Assessing Genetic Structure with Multiple Classes of Molecular Markers: A Case Study Involving the Introduced Fire Ant *Solenopsis invicta*

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We used 30 genetic markers of 6 different classes to describe hierarchical genetic structure in introduced populations of the fire ant *Solenopsis invicta*. These included four classes of presumably neutral nuclear loci (allozymes, codominant random amplified polymorphic DNAs (RAPDs), microsatellites, and dominant RAPDs), a class comprising two linked protein-coding nuclear loci under selection, and a marker of the mitochondrial DNA (mtDNA). Patterns of structure revealed by *F* statistics and exact tests of differentiation were highly concordant among the four classes of neutral nuclear markers, although the microsatellites were the most effective markers for detecting structure. The results from the mtDNA complemented those from the neutral nuclear markers by revealing that strong limitations to female-mediated gene flow were the cause of the local structure registered by the nuclear markers. The pattern of structure inferred from the selected nuclear loci was markedly different from the patterns derived from the other sets of markers but was predictable on the basis of the presumed mode of selection acting on these loci. In general, the results for all six classes of markers can be explained by known features of the social and reproductive biology of fire ants. Thus, the results from these diverse sets of markers, combined with detailed natural history data, provide an unusually complete picture of how the fundamental evolutionary forces of gene flow, drift, and selection govern the distribution of genetic variation within and between fire ant populations.

Introduction

Studies of population genetic structure provide windows to the roles that the fundamental evolutionary forces of selection, gene flow, and drift play in processes such as local adaptation and speciation (Barton and Clark 1990; Avise 1994, pp. 204–233; Slatkin 1994; Foster, Scott, and Cresko 1998). Recent empirical and theoretical advances have led to the increased availability of an assortment of molecular markers and new methods for analyzing data derived from such markers, raising hopes of gleaning more comprehensive pictures of genetic structure and deeper insights into its evolutionary causes and consequences (Avise 1994, pp. 3–15; Mitton 1994; Roderick 1996). Although many newer methods employ information on the evolutionary relationships of the variants surveyed at each marker (Templeton 1998), most currently available nuclear markers yield data that must be analyzed using only information on allele frequencies and distributions. Given that diverse types of molecular markers can be used for this purpose, considerable interest has arisen in comparing the patterns of structure revealed by different classes of markers in focal study populations.

Patterns of structure may differ among loci depending on the type of mutation process generating variation, the magnitude of variation, the mode of inheritance, the nature of genetic information obtained (genotypic or not), the effects of selection, and stochastic variation (Mitton 1994; Palumbi and Baker 1994; Roderick 1996; Latta and Mitton 1997; Charlesworth 1998). This diversity of factors influencing genetic structure, if sufficiently well understood, can provide a powerful means of disentangling the effects of different evolutionary forces by combining analyses of complementary markers in single studies (Mitton 1994; Palumbi and Baker 1994; Pogson, Mesa, and Boutilier 1995; Neigel 1997). For instance, concordance in patterns of structure among nuclear markers of different classes can be inferred to signal that gene flow and drift, which affect all neutral nuclear elements similarly, are the major causes of the observed structure (Lewontin and Krakauer 1973; Mitton 1994; Scribner, Arntzen, and Burke 1994; Lehmann et al. 1996; Estoup et al. 1998). Discordance between presumed neutral nuclear markers and markers for which other evidence suggests a role for selection (e.g., important physiological or behavioral genes) provides strong ancillary evidence that selection on the latter markers is a primary force molding their unique patterns of structure (Koehn, Milkman, and Mitton 1976; Chevillon et al. 1995; Long and Singh 1995; Bonnin, Prosperi, and Olivieri 1996; Lawson and King 1996; Yang, Yeh, and Yanchuk 1996). Finally, discordance between nuclear markers and various organellar markers can implicate differences in the strength of maternal, paternal, and biparental components of gene flow (FitzSimmons et al. 1997; Latta and Mitton 1997; Rassmann et al. 1997; Latta et al. 1998; McCauley 1998).

Given the importance of studying genetic structure using diverse markers, it is surprising that relatively few empirical studies using multiple sets of loci in single species have been published (reviewed in Mitton 1994; Bonnin, Prosperi, and Olivieri 1996; Yang, Yeh, and Yanchuk 1996; Thompson, Taylor, and McPhail 1997; Bossert and Pashley Prowell 1998). Among these, usually only two classes of markers were compared, and seldom have multiple classes of markers of the same general type been studied (e.g., presumed neutral nuclear markers). Moreover, few studies of structure using

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multiple classes of markers have been conducted on organisms with well-known natural histories. The result is that discordant results among markers or marker sets in some cases cannot be given meaningful biological explanations (see Mitton 1994; Pogson, Mesa, and Boutilier 1995; Bossart and Pashley Prowell 1998).

The fire ant *Solenopsis invicta* represents an excellent model system for undertaking detailed studies of genetic structure using many markers of multiple classes. This insect, which was inadvertently introduced into the U.S.A. from South America in the 1930s (Lofgren 1986), is the subject of a vast literature concerning the natural history, social behavior, and breeding biology in the introduced range (reviewed in Lofgen, Banks, and Glancey 1975; Lofgen 1986; Vinson and Greenberg 1986; Ross and Keller 1995*a*; Tschinkel 1998). Furthermore, a considerable amount of information is now available on the basic population genetics of both native and introduced fire ants (Ross 1993; Ross et al. 1993, 1997; Ross and Shoemaker 1997). The large number of markers developed for these earlier studies are combined with newly developed markers in the present study to provide detailed descriptions of genetic structure at several hierarchical levels in the introduced range. The descriptions derived from the different markers are interpreted in light of the properties of the markers and the features of the reproductive and social behavior of *S. invicta* expected to influence structure at the scales examined. The results are used further to evaluate the effectiveness of different types of neutral markers in detecting structure caused by variation in gene flow and drift regimes.

Six classes of markers are used in this study: presumed neutral allozyme loci, codominant random amplified polymorphic DNA (RAPD) loci, microsatellite loci, dominant RAPD loci, protein-encoding loci under selection, and a segment of the mitochondrial DNA (mtDNA) (see Williams et al. 1990; Avise 1994, pp. 44–91; Mitton 1994; Davis et al. 1995; Jarne and Lagoda 1996; Roderick 1996; and Parker et al. 1998 for overviews of these marker types). Allozyme loci, which encode functional enzymes, exhibit variation detectable by electrophoresis that results primarily from point mutations in exons that cause charged amino acid substitutions. Codominant RAPD loci represent relatively short segments of DNA in which length variants occur in the amplicon because of mutational insertion or deletion events in the template DNA. Microsatellite loci are segments of DNA containing variable numbers of short repeat units, with this variation thought to arise primarily from polymerase slippage during replication. Dominant RAPD loci are segments of DNA for which PCR-based amplification succeeds or fails as a result of point mutations at the priming sites or insertion or deletion events in the amplified portion of the template.

Several additional points about these first four classes of markers, which are assumed at the outset to constitute neutral markers of the nuclear genome, bear mentioning. First, the allozymes are the products of coding DNA, whereas the other three classes of markers may occur commonly or primarily in noncoding DNA (Williams et al. 1990; Jarne and Lagoda 1996; Lu and Rank 1996; Harr et al. 1998). This distinction has frequently been used to argue that allozymes are more likely than the other markers to experience selection of one form or another (Mitton 1994; Neigel 1997; Parker et al. 1998). Second, the dominant RAPDs differ from the other three types of markers in that complete genotypic information and allele counts cannot be obtained directly, because diploid individuals bearing one copy of amplifiable template (heterozygotes) cannot be distinguished from those bearing two copies (homozygotes). Finally, diversity at individual loci, as measured by numbers of alleles and heterozygosity, ranges from very low, for the allozymes and dominant RAPDs, to relatively high, for the microsatellites (see below), with these differences tied to the different mutational processes characteristic of each marker class. (All of the markers used in this study likely possess reduced variation due to population bottlenecks during initial colonization of the U.S.A. by *S. invicta* [e.g., Ross et al. 1993].)

In addition to these four classes of presumably neutral nuclear loci, a class comprising two linked protein-coding nuclear loci previously shown to experience strong selection in fire ants is used to generate descriptions of genetic structure (Ross 1997). The form of selection acting on these two genes and the expected impact of such selection on patterns of gene flow are discussed below.

Finally, an organellar genome, the mtDNA, provides the sixth class of marker for this study. Variation was assessed using restriction enzyme digestion to detect point mutations in an amplified portion of the molecule (no indels have been detected in this region of fire ant mtDNA). As in most animals, the mtDNA of fire ants is inherited maternally (Shoemaker and Ross 1996), such that comparisons of structure based on mitochondrial and nuclear markers can provide information on sex-biased patterns of gene flow.

Attempts to describe structure in fire ants or any other highly social animal must account for the role that social behavior can play in governing gene flow and shaping selection domains (Ross and Keller 1995*a*). A remarkable social feature of *S. invicta* that profoundly influences these forces is the presence of two alternative types of social organization. These two types differ primarily in the number of fertile queens per colony, with colonies of the monogyne (**M**) social form containing only a single queen and colonies of the polygyne (**P**) form containing multiple queens (Ross and Keller 1995*a*). Other differences in the reproductive, dispersal, and social biology of the two forms are associated with variation in queen number; these differences reflect the nature of social selection acting on each form and, in turn, influence patterns of gene flow within and between the forms (Ross and Keller 1995*a*, 1995*b*; Shoemaker and Ross 1996; Ross et al. 1997; Ross and Shoemaker 1997; DeHeer, Goodisman, and Ross 1999). Importantly, social constraints on gene flow appear to be associated with variation at the selected protein loci included in this study, such that descriptions of genetic structure

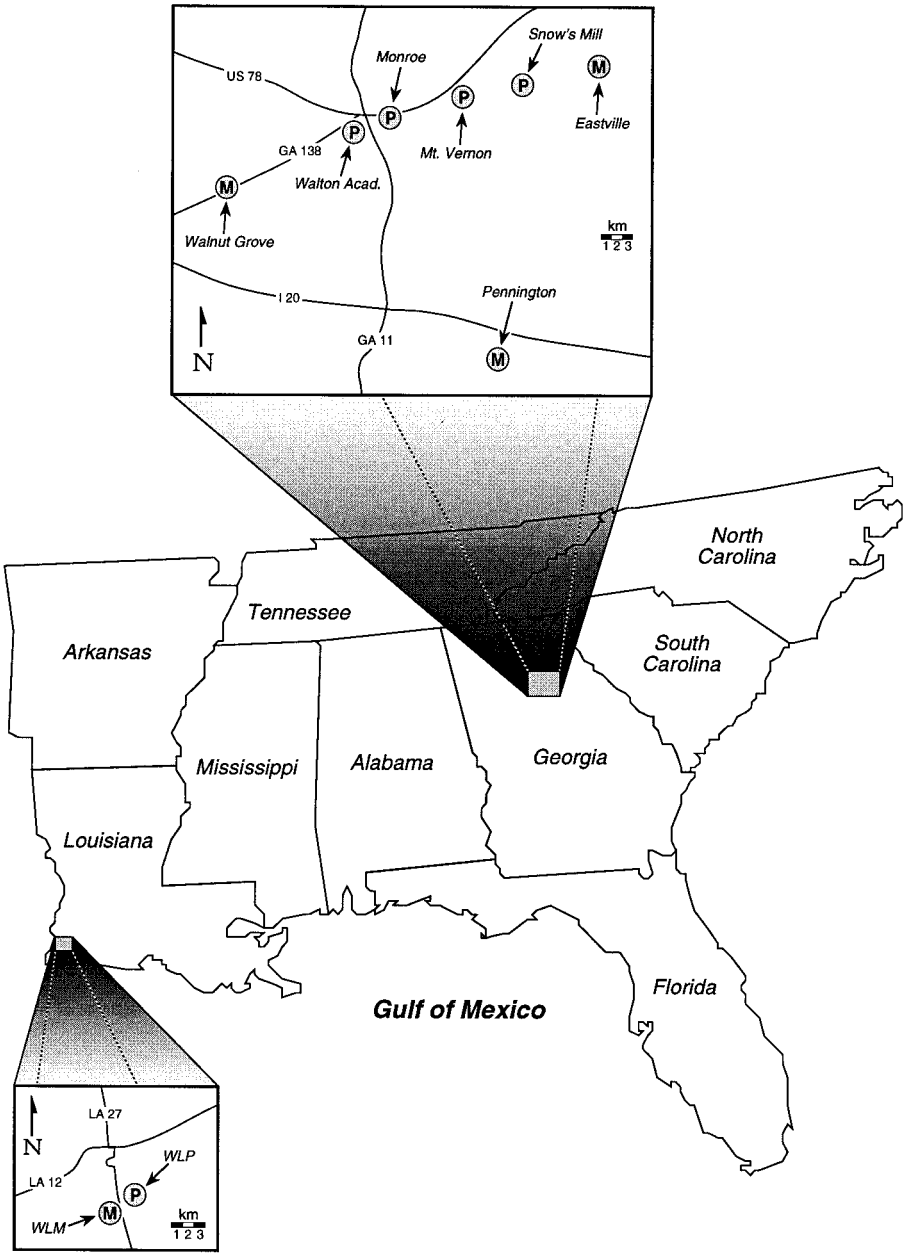

FIG. 1.—Locations of nine sites in the U.S.A. from which samples of *Solenopsis invicta* were collected. Sites at which only polygyne nests were sampled are indicated by ''P'' and sites where only monogyne nests were sampled are indicated by ''M.'' Sample sizes for each site and genetic marker are shown in the appendix.

based on the latter markers, when combined with descriptions from the neutral markers, yield especially fruitful insights into the evolutionary forces generating structure in populations of this highly social insect.

Materials and Methods

Samples Collected

Samples of *S. invicta* were obtained from nine sites in the introduced range, seven in Georgia (Clarke, Morgan, Oconee, and Walton Counties) and two in Louisiana (Calcasieu Parish), from fall 1995 to spring 1996 (fig. 1). The seven sites in Georgia include three sites at which only **M** nests were sampled and four where only **P** nests were sampled. The four Georgia **P** sites are a subset of the sites surveyed in Ross and Shoemaker (1997), whereas all three Georgia **M** sites were included in this earlier study. (Data for six of the allozymes and the mtDNA from these sites were reported in Ross and Shoemaker [1997], but data for all other markers from these sites have not been reported.) One site in Louisiana included only nests of the **M** form, whereas the other included only nests of the **P** form. The social form of each nest was confirmed as detailed in Ross and Shoemaker (1997). From 40 to 75 nests were sampled at each site; several wingless reproductive queens (**P** nests) or winged nonreproductive queens (**M** nests) were

collected from each nest, but genetic data for each marker were obtained from only a single randomly chosen individual per nest. The Louisiana sites and many of the sites within Georgia are located within a few kilometers of one another, within the distances over which fire ant sexuals are thought to be capable of dispersing (e.g., Markin et al. 1971; DeHeer, Goodisman, and Ross 1999).

Classes of Genetic Markers Employed

Neutral Allozymes

Data were obtained for eight polymorphic allozyme loci using starch gel electrophoresis coupled with specific histochemical staining. Procedures for scoring these markers and evidence that they are inherited in Mendelian, codominant fashion are presented in Shoemaker, Costa, and Ross (1992). These loci typically exhibit stable allele frequencies between different life stages and castes, and the genotype frequencies generally conform to Hardy-Weinberg expectations, suggesting that they behave as neutral markers in *S. invicta* (e.g., Ross 1993; Ross and Shoemaker 1997; Ross et al. 1997); a conspicuous exception is *Pgm-3* in the **P** form (below). Each locus possesses two or three alleles (table 1), the frequencies of which are listed by site in the appendix.

Codominant RAPDs

Sixty-five random 10-bp primers were used individually in the polymerase chain reaction (PCR) following standard RAPD reaction protocols (Shoemaker, Ross, and Arnold 1994). The PCR products were separated in 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light to screen for length variation in the amplified regions. Haploid (hemizygous) males were used for this initial identification of length variants, because heteroduplex bands commonly produced in codominant RAPD heterozygotes complicate interpretation of the genetic basis of the banding patterns (Hunt and Page 1992; Davis et al. 1995; Novy and Vorsa 1996). Five primers yielding bands of varying mobility that were produced consistently under various template concentrations subsequently were screened for suitability as codominant markers by surveying females. This allowed identification of putative heterozygote banding patterns, which in all cases included the two homoduplex bands representing each allele and one or two heteroduplex bands (Davis et al. 1995). The assumed allelic composition of each heterozygous genotype at the five markers was confirmed by comparing the banding patterns for such genotypes with patterns for synthetic heterozygotes obtained by mixing equal amounts of DNA template from haploid males of known genotype prior to PCR (e.g., Hunt and Page 1992; Davis et al. 1995). Modifications to the basic RAPD protocol used for each marker and progeny data confirming Mendelian, codominant inheritance of the amplification products will be reported elsewhere (unpublished data). Several genotypic and DNA size standards were run on every gel to aid in identifying genotypes. Samples that could not be scored reliably were subjected to reamplification and, if still unsatisfactory, were excluded. From two to four alleles were found at each of the five codominant RAPD loci (table 1); their frequencies are listed in the appendix.

Table 1
Measures of Genetic Diversity for Four Classes of Neutral Nuclear Markers in Introduced Populations of *Solenopsis invicta*

Locus	Observed Number of Alleles	Effective Number of Alleles	Expected Heterozygosity
Neutral allozymes			
Aat-2	2	1.15	0.127
Acoh-1	2	1.33	0.249
Acoh-5	2	1.62	0.384
Acyl	2	1.33	0.248
Est-4	2	1.89	0.471
G3pdh-1	2	1.79	0.443
Pgm-1	3	1.34	0.253
Pgm-3[a]	2	1.74	0.425
Mean for class	2.1	1.52	0.325
Codominant RAPDs			
Opc11-1	2	1.14	0.125
Opc13-1	2	1.49	0.328
Opc19-1	4	2.28	0.561
Ubc406-1	2	1.82	0.451
Ubc414-1	3	2.72	0.633
Mean for class	2.6	1.89	0.420
Microsatellites			
Sol-6	5	1.88	0.469
Sol-11	8	3.90	0.744
Sol-18	3	1.32	0.240
Sol-20	11	3.95	0.747
Sol-42	11	4.17	0.760
Sol-49	7	3.56	0.719
Sol-55	7	2.94	0.659
Mean for class	7.4	3.10	0.620
Dominant RAPDs[b]			
$Opa9_{410}$	2	1.93	0.483
$Opa9_{680}$	2	1.26	0.207
$Opa9_{1100}$	2	1.59	0.373
$Opc8_{480}$	2	1.24	0.196
$Opc11_{960}$	2	1.55	0.356
$Opc13_{1600}$	2	1.82	0.451
$Opc13_{1800}$	2	1.39	0.279
$Ubc414_{1600}$	2	1.14	0.119
Mean for class	2	1.49	0.308

[a] *Pgm-3* is regarded as a neutral locus only in the monogyne form, so data are from the four sampling sites containing only nests of this form.

[b] Dominant RAPD loci are assumed to have two alleles. Estimates of the effective number of alleles and expected heterozygosity at these loci assume Hardy-Weinberg equilibrium genotype proportions (see text).

Microsatellites

Data were obtained for seven polymorphic dinucleotide microsatellite loci by means of PCR amplification of target DNA using specific pairs of primers, followed by separation of the PCR products in polyacrylamide sequencing gels and visualization using autoradiography (specific procedures in Krieger and Keller 1997). From 3 to 11 alleles were recorded at each microsatellite locus (table 1); the allele frequencies are listed in the appendix.

Dominant RAPDs

The survey for codominant RAPD loci led also to the identification of eight variable RAPD markers char-

acterized by the presence or absence of a specific band. Of many such potential dominant markers observed in the initial survey, these eight were selected because they displayed consistent amplification that yielded brightly staining products, their amplification was minimally affected by variation in template concentration, relatively few nontarget bands resulted from amplification with each primer, and scoring was consistent across replicate PCRs (e.g., McClelland and Welsh 1994; Stewart and Excoffier 1996; Palacios and González-Candelas 1997). Moreover, all eight markers exhibited overall frequencies of band presence of less than $1 - 3/N$ (where N is the number of individuals sampled), reducing potential bias in the allele frequency estimates stemming from low counts of the band absence phenotype (Lynch and Milligan 1994; Stewart and Excoffier 1996). The protocol used for scoring these RAPD markers and progeny data confirming their Mendelian, dominant inheritance will be reported elsewhere (unpublished data). DNA size standards were run on every gel to aid in identifying the target band, and samples were reamplified or excluded when they could not be scored reliably. Dominant RAPD loci are assumed to possess only two alleles (table 1), the frequencies of which are listed in the appendix.

Protein Loci Under Selection

Data were obtained for the locus *Gp-9* using starch gel electrophoresis coupled with nonspecific protein staining. The product of *Gp-9* is inherited in Mendelian, codominant fashion, and it has been shown that this gene (or a chromosome segment marked by it) is under strong differential selection in the two social forms of *S. invicta* (Ross 1997; Keller and Ross 1998; Ross and Keller 1998). The most striking aspect of selection at this biallelic locus is almost complete overdominance in egg-laying queens of the **P** form, which is absent in the **M** form. This differential selection results in strong allele frequency differences between the forms (the **M** form is fixed for one of the alleles), as well as strong departures of genotype frequencies from Hardy-Weinberg equilibrium proportions (HWEP) in the **P** form (Ross 1997). *Gp-9* is tightly linked to and in strong gametic disequilibrium with the polymorphic allozyme locus *Pgm-3* (Ross 1997). As expected by virtue of this association, strong allele frequency differences exist between the two forms at *Pgm-3,* and genotype frequencies at this locus do not match HWEP in the **P** form (Ross 1992). Variation at *Gp-9* is more strongly associated with the phenotypic differences on which selection acts than is variation at *Pgm-3,* presumably because *Gp-9* is in stronger disequilibrium with the actual gene(s) causing the effects (Keller and Ross 1999). This fact, combined with the generally higher genotypic variability at *Pgm-3* than *Gp-9,* means that *Pgm-3* provides additional information to that available from *Gp-9* alone; therefore, both markers are employed in most of the analyses of genetic structure involving loci under selection. Because no evidence has been found for selection acting on *Pgm-3* in the **M** form, this locus is treated as a neutral marker in analyses involving only this form. Allele frequencies at *Gp-9* and *Pgm-3* are shown for each site in the appendix.

MtDNA

Haplotypes of the mtDNA were scored following PCR amplification of a 4-kb segment, digestion of the PCR product with 13 enzymes, separation of the digestion products in 1.5% agarose gels, staining with ethidium bromide, and visualization under UV light (specific procedures in Ross and Shoemaker 1997). Maternal inheritance of the PCR product has previously been demonstrated in family studies (Shoemaker and Ross 1996). Composite haplotypes are defined by possession of unique sets of restriction sites across all enzymes; only five such haplotypes were identified in this study, the frequencies of which are shown in the appendix.

Population Genetic Data Analyses

All genetic data were obtained from the same single individual from each nest for all of the Louisiana samples and many of the Georgia **M** samples (43% of the total). For the remaining samples, markers of two different classes may have been scored from two different individuals in a given nest. However, in all cases, only a single genotype or haplotype per marker was scored from each nest, thus avoiding possible bias in the estimates of genotype and allele frequencies attributable to family structure and nonindependence of genotypes (e.g., Ross et al. 1997).

Test for Hardy-Weinberg Equilibrium Genotype Proportions

Conformity of genotype frequencies to HWEP was tested for each codominant nuclear marker at each site by means of exact tests implemented in the program GENEPOP v3.1b (Raymond and Rousset 1995*a*; Rousset and Raymond 1995). (*Gp-9* and *Pgm-3* were not tested in the **P** form, because they were previously shown to depart strongly from HWEP). Fisher's method of combining independent test results (Manly 1985, pp. 432–433) was used to determine overall significance of departures from HWEP for each locus, each site, and each social form in Georgia.

Partitioning of Genetic Variance among Hierarchical Levels of Structure

Total genetic variance was partitioned among the different levels of structure for each marker class by calculating hierarchical F_{ST} values. These values were estimated for a three-level hierarchy (sites within forms within states) using the methods of Weir and Cockerham (1984), as implemented in the program TFPGA (available from M. Miller, Northern Arizona University). For the dominant RAPDs, allele frequencies were estimated using the Taylor expansion method of Lynch and Milligan (1994); the assumption of HWEP that underlies this method is supported by its general occurrence at the 19 or 20 codominant neutral markers (below; see also Buso, Rangel, and Ferreira 1998). For the selected protein loci *Gp-9* and *Pgm-3,* use was made of the fact that the recombination frequency between the loci is extremely low and that one of the two-locus haplotypes

appears to be absent in the wild (Ross 1997). Thus, the linkage phase of double heterozygotes could be inferred, and two-locus gamete haplotype counts could be obtained from each site and used to estimate F_{ST}. The complete hierarchical analysis for the selected nuclear loci yielded large negative F_{ST} values for between-state differentiation (see Weir 1996, pp. 175–176, for possible reasons), making it infeasible to partition the variance at the lower levels. For this reason, the hierarchical F_{ST} analyses were broken down into separate subanalyses, the first identifying the variance residing between the forms within each state, and the second identifying the variance among sites within the forms in Georgia. Average F_{ST} values for each class of neutral nuclear markers were obtained by jackknifing over loci.

Information on the presumed genealogical relationships of the microsatellite alleles and mtDNA haplotypes was not employed in the analyses of genetic structure (e.g., Excoffier, Smouse, and Quattro 1992; Neigel 1997), because the loss of genetic variation and extensive human-mediated dispersal characterizing the early phases of fire ant colonization violate the assumption of mutation-drift equilibrium underlying incorporation of such information in analyses of structure (Goodman 1998).

Variability of Neutral Nuclear Markers for Detecting Genetic Structure

The variance in F_{ST} values among loci constituting each class of neutral nuclear markers was assessed by bootstrapping the values obtained from single-level analyses (using GENEPOP) across the loci and determining which point estimates fell outside the middle 95% of the 5,000 bootstrap values (see Van Dongen 1995; Rousset and Raymond 1997). Bootstrapping was conducted for sites within the Georgia **M** form, for sites within the Georgia **P** form, for the two forms within Georgia, for the two forms within Louisiana, and for ants in the separate states. These analyses were used to determine whether the different classes of markers differed in the consistency with which they detected structure. Additionally, individual outlier loci giving extreme values of F_{ST} were identified among the entire set of neutral nuclear markers using a modification of the approach of Beaumont and Nichols (1996). These authors showed by simulation that expected F_{ST} values are little affected by individual-locus heterozygosity under a variety of conditions, given that heterozygosity is always greater than 0.1 (as in our study). Therefore, we treated the markers of all four classes as a group and used exploratory data analysis (Tukey 1977) to identify loci returning unusually low or high single-level F_{ST} values. Loci yielding values more than two interquartile ranges lower than the 25th percentile or higher than the 75th percentile were defined as outliers. These analyses were used to identify single presumed neutral markers that may be subject to selection, systematic scoring errors, or other factors affecting their usefulness as markers of gene flow and drift.

Evaluation of Significance of Hierarchical Genetic Structure

The significance of structure detected for each marker at each hierarchical level was evaluated by conducting Fisher's exact tests of allele or haplotype frequency differentiation among sites or groups of sites using the program GENEPOP (Raymond and Rousset 1995*b*). For the dominant RAPDs, band presence/absence data for each locus were converted into genotype frequencies by assuming HWEP (e.g., Lynch and Milligan 1994). The exact tests for the dominant RAPDs were supplemented by permutation tests for detecting significant structure. For these latter tests, interindividual phenotypic distances were calculated from the numbers of band presence/absence differences across all eight dominant RAPD loci using the program AMOVA-PREP (available from M. Miller, Northern Arizona University) (see Huff, Peakall, and Smouse 1993; Stewart and Excoffier 1996). These multilocus RAPD distances then were permuted across populations using the program WINAMOVA v1.55 (Excoffier, Smouse, and Quattro 1992), resulting in 10,000 random distributions for each level of structure. A conservative indication of significant differentiation at a given level was obtained when the calculated ϕ_{ST} ($=F_{ST}$) values were greater than 95% of the values derived from the randomized data (Michalakis and Excoffier 1996; Stewart and Excoffier 1996). Each of these permuation analyses for the dominant RAPDs was broken down into two subanalyses as described above for the selected nuclear loci. Fisher's method of combining test results was used to determine the overall significance of differentiation for each class of markers, as well as for all 28 neutral nuclear markers combined, at each level of structure.

Estimation of Genetic Divergence Between Pairs of Sites

Genetic divergence between each pair of sites was quantified for all six classes of markers by estimating values of the coancestry distance (Reynolds, Weir, and Cockerham 1983; Weir 1996, pp. 190–198). The pairwise distances for each class were grouped for display according to whether sites were of the same or different social forms and in the same or different states.

Results

Marker Diversity

Levels of diversity at the 28 neutral nuclear markers employed to describe genetic structure are shown in table 1. The neutral allozymes and dominant RAPDs display the lowest diversity, with only two alleles normally segregating per locus and average heterozygosities of little more than 30%. The microsatellites display the highest diversity, with 3–11 alleles segregating per locus and heterozygosities averaging about twice those of the allozymes and dominant RAPDs.

Hardy-Weinberg Equilibrium Genotype Proportions

Results of the tests for HWEP at each neutral codominant nuclear marker at each site are shown in the

appendix. For the neutral allozymes, only two significant departures from HWEP ($P < 0.05$) were detected (both at locus *Acy1*), while for the codominant RAPDs, only three departures were detected (two at locus *Ubc406-1*). For each of these classes, the total number of departures observed is close to the number expected due to chance. Also, the occurrence of two deviations at any single locus of one of these classes is not highly unlikely given the total number found, this probability being 0.07 for *Acy1* and 0.51 for *Ubc406-1* (Monte Carlo simulations). The combined probability of conformity to HWEP over all sites is greater than 0.11 for each of the allozyme and codominant RAPD loci, and the combined probability over all loci of each of these two classes is greater than 0.28 for each of the sites. Finally, the combined probability over all loci of each class is greater than 0.17 for each social form in Georgia. This general conformity to HWEP at the allozyme and codominant RAPD loci is consistent with the assumption of neutrality of these markers and suggests that strong deviations from panmixis or pronounced substructure within sites are unlikely.

For the microsatellite loci, about twice as many significant departures from HWEP occur over the various study sites as are expected by chance (appendix). Two loci, *Sol-20* and *Sol-42,* are responsible for the majority of these departures, and only these loci display a combined probability of conformity to HWEP across all sites of less than 0.05. One possible explanation for the discrepancy between these two loci, which have the highest allelic richness of any of the markers (table 1), and the other codominant loci is that they are more sensitive to subtle deviations from HWEP than the less polymorphic markers. This seems doubtful for *Sol-42,* given that both heterozygote and homozygote excesses are detected at different sites. Moreover, two other microsatellite loci with high diversity, *Sol-11* and *Sol-55,* do not depart from HWEP at any of the study sites. Another possible explanation for the discrepancies is that *Sol-20* and *Sol-42* possess null alleles (Pemberton et al. 1995), but, again, this seems unlikely for *Sol-42,* because the presence of such alleles should always produce apparent excess homozygosity. These two loci may be particularly susceptible to scoring errors due to sensitivity of the amplifications to template quality or quantity, production of artifact (stutter) bands, or differential amplification of alleles differing greatly in length (Wattier et al. 1998). A final possibility is that these loci are affected by selection. Regardless of the source of departures from HWEP at these loci, their exclusion from the analyses of structure has little impact on the results (below), so they are provisionally assumed to be informative markers for inferring the effects of gene flow and drift in the study populations.

The combined probability of conformity to HWEP over all seven microsatellite loci is less than 0.05 for two of the nine study sites, Walton Academy and WLM. However, removal of *Sol-20* and *Sol-42* renders the combined probabilities for these sites nonsignificant. For the social forms in Georgia, the combined probabilities of conformity over all microsatellite loci are 0.21 for the **M** form and 0.02 for the **P** form. In the latter case, again, the combined probability becomes nonsignificant when *Sol-20* and *Sol-42* are eliminated. Thus, the evidence suggests that the microsatellite loci we studied (with the possible exceptions of *Sol-20* and *Sol-42*) behave as neutral markers in our study populations.

Partitioning of Genetic Variance

The distribution of total genetic variance among the three hierarchical levels of sampling is depicted for each class of markers in figure 2. The overall patterns are very similar for the four classes of neutral nuclear markers (upper four pie diagrams), with relatively small and constant parts of the variance for each class partitioned between the states, between the forms within each state, and among the sites of each form in Georgia. The magnitude of the total variance found at each level varies to some extent among these four marker classes, with the magnitude generally paralleling the genetic diversity of the markers. Thus, the allozymes have the smallest proportion of the total variance residing at each level (no more than 1.7%), while the microsatellites have the largest (about 5%). The great bulk of the total genetic variance for these four classes (85%–95%) resides within the study sites. The extent of genetic structure at the levels we investigated therefore is relatively modest in introduced *S. invicta,* especially at the smallest scales (absolute differentiation is cumulative and so is greater at the larger scales than is apparent from fig. 2).

Although these presumably neutral nuclear loci display broadly similar signals of hierarchical structure, it is of interest to know how variable the signals are among the markers constituting each class. Within-class variance appears to be somewhat lower for the microsatellites than for the other classes, with 43% of the single-locus F_{ST} estimates for the microsatellites falling outside of the bootstrap 95% confidence interval (CI) and 52%–60% of the estimates for the other classes falling outside of their respective 95% CIs. The dominant RAPDs exhibited the highest amount of among-locus variability, as might be expected given the inability to obtain direct genotypic information from these markers (Lynch and Milligan 1994; Latta and Mitton 1997).

The exploratory data analyses employed to identify outlier loci of any of these four marker classes indicated that unusually large F_{ST} estimates were obtained for the microsatellite *Sol-55* and the dominant RAPDs $Opc8_{480}$ and $Opc13_{1600}$ among sites in the Georgia **P** form, and for the microsatellite *Sol-11* and the dominant RAPD $Opc11_{960}$ between the states. These outliers may represent loci that are subject to differential selection at these levels, to systematic scoring errors, or to large stochastic variation.

The distribution of variation at the selected, linked protein loci *Gp-9* and *Pgm-3* differs strikingly from the distributions at the four classes of neutral nuclear loci (fig. 2). An extraordinarily large portion of the variance (88%) resides between the forms within each state, whereas effectively zero variance occurs between the states or among the sites of each form in Georgia. These patterns are understandable in terms of the strong dif-

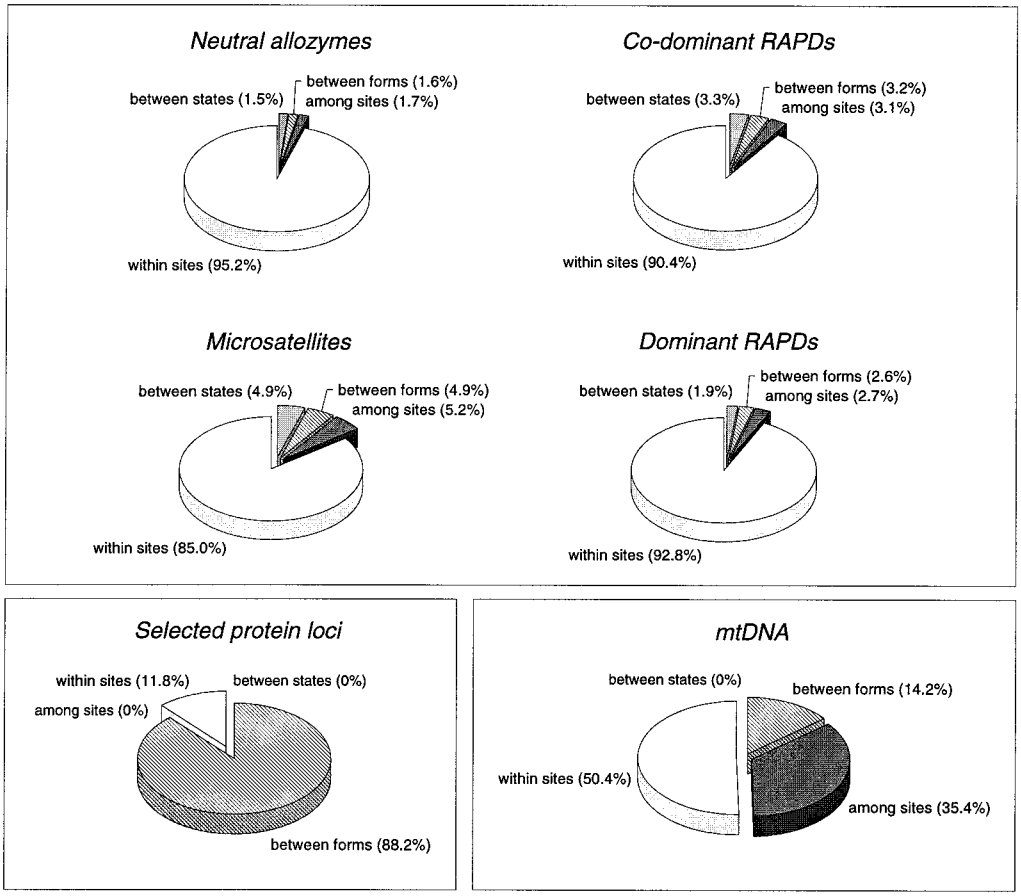

FIG. 2.—Partitioning of total genetic variance among the different hierarchical levels of sampling in the study populations for each of six classes of molecular markers. The upper four pie diagrams represent the four classes of neutral nuclear markers.

ferential selection between the forms acting on these linked genes (Ross 1992, 1997), which creates dissimilarities between any populations differing in social form but homogenizes sites within each form by overcoming the effects of drift associated with limited dispersal.

MtDNA haplotype variation is distributed in yet another unique pattern (fig. 2). In this case, none of the variance in the hierarchical analysis resides between states, yet very large variance components exist between sympatric forms and among sites within forms. The mtDNA differentiation at the latter levels stands in strong contrast to the results from the neutral nuclear markers, and presumably results from greater restrictions on female-mediated than on male-mediated gene flow at these levels (e.g., Ross and Shoemaker 1997; Goodisman and Ross 1998).

Significance of Genetic Structure

Permutation tests for differentiation at the dominant RAPDs yielded results similar to those of the exact tests in all cases, so only results of the latter tests are reported. Genetic differentiation between the states is highly significant for all four classes of neutral nuclear markers as well as the mtDNA (table 2). In contrast, no significant differentiation between the states was found for the selected protein loci *Gp-9* and *Pgm-3,* as expected given that a negative F_{ST} value was estimated for this highest sampling level. The strong differentiation between states inferred for the mtDNA using exact tests (table 2) is somewhat surprising given that the hierarchical F_{ST} analyses revealed that none of the total variance for this genome resides at this level (fig. 2). In contrast, a single-level F_{ST} analysis indicated that 14% of the total mtDNA variance occurs between the states when lower-level structure is ignored, with this differentiation significant at $P < 0.0001$ using a permutation test with 10,000 permutations (Excoffier, Smouse, and Quattro 1992). Clearly, by disregarding significant lower-level structure in the analyses of differentiation at higher levels, the potential exists for somewhat misleading conclusions regarding the sources of differentiation (see also Lewontin 1972). In the present case, the substantial mtDNA differentiation between sympatric social forms and among the Georgia **P** sites (below) generates most of the apparent strong between-state differentiation.

Differentiation between the sympatric social forms in Georgia generally is significant for the neutral nuclear markers and mtDNA, whereas the social forms in Louisiana are less obviously differentiated at these markers (table 2). Exceptions to these generalizations are the allozymes in Georgia, which show marginally nonsignificant between-form differentiation, and the microsatel-

Table 2
Probability Values from Exact Tests Indicating Genetic Differentiation at Different Sampling Levels for Six Classes of Molecular Markers in Introduced Populations of *Solenopsis invicta*

Between States		
Neutral allozymes	**<0.0001**	
Codominant RAPDs	**<0.0001**	
Microsatellites	**<0.0001**	
Dominant RAPDs	**<0.0001**	
Selected protein loci	0.733	
mtDNA	**<0.0001**	
Between Social Forms	**Georgia**	**Louisiana**
Neutral allozymes	0.060	0.165
Codominant RAPDs	**0.029**	0.725
Microsatellites	**0.004**	**<0.0001**
Dominant RAPDs	**<0.0001**	0.116
Selected protein loci	**<0.0001**	**<0.0001**
mtDNA	**<0.0001**	**0.001**
Among Sites	**Georgia M Form**	**Georgia P Form**
Neutral allozymes	0.673	0.378
Codominant RAPDs	0.224	0.441
Microsatellites	**0.001**	**0.001**
Dominant RAPDs	0.919	**0.010**
Selected protein loci	0.556	0.475
mtDNA	0.274	**<0.0001**

NOTE.—Probability values less than 0.05, which signify significant differentiation, are shown in bold.

lites and mtDNA in Louisiana, which show highly significant between-form differentiation. Disregarding the loci *Sol-20* and *Sol-42*, which do not conform to HWEP, has little effect on the latter result for the microsatellites (*P* remains less than 0.001). Given the large among-site component of variance found for the mtDNA (fig. 2), the possibility that the significant between-form differentiation in Georgia simply reflects differentiation within one or both forms was investigated using a two-level F_{ST} analysis (sites nested within the forms in Georgia) coupled with permutation testing. The forms remain significantly differentiated in this analysis ($P < 0.05$), suggesting that the mtDNA differentiation between the forms cannot be completely explained by variation among sites. Finally, the sympatric social forms in both states are highly significantly differentiated at the selected protein loci (table 2), as expected given that almost 90% of the total variance at these loci occurs at the between-form level (fig. 2).

Considering differentiation within each of the social forms in Georgia, there is no evidence for significant among-site structure in the **M** form at the neutral nuclear markers and mtDNA, except at the microsatellites (table 2). In the alternate **P** form, in contrast, the microsatellites, dominant RAPDs, and mtDNA show significant among-site differentiation. Disregarding the loci *Sol-20* and *Sol-42* again has little impact on the finding of significant among-site differentiation at the microsatellites in the two forms ($P < 0.018$ for the **M** form and $P < 0.008$ for the **P** form). Neither form displays significant among-site differentiation at the selected protein loci.

Combining the results of the exact tests for all 28 neutral allozyme, RAPD, and microsatellite loci reveals the following general patterns for presumably neutral elements of the nuclear genome. Fire ants from the different states are highly significantly differentiated ($P < 0.0001$), the sympatric social forms in each state are highly significantly differentiated (both $P < 0.0001$), sites within the Georgia **M** form are marginally undifferentiated ($P = 0.054$), and sites within the Georgia **P** form are highly significantly differentiated ($P < 0.001$). Exclusion of *Sol-20* and *Sol-42* changes these results little, other than to increase the probability that the Georgia **M** sites are undifferentiated to $P = 0.208$.

Genetic Divergence Between Specific Sites

Values of the coancestry distances between pairs of sites grouped into four different categories are presented for each marker class in figure 3. For each of the four classes of neutral nuclear markers (four upper panels), the distances generally fall into two clusters, with very short distances between sites in the same state and longer distances between sites in the different states. Whether or not a pair of sites belongs to the same social form has little impact on the genetic distance between them relative to the effect of whether or not they are in the same state. This suggests that the major genetic discontinuities found in this study occur at the between-state level, a conclusion apparent also from the significance testing for allelic differentiation at each level (table 2).

A very different pattern is seen for the selected protein loci (fig. 3). Between-site distances based on *Gp-9* and *Pgm-3* cluster according to social form rather than according to state, a pattern that is little affected by among-site variation within each form. Such a pattern is expected if strong differential selection on each form dominates any effects of gene flow and drift, homogenizing allele frequencies within and causing divergence between the forms that is independent of geographic location.

Yet another distinctive pattern of pairwise distances is found for the mtDNA (fig. 3). Two clusters of distance values are seen for each category, one comprising values around zero and the other comprising considerably higher values. For sites of the same form in the same state (Georgia), all of the values for the **M** form are clustered around zero, whereas all of the values over 0.5 are for pairs of **P** sites. This pattern is consistent with the finding of significant haplotype differentiation among **P** sites but not among **M** sites (above). For sites of different forms in the same state, many of the distances are greater than 1.0 for the Georgia ants, but the large variation in haplotype frequencies among **P** sites also results in some between-form distances that are close to zero. The large variation in haplotype frequencies at the Georgia **P** sites also explains the two clusters of values for the remaining two categories (fig. 3), because it means that some Georgia **P** sites must be quite similar to, and others quite dissimilar to, sites of either form in Louisiana (most of the variation occurs among just three haplotypes). This explanation also reveals how the sig-

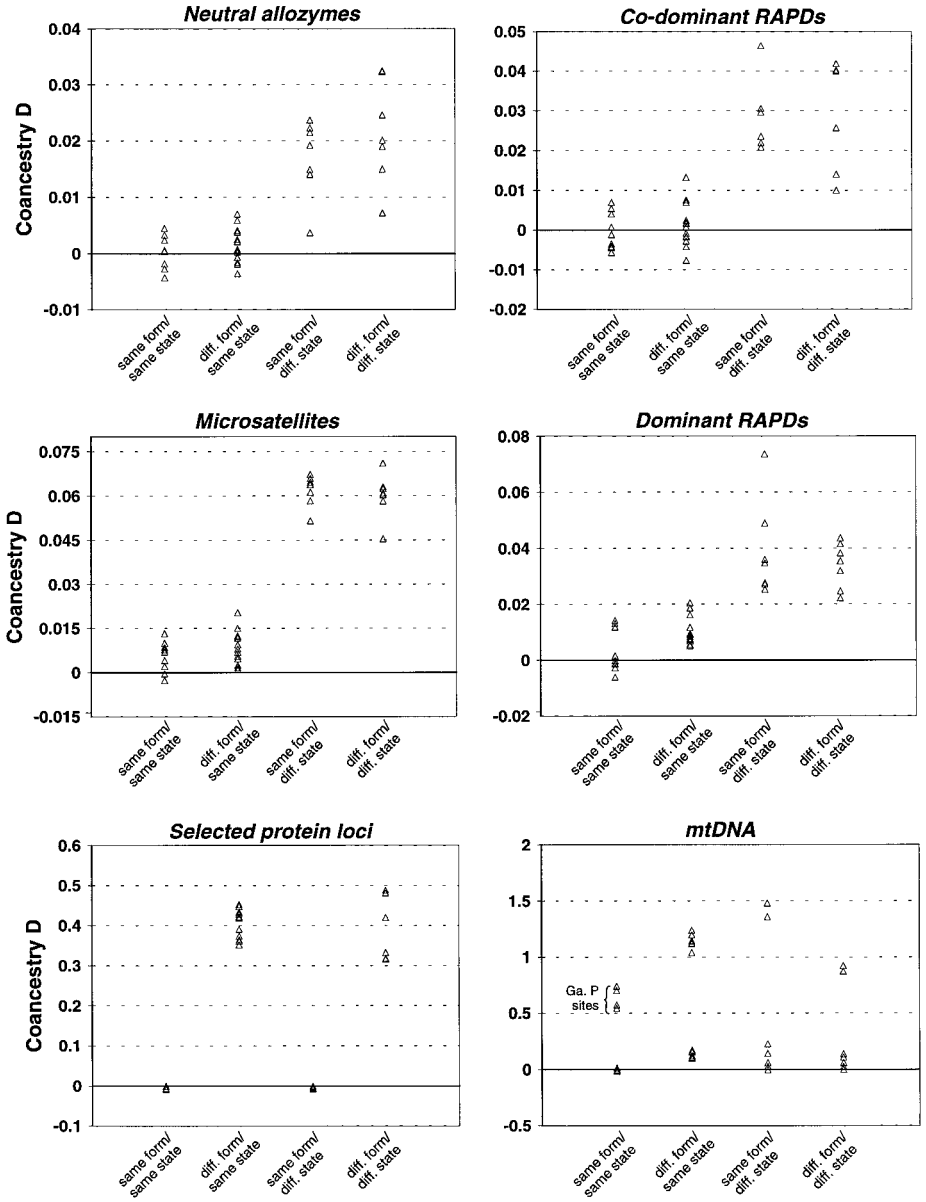

FIG. 3.—Values of the coancestry distance (D) between each pair of study sites for each of six classes of molecular markers. The upper four panels represent the four classes of neutral nuclear markers. The first two categories in each panel represent distances between sites within the same state (same or different social forms), whereas the last two categories represent distances between sites in different states (same or different social forms).

nificant mtDNA differentiation found between the states (table 2) can be ascribed largely to among-site differentiation in the Georgia **P** form.

Discussion

Comparison of Results from Different Classes of Markers

A principal objective of this study was to compare descriptions of genetic structure from different classes of markers in introduced fire ants to learn if coherent and complementary views of the roles of different evolutionary forces would be obtained. The first step toward meeting that objective was to compare the patterns of structure revealed by the different classes of presumed neutral nuclear markers. The four classes of such markers were relatively concordant in showing small ($\leq$5%) and quite even amounts of the total genetic variance distributed among each of the three levels of structure investigated (fig. 2). Moreover, markers of these four classes were generally in agreement with respect to the location of significant differentiation between sites or groups of sites (table 2). For instance, markers of all four classes indicated significant differentiation between the states and (with the possible exception of the allozymes) between the social forms in Georgia, whereas there was a general inability to detect differentiation between the social forms in Louisiana and among **M** sites in Georgia.

The general concordance in structure revealed by the allozyme loci, which are genes that encode metabolically important protein products, and by the DNA loci, many of which presumably mark noncoding segments, indicates that selection on the allozymes is unlikely to be an important factor influencing genetic structure in the study populations. Of course, one allozyme locus, *Pgm-3,* and the protein-coding locus *Gp-9* were excluded from consideration as ''neutral markers'' a priori because of earlier evidence that they are affected by selection. This evidence came from evaluating individual markers to confirm the mode of inheritance, determine the stability of gene frequencies across life stages and castes, and test for Hardy-Weinberg genotype proportions (e.g., Ross 1992, 1993, 1997; Shoemaker, Costa, and Ross 1992). At least this degree of evaluation of individual markers will likely be necessary to resolve the unexplained disparities in patterns of structure revealed by allozymes and other classes of nuclear markers that have occasionally been reported (Mitton 1994; Pogson, Mesa, and Boutilier 1995; Ayres and Ryan 1997; Neigel 1997; Raybould, Mogg, and Gliddon 1997; Thompson, Taylor, and McPhail 1997). Our data from introduced and native fire ants (Ross et al. 1997), taken with results from other comparative studies (e.g., Scribner, Arntzen, and Burke 1994; Lehmann et al. 1996; Le Corre, Dumolin-Lapègue, and Kremer 1997; Estoup et al. 1998; Streiff et al. 1998), suggest that properly evaluated allozymes are no more likely than other classes of markers to give biased estimates of gene flow due to the action of selection.

The general concordance observed between the dominant RAPD loci and the neutral nuclear loci for which complete genotypic information is available confirms that markers of the former class can be informative for describing genetic structure (see also Le Corre, Dumolin-Lapègue, and Kremer 1997; Aagaard, Krutovskii, and Strauss 1998; Buso, Rangel, and Ferreira 1998). Importantly, the eight dominant RAPD loci we chose for study were carefully appraised beforehand to ensure that they were inherited in Mendelian fashion and minimally subject to artifactual scoring errors (see also Stewart and Excoffier 1996; Palacios and González-Candelas 1997). In our study, the inevitable loss of information and relatively large variance in single-locus F_{ST} estimates due to dominance apparently were adequately compensated for by the relatively large numbers of loci used and samples scored (Aagaard, Krutovskii, and Strauss 1998), and probably also by our ability to assume Hardy-Weinberg equilibrium and thus to specify genotype and allele frequencies at each site.

Although the four classes of neutral nuclear markers were largely concordant in describing structure, the microsatellites consistently had the greatest proportion of total variance partitioned at each sampling level and detected significant differentiation among sites or groups of sites, even when few or no other neutral nuclear markers did (table 2). Moreover, the consistency of the single-locus F_{ST} estimates apparently was greater for the microsatellites than for the other classes of markers (cf. Streiff et al. 1998). The greater effectiveness of the microsatellites in detecting structure seems not to be an artifact of inclusion of the loci departing from Hardy-Weinberg equilibrium (*Sol-20* and *Sol-42*), nor is it due to the microsatellites having an unusually high proportion of outlier loci registering extreme differentiation. The usual explanation given for superior detection power of microsatellites—that the high mutation rates generating their large allelic diversity make them especially sensitive to recent sunderings of gene flow (Mitton 1994; Pogson, Mesa, and Boutilier 1995; FitzSimmons et al. 1997; Estoup et al. 1998)—cannot be true in the present case, because the time span since the introduction of fire ants and separation of the study populations (no more than 50 years) is too short for differences in mutation rates to have had any effect. Rather, the explanation seems to be that the higher amount of variation at the microsatellite markers affords greater statistical power to the exact tests used to detect differentiation (Estoup et al. 1998). Also, although the proportions of microsatellite variance partitioned among different levels are not much greater than the proportions partitioned using the other neutral nuclear markers, highly variable markers return downward-biased F_{ST} values compared with less variable markers given the same magnitude of absolute divergence (Charlesworth 1998).

Outlier loci producing extreme F_{ST} values were relatively rare in our set of neutral nuclear markers, with only 5 of the 136 values obtained (3.7%) regarded as being unusually large for a given level of structure. It is possible that the five loci involved mark chromosomal regions subject to weak differential selection. However, three of the anomalous values occur among sites of the Georgia polygyne form, and it is difficult to imagine how selection could produce differentiation among these sites given their geographic proximity and the recency of colonization of this area. No outlying F_{ST} estimates were found between the sympatric social forms in Georgia or Louisiana, even though this is the most likely context for differential selection to occur. The outlying values thus may best be interpreted as resulting from stochastic variation, perhaps in association with range expansion and secondary contact (e.g., Latta and Mitton 1997). The overall concordance of structure among the presumably neutral nuclear markers of all four classes, taken with the finding that the genotypes at these markers generally appear in HWEP, suggest that recent or contemporary patterns of gene flow coupled with drift explain the major features of fire ant genetic structure detected by the majority of our nuclear markers.

The partitioning of variance for the selected loci *Gp-9* and *Pgm-3* differs strikingly from that for the neutral nuclear loci and mtDNA, as expected if selection acting on one or both of these linked loci dominates the effects of gene flow and drift. Recent studies have shown that the allele $Gp\text{-}9^b$ ($=Gp\text{-}9^{95}$) is associated with several key features of polygyny in fire ants, including the tendency of queens to exhibit restricted dispersal and the tendency of workers to accept multiple nestmate queens (Ross and Keller 1998; DeHeer, Goodisman, and Ross 1999). Thus, this allele seems to be required for the expression of the **P** social organization (Ross 1997). *Pgm-3* is in strong linkage disequilibrium with *Gp-9* in the introduced range

and similarly shows an association of different alleles with the different social organizations (Ross 1997). Strong and uniform selection acts on these genes in the **P** form because workers adopt new egg-laying queens only if they bear the *Gp-9*^{b} allele (Keller and Ross 1998; Ross and Keller 1998). Given the apparent intensity and uniformity of selection in the **P** form and the strong association between allelic variation and social variation, the expectation is that little differentiation should exist among sites of the same form at these two loci, regardless of the geographic distance separating them, whereas strong differentiation should always exist between sites differing in social form. These expected patterns of structure were observed (fig. 2). Clearly, selection overwhelms any effects of gene flow and drift in determining the distribution of genetic variation at *Gp-9* and *Pgm-3*, a conclusion evident also from the fact that identity of social organization but not geographic proximity of study sites influences the pairwise genetic distances calculated from these loci (fig. 3).

The pattern of genetic structure for the mtDNA is complementary to that for the neutral nuclear markers because it allows separation of the maternal and biparental components of gene flow (assuming that selection does not affect the haplotype frequencies). Among-site structure constitutes a much larger proportion of the mtDNA variance than of the nuclear DNA variance (fig. 2), with the strong mtDNA differentiation at this level wholly attributable to variation among sites in the **P** form (table 2). Similarly, differentiation between the social forms appears to be more pronounced for the mitochondrial than for the nuclear genome in both Georgia and Louisiana. The relatively greater mtDNA differentiation at these two levels is unlikely to be due solely to a smaller effective population size for the mitochondrial genome and its consequently greater susceptibility to drift compared with the nuclear genome (e.g., Mitton 1994; Neigel 1997). Rather, at least part of the relatively stronger mtDNA structure within the Georgia **P** form and between sympatric social forms probably arises from greater restrictions on queen-mediated than on male-mediated gene flow predicted on the basis of the social and breeding biology of fire ants (discussed below; see also Ross and Keller 1995*a*; Ross and Shoemaker 1997).

Final considerations in evaluating the descriptions of structure derived from the different marker sets concern the unique features of the study populations. These populations undoubtedly are not at equilibrium. Fire ants have only recently colonized the study areas; the introduced populations have suffered considerable losses of genetic variation during the colonization process; and the natural dispersal has, to an unknown extent, been enhanced by human-mediated dispersal. It is possible that as the populations approach equilibrium, the magnitude of structure that we observed at the various levels could change substantially (e.g., Barton and Clark 1990; Pogson, Mesa, and Boutilier 1995). However, patterns and magnitudes of differentiation similar to those detailed here have been observed within and between the social forms of *S. invicta* in two separate regions of the native range using allozymes, microsatellites, and mtDNA (Ross et al. 1997). Differentiation between these native regions, on the other hand, generally greatly exceeds the differentiation found between Georgia and Lousiana, even though the native regions are less distant from one another than are these two states. These results are expected if gene flow regimes in fire ants are such that structure equilibrates rather quickly at localized scales but much more slowly at regional scales (e.g., Porter and Geiger 1995) and/or there is a strong historical component to regional differentiation in the native range.

Hierarchical Genetic Structure

Hierarchical F_{ST} analysis partitions total genetic variance into components occurring at each level of sampling. The value of this approach is that it allows identification of the specific scales at which limited gene flow favors differentiation. A possible disadvantage is that the approach can obscure the absolute magnitude of divergence at a given level if this divergence is attributable primarily to structure at lower levels. Thus, for instance, the use of exact tests revealed strong mtDNA differentiation between fire ants in Georgia and Louisiana (table 2), yet the hierarchical F_{ST} analysis indicated no variance at this level but very strong structure at the two subordinate levels. Biologically, the hierarchical analysis is valuable because it suggests that the mtDNA differentiation between the states can be explained by limited queen-mediated gene flow within the **P** form and between the social forms, so there is no need to invoke any additional biological factors that constrain queen dispersal at larger scales. Nonetheless, it is useful from a genetic perspective to recognize the existence of strong mtDNA differentiation at larger scales, no matter what the biological cause, and such recognition is hampered by exclusive reliance on a hierarchical F_{ST} analysis. Thus, such hierarchical analyses should always be accompanied by single-level analyses estimating exact probabilities, F_{ST} values, or pairwise genetic distances.

Gene Flow and Selection in Fire Ants

Results of this study of genetic structure confirm earlier predictions about patterns of gene flow and selection based on the social biology and breeding behavior of fire ants. The significant differentiation between Georgia and Louisiana fire ants found using neutral nuclear markers and mtDNA was anticipated because of the long distance between these states, which is orders of magnitude greater than typical dispersal distances of fire ant sexuals (Markin et al. 1971). Similar significant differentiation between fire ants from Georgia and Texas, which are separated by an even greater distance, was found earlier using six of the neutral allozyme loci used here (Ross and Shoemaker 1997). These patterns are evidence for isolation by distance that is expected to preserve for some time, or even allow a further buildup of, stochastic variation in the genetic composition of the ants that first colonized these widely separated areas.

The significant differentiation that we detected between sympatric social forms at the microsatellites (Louisiana) or at the majority of neutral nuclear markers (Georgia), together with the strong mtDNA differentiation be-

tween the forms in both states, is compatible with the earlier prediction that significant gene flow between the social forms occurs by only one of four possible routes: **M** males mating with **P** queens (Ross and Shoemaker 1993). This prediction is based partly on the finding that the phenotypes and behaviors of queens of each form, combined with form-specific worker preferences for particular queen phenotypes during queen recruitment, largely render queens of each form incapable of becoming reproductives in nests of the alternate form (Keller and Ross 1993; Ross and Keller 1995*a*; Shoemaker and Ross 1996; Ross and Shoemaker 1997; DeHeer, Goodisman, and Ross 1999). That is, queens appear not to serve as effective agents of between-form gene flow. Moreover, based on the rarity of fertile males produced by **P** nests, **P** males are unlikely to commonly mate with **M** queens, a conclusion supported by direct genetic evidence from the markers *Gp-9* and *Pgm-3* (Shoemaker and Ross 1996). On the other hand, direct genetic data from these same markers suggest that **P** queens commonly mate with **M** males at our Georgia study sites (Ross 1992, 1997; Ross and Keller 1995*b*). The magnitude of between-form gene flow via this single route is clearly not sufficient to completely homogenize variation across the two forms at the neutral nuclear loci, a conclusion that was reached also for native fire ants using allozymes and microsatellites (Ross et al. 1997) but that was not apparent from earlier studies in the introduced range using only allozymes (Ross, Vargo, and Fletcher 1987; Ross and Shoemaker 1993, 1997). Pronounced between-form mtDNA differentiation similar to that occurring in Georgia and Louisiana has also been found in native populations (Ross et al. 1997). Taken together, these results indicate that weak but significant differentiation at neutral nuclear loci, coupled with strong differentiation at the mtDNA, is a common feature of the social forms of *S. invicta* where they occur in sympatry. This differentiation stems from relatively well understood differences in the social biologies of the two forms that lead to socially induced barriers to gene flow between them.

Significant differentiation was detected among sites in the **P** form but not in the **M** form in Georgia by using mtDNA and by combining data from all of the neutral nuclear markers, a finding made more noteworthy by the fact that the **P** sites are much more closely spaced than are the **M** sites (fig. 1). These results, which parallel those found earlier for this locale and for native populations (Ross and Shoemaker 1997; Ross et al. 1997), suggest greater limitations on local gene flow in the **P** form than in the **M** form, due primarily or solely to relatively restricted queen-mediated gene flow in the **P** form. Polygyne queens that become egg-layers appear to mate in their natal nests or to disperse only short distances when they participate in mating flights (Ross and Keller 1995*a*; unpublished data), and **P** nests reproduce mainly by localized budding (Vargo and Porter 1989). In contrast, **M** queens disperse widely during their mating flights (Markin et al. 1971), and nests of this form are founded independently by such newly mated queens. Thus, differences in the reproductive and dispersal behaviors of the two forms predict the existence of the smaller-scale patterns of structure we observed. Despite presumed extensive dispersal by **M** queens, we did find significant local structure in this form using the microsatellites, consistent with long-term restrictions on gene flow at scales on the order of tens of kilometers.

Genetic variation at the selected loci *Gp-9* and *Pgm-3* is closely associated with the phenotypic and behavioral variation in queens and workers that affects patterns of intra- and interform gene flow (Ross 1997; Ross and Keller 1998; DeHeer, Goodisman, and Ross 1999). For this reason, it is illuminating to compare patterns of hierarchical structure between the selected markers and both the neutral nuclear and the mtDNA markers. The perspective provided is that selection acting on a restricted set of nuclear genes has indirect but consequential effects on a large number of unlinked neutral elements of the nuclear and mitochondrial genomes. Other population studies have identified putative loci under selection, but seldom have the effects of selection at such loci been tied directly to the distribution of variation at other unlinked, presumably neutral markers (see Koehn, Milkman, and Mitton 1976; Hasson et al. 1998; Parker et al. 1998).

Many studies of genetic structure that make use of *F* statistics or similar measures of differentiation are subject to limitations that can affect interpretations of the results. Among these, biases may arise from the choice of markers or methods of analysis, specification of the hierarchical levels of sampling may be arbitrary and immaterial to the population biology of the study organism, the partitioning of variance at each level is an average for all of the population units assigned to that level, equilibrium assumptions that underly the analyses may not be met, and patterns of gene flow and selection are likely to be dynamic (Lewontin 1972; Urbanek, Goldman, and Long 1996; Bossart and Pashley Prowell 1998; Yang 1998). These potential problems have been largely overcome in studies of fire ant structure by using many well-characterized markers of different classes, by comparing patterns of structure at various scales from several different areas in the native and introduced ranges, by looking for the specific locations of significant differentiation, and by exploiting the extensive information available on fire ant biology both to design sampling schemes that are biologically meaningful and to make clear predictions against which the results can be compared. Detailed knowledge of the natural history of a study organism coupled with the use of multiple, diverse, well-understood genetic markers may be a prerequisite for future studies if these are to avoid the criticism of being irrelevant to understanding the evolutionary causes and consequences of population genetic structure (Bossart and Pashley Prowell 1998).

Acknowledgments

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APPENDIX

Allele and Haplotype Frequencies for 29 Nuclear Loci and the mtDNA in *Solenopsis invicta* Sampled at Nine Sites in its Introduced Range

	Georgia							Louisiana	
	M Form			**P** Form				**M** Form	**P** Form
	Walnut Grove	Pennington	Eastville	Walton Academy	Monroe	Mount Vernon	Snow's Mill	WLM	WLP
Nuclear loci									
Neutral Allozymes									
Aat-2	**84**	**80**	**140**	**126**	**110**	**130**	**130**	**148**	**118**
100	0.929	0.863	0.900	0.960	0.918	0.915	0.962	0.966	0.949
144	0.071	0.138	0.100	0.040	0.082	0.085	0.038	0.034	0.051
Acoh-1	**84**	**80**	**140**	**126**	**110**	**130**	**130**	**140**	**118**
82	0.214	0.112	0.207	0.111	0.127	0.115	0.108	0.214	0.093
100	0.786	0.887	0.793	0.889	0.873	0.885	0.892	0.786	0.907
Acoh-5	**84**	**80**	**140**	**126**	**108**	**130**	**130**	**144**	**118**
93	0.286	0.262	0.286	0.278	0.306	0.192	0.246	0.292	0.195
100	0.714	0.738	0.714	0.722	0.694	0.808	0.754	0.708	0.805
Acyl	**78**	**68**	**120**	**126**	**110**	**130***	**92**	**146***	**116**
93	0.128	0.132	0.150	0.143	0.182	0.115	0.185	0.151	0.121
100	0.872	0.868	0.850	0.857	0.818	0.885	0.815	0.849	0.879
Est-4	**82**	**80**	**140**	**128**	**108**	**126**	**130**	**146**	**116**
100	0.659	0.613	0.586	0.633	0.537	0.627	0.662	0.651	0.612
160	0.341	0.387	0.414	0.367	0.463	0.373	0.338	0.349	0.388
G3pdh-1	**82**	**80**	**140**	**130**	**106**	**128**	**130**	**146**	**118**
40	0.232	0.375	0.307	0.346	0.292	0.273	0.262	0.438	0.424
100	0.768	0.625	0.693	0.654	0.708	0.727	0.738	0.562	0.576
Pgm-1	**82**	**80**	**140**	**130**	**110**	**130**	**130**	**146**	**118**
96	0.195	0.237	0.179	0.138	0.200	0.215	0.138	0.034	0.042
100	0.793	0.762	0.814	0.862	0.800	0.785	0.862	0.966	0.958
102	0.012	0	0.007	0	0	0	0	0	0
Pgm-3[a]	**82**	**80**	**140**	—	—	—	—	**146**	—
89	0.207	0.275	0.257	—	—	—	—	0.425	—
100	0.793	0.725	0.743	—	—	—	—	0.575	—
Codominant RAPDs									
Opc11-1	**84***	**80**	**136**	**130**	**110**	**130**	**130**	**148**	**118**
S	0.036	0.125	0.096	0.046	0.027	0.062	0.062	0.068	0.085
F	0.964	0.875	0.904	0.954	0.973	0.938	0.938	0.932	0.915
Opc13-1	**84**	**80**	**138**	**130**	**110**	**130**	**130**	**148**	**118**
S	0.774	0.813	0.732	0.792	0.718	0.792	0.823	0.851	0.831
F	0.226	0.188	0.268	0.208	0.282	0.208	0.177	0.149	0.169
Opc19-1	**82**	**80**	**132**	**130**	**108**	**130**	**130**	**144**	**114**
S	0.134	0.225	0.205	0.131	0.157	0.162	0.108	0.125	0.184
M	0.671	0.663	0.636	0.700	0.676	0.700	0.700	0.431	0.491
F	0.037	0.013	0.038	0.038	0.102	0.069	0.085	0.306	0.237
xF	0.159	0.100	0.121	0.131	0.065	0.069	0.108	0.139	0.088
Ubc406-1	**78**	**80**	**126***	**124**	**106**	**130***	**130**	**120**	**104**
S	0.628	0.600	0.667	0.653	0.736	0.677	0.692	0.625	0.596
F	0.372	0.400	0.333	0.347	0.264	0.323	0.308	0.375	0.404
Ubc414-1	**82**	**80**	**134**	**130**	**108**	**130**	**130**	**146**	**118**
S	0.207	0.225	0.127	0.146	0.278	0.185	0.162	0.260	0.288
M	0.329	0.275	0.425	0.362	0.315	0.392	0.400	0.192	0.246
F	0.463	0.500	0.448	0.492	0.407	0.423	0.438	0.548	0.466
Microsatellites									
Sol-6	**76**	**80**	**138**	**128**	**108**	**130**	**130**	**148**	**116**
95	0.013	0	0	0	0	0	0	0	0
109	0.079	0.150	0.145	0.086	0.120	0.100	0.131	0.027	0.017
111	0	0	0	0.016	0	0	0	0	0
113	0.671	0.688	0.652	0.703	0.731	0.692	0.700	0.628	0.759
115	0.237	0.162	0.203	0.195	0.148	0.208	0.169	0.345	0.224
Sol-11	**78**	**80**	**138**	**130**	**108**	**130**	**130**	**146**	**118**
123	0	0	0	0	0.009	0	0	0	0
143	0.372	0.237	0.413	0.423	0.426	0.462	0.438	0.027	0.068
145	0.115	0.150	0.051	0.062	0.074	0.069	0.023	0	0
147	0.103	0.162	0.101	0.131	0.093	0.138	0.108	0	0.144

APPENDIX
Continued

	Georgia							Louisiana	
	M Form			**P** Form				**M** Form	**P** Form
	Walnut Grove	Pennington	Eastville	Walton Academy	Monroe	Mount Vernon	Snow's Mill	WLM	WLP
149	0	0	0	0	0.009	0	0	0	0
151	0.167	0.262	0.254	0.185	0.176	0.138	0.238	0.623	0.661
155	0.244	0.188	0.181	0.200	0.213	0.192	0.192	0.342	0.127
171	0	0	0	0	0	0	0	0.007	0
Sol-18	**78**	**80**	**138**	**130**	**108**	**130***	**130**	**148**	**118**
125	0.833	0.837	0.833	0.869	0.815	0.838	0.854	0.919	0.924
127	0.167	0.150	0.167	0.131	0.185	0.162	0.146	0.081	0.076
129	0	0.013	0	0	0	0	0	0	0
Sol-20‡	**80**	**80**	**138**	**130**	**108***	**130**	**130**	**148***	**118**
114	0	0	0	0.008	0	0	0	0	0
124	0.237	0.138	0.094	0.169	0.093	0.138	0.115	0.115	0.212
126	0.400	0.475	0.493	0.408	0.426	0.462	0.377	0.345	0.322
128	0.225	0.075	0.167	0.177	0.222	0.100	0.200	0.264	0.085
130	0	0.025	0	0	0	0	0.023	0	0.008
132	0	0	0	0	0	0.008	0	0	0
136	0	0	0	0	0	0	0	0.014	0
144	0.025	0.112	0.094	0.100	0.176	0.177	0.100	0.264	0.347
146	0	0	0.007	0	0	0	0	0	0
150	0.038	0	0	0.031	0	0	0.008	0	0
152	0.075	0.175	0.145	0.108	0.083	0.115	0.177	0	0.025
Sol-42‡	**78†**	**80**	**138**	**130***	**108**	**130**	**130***	**148***	**118**
117	0.397	0.338	0.457	0.338	0.306	0.354	0.362	0.297	0.466
119	0.205	0.237	0.181	0.177	0.241	0.215	0.169	0.169	0.220
121	0.115	0.150	0.094	0.131	0.157	0.092	0.085	0.088	0.059
123	0	0	0.007	0	0	0	0	0	0
125	0	0	0.007	0.023	0	0	0.015	0	0.008
127	0	0	0	0.008	0	0	0	0	0
129	0	0	0	0.008	0.009	0	0	0	0
131	0.231	0.162	0.181	0.215	0.176	0.208	0.262	0.284	0.161
141	0	0	0.007	0	0	0.031	0.015	0	0
143	0.051	0.112	0.058	0.100	0.083	0.100	0.092	0.155	0.085
145	0	0	0.007	0	0.028	0	0	0.007	0
Sol-49	**78**	**80**	**138**	**130†**	**108**	**130**	**130**	**146**	**118**
142	0.218	0.213	0.174	0.177	0.176	0.100	0.123	0.096	0.085
148	0.115	0.125	0.116	0.062	0.056	0.023	0.062	0.151	0.220
160	0.436	0.325	0.377	0.477	0.444	0.515	0.523	0.404	0.373
162	0.064	0.150	0.159	0.046	0.056	0.131	0.092	0.021	0.042
164	0.013	0	0	0	0.009	0.008	0.008	0	0
166	0.154	0.188	0.174	0.238	0.259	0.223	0.192	0.322	0.280
168	0	0	0	0	0	0	0	0.007	0
Sol-55	**74**	**78**	**138**	**82**	**108**	**128**	**126**	**144**	**118**
149	0.662	0.474	0.391	0.415	0.620	0.641	0.389	0.597	0.636
151	0.108	0.090	0.036	0.061	0.046	0.070	0.079	0.063	0.042
152	0.014	0.090	0.116	0.049	0.056	0.047	0.119	0.097	0.076
153	0.014	0.013	0.014	0.012	0.009	0.008	0.008	0.007	0
155	0.081	0.103	0.138	0.098	0.130	0.070	0.143	0.049	0.085
157	0	0.038	0.029	0.085	0.019	0.070	0.024	0.007	0.025
159	0.122	0.192	0.275	0.280	0.120	0.094	0.238	0.181	0.136
Dominant RAPDs[b]									
$Opa9_{410}$	**82**	**80**	**132**	**130**	**108**	**130**	**130**	**140**	**114**
−	0.357	0.362	0.430	0.499	0.413	0.451	0.484	0.434	0.331
+	0.643	0.639	0.570	0.501	0.587	0.549	0.517	0.566	0.669
$Opa9_{680}$	**80**	**80**	**126**	**130**	**108**	**128**	**130**	**142**	**114**
−	0.923	0.936	0.900	0.903	0.893	0.867	0.903	0.806	0.839
+	0.078	0.064	0.100	0.097	0.107	0.133	0.097	0.194	0.162
$Opa9_{1100}$	**80**	**78**	**126**	**130**	**108**	**128**	**130**	**136**	**114**
−	0.238	0.366	0.339	0.187	0.155	0.258	0.256	0.278	0.239
+	0.762	0.634	0.661	0.813	0.845	0.743	0.744	0.722	0.761
$Opc8_{480}$	**78**	**70**	**128**	**124**	**102**	**126**	**126**	**138**	**112**
−	0.848	0.879	0.858	0.843	0.919	0.960	0.873	0.941	0.846
+	0.152	0.121	0.142	0.157	0.081	0.040	0.127	0.060	0.154

APPENDIX
Continued

	Georgia							Louisiana	
	M Form			**P** Form				**M** Form	**P** Form
	Walnut Grove	Pennington	Eastville	Walton Academy	Monroe	Mount Vernon	Snow's Mill	WLM	WLP
Opc11$_{960}$	**82**	**80**	**134**	**130**	**110**	**130**	**130**	**148**	**118**
−	0.767	0.792	0.793	0.842	0.776	0.869	0.785	0.547	0.524
+	0.233	0.208	0.207	0.158	0.224	0.131	0.215	0.453	0.476
Opc13$_{1600}$	**84**	**80**	**138**	**130**	**108**	**130**	**130**	**148**	**118**
−	0.278	0.325	0.276	0.396	0.413	0.256	0.433	0.389	0.473
+	0.722	0.675	0.724	0.604	0.587	0.744	0.567	0.611	0.527
Opc13$_{1800}$	**84**	**80**	**138**	**130**	**108**	**130**	**130**	**148**	**118**
−	0.818	0.823	0.799	0.833	0.893	0.869	0.869	0.823	0.738
+	0.182	0.177	0.201	0.167	0.107	0.131	0.131	0.177	0.262
Ubc414$_{1600}$	**82**	**80**	**134**	**130**	**108**	**130**	**130**	**146**	**118**
−	0.976	0.895	0.906	0.953	0.972	0.937	0.937	0.937	0.912
+	0.025	0.105	0.094	0.047	0.028	0.063	0.063	0.063	0.088
Selected protein loci									
Gp-9[c]	**84**	**80**	**140**	**130**	**110**	**128**	**130**	**146**	**118**
95	0	0	0	0.500	0.500	0.492	0.500	0	0.500
100	1.0	1.0	1.0	0.500	0.500	0.508	0.500	1.0	0.500
Pgm-3[a]	—	—	—	**130**	**106**	**130**	**128**	—	**118**
89	—	—	—	0.623	0.566	0.608	0.586	—	0.653
100	—	—	—	0.377	0.434	0.392	0.414	—	0.347
mtDNA	**43**	**38**	**68**	**63**	**54**	**60**	**65**	**72**	**59**
A	0.837	0.895	0.868	0.159	0.167	0.700	0.769	0.750	0.949
B	0.163	0.053	0.118	0.032	0	0	0	0.222	0.034
C	0	0	0	0.810	0.833	0.300	0.231	0.014	0.017
D	0	0.053	0.015	0	0	0	0	0	0
Y	0	0	0	0	0	0	0	0.014	0

NOTE.—The numbers of haploid genomes on which each estimate is based are shown in bold. For the codominant neutral nuclear markers, significant excesses of homozygotes above Hardy-Weinberg equilibrium proportions (HWEP) are indicated by *, whereas significant excesses of heterozygotes are indicated by †. Loci with a combined probability of conformity to HWEP over all sites of less than 0.05 are indicated by ‡. Allele designations for the allozymes, codominant RAPDs, and *Gp-9* indicate relative band mobilities for homozygotes, designations for the dominant RAPDs indicate the allele enabling amplification (+) and the allele precluding it (−), and designations for the microsatellites indicate the length of the amplification product.

[a] *Pgm-3* is regarded as a neutral locus in the monogyne (**M**) form and as a selected locus in the polygyne (**P**) form.

[b] Allele frequencies for the dominant RAPDs were estimated from the frequencies of band absence using the Taylor expansion method of Lynch and Milligan (1994).

[c] The *95* allele of *Gp-9* is referred to as the *b* allele, and the *100* allele as the *B* allele, in previous papers (Ross 1997; Keller and Ross 1998; DeHeer, Goodisman, and Ross 1999).

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