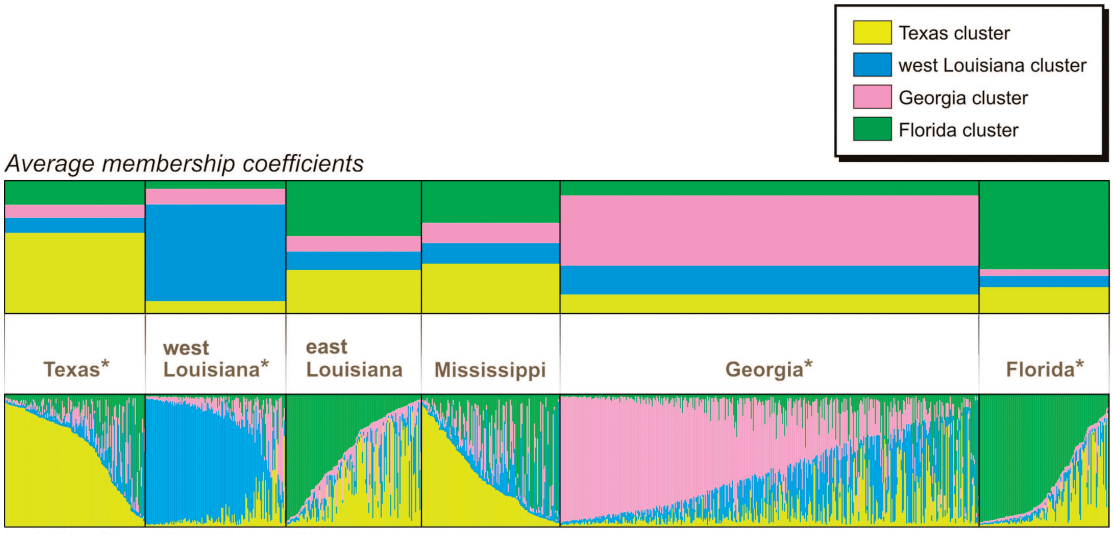

Fig. 6. Membership coefficients from Bayesian cluster analyses for six predefined regional populations of *S. invicta* in the southern United States based on combined genetic data from 16 nuclear genes and the mtDNA. The different colors represent the four distinct clusters hypothesized by this method without prior information on sample locality or social form. For the individual membership coefficients, each individual is represented by a vertical line divided into parts proportional to the individual's proposed ancestry in each cluster. Asterisks signify regional populations with average membership coefficients >0.5 in a given hypothetical cluster.

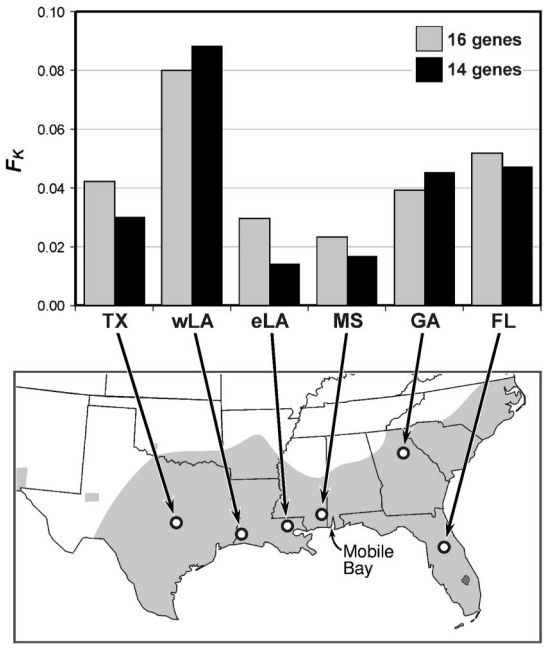

Fig. 7. Estimates of F_K for introduced *S. invicta* from six regions in the southern United States obtained from Bayesian cluster analyses conducted on data from 14 or 16 nuclear genes. The 14-gene analysis excluded the microsatellite loci *Sol-20* and *Sol-42* (see text for details). Regional collection localities are shown on the range map of introduced *S. invicta*.

Bayesian simulations incorporating prior information on colony locality and social form showed that most individuals could be assigned specifically to their appropriate sample population with very high probability, regardless of whether the data from both genomes or just the nuclear data were used (data not shown). Similarly high overall probabilities of assignment resulted when the data from the two social forms in each region were pooled. These results suggest that our multi-locus data contain unique genetic signatures for each sampled population that, when coupled with prior geographic and biological information, allow most individuals to be assigned with high probability to their population of origin. However, a substantial proportion of individuals in each population were inferred to have some admixed ancestry, which for some individuals approached or even exceeded 0.5.

Finally, estimates of F_K for each of the six regions, obtained from the Bayesian analyses conducted on the nuclear data, are presented in Fig. 7. The two lowest estimates are for the two populations closest to Mobile (Mississippi and eastern Louisiana), with the lowest value for all 16 genes obtained for the Mississippi locality. Thus, the ants closest to the point of initial establishment in the United States seem to have diverged the least from the original founder population. Somewhat unexpectedly, however, the most divergent samples come not from the most peripheral (and presumably most recently colonized) regions but from western Louisiana, a pattern also reflected in the NMDS and Bayesian cluster analyses (Figs. 4–6).

Discussion

Patterns of Population Genetic Structure in Invasive *S. invicta*. We surveyed genetic variation in *S. invicta* from six widely separated locations in the United States by using several classes of markers to infer patterns of structure in both social forms at differing scales across the introduced range. Although previous studies have analyzed genetic structure in *S. invicta* in the United States (Ross and Fletcher 1985b; Ross et al. 1987, 1996, 1999; Ross and Shoemaker 1993, 1997; Shoemaker and Ross 1996; Chen et al. 2003), none has examined it at multiple scales in both social forms over such a broad geographic area. The general patterns revealed by our analyses are 1) significant mtDNA differentiation among local sites within the P but not the M social form, with no local nuclear differentiation within either form; 2) weak nuclear but pronounced mtDNA differentiation between sympatric populations of the two social forms (interform nuclear differentiation evident only in the geographically peripheral populations); 3) significant nuclear but no mtDNA regional differentiation (apparent mtDNA differentiation at this level is attributable to differentiation between sympatric forms); and 4) a weak correlation between nuclear divergence and geographic distance between populations (i.e., pattern of isolation by distance) with no such association for the mtDNA.

These patterns reveal that variation at our scales of sampling is partitioned very differently at the nuclear and mtDNA genomes. In general, the different patterns at the most localized scales can be understood as arising from the unique social and breeding biology of each social form, coupled with the different transmission dynamics of the two genomes. Considering first the patterns of local structure within each form, the demonstration that significant mtDNA differentiation occurs at this level in the P form but not the M form in Georgia, and that nuclear differentiation is absent in both forms, follows from the known dispersal and mating habits of the ants. Patterns of dispersal contrast sharply between queens of the two forms, with M queens traveling relatively long distances from their natal nest during nuptial flights (Markin et al. 1971, DeHeer et al. 1999), whereas P queens travel shorter distances or not at all for mating (Porter 1991, Goodisman and Ross 1998, DeHeer et al. 1999, Goodisman et al. 2000). Moreover, nests of the P form propagate by budding (Vargo and Porter 1989), a process in which groups of workers and queens from an existing nest travel by foot to establish a new nest. The resulting restriction on long-distance gene flow via queens within the P form leads to patterns of pronounced local structure of the maternally inherited mtDNA in this social form that is not, however, registered at the nuclear genome (Shoemaker and Ross 1996, Goodisman and Ross 1998, Goodisman and Ross 1999). At least part of the reason is that most P queens seem to mate with widely dispersing M males (see below), a behavior expected to act as a homogenizing force on nuclear variation among local P sites.

Considering next the pronounced mtDNA but weak nuclear differentiation between the forms in sympatry, several studies have shown that young queens of each social form seldom, if ever, succeed in becoming reproductives in colonies of the alternate form (Shoemaker and Ross 1996, Ross and Shoemaker 1997, Keller and Ross 1998, DeHeer et al. 1999, Goodisman et al. 2000, Ross and Keller 2002). This constraint seems to result from behavioral decisions made by both queens and workers. Specifically, queens of the M form attempt to found colonies independently after mating, whereas queens of the P form generally attempt to gain entry into existing polygyne colonies to initiate reproduction (Glancey and Lofgren 1988, Goodisman and Ross 1998, DeHeer et al. 1999, Goodisman et al. 2000). Moreover, workers selectively eliminate queens of the alternate social form that attempt to enter a colony (Keller and Ross 1998, Vander Meer and Porter 2001, Krieger and Ross 2002, Ross and Keller 2002). Despite some potential mechanisms for female-mediated interform gene flow (Ross and Shoemaker 1997, DeHeer 2002), our data suggest such gene flow is sufficiently rare that any initial differences in mtDNA haplotype frequencies between the forms where they co-occur are preserved or enhanced through drift over time.

The contrasting weak or nonexistent interform divergence at nuclear genes is consistent with earlier studies suggesting that queens of the P form mate commonly, or even predominantly, with males of the M form where the two forms co-occur (Ross and Shoemaker 1993, Ross and Keller 1995a, Shoemaker and Ross 1996). (P colonies in the introduced range produce few fertile males, so that P queens often must rely largely on immigrant M males for mating opportunities; Ross and Fletcher 1985a, Ross and Shoemaker 1993.) Such interform matings provide a conduit for nuclear but not mtDNA gene flow between the forms where they occur in sympatry and so act as a homogenizing force only for nuclear markers. Our present results extend this previously documented pattern of strong mtDNA differentiation coupled with minimal nuclear differentiation between the forms to multiple sites of sympatry across the introduced range in the United States (also see Shoemaker and Ross 1996, Ross and Shoemaker 1997, Ross et al. 1999). Confirmation of the robustness of this pattern lends further credence to the idea that differences in social behavior within ant species can act as strong constraints on particular routes of gene flow and drive population genetic divergence (West-Eberhard 1986, Ross and Keller 1995b, Shoemaker and Ross 1996).

The two remaining general patterns of structure we observed at larger geographic scales can be interpreted in light of the invasion dynamics of the two social forms in the United States and differences in the genetic properties of the different classes of markers used. We found consistent, statistically significant nuclear differentiation among regional populations, with ≈4% of the total nuclear variance distributed among regions (Fig. 3; also see Ross et al. 1987, 1999). Moreover, the extent of this differentiation scales weakly with geographic distance, i.e., there is some indication of isolation by distance. Nonetheless, the NMDS analyses show that the regional populations tend not to cluster appreciably according to geography. Much of the initial expansion of *S. invicta* involved isolated introductions into previously uninhabited territory via human-mediated long-distance dispersal (Lofgren 1986a). The expected result of such events is the appearance of regional fire ant populations with relatively unique genetic compositions that may or may not be most similar to geographically adjacent populations (depending on the source of the founders). However, at least occasional anthropogenic dispersal between adjacent regions, perhaps coupled with resumption of extensive gene flow once subfounder populations regain contact with each other or the main population, may explain the observed weak signal of isolation by distance. An important point in this context is that dispersal occurring since the period of principal range expansion seems to have been primarily natural rather than anthropogenic (Lofgren 1986a); that natural dispersal occurs over distances orders of magnitude shorter than the distances between our study populations (Markin et al. 1971) means that the observed higher level differentiation established during range expansion may persist for some time.

Our pairwise population F_{ST} estimates suggest pronounced mtDNA differentiation among regional sites, but such differentiation is no greater than the differentiation between sympatric social forms (Fig. 2). Indeed, the analysis of molecular variance, which partitions the total variance hierarchically at multiple levels, identified no residual among-region structure once between-form structure was accounted for (Fig. 3). The overwhelming interform differentiation at the mtDNA presumably has two causes, the lack of queen-mediated gene flow between the forms in sympatry (discussed above) and the derivation of subfounder populations of each form primarily from other populations of the same form. This latter trend, evidence for which is the tendency of populations to cluster by social form in the NMDS analyses of the mtDNA (Fig. 4, inset), is expected to pertain especially to P populations, because a particular allele required for the expression of polygyny (the *b* allele of the gene *Gp-9*) is not present in M populations (Ross 1997, Krieger and Ross 2002). (The converse is not true because the P form is polymorphic for both the *b* allele and the *Gp-9* allele fixed in the M form, allele *B;* therefore, M populations conceivably could arise from polygyne founders.)

The absence of any clustering of regional populations or signal of isolation by distance for the mtDNA data, which seems to conflict with the nuclear data to some extent, may result from the failure of the mtDNA to register patterns of colonization and gene flow detectable at larger scales with the numerous nuclear markers. This failure may stem from the reduced effective population size of the mtDNA compared with the nuclear genome and its correspondingly greater susceptibility to stochastic frequency changes after

subfounding events, or from the mtDNA having been surveyed at only a single region with a technique that resolved only limited polymorphism. Alternatively, gene flow reestablished after regional subfounder populations regain contact with other populations may be more effectively mediated by males than females, a plausible idea given that male fire ants are smaller, and hence potentially more easily dispersed on wind currents, than queens. In this latter scenario, actual patterns of gene flow differ between the two genomes at the larger geographic scales.

Further Population Genetic Insight into the Invasion History of *S. invicta*. The invasion and subsequent spread of *S. invicta* throughout the southern United States is expected to have generated distinctive genetic footprints that reflect the colonization process. For example, a simple model of range expansion invoking a single introduction followed by repeated subfounder events occurring in a stepping-stone–like process would be supported if the more peripheral localities in the introduced range harbor reduced genetic variation compared with areas nearer the site of introduction at Mobile, AL (Ramachandran et al. 2005). We did not detect a pattern of high diversity around Mobile and reduced diversity in the most peripheral populations (those in Texas, Georgia, and Florida), suggesting that some feature of this simple model is incorrect. Frequent anthropogenic transport of ants over long distances clearly is at odds with the assumption of a stepping-stone–like process. Moreover, subfounding of peripheral populations by natural dispersal may often have involved relatively large numbers of individuals, in which case significant reductions in diversity would not be expected even in the absence of anthropogenic dispersal.

However, genetic footprints of the colonization process are evident from the Bayesian analyses of the nuclear data, which revealed that the study populations located nearest Mobile resemble the original founder population more closely than populations more distant from the point of entry. Specifically, the estimated F_K values generally approximate the U-shaped distribution expected under a general model of outward colonization from Mobile via sequential subfounder events, with the conspicuous exception of the western Louisiana population (Fig. 7). Indeed, the eastern Louisiana and Mississippi populations, located nearest Mobile, seem to have diverged least from the hypothetical founder population based on the F_K estimates. Moreover, these two populations are the least genetically distinctive of the sampled populations according to both the NMDS and Bayesian cluster analyses of the nuclear genes, a further indication that they most closely resemble the original founders. Thus, regional population differentiation may have developed largely according to the pattern predicted by range expansion outward from Mobile, with this expansion involving repeated reductions in effective population size during subfounding that significantly altered allele frequencies but not overall diversity in peripheral populations.

The pronounced mtDNA differentiation evident between paired sympatric populations of the two social forms of *S. invicta* is a hallmark of both introduced and native populations (Shoemaker and Ross 1996; Ross 1997; Ross et al. 1997, 1999; Ross and Shoemaker 1997) and is expected on the basis of the inability of queens to become reproductives in colonies of the alternate form, as discussed above. Significant nuclear differentiation between such paired populations may be expected as well if interform matings are not too common, and, indeed, such differentiation has been reported previously in the native range (Ross et al. 1997) and the United States in some instances (e.g., Ross et al. 1999). An important finding of the current study is that only the geographically peripheral populations (those most distant from Mobile) exhibited significant interform nuclear differentiation. This result can be explained if these represent more recently established populations in which interform gene flow mediated by P queens mating with M males has not occurred over as long a period as in the earlier established Mississippi and eastern Louisiana populations.

Comprehensive molecular and population genetic analyses implicate a single gene of major effect in the expression of polygyny in *S. invicta* (for review, see Krieger 2005). Specifically, the *b* allele of the gene *Gp-9* must be present in a colony for it to exhibit polygyne social organization in the introduced range. (A related allele that also induces polygyny in the native range is not found in the United States.) Earlier results showing that co-occurring populations of the alternate forms are highly similar at their nuclear genomes were taken as evidence that local P populations arise from preexisting M populations under specific ecological conditions, such as habitat saturation (Ross et al. 1987, Ross and Keller 1995b), but such similarity has been interpreted more recently as resulting from ongoing male-mediated gene flow between the social forms (Ross and Shoemaker 1993, Ross and Keller 1995a, Shoemaker and Ross 1996). Our mtDNA data provide additional compelling evidence that new P populations cannot arise "spontaneously" from the M form under environmental induction, but instead can only be founded by queens produced in preexisting P populations that bear the appropriate variation at *Gp-9* (the *b* allele). This mtDNA evidence is the strong differentiation that exists between co-occurring forms as well as the overall similarity of all P populations throughout the introduced range. The latter data represent an important population genetic footprint of the spread of the P form throughout the introduced range by means of the dispersal of queens (by natural and/or anthropogenic means) from previously established invasive populations of this same form.

Possible Secondary Introduction(s) of *S. invicta* into the United States. The requirement for P populations to originate from other P populations raises the issue of how this social form came to exist in the United States. One potential explanation is that the original founders included or consisted solely of ants of the P form. Even if the original founders comprised exclusively ants of this form, M colonies could quickly arise,

because the *B* allele of *Gp-9* that is fixed in the M form segregates along with the *b* allele in the P form. The M form presumably had a reproductive advantage over the P form during initial colonization because of its superior dispersal abilities (Ross and Keller 1995b), which could account for the obvious dominance of this form during the first few decades after the introduction. A problem with this explanation is that polygyny was not observed during a period of almost 40 yr after the initial appearance of the ant (Glancey et al. 1973, 1975; Hung et al. 1974; Fletcher et al. 1980), despite the considerable number of studies conducted over that period and the readily detectable differences between colonies of the two forms.

An alternative explanation is that the P form was introduced secondarily at some point after the initial arrival of *S. invicta* in Mobile. The occurrence of such multiple introductions is not as unlikely as may first seem, given that another South American fire ant species was introduced into the United States before *S. invicta* (the closely related *Solenopsis richteri* Forel in 1918, also at Mobile; Lofgren et al., 1975) and that both *S. invicta* and the fire ant *Solenopsis geminata* (F.) have been introduced to many places throughout the world (Trager 1991). If the original inoculum of *S. invicta* at Mobile consisted solely of M ants, as the lack of discovery of polygyny for 40 yr might suggest, then a secondary introduction is required to explain the later appearance of polygyny. The reason, again, is that the *b* allele of *Gp-9* required for expression of polygyny is absent from the M form (Krieger 2005). If a secondary introduction involving the polygyne form occurred, any nuclear genetic signature of it may have been largely eroded by the considerable ongoing gene flow from the M to the P form through males. However, the rather substantial difference in overall mtDNA haplotype composition between the two forms (Fig. 4) is perhaps best understood as reflecting differences in the original haplotype composition of colonizers of the two forms rather than in situ divergence between the forms during the brief period they have inhabited the United States.

Finally, the unique nuclear genetic composition of the western Louisiana population hints at another possible secondary introduction of *S. invicta* into the United States. Evidence for this comes from the NMDS and Bayesian clustering analyses (Figs. 4–6), which reveal that western Louisiana contains the genetically most distinctive fire ants we sampled. Indeed, the F_K values estimated for western Louisiana by the Bayesian method depart strongly from the general U-shaped distribution expected if populations increasingly distant from Mobile resemble the founder population to a lesser degree; that is, western Louisiana ants are surprisingly divergent from this inferred ancestral population given their location. Moreover, the western Louisiana samples were unique among all our samples in that the Bayesian clustering analyses gave a higher posterior probability for two nuclear genetic clusters than for a single cluster at this site (these two clusters do not correspond to the social forms). A secondary introduction of *S. invicta* near this site, such that individuals there now represent two admixed populations, might be expected to yield such a result. Finally, this sampling site is located close (30 km) to Port Arthur, TX, a major deepwater port of entry likely to be particularly susceptible to a fire ant introduction.

In conclusion, the patterns of genetic variation we detected, combined with historical and other observational data, suggest the following general narrative of range expansion and contemporary gene flow for *S. invicta* in the United States. Early colonization from the initial site of establishment (Mobile Bay) often involved long-distance, human-mediated dispersal. Subfounder populations of each social form typically were established by queens of the same form, perhaps often derived from very distant localities, and the ensuing strong mtDNA differentiation between the forms both in allopatry and sympatry persists because of the failure of queens to commonly serve as conduits of interform gene flow. Males apparently are effective agents of nuclear gene flow between the forms where they co-occur, which has allowed the homogenization of interform nuclear variation at localities where the forms have been in contact for the longest period (localities nearest Mobile). Range expansion from Mobile generally occurred by means of sequential outward subfounder events, with the result that more peripheral populations are the most genetically distinctive and least resemble the hypothesized original founder population. Secondary introductions of *S. invicta* also may have occurred, the best evidence for which is the genetic distinctiveness and signal of admixture between two discrete populations discerned for ants sampled near the deepwater port of Port Arthur, TX. Whether the inferred introduction in this area corresponds to the presumed secondary introduction of the polygyne form into the United States or represents yet another introduction is presently unclear.

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Appendix 1. Allele and haplotype frequencies for 16 nuclear loci and the mtDNA in *S. invicta* sampled from 17 sites in the United States

	Texas		Western Louisiana		Eastern Louisiana		Mississippi		Georgia							Florida	
									M[a]			P[a]					
	M	P	M	P	M	P	M	P	1	2	3	1	2	3	4	M	P
Allozyme loci																	
Aat-2	**72**	**63**	**75**	**60**	**70**	**42**	**69**	**61**	**42**	**40**	**70**	**65**	**63**	**65**	**55**	**65**	**59**
100	0.965	0.944	0.960	0.942	0.957	0.905	0.935	0.959	0.929	0.863	0.900	0.962	0.960	0.915	0.918	0.838	0.881
144	0.035	0.056	0.040	0.058	0.043	0.095	0.065	0.041	0.071	0.138	0.100	0.038	0.040	0.085	0.082	0.162	0.119
Acoh-1	**70**	**61**	**71**	**60**	**68**	**39**	**65**	**62**	**42**	**40**	**70**	**65**	**63**	**65**	**55**	**64**	**60**
82	0.093	0.033	0.225	0.100	0.096	0.051	0.100	0.032	0.214	0.112	0.207	0.108	0.111	0.115	0.127	0.109	0.050
100	0.907	0.967	0.775	0.900	0.904	0.949	0.900	0.968	0.786	0.887	0.793	0.892	0.889	0.885	0.873	0.891	0.950
Acoh-5	**71**	**63**	**73**	**60**	**65**	**40**	**69**	**61**	**42**	**40**	**70**	**65**	**63**	**65**	**54**	**62**	**58**
93	0.232	0.230	0.288	0.192	0.323	0.275	0.246	0.295	0.286	0.262	0.286	0.246	0.278	0.192	0.306	0.444	0.578
100	0.768	0.770	0.712	0.808	0.677	0.725	0.754	0.705	0.714	0.738	0.714	0.754	0.722	0.808	0.694	0.556	0.422
Acy1	**70**	**62**	**74**	**59**	**65**	**43**	**68**	**63**	**39**	**34**	**60**	**46**	**63**	**65**	**55**	**63**	**58**
93	0.079	0.073	0.155	0.119	0.085	0.058	0.081	0.063	0.128	0.132	0.150	0.185	0.143	0.115	0.182	0.056	0.129
100	0.921	0.927	0.845	0.881	0.915	0.942	0.919	0.937	0.872	0.868	0.850	0.815	0.857	0.885	0.818	0.944	0.871
Ddh-1	**71**	**19**	**70**	**58**	**59**	**31**	**69**	**20**									**16**
88	0.261	0.368	0.293	0.267	0.280	0.355	0.319	0.300	—	—	—	—	—	—	—	—	0.438
100	0.739	0.632	0.707	0.733	0.720	0.645	0.681	0.700	—	—	—	—	—	—	—	—	0.562
Est-4	**72**	**59**	**74**	**59**	**67**	**47**	**69**	**62**	**41**	**40**	**70**	**65**	**64**	**63**	**54**	**63**	**60**
100	0.465	0.542	0.642	0.602	0.634	0.617	0.529	0.556	0.659	0.613	0.586	0.662	0.633	0.627	0.537	0.381	0.425
160	0.535	0.458	0.358	0.398	0.366	0.383	0.471	0.444	0.341	0.387	0.414	0.338	0.367	0.373	0.463	0.619	0.575
G3pdh-1	**72**	**58**	**74**	**60**	**68**	**47**	**68**	**62**	**41**	**40**	**70**	**65**	**65**	**64**	**53**	**64**	**58**
40	0.444	0.405	0.432	0.425	0.294	0.372	0.397	0.363	0.232	0.375	0.307	0.262	0.346	0.273	0.292	0.273	0.414
100	0.556	0.595	0.568	0.575	0.706	0.628	0.603	0.637	0.768	0.625	0.693	0.738	0.654	0.727	0.708	0.727	0.586
Pgm-1	**71**	**63**	**74**	**60**	**67**	**47**	**67**	**63**	**41**	**40**	**70**	**65**	**65**	**65**	**55**	**65**	**60**
96	0.134	0.063	0.034	0.050	0.097	0.128	0.142	0.159	0.195	0.237	0.179	0.138	0.138	0.215	0.200	0.038	0.025
100	0.866	0.937	0.966	0.950	0.903	0.872	0.858	0.841	0.793	0.762	0.814	0.862	0.862	0.785	0.800	0.962	0.975
102	0	0	0	0	0	0	0	0	0.012	0	0.007	0	0	0	0	0	0
Pgm-3[b]	**72**		**74**		**67**		**67**		**41**	**40**	**70**					**65**	
89	0.278	—	0.432	—	0.239	—	0.194	—	0.207	0.275	0.257	—	—	—	—	0.238	—
100	0.722	—	0.568	—	0.761	—	0.806	—	0.793	0.725	0.743	—	—	—	—	0.762	—
Microsatellite loci																	
Sol-6	**68**	**63**	**74**	**58**	**69**	**60**	**63**	**63**	**38**	**39**	**69**	**65**	**64**	**65**	**54**	**62**	**59**
95	0	0	0	0	0	0	0	0	0.013	0	0	0	0	0	0	0	0
109	0.147	0.190	0.027	0.017	0.152	0.133	0.127	0.111	0.079	0.154	0.145	0.131	0.086	0.100	0.120	0.024	0.042
111	0	0	0	0	0	0	0	0	0	0	0	0	0.016	0	0	0	0
113	0.676	0.619	0.628	0.759	0.623	0.658	0.738	0.675	0.671	0.679	0.652	0.700	0.703	0.692	0.731	0.847	0.831
115	0.176	0.190	0.345	0.224	0.217	0.208	0.119	0.183	0.237	0.167	0.203	0.169	0.195	0.208	0.148	0.121	0.127
119	0	0	0	0	0.007	0	0.016	0.032	0	0	0	0	0	0	0	0.008	0
Sol-11	**68**	**63**	**73**	**59**	**69**	**60**	**62**	**63**	**39**	**39**	**69**	**65**	**65**	**65**	**54**	**62**	**59**
123	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.009	0	0
143	0.169	0.230	0.027	0.068	0.210	0.233	0.210	0.183	0.372	0.244	0.413	0.438	0.423	0.462	0.426	0.194	0.263
145	0.081	0.127	0	0	0.138	0.108	0.040	0.103	0.115	0.154	0.051	0.023	0.062	0.069	0.074	0.032	0.127
147	0.110	0.063	0	0.144	0.152	0.175	0.169	0.151	0.103	0.154	0.101	0.108	0.131	0.138	0.093	0.290	0.119
149	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.009	0	0
151	0.441	0.397	0.623	0.661	0.391	0.358	0.427	0.405	0.167	0.269	0.254	0.238	0.185	0.138	0.176	0.427	0.390

(continued)

Appendix 1. Continued

	Texas		Western Louisiana		Eastern Louisiana		Mississippi		Georgia							Florida	
									M[a]			P[a]					
	M	P	M	P	M	P	M	P	1	2	3	1	2	3	4	M	P
Microsatellite loci																	
155	0.199	0.183	0.342	0.127	0.109	0.125	0.153	0.159	0.244	0.179	0.181	0.192	0.200	0.192	0.213	0.056	0.102
171	0	0	0.007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sol-18	**68**	**63**	**74**	**59**	**69**	**60**	**62**	**63**	**39**	**39**	**69**	**65**	**65**	**65**	**54**	**62**	**59**
125	0.691	0.675	0.919	0.924	0.746	0.700	0.766	0.786	0.833	0.833	0.833	0.854	0.869	0.838	0.815	0.750	0.839
127	0.309	0.325	0.081	0.076	0.254	0.300	0.185	0.151	0.167	0.154	0.167	0.146	0.131	0.162	0.185	0.242	0.127
129	0	0	0	0	0	0	0.048	0.063	0	0.013	0	0	0	0	0	0.008	0.034
Sol-20	**65**	**62**	**74**	**59**	**69**	**60**	**63**	**63**	**40**	**39**	**69**	**65**	**65**	**65**	**54**	**57**	**57**
114	0	0	0	0	0	0	0	0	0	0	0	0	0.008	0	0	0	0
122	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.009	0
124	0.262	0.177	0.115	0.212	0.217	0.067	0.246	0.183	0.237	0.141	0.094	0.115	0.169	0.138	0.093	0.237	0.167
126	0.485	0.435	0.345	0.322	0.420	0.683	0.595	0.500	0.400	0.462	0.493	0.377	0.408	0.462	0.426	0.579	0.614
128	0.254	0.387	0.264	0.085	0.362	0.250	0.159	0.317	0.225	0.077	0.167	0.200	0.177	0.100	0.222	0.175	0.219
130	0	0	0	0.008	0	0	0	0	0	0.026	0	0.023	0	0	0	0	0
132	0	0	0	0	0	0	0	0	0	0	0	0	0	0.008	0	0	0
136	0	0	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
144	0	0	0.264	0.347	0	0	0	0	0.025	0.115	0.094	0.100	0.100	0.177	0.176	0	0
146	0	0	0	0	0	0	0	0	0	0	0.007	0	0	0	0	0	0
150	0	0	0	0	0	0	0	0	0.038	0	0	0.008	0.031	0	0	0	0
152	0	0	0	0.025	0	0	0	0	0.075	0.179	0.145	0.177	0.108	0.115	0.083	0	0
Sol-42	**68**	**63**	**74**	**59**	**69**	**60**	**62**	**63**	**39**	**39**	**69**	**65**	**65**	**65**	**54**	**61**	**59**
107	0	0	0	0	0.007	0	0	0	0	0	0	0	0	0	0	0	0
117	0.250	0.183	0.297	0.466	0.188	0.142	0.323	0.294	0.397	0.346	0.457	0.362	0.338	0.354	0.306	0.172	0.220
119	0.132	0.254	0.169	0.220	0.094	0.075	0.113	0.103	0.205	0.244	0.181	0.169	0.177	0.215	0.241	0.172	0.161
121	0.235	0.135	0.088	0.059	0.225	0.233	0.161	0.079	0.115	0.141	0.094	0.085	0.131	0.092	0.157	0.041	0.093
123	0	0	0	0	0	0	0	0	0	0	0.007	0	0	0	0	0.049	0
125	0.044	0.032	0	0.008	0.094	0.058	0.016	0.056	0	0	0.007	0.015	0.023	0	0	0.205	0.254
127	0	0	0	0	0	0	0	0.008	0	0	0	0	0.008	0	0	0	0
129	0	0	0	0	0	0	0	0.024	0	0	0	0	0.008	0	0.009	0	0
131	0.147	0.079	0.284	0.161	0.261	0.400	0.226	0.325	0.231	0.167	0.181	0.262	0.215	0.208	0.176	0.287	0.229
133	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.008
137	0	0	0	0	0.007	0	0	0	0	0	0	0	0	0	0	0	0
141	0	0.056	0	0	0	0	0	0	0	0	0.007	0.015	0	0.031	0	0.008	0
143	0.191	0.262	0.155	0.085	0.123	0.092	0.153	0.095	0.051	0.103	0.058	0.092	0.100	0.100	0.083	0.066	0.034
145	0	0	0.007	0	0	0	0.008	0.016	0	0	0.007	0	0	0	0.028	0	0
Sol-49	**68**	**63**	**73**	**59**	**69**	**60**	**62**	**63**	**39**	**39**	**69**	**65**	**65**	**65**	**54**	**62**	**59**
141	0.250	0.246	0	0	0.145	0.167	0.129	0.127	0	0	0	0	0	0	0	0.073	0.237
142	0	0	0.096	0.085	0	0	0	0	0.218	0.218	0.174	0.123	0.177	0.100	0.176	0	0
148	0.096	0.087	0.151	0.220	0.094	0.192	0.073	0.063	0.115	0.128	0.116	0.062	0.062	0.023	0.056	0.194	0.127
156	0.007	0.008	0	0	0	0	0	0.008	0	0	0	0	0	0	0	0	0
158	0	0	0	0	0	0.008	0	0	0	0	0	0	0	0	0	0	0
160	0.331	0.254	0.404	0.373	0.442	0.358	0.444	0.516	0.436	0.321	0.377	0.523	0.477	0.515	0.444	0.323	0.263
162	0.081	0.079	0.021	0.042	0.239	0.158	0.145	0.151	0.064	0.154	0.159	0.092	0.046	0.131	0.056	0.347	0.237
164	0	0	0	0	0.007	0	0	0	0.013	0	0	0.008	0	0.008	0.009	0	0

(continued)

Appendix 1. Continued

	Texas		Western Louisiana		Eastern Louisiana		Mississippi		Georgia							Florida	
									M[a]			P[a]					
	M	P	M	P	M	P	M	P	1	2	3	1	2	3	4	M	P
Microsatellite loci																	
166	0.235	0.317	0.322	0.280	0.065	0.117	0.210	0.135	0.154	0.179	0.174	0.192	0.238	0.223	0.259	0.065	0.127
168	0	0.008	0.007	0	0	0	0	0	0	0	0	0	0	0	0	0	0.008
170	0	0	0	0	0.007	0	0	0	0	0	0	0	0	0	0	0	0
Sol-55	**68**	**63**	**72**	**59**	**69**	**60**	**62**	**63**	**37**	**38**	**69**	**63**	**41**	**64**	**54**	**62**	**59**
149	0.331	0.651	0.597	0.636	0.406	0.767	0.419	0.770	0.662	0.461	0.391	0.389	0.415	0.641	0.620	0.702	0.720
151	0.066	0.008	0.062	0.042	0.036	0.017	0.040	0.008	0.108	0.092	0.036	0.079	0.061	0.070	0.046	0.032	0.025
152	0.213	0.095	0.097	0.076	0.138	0.033	0.105	0.024	0.014	0.092	0.116	0.119	0.049	0.047	0.056	0.073	0.068
153	0	0.008	0.007	0	0	0	0.040	0.056	0.014	0.013	0.014	0.008	0.012	0.008	0.009	0.065	0.025
155	0.154	0.111	0.049	0.085	0.116	0.050	0.137	0.040	0.081	0.105	0.138	0.143	0.098	0.070	0.130	0.024	0.068
157	0.059	0.040	0.007	0.025	0.072	0.025	0.032	0.024	0.000	0.039	0.029	0.024	0.085	0.070	0.019	0.056	0.068
159	0.176	0.087	0.181	0.136	0.232	0.092	0.226	0.079	0.122	0.197	0.275	0.238	0.280	0.094	0.120	0.048	0.025
161	0	0	0	0	0	0.017	0	0	0	0	0	0	0	0	0	0	0
mtDNA	**68**	**63**	**69**	**60**	**70**	**46**	**69**	**62**	**43**	**38**	**68**	**65**	**63**	**60**	**54**	**64**	**58**
A	0.647	0.111	0.754	0.950	0.600	0.957	0.797	0.097	0.837	0.895	0.868	0.769	0.159	0.700	0.167	0.406	0.224
B	0.029	0	0.217	0.033	0.400	0	0.145	0	0.163	0.053	0.118	0	0.032	0	0	0.594	0.224
C	0.309	0.889	0.014	0.017	0	0.043	0.043	0.887	0	0	0	0.231	0.810	0.300	0.833	0	0.552
D	0	0	0	0	0	0	0	0	0	0.053	0.015	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0.016	0	0	0	0	0	0	0	0	0
W	0	0	0	0	0	0	0.014	0	0	0	0	0	0	0	0	0	0
X	0.015	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Y	0	0	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Note that M is monogyne social form, and P is polygyne social form. The sample sizes (numbers of individuals) on which each frequency estimate is based are shown in bold. Allele designations for the allozyme loci indicate relative band mobilities for homozygotes and allele designations for the microsatellite loci indicate the length of the amplification product. Dashes indicate that the locus was not scored for these samples.

[a] Samples for each social form in Georgia were obtained from multiple sites.

[b] Data from *Pgm-3* are not used for the polygyne form, because this locus is linked to *Gp-9*, a gene under strong selection in this social form (see text).