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Alternative Genetic Foundations for a Key Social Polymorphism in Fire Ants

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ABSTRACT

Little is known about the genetic foundations of colony social organization. One rare example in which a single major gene is implicated in the expression of alternative social organizations involves the presumed odorant-binding protein gene *Gp-9* in fire ants. Specific amino acid substitutions in this gene invariably are associated with the expression of monogyny (single queen per colony) or polygyny (multiple queens per colony) in fire ant species of the *Solenopsis richteri* clade. These substitutions are hypothesized to alter the abilities of workers to recognize queens and thereby regulate their numbers in a colony. We examined whether these same substitutions underlie the monogyny/polygyny social polymorphism in the distantly related fire ant *S. geminata*. We found that *Gp-9* coding region sequences are identical in the polygyne and monogyne forms of this species, disproving our hypothesis that one or a few specific amino acid replacements in the protein are necessary to induce transitions in social organization in fire ants. On the other hand, polygyne *S. geminata* differs genetically from the monogyne form in ways not mirrored in the two forms of *S. invicta*, a well-studied member of the *S. richteri* clade, supporting the conclusion that polygyny did not evolve via analogous routes in the two lineages. Specifically, polygyne *S. geminata* has lower genetic diversity and different gene frequencies than the monogyne form, suggesting that the polygyne form originated via a founder event from a local monogyne population. These comparative data suggest an alternative route to polygyny in *S. geminata* in which loss of allelic variation at genes encoding recognition cues has led to a breakdown in discrimination abilities and the consequent acceptance of multiple queens in colonies.

A major goal in evolutionary biology is to understand the genetic architecture underlying complex adaptations (Orr and Coyne 1992; Orr 1998; Glazier *et al.* 2002). Nowhere is this interest in the architecture of adaptation stronger than in the study of animal social behavior (Robinson *et al.* 1997; Robinson 1999; Young *et al.* 1999; Ross and Keller 2002; Ben-Shahar *et al.* 2002). A number of studies that demonstrate heritable influences on key traits associated with complex social behavior now exist (*e.g.*, Page and Robinson 1994; O'Donnell 1996; Hunt *et al.* 1998; Osborne and Oldroyd 1999; Fraser *et al.* 2000; Plateaux-Quénu *et al.* 2000; Buschinger and Schreiber 2002; Hughes *et al.* 2003; reviewed in Robinson *et al.* 1997; Ross and Keller 2002). However, progress in identifying the numbers and effects of specific genes influencing these traits has been slow. Continued progress in these efforts is critical for understanding how specific gene products function to shape social behavior, as well as for constructing improved evolutionary models of the origins of eusociality, one of relatively few major transitions in the history of life (Maynard Smith and Szathmáry 1995).

One rare example in which a candidate gene of major effect on a key social trait has been identified involves the gene *Gp-9* in fire ants. Many fire ant species display two fundamentally different types of social organization distinguished by, among other features, the number of egg-laying queens in a colony (Ross and Keller 1995). Colonies of the monogyne type are headed by a single egg-laying queen, whereas those of the polygyne type contain multiple queens. In *Solenopsis invicta*, the best-studied fire ant species, the form of social organization displayed by a colony depends on the *Gp-9* genotypic composition of the worker force (Ross 1997; Ross and Keller 1998). Two major classes of coding region variants exist at *Gp-9*, designated the *B*-like and *b*-like classes (Krieger and Ross 2002). Colonies of the monogyne form of *S. invicta* contain workers bearing only *B*-like alleles, whereas colonies of the polygyne form contain substantial proportions of workers bearing *b*-like alleles. Krieger and Ross (2002) determined that the product of *Gp-9* is a member of a family of proteins that mediate perception of chemical cues from conspecifics in insects, known as odorant-binding proteins (Pelosi and Maida 1995; Hekmat-Scafe *et al.* 2002). Fire ant workers regulate colony queen number on the basis of cuticular chemical signals produced by individual queens

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(Keller and Ross 1998; Ross and Keller 2002), so it has been hypothesized that workers with *Gp-9* alleles of the different classes may differ in their abilities to recognize queens, leading to different tolerance thresholds for queen number in monogyne and polygyne colonies. Apart from *Gp-9*, the two forms of *S. invicta* strongly resemble one another genetically where they occur in sympatry, typically harboring the same alleles and levels of variation at numerous marker genes. This similarity between the forms over many genes, interpreted as the signature of substantial interform gene flow (Shoemaker and Ross 1996; Ross *et al.* 1997, 1999; Ross 2001), provides strong ancillary evidence that *Gp-9* (and, perhaps, genes with which it is in strong disequilibrium) plays a direct role in determining colony social organization in *S. invicta*.

Krieger and Ross (2002) also sequenced *Gp-9* from polygyne fire ants of three other species closely related to *S. invicta* (all are members of the *S. richteri* clade; see Figure 1). In every case, these specimens possessed *b*-like alleles, suggesting that particular substitutions at *Gp-9* induce the expression of polygyny in fire ants. Specifically, all *b*-like alleles share three amino acids that are absent from all *B*-like alleles over the 134 residues of the mature protein (see Figure 2), implying that one or more of these amino acids may be crucial to the functional role of GP-9 protein in queen discrimination. The four species of the *S. richteri* clade studied by Krieger and Ross (2002) are the only members of a larger group of South American fire ant species, known as the *S. saevissima* species group (Trager 1991; Pitts 2002), that are reported to exhibit polygyny as an alternative form of social organization. Outside of this group, polygyny in the genus is well documented only for *S. geminata* (Adams *et al.* 1976; Mackay *et al.* 1990), a member of a primarily North American clade of fire ants (Figure 1). We wished to determine whether the conserved *b*-like residues invariably associated with polygyny in the *S. richteri* clade are associated with polygyny in *S. geminata* as well. Because polygyny in *S. geminata* apparently evolved independently from polygyny in the *S. richteri* clade, comparative sequence analysis of *Gp-9* in this species provides a test of the hypothesis that one or few specific amino acid replacements in the protein product are necessary determinants of colony social organization in Solenopsis and, perhaps, in other groups of ants.

The *Gp-9* sequence of a monogyne *S. geminata* specimen was determined previously by Krieger and Ross (2002). As expected, this sequence features the characteristic *B*-like residues methionine and valine at positions 95 and 139, respectively (Figure 2). However, the sequence includes the *b*-like glycine residue at position 42, suggesting that the amino acid at this position may not be essential to the function of GP-9 protein in determining social organization. This information allows us to further refine our working hypothesis to predict that polygyny in *S. geminata* is associated with a *Gp-9* allele coding the characteristic *b*-like isoleucine residue at position 95, position 139, or both positions.

In this article we characterize the *Gp-9* sequences of polygyne *S. geminata* from northern Florida. We also describe in detail the population genetic structure of both social forms of this species in this area, and we compare these results with those from *S. invicta* in order to comprehensively evaluate the likelihood that polygyny evolved via analogous routes in both lineages. We find that the polygyne *S. geminata* that we studied possess the same *Gp-9* allele reported previously for the monogyne form, indicating that the molecular mechanisms underlying regulation of social organization in fire ants are not universal. On the other hand, the polygyne study population appears to have experienced a recent population bottleneck that resulted in a loss of genetic diversity and the emergence of reproductive isolation from the monogyne form. These features, which are uncharacteristic of polygyny in *S. invicta*, support the conclusion that the origin of polygyny in the genus Solenopsis has followed at least two different evolutionary routes, with alternative genetic foundations for regulation of colony queen number characteristic of each.

MATERIALS AND METHODS

Sample collection and processing: Samples of *S. geminata* were collected from 37 nests in Gainesville, Florida and the immediately surrounding area (Alachua County) in the spring of 2002. Samples of 8 additional nests of this species were collected in Leon County, Florida, some 200 km distant from the other nests, during the same period. Many adult workers were collected from each nest. In addition, worker brood and wingless (presumably egg-laying) queens were collected opportunistically from 22 and 7 of the nests, respectively. Collected ants either were frozen in the field on dry ice or were returned alive to the laboratory for further processing. All samples were stored in a laboratory freezer at −80° pending genetic analysis. Collected ants were identified as *S. geminata* by J. P. Pitts on the basis of the morphological characteristics of voucher specimens from several nests (Trager 1991). Species identity was further confirmed by use of diagnostic allozyme markers on samples from every nest (see below).

The mating status of a subset of the wingless queens collected from polygyne nests was determined by one of two methods. Nine queens from three nests were dissected and the spermatheca (sperm storage organ) of each was examined; mated queens possessed opaque, whitish spermathecae whereas unmated queens possessed translucent spermathecae (*e.g.*, Goodisman and Ross 1999). Fourteen queens from four additional nests were isolated in laboratory rearing units with workers and brood from their parent colony (*e.g.*, Ross 1997) and brood production patterns were tracked over the ensuing 6-week period; mated queens produced worker brood by the end of this period whereas unmated queens produced males or no brood.

Total DNA was extracted from individual worker ants from each nest with the Puregene kit (Gentra Systems, Research Triangle Park, NC) by following the manufacturer's instructions for solid tissue samples.

Allozyme electrophoresis: Genotypes of *S. geminata* workers were determined at eight allozyme loci. Electrophoresis was conducted in 14% horizontal starch gels using extracts of soluble proteins derived from individual larval (*Hexa*), pupal (*Acoh-5*

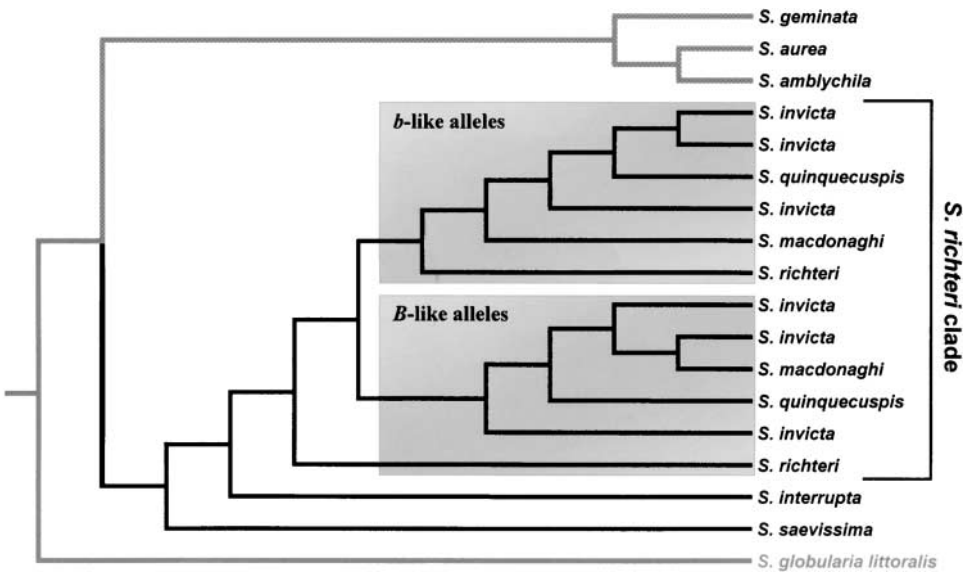

FIGURE 1.—Phylogeny of *Gp-9* alleles in fire ants (from KRIEGER and ROSS 2002). The 2200 bp of sequence determined for each allele includes the coding regions, introns, and 3′ flanking region. Each allele is labeled with the name of the species in which it was found (several alleles of each class were discovered in the multiple samples of *S. invicta* sequenced). Branches for the native South American fire ant species placed in the *S. saevissima* species group are black; the socially polymorphic members of this group comprise the *S. richteri* clade. Branches for the primarily North American clade of fire ants that includes *S. geminata* are stippled. The two classes of *Gp-9* alleles found in the *S. richteri* clade are identified by shaded boxes. The phylogeny is rooted using the sequence from the non-fire-ant species *S. globularia littoralis* as an outgroup.

and *Est-6*), or adult (*Est-2*, *G3pdh-1*, *Gpi*, *Fbp-2*, and *Odh*) workers (for methods, see SHOEMAKER *et al.* 1992). Among the loci polymorphic in *S. geminata*, *Gpi* and *Odh* were developed by ROSS *et al.* (1987), whereas *Acoh-5*, *Est-6*, *Fbp-2*, and *Hexa* were developed in the course of this study. Mendelian inheritance of the allelic enzymes distinguishable by electrophoresis was demonstrated by ROSS *et al.* (1988) and by virtue of the genotype distributions observed in monogyne colonies in this study. The loci *Est-2* and *G3pdh-1* were scored because alleles fixed in *S. geminata* are not found in the only other fire ant species that also occur in the study area, *S. invicta* and *S. xyloni* (ROSS *et al.* 1987; our unpublished data). Thus, these loci were used to check our *S. geminata* samples for evidence of introgression from these other species. Between 8 and 60 (mean is 22) workers from each nest were genotyped at each allozyme locus.

Microsatellite analysis: Genotypes of *S. geminata* workers were determined at three microsatellite loci (*Sol-11*, *Sol-42*, and *Sol-49*). These loci constitute a subset of the polymorphic dinucleotide-repeat loci developed for *S. invicta* by KRIEGER and KELLER (1997). Genotypes were determined by means of multiplex PCR reactions performed with fluorescently labeled primers (Applied Biosystems, Foster City, CA), followed by laser detection of the denatured PCR products separated in polyacrylamide gels. Primers used to amplify the loci were those described by KRIEGER and KELLER (1997), with one primer of each pair distinctively labeled at the 5′ end [*Sol-11.rev* (6FAM), *Sol-42.for* (NED), and *Sol-49.rev* (HEX)] so that the products of all three loci could be detected simultaneously on single gels. The three loci were amplified together in 20-μl reaction mixtures containing 1.6× PCR buffer (2.4 mM $MgCl_2$), 1× Q-Solution (QIAGEN, Valencia, CA), 300 μM dNTPs, 0.25 μM *Sol-11* primers, 0.38 μM *Sol-42* primers, 0.25 μM *Sol-49* primers, 1.5 units of *Taq* polymerase, and 2 μl of hydrated template DNA, using the cycling profile of KRIEGER and KELLER (1997). Following PCR, 2.0 μl of product from each multiplex reaction was combined with 2.0 μl deionized formamide, 0.5 μl GeneScan-400 (ROX) size standard (Applied Biosystems), and 0.5 μl loading dye in a 0.5-ml tube. The mixture was denatured for 3 min at 95°, and then 1.3 μl was loaded in a polyacrylamide gel (4.8% acryl-bisacrylamide, 6 M urea). In addition to the 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 genotypes were scored using GENESCAN 3.1.2 software (Applied Biosystems). Between 4 and 10 (mean is 8.5) workers from each nest were genotyped at each microsatellite locus.

mtDNA sequencing: A 784-bp portion of the COI gene from the mtDNA genome of *S. geminata* workers was sequenced. The PCR reaction mixes, sequences of external PCR and sequencing primers, and thermal cycling conditions are described in SHOEMAKER *et al.* (2000). mtDNA amplicons were purified for sequencing using Agencourt magnetic beads, and the purified products were used directly in standard fluorescent cycle-sequencing PCR reactions (ABI Prism Big Dye terminator chemistry, Applied Biosystems). Because monogyne colonies comprise simple family groups of single matrilines (ROSS *et al.* 1988), the mtDNA of only a single worker per monogyne nest was sequenced. Polygyne colonies comprise multiple matrilines, so the mtDNA of four workers per nest was sequenced.

***Gp-9* sequence analyses:** We sequenced the entire coding regions of the *Gp-9* gene from two wingless (presumably egg-laying), mated *S. geminata* queens collected from separate confirmed polygyne nests. Wingless queens were selected as source material because such females invariably bear *b*-like alleles in the polygyne form of *S. invicta* (ROSS and KELLER 1998) and, presumably, other *S. richteri*-clade species (KRIEGER and ROSS 2002). The initial template DNA for PCR amplification of *Gp-9* was single-stranded cDNA synthesized from oligo(dT_{18})-primed RNA. Total RNA was isolated (RNeasy Mini kit, QIAGEN) and reverse transcribed with avian myeloblastosis reverse transcriptase (Roche Molecular Biochemicals, Indianapolis) for 30 min at 42°. PCR reactions were set up in 20-μl reaction mixtures containing 1× PCR buffer (1.5 mM $MgCl_2$), 500 μM dNTPs, 0.5 μM each of primers *Gp-9/-33.for* (5′-CATTCAAAG TACAGTAGAATAACTGCC-3′) and *Gp-9_490.rev* (5′-GTATG CCAGCTGTTTTTAATTGC-3′), located in the 5′ and 3′ flanking sequences of *Gp-9*, respectively, 1.5 units of proofreading DNA polymerase (TaqPlus Precision, Stratagene, La Jolla, CA), and 1 μl of template DNA. Amplification was performed using the following cycling profile: 30 cycles of 92° (20 sec),

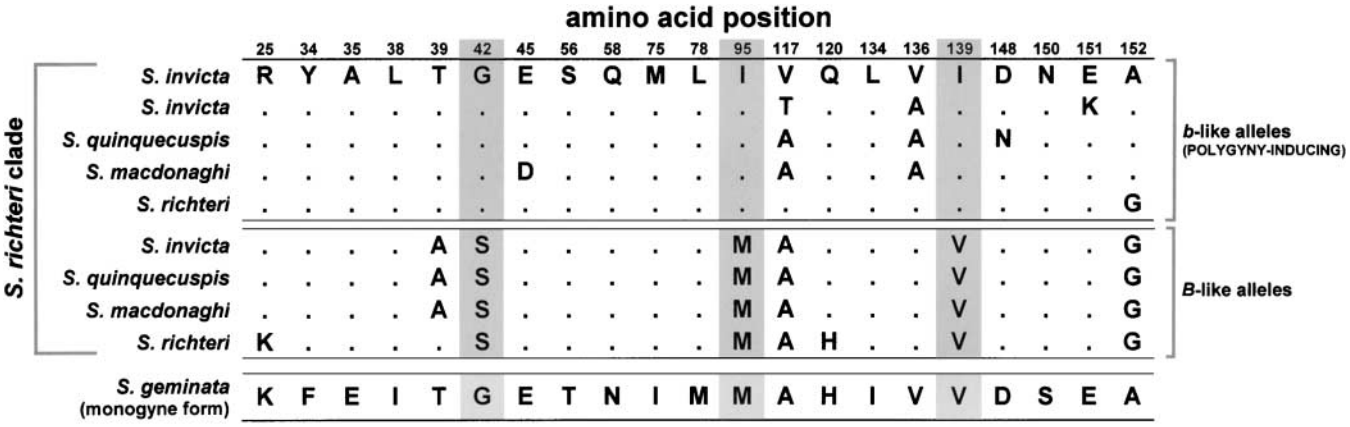

FIGURE 2.—Consensus of amino acid sequences (variable sites only) for *Gp-9* alleles in the socially polymorphic *S. richteri*-clade species and in monogyne *S. geminata* (from KRIEGER and ROSS 2002). Residues identical to those in the first allele listed are indicated by periods. Alleles of the *b*-like class invariably are associated with polygyny in the *S. richteri* clade. Positions of the three amino acid residues that distinguish all *b*-like from all *B*-like alleles in this clade are shaded. No synonymous substitutions occur among sequences at the sites depicted.

58° (30 sec), and 72° (1 min), and a final elongation step at 72° (10 min). The resulting PCR product was gel purified (QIAquick gel extraction kit, QIAGEN) and cloned into a pCR 4-TOPO vector (Invitrogen, San Diego), which was used to transfect competent *Escherichia coli* cells (TOP10F, Invitrogen). Eight clones representing the PCR amplicon of each queen were sequenced using ABI Prism Big Dye terminator chemistry. This number of clones was sequenced to detect alternate alleles if the focal individuals were heterozygous (the binomial probability of not finding two sequences in such individuals is <0.005).

To efficiently screen *S. geminata* workers for the presence of *b*-like nucleotide substitutions at *Gp-9*, we designed two different two-stage PCR assays that distinguish *b*-like from *B*-like sequences in all fire ant species studied to date. One assay detects the *b*-like substitution at codon 95, whereas the other detects the *b*-like substitution at codon 139 (Figure 2). We screened for these two substitutions separately because either or both may underlie the functional differences in the allelic GP-9 proteins. In the first stage of both assays, a segment of *Gp-9* was amplified using primers that bind to invariant sites in all fire ant species just upstream and downstream of codons 95 and 139, respectively. PCR reactions were set up in 20-μl reaction mixtures containing 1.6× PCR buffer (2.4 mM $MgCl_2$), 1× Q-solution, 100 μM dNTPs, 0.5 μM each of primers *Gp-9.for* (5′-GGAGCTGATTATGATGAAGAGAAAAT-3′) and *Gp-9.rev* (5′-AGCACAGCTTCAGCTGCTAAGA-3′), 1 unit of *Taq* DNA polymerase, and 2 μl of template DNA. The following cycling profile was used: initial denaturation at 92° (2 min); 35 cycles of 92° (45 sec), 57° (45 sec), and 72° (1 min); and a final step at 72° (5 min). After confirming successful amplification of the predicted 380-bp product via electrophoresis of an aliquot in an ethidium bromide-stained agarose gel, a 1:200 dilution of this product was used as the template for two separate second-stage PCRs. The stage-1 PCR product was used as the template for the stage-2 PCRs (rather than genomic DNA) because this enhanced the sensitivity of and reduced variation in the assays.

In the two separate stage-2 PCRs, primers fully complementary to *b*-like but not *B*-like sequences at either codon 95 or 139 were employed to achieve selective amplification of the same gene segment amplified in the first stage. The selective stage-2 primers are identical to those used initially, but include additional 3′ nucleotides matching the diagnostic nucleotides of codons 95 and 139 in all *b*-like alleles. Each of these selective stage-2 primers was paired with the appropriate general primer from the stage-1 PCR to amplify templates with *b*-like sequences at either codon 95 or 139; thus, the presence of the predicted 380-bp amplicon signified the presence of a *b*-like sequence at either codon (depending on the particular assay) in the source individual. Reactions in the stage-2 PCR for *b*-like codon 139 were set up in 20-μl reaction mixtures containing 1× PCR buffer, 100 μM dNTPs, 0.08 μM each of primers *Gp-9.for* and *all_b.rev* (5′-AGCACAGCTTCAGCTGCTAAGAT-3′), 1 unit of *Taq* DNA polymerase, and 2 μl of diluted product from the stage-1 PCR. The cycling profile was identical to that for the stage-1 PCR, but with an annealing temperature of 69.3°. Reactions in the stage-2 PCR for *b*-like codon 95 were set up in 20-μl reaction mixtures containing 1× PCR buffer, 100 μM dNTPs, 0.04 μM each of primers *all_b.for* (5′-GGAGCT GATTATGATGAAGAGAAAATA-3′) and *Gp-9.rev*, 1 unit of platinum *Taq* DNA polymerase (Invitrogen), and 2 μl of diluted product from the stage-1 PCR. The following cycling profile was used for this reaction: initial denaturation at 94° (2 min) and 16 cycles of 94° (30 sec), 65° (30 sec), and 72° (35 sec). Preliminary experiments using template DNA from fire ants of eight species whose *Gp-9* was sequenced by KRIEGER and ROSS (2002) confirmed that ants with *b*-like alleles consistently yielded an intense 380-bp gel band in the specific assay for each codon, whereas ants lacking alleles of this class did not. Several such individuals bearing or lacking *b*-like alleles were included in each assay as positive or negative controls, respectively.

Three *S. geminata* workers from each polygyne nest (n = 51), as well as two from each monogyne nest (n = 54), were surveyed for *b*-like sequences at each of the two crucial codons using these PCR assays.

Phylogenetic analysis of mtDNA sequences: A minimum spanning tree (ROHLF 1973) representing the relationships of the *S. geminata* sequence haplotypes was constructed using the program ARLEQUIN 2.000 (SCHNEIDER *et al.* 2000). The distance matrix used in this procedure was composed of the square of the nucleotide differences between haplotypes. Sequences obtained from the congeners *S. aurea* and *S. xyloni*, which are also members of the primarily North American fire ant clade (TRAGER 1991), were included as outgroups.

Assay for Wolbachia: *S. geminata* workers were screened for the presence of the bacterial endosymbiont Wolbachia by means of a diagnostic PCR assay, using the primers *Wsp*81F

and *Wsp*691R [for methods, see Zhou *et al.* (1998) and Shoemaker *et al.* (2000)]. These primers amplify a 575- to 625-bp portion of a gene encoding the major surface protein of Wolbachia (Braig *et al.* 1998; Zhou *et al.* 1998). Primers for a nuclear EF-1α gene were included in the PCRs to provide an internal positive control for successful amplification. All samples for which the mtDNA was sequenced were screened for Wolbachia.

Determination of colony social organization: The study of Ross *et al.* (1988) revealed that monogyne *S. geminata* colonies have a simple family structure in which all workers are the daughters of a single queen mated to a single (haploid) male. Thus, we determined that a given nest was monogyne after inspecting the arrays of worker genotypes at all nine polymorphic nuclear loci and confirming that the genotype identities and proportions were consistent with simple family structure. Nests with more complex genotype arrays were considered to be polygyne. Polygyny was further confirmed in several such nests by recovering multiple wingless, mated queens in the field.

Colony and population genetic data analyses: Allele frequencies at the nine nuclear loci were estimated for the monogyne and polygyne forms separately, as well as for the metapopulation as a whole, using the program RELATEDNESS 5.0 (Queller and Goodnight 1989); nests were weighted equally for these calculations. Relatedness of nestmate workers in each form was estimated from genotype distributions using the same program. For the monogyne form, the reference population for relatedness estimation was defined as all monogyne nests, with allele frequency differentiation between the Alachua County and Leon County nests taken into account (by employing the "deme" option in RELATEDNESS 5.0). For the polygyne form, the reference population initially was defined as the entire metapopulation, and, because of differences in levels of variation between the two classes of nuclear markers, relatedness was estimated using just the microsatellites as well as all loci combined. Polygyne nestmate relatedness also was estimated while excluding monogyne nests from the reference population. Finally, relatedness between polygyne workers collected from different nests was estimated using the metapopulation as the reference population. For all relatedness calculations, nests were weighted equally and standard errors and their derivative 95% confidence limits were obtained by jackknifing over nests or loci.

Genotype proportions in the two forms were tested for conformity to Hardy-Weinberg expectations (HWE) using exact tests implemented in the program GENEPOP 3.3 (Raymond and Rousset 1995a). To avoid the use of nonindependent genotypes, a single genotype per nest was drawn at random (with replacement) for each of 100 tests conducted (*e.g.*, Ross *et al.* 1997). Fisher's method of combining test results (Manly 1985) was used to evaluate the overall significance of departures from HWE across all nine loci in each test. Values of F_{IS} were estimated for each locus in each test using the method of Weir and Cockerham (1984).

Genetic differentiation between the monogyne and polygyne forms was evaluated by conducting exact tests implemented in GENEPOP 3.3 (see Raymond and Rousset 1995b). For the nuclear loci, a single genotype per nest was drawn at random (with replacement) for each of 25 tests conducted, and Fisher's method of combining results was used to assess the significance of differentiation across all loci in each test. For the mtDNA, a single test was conducted using only one of the four identical haplotypes scored from each polygyne nest (to avoid inflating sample sizes). Values of F_{ST} for marker genes of each class (allozymes, microsatellites, and mtDNA) also were estimated for each of the subsampled data sets, using the method of Weir and Cockerham (1984) implemented in GENEPOP 3.3. Sequence differences among microsatellite alleles or mtDNA haplotypes were ignored in these estimates.

A number of procedures were conducted to assess the probability that the polygyne study population was derived from the monogyne form after having experienced a reduction in effective population size (a population bottleneck). Allelic richness (A, mean number of alleles per nuclear locus) was estimated for each form. For the monogyne form this was done by means of repeated random subsampling (Leberg 2002) to correct for the difference in number of nests of each form sampled; 50 subsamples of the genotypes in 17 randomly selected monogyne nests were drawn, and the mean value of A over the 50 subsamples was calculated. Numbers of alleles at the nine nuclear loci were compared between the social forms by means of paired t-tests, with the counts transformed as $(x + 1)^{1/2}$ (Snedecor and Cochran 1980). Expected heterozygosity (gene diversity) was calculated for each nuclear locus (h_{exp}) and for the loci combined (H_{exp}) from the allele frequency estimates for the two forms using Equations 8.4 and 8.6, respectively, of Nei (1987). Estimates of H_{exp} were compared between the forms using paired t-tests on the h_{exp} values; values of zero were first converted to $1/(4n)$, where n is the number of nests sampled, and all values were then angular-transformed for the tests (Snedecor and Cochran 1980; Archie 1985). Haplotype diversity (h) was calculated from the mtDNA sequence data for each social form using Equation 8.4 of Nei (1987). For comparative purposes, many of these same statistics were calculated for the two social forms of *S. invicta*, using data collected from two separate native populations in Argentina [the Corrientes and Formosa populations studied by Ross *et al.* (1997)]. Genetic data for *S. invicta* were obtained from 15 nuclear loci (8 allozyme and 7 microsatellite loci) and from restriction fragment length polymorphism variants of the mtDNA control region.

Two statistics developed to detect past bottlenecks and/or the population expansions expected to follow such events were estimated from the *S. geminata* microsatellite allele size and frequency data, and their values were compared between the social forms. The M ratio of Garza and Williamson (2001) is the ratio of the allele number to the range in allele sizes at a locus estimated from population samples. Reductions in effective population size increase the relative force of drift, leading to an expected disproportionate decrease in allele numbers compared to the size ranges they encompass, with a commensurate decrease in the estimate of M. The imbalance index (β-statistic) of Kimmel *et al.* (1998) is the ratio of the variance in allele sizes to the expected heterozygosity based on allele frequencies at a locus estimated from population samples. Under a general asymmetric stepwise mutation model for an equilibrium population, the two measures of microsatellite variability used in this ratio are expected to be equal ($\beta = 1$). Bottlenecks followed by population growth cause transient inflations in the allele size variance relative to the heterozygosity, leading to the expectation that estimates of β exceed one and are greater than values estimated for nonbottlenecked reference populations.

Computer simulations of a founder event: We wished to learn whether a population bottleneck and subsequent expansion, as would have occurred if the polygyne study population was derived via a founder event from the local monogyne form, could reasonably explain the specific patterns of variation observed in the polygyne form at all three classes of markers. Specifically, we asked whether such an event could give rise to the different levels of polymorphism between the social forms of *S. geminata*, as well as among the three classes of markers within the polygyne form. Computer simulations were carried out to model the effects of such an event on allele/haplotype counts (see appendix a). The models simulated the founding of an ancestral polygyne population by mated queens originating from the local monogyne source popula-

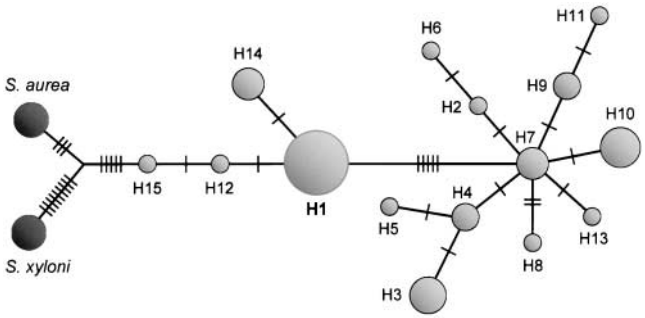

Figure 3.—Relationships of mtDNA sequence haplotypes from *S. geminata* depicted as a minimum spanning tree. Areas of the circles are proportional to the frequency of occurrence of each *S. geminata* haplotype in the sample of 44 nests. Ticks on the branches correspond to presumed single-nucleotide substitutions. H1 is the haplotype that occurs uniquely in the polygyne form of *S. geminata*. Sequences of *S. aurea* and *S. xyloni* are included as outgroups.

tion, using the observed allele frequencies to specify the genotypes of founder queens and their mates. Subsequent generations of descendant queens mated either with males produced in the newly founded population or with males immigrating from the neighboring monogyne population. The models assumed Hardy-Weinberg genotype proportions at the nuclear markers and random mating with respect to genotype/haplotype. We varied several model parameters, including numbers of founder queens, population growth rates, extent of gene flow from the monogyne form, and the presence of mutation at the microsatellites. The numbers of alleles/haplotypes at each marker were tracked through time as the simulated populations grew and were compared with the actual counts obtained from the polygyne study population.

RESULTS

General results: Each of the six polymorphic allozyme loci scored in *S. geminata* was found to have two electrophoretically distinguishable alleles. The three microsatellite loci possessed from 7 to 17 alleles, with single dinucleotide differences separating almost all of the most similar alleles at each locus. Fifteen unique sequence haplotypes were detected at the COI gene of the mtDNA (GenBank accession nos. AY254475–AY254489), which encompass a total of 19 synonymous point substitutions over the 784 bp of sequence. Average pairwise sequence divergence among the 15 haplotypes is modest at 0.576% (SD = 0.076%). Considerable AT bias exists in the base composition of these sequences (average of 63.44% AT), consistent with previous studies of this mtDNA gene in other fire ants (Shoemaker *et al.* 2003). The relationships of the haplotypes are depicted as a minimum spanning tree in Figure 3. Frequencies of all alleles and haplotypes detected in the two forms of *S. geminata* are reported in appendix b.

Colony social organization: Seventeen nests of the 44 studied were determined to constitute polygyne colonies on the basis of complex worker genotype arrays observed at the nuclear markers; such complex arrays are inconsistent with the simple family structure characteristic of monogyne colonies (*e.g.*, Ross *et al.* 1988). Only the three microsatellite loci were useful for this analysis, because the six allozyme loci are essentially monomorphic in the polygyne form (see below). Using the most polymorphic microsatellite locus (*Sol-42*) as an example, workers from each of these presumed polygyne nests typically possessed four to six different genotypes, whereas a maximum of only two genotypes can occur at any given locus in a simple family with male haploidy. Multiple wingless (presumably egg-laying) queens were collected from 7 of the polygyne nests, and most of these queens (19/23) were mated judging from the appearance of their spermathecae or their brood production patterns in the laboratory. This constitutes independent evidence of functional polygyny in this subset of nests. All 17 polygyne nests occurred in a restricted (~2000 m^2) patch on a roadside in the city of Gainesville (Alachua County). Each of the remaining 27 nests sampled possessed worker genotype arrays at the allozyme and microsatellite loci indicative of monogyny (*e.g.*, Ross *et al.* 1988); these include 3 nests found at the periphery of the polygyne patch in Gainesville, 16 nests sampled at varying distances from this patch in Alachua County, and the 8 nests from Leon County. Wingless queens were not found in any of these monogyne nests.

Colony and population genetic structure: As expected given the genotype arrays observed, average worker nestmate relatedness in the 27 monogyne nests is statistically indistinguishable from the value of 0.75 expected for daughters in simple families (Figure 4). Surprisingly, nestmate relatedness in the 17 polygyne nests averages even higher than this (0.88), despite the evidence for multiple egg-laying queens in each nest. However, this value is inflated by two factors. First, the allozyme loci are virtually monomorphic in the polygyne form (below), contributing to elevation of the relatedness estimates based on all loci. When only the three microsatellite loci were employed, the estimate of nestmate relatedness in this form falls significantly below the simple-family value of 0.75 (Figure 4). Second, substantial microsatellite allele frequency differences exist between the forms (below), contributing to an elevation of relatedness estimates over the expected pedigree values when the entire metapopulation is used as the reference population (*e.g.*, Pamilo 1989). When monogyne nests were excluded from the reference population, polygyne worker nestmate relatedness dropped further to ~0.2 (Figure 4). Average relatedness between workers from different polygyne nests, estimated by necessity using the metapopulation as the reference population, again is substantial, exceeding 0.75 when all nine loci are used and 0.5 when just the microsatellites are employed (Figure 4). In aggregate, the relatedness data indicate that the entire polygyne study population may represent an extended family derived from relatively few ancestors, with each nest likely inhabited by a mixture of close and more distantly related kin.

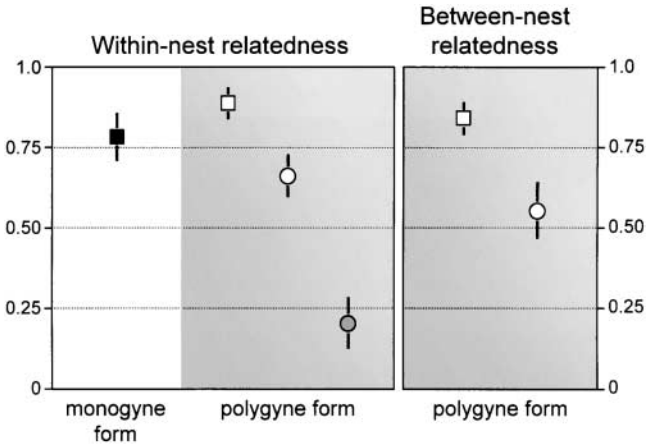

FIGURE 4.—Estimates of worker relatedness within and between nests of *S. geminata*. Squares indicate estimates derived from all nine nuclear loci studied, whereas circles indicate estimates derived from the three microsatellite loci only. For the polygyne form, open shapes indicate estimates obtained by defining the entire metapopulation as the reference population, whereas the shaded circle indicates the within-nest relatedness estimate obtained by excluding monogyne nests from the reference population. Vertical bars indicate 95% confidence intervals around the point estimates.

Analyses of genotype proportions in the monogyne form revealed that the genotypes at the nine nuclear loci departed significantly from HWE in 7 of the 100 tests conducted, a slight excess over the 5% of significant tests expected by chance. The single-locus F_{IS} values associated with these 7 significant tests typically were negative, indicating that the departures were due to an excess of heterozygotes at some loci. These results suggest a general conformity of the genotypes observed in the monogyne form to HWE and rule out widespread local inbreeding as a feature of the mating biology. Similar analyses in the polygyne form were limited to the three microsatellite loci, for which variation was sufficient to conduct such tests. The combined significance of departure from HWE at these loci reached the $P < 0.05$ level in just 4 of the 100 tests conducted, an outcome expected by chance. Thus, there also is no evidence for extensive consanguineous mating in the polygyne population of *S. geminata* that we studied.

Clear differences exist between the monogyne and polygyne forms in their allele and haplotype frequencies (see APPENDIX B). This is most obvious for the allozyme loci and the mtDNA. A single allele is fixed or virtually fixed in the polygyne form at each of the six allozyme loci, yet at four of these loci (*Acoh-5*, *Est-6*, *Gpi*, and *Odh*) these same alleles occur at only moderate frequencies in the monogyne form. A single mtDNA haplotype is fixed in the polygyne form, yet this haplotype was not among the 14 haplotypes found in the monogyne form. Allele frequency disparities also are apparent between the forms at the microsatellite loci, in the frequencies of $Sol\text{-}11^{153}$, $Sol\text{-}42^{161}$, and $Sol\text{-}49^{158}$, for instance. Exact tests of the allele and haplotype frequency differentiation between the social forms confirmed that they are highly genetically divergent. At the nuclear loci, all 25 tests conducted indicated highly significant between-form differentiation across all loci (all $P < 0.0001$), with the same results found individually for each of the seven most polymorphic loci. Not surprisingly, the single test conducted on the mtDNA haplotype frequencies similarly revealed highly significant between-form differentiation ($P < 0.0001$). Results of these tests were similar when the monogyne sample was composed of all monogyne nests or just the Alachua County nests (which can reasonably be considered to be sympatric with the polygyne population).

Estimates of F_{ST} between the social forms, averaged over the 25 samples of single individuals per nest, were 0.27 for the allozymes and 0.17 for the microsatellites, with an estimate of 0.23 over all nine nuclear loci (Alachua County nests only). For the mtDNA haplotypes, F_{ST} was estimated at 0.51 (Alachua County nests only). Thus, about one-quarter of the total nuclear gene diversity and one-half of the mtDNA diversity reside between rather than within the social forms where they occur in effective sympatry.

Genetic diversity in the two social forms: A comparison of the numbers of variants found at each class of marker gene in each form of *S. geminata* is shown in the top panel of Figure 5. A total of 44 nuclear alleles were detected in the monogyne form (40 in the Alachua County samples), whereas 33 alleles were found in the polygyne form. Fourteen mtDNA haplotypes were detected in the monogyne form (12 in the Alachua County samples), whereas only a single haplotype was found in the polygyne form. The differences in numbers of nuclear alleles between the forms are reflected in the higher estimates of allelic richness (A) for the monogyne than for the polygyne form (Table 1, top). None of the 50 values of A obtained by subsampling the monogyne form was as low as the value obtained for the polygyne form, regardless of whether only the allozymes, only the microsatellites, or all nuclear markers were considered. Paired t-tests over the nine nuclear loci formally confirmed that the monogyne form has higher allelic richness than the polygyne form ($t = 3.04$, $P = 0.016$), even when just the Alachua County samples were considered ($t = 3.00$, $P = 0.017$).

Estimates of expected heterozygosity at the nuclear loci (H_{exp}), which reflect the frequencies as well as numbers of alleles, also are higher in the monogyne form than in the polygyne form (Table 1, top). Paired t-tests over the individual-locus heterozygosities again confirmed that this difference is significant ($t = 4.98$, $P = 0.001$ for the Alachua County samples). Haplotype diversity (h), which is zero in the polygyne form, is very high in the monogyne form (Table 1), reflecting the large number of haplotypes discovered in our limited number of samples.

The relatively low genetic variation in the polygyne form of *S. geminata* suggests that this population may

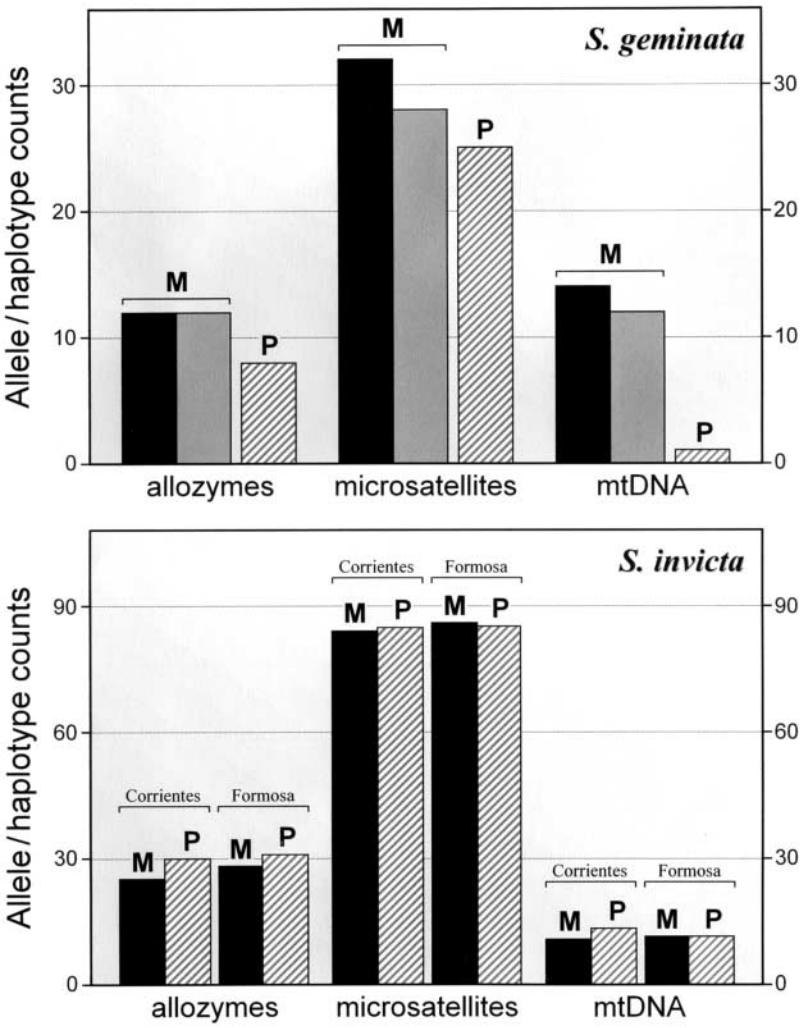

Figure 5.—Numbers of alleles/haplotypes found in the monogyne (M) and polygyne (P) forms of *S. geminata* and *S. invicta*. For *S. geminata*, black bars represent data for all monogyne nests sampled, whereas gray bars represent data for monogyne nests from Alachua County only. Samples of *S. invicta* come from two geographic populations in the native range [Corrientes and Formosa Provinces, Argentina; see Ross *et al.* (1997)]. Sample sizes (numbers of nests sampled) are: all monogyne *S. geminata*, 27; Alachua County monogyne *S. geminata*, 19; polygyne *S. geminata*, 17; Corrientes monogyne *S. invicta*, 37; Corrientes polygyne *S. invicta*, 44; Formosa monogyne *S. invicta*, 35; and Formosa polygyne *S. invicta*, 35.

have suffered a reduction in effective population size (bottleneck), perhaps associated with its derivation via a founder event from the local monogyne population or similar ancestral population. The results of two tests designed to detect the genetic signatures of such an event in the microsatellite data are presented in Table 2. Values of the *M* ratio generally are lower in polygyne than in monogyne *S. geminata*, reflecting the disproportionate reduction in allele numbers relative to allele size ranges in the polygyne form expected following a bottleneck (Garza and Williamson 2001). Although values of the imbalance index (β-statistic) typically are slightly >1 in the monogyne form, suggesting a possible mild bottleneck and period of subsequent growth in the history of this form that inflated the allele size variance relative to the heterozygosity (Kimmel *et al.* 1998), estimates of this statistic for the polygyne form are considerably higher, consistent with the occurrence of a more recent or more pronounced bottleneck and subsequent expansion in the polygyne study population.

Computer simulations of a founder event: Computer simulations were undertaken to determine whether a founder event in which the ancestral polygyne *S. geminata* population was derived from the local monogyne population could reasonably explain the different levels of variation observed between the forms, as well as the different levels observed among the three classes of markers within the polygyne form. A relatively narrow window of model parameter values led to allele/haplotype counts in such simulated populations that were compatible with actual counts in the polygyne study population for all of the markers (see appendix a). The highest probabilities of the simulated and observed data being compatible were reached when the modeled population was founded by a small number of mated queens (7–15), had a low growth rate (≤20%), and experienced a small amount of gene flow from the monogyne source population (2–5%). Incorporating mutation at the microsatellites improved the probability of the simulation results being compatible with the observed data but did not substantially widen the range of parameter values leading to such results. The most significant conclusion from these simulations is that the particular patterns of genetic variation we observed in the polygyne study population can be explained by assuming that it is descended from a founder population derived from the

TABLE 1

Estimates of genetic diversity statistics for *S. geminata* and *S. invicta*

Statistic	Monogyne form: All nests	Monogyne form: Alachua County	Polygyne form
	S. geminata		
Allelic richness (A)			
Allozymes	2.00[a]	2.00	1.33
Microsatellites	9.83[a]	9.33	8.33
All nuclear loci	4.61[a]	4.44	3.67
Expected heterozygosity (H_{exp})			
Allozymes	0.365	0.313	0.006
Microsatellites	0.800	0.797	0.524
All nuclear loci	0.510	0.475	0.179
Haplotype diversity (h)	0.914	0.916	0

Statistic	Corrientes population: Monogyne form	Corrientes population: Polygyne form	Formosa population: Monogyne form	Formosa population: Polygyne form
	S. invicta			
Allelic richness (A)				
Allozymes	3.13	3.63	3.38	3.88
Microsatellites	12.00	12.14	12.29	12.14
All nuclear loci	7.27	7.60	7.53	7.73
Expected heterozygosity (H_{exp})				
Allozymes	0.279	0.273	0.362	0.327
Microsatellites	0.791	0.766	0.763	0.715
All nuclear loci	0.518	0.503	0.549	0.508
Haplotype diversity (h)	0.462	0.559	0.860	0.863

Sample sizes are reported in the legend to Figure 5. The two populations of native *S. invicta* are located in Corrientes and Formosa Provinces, Argentina (see Ross *et al.* 1997).

[a] Corrected for sample size bias by method of repeated random subsampling (Leberg 2002).

neighboring monogyne form. The simulation results further suggest that such a founder event is likely to have occurred within the past 50 generations (<100 years).

Examination for hybridization and infection by Wolbachia: We examined whether interspecific hybridization or infection with Wolbachia may be associated with polygyny in *S. geminata* at our study site. No alleles diagnostic for the sympatric fire ant species *S. invicta* or *S. xyloni* were observed in the samples of *S. geminata* obtained for this study. Rather, all 882 workers surveyed at the two diagnostic allozyme loci possessed only the *null* allele at *Est-2* and the *105* allele at *G3pdh-1*, alleles characteristic of *S. geminata* but not known to occur in the other species (Ross *et al.* 1987; our unpublished data). Thus, there is no evidence of introgression from other fire ant species into the *S. geminata* study populations of either form, in contrast to the widespread hybridization that occurs between related fire ant species in some areas of the United States (Shoemaker *et al.* 1996; Helms Cahan and Vinson 2003).

Application of a diagnostic PCR assay for Wolbachia resulted in the lack of amplification of the major surface protein gene in all *S. geminata* samples. Thus, there is no evidence that the study populations of either form harbor this common bacterial endosymbiont of other fire ant species (*e.g.*, Shoemaker *et al.* 2000, 2003).

***Gp-9* sequence of polygyne *S. geminata*:** All eight *Gp-9* coding region sequences from each of two polygyne *S. geminata* queens were identical to the sequence obtained from a monogyne queen of this species from Florida in a previous study (Krieger and Ross 2002; GenBank accession no. AF427905). This *Gp-9* allele encodes a protein with amino acids typical of the *B*-like rather than the *b*-like alleles of the *S. richteri* clade at the key positions 95 and 139 (Figure 2). Results of our PCR assays for detecting *b*-like sequences at these two codons confirm that only alleles encoding *B*-like residues are likely to exist in *S. geminata* from Florida: none of the 51 polygyne or 54 monogyne workers assayed yielded the 380-bp amplicons indicative of *b*-like sequences at position 95 or position 139. Thus, there is no evidence that parallel amino acid replacements in the

TABLE 2

Estimates of statistics to detect bottlenecks in *S. geminata* using data from three microsatellite loci

Statistic	Monogyne form: All nests	Monogyne form: Alachua County	Polygyne form
M[a]			
Sol-11	1.000	1.125	0.857
Sol-42	1.067	0.933	0.933
Sol-49	1.250	1.250	0.833
All loci[b]	1.106	1.103	0.875
β[c]			
Sol-11	2.149	2.785	5.198
Sol-42	0.958	1.008	19.750
Sol-49	1.948	1.599	8.394
All loci[d]	4.050	4.421	28.289

[a] *M* ratio of Garza and Williamson (2001).
[b] Estimated as the mean across loci.
[c] Imbalance index of Kimmel *et al.* (1998).
[d] Estimated using the formula of Kimmel *et al.* (1998, p. 1927).

GP-9 protein have occurred between *S. geminata* and the *S. richteri*-clade species in association with the origin of polygyny. Indeed, there apparently is no association whatsoever between colony social organization and *Gp-9* genotype in *S. geminata*, given that the sequenced individuals representing both social forms were all homozygotes for the identical allele.

DISCUSSION

The objective of this study was to compare sequences of the *Gp-9* gene from the polygyne social form of the fire ant *S. geminata* with the sequence found in the monogyne form of this species, as well as with sequences from fire ant species in the *S. richteri* clade displaying a similar polymorphism in colony social organization. We hypothesized that if polygyny in *S. geminata* originated in the same manner as in *S. invicta*, a well-studied member of the *S. richteri* clade, then the *Gp-9* variant associated with polygyny in *S. geminata* would code for particular amino acid residues characteristic of the polygyny-inducing *b*-like alleles in the *S. richteri*-clade species. Specifically, we reasoned that polygyne *S. geminata* would bear a *Gp-9* sequence coding for isoleucine at position 95 or 139 (or both); these residues are unique to all *b*-like alleles, and thus one or both are hypothesized to be responsible for the altered queen-recognition capabilities of workers in polygyne nests (Krieger and Ross 2002; see Figure 2). To establish if polygyny likely arose in an analogous fashion in *S. geminata* and *S. invicta*, an assumption implicit in the comparative analysis of *Gp-9* sequences, we also characterized the colony and population genetic structure of both social forms of *S. geminata* for comparison with *S. invicta*. Finally, we examined whether interspecific hybridization or infection with the endosymbiont Wolbachia, both of which are common in some fire ants, might be associated with the expression of polygyny in *S. geminata*.

Gp-9 sequences in the polygyne *S. geminata* population we studied were found to be identical to the coding region sequence previously determined for the monogyne form of this species (Krieger and Ross 2002). Furthermore, application of our specific PCR assays on numerous polygyne specimens suggests that no sequences with *b*-like codons at positions 95 or 139 are likely to be present at our study site. Thus, the predicted amino acid replacements characteristic of all *b*-like alleles are not present in polygyne *S. geminata*, refuting our working hypothesis that one or both of these substitutions are necessary for the expression of polygyny in fire ants. Moreover, because no nucleotide substitutions of any kind distinguish the *Gp-9* coding region sequences of polygyne and monogyne *S. geminata*, variation in this gene apparently does not obligately correspond with variation in colony social organization in this species, as it does in the *S. richteri* clade. From this we conclude that sequence polymorphism at *Gp-9* does not always underlie social polymorphism involving colony queen number in the genus Solenopsis.

Gp-9 notwithstanding, we found marked genetic differences between the polygyne and monogyne forms of *S. geminata*, and these differences are not paralleled in the two social forms of *S. invicta*. These findings are taken to support the necessary conclusion from the *Gp-9* sequence data that polygyny has not evolved in analogous fashion in *S. geminata* and the *S. richteri* clade. The social forms of *S. geminata* are strongly differentiated at both their nuclear and mitochondrial genomes, a pattern not found in *S. invicta*. Whereas <2% of the total nuclear diversity in native *S. invicta* from the Corrientes and Formosa populations in Argentina resides between the social forms (Ross *et al.* 1997; Ross 2001), almost one-quarter of the nuclear diversity in *S. geminata* occurs at this level. Differentiation between the forms of *S. invicta* is somewhat more pronounced at the mtDNA than at the nuclear genome, ranging from 4 to 32% of the total haplotype variation (Ross *et al.* 1997; Shoemaker *et al.* 2003), but this still is considerably less than the mtDNA differentiation found between the forms of *S. geminata* (51%). These and other results are consistent with the idea that substantial nuclear gene flow occurs between the social forms of *S. invicta* where they occur in sympatry (Shoemaker and Ross 1996; Ross *et al.* 1997, 1999; Ross 2001), while gene flow between the forms of *S. geminata* appears from our data to be minimal. We speculate that the events inducing a shift in social organization in the ancestral polygyne *S. geminata* population also caused a change in the breeding biology of this form that has resulted in its more or less complete reproductive isolation from the monogyne form. Evidently, this change did not involve the adoption of localized inbreeding, once thought to be an important factor in the evolution of polygyny in ants and other social

Hymenoptera (Hamilton 1972), because both forms of *S. geminata* display genotype distributions in general agreement with Hardy-Weinberg expectations.

The two forms of *S. geminata* differ further in that genetic diversity is significantly reduced in the polygyne form relative to the monogyne form, with this reduction apparently due to a substantial bottleneck in the history of the polygyne population. Numbers of nuclear alleles and mtDNA haplotypes are significantly lower in the polygyne than in the monogyne form of *S. geminata*, as are the expected heterozygosity and haplotype diversity. Again, these differences are not characteristic of *S. invicta*: numbers of alleles (allelic richness) and numbers of haplotypes are virtually identical between the sympatric forms in each of two native populations (Figure 5, bottom; Table 1, bottom), and the expected heterozygosity and haplotype diversity in the paired forms are similar as well (Table 1, bottom; heterozygosity does not differ significantly between sympatric *S. invicta* forms: paired *t*-tests, both $P > 0.05$).

These comparative data lead us to speculate that the polygyne form of *S. geminata* originated from a small, isolated founder population derived from the nearby monogyne population, with the inception of polygyny driven by a loss of genetic variation rather than by specific amino acid replacements at *Gp-9*, the crucial evolutionary event presumed to have driven the origin of polygyny in the *S. richteri*-clade species. Monogyny generally is assumed to be ancestral to polygyny in ants and other social Hymenoptera (*e.g.*, Ross and Carpenter 1991; Bourke and Franks 1995), and *Gp-9* sequence data specifically support this evolutionary polarity in the transition of social organization in the *S. richteri* clade (Krieger and Ross 2002). Additional evidence for the derivation of the polygyne *S. geminata* population from the monogyne form is that, in general, the polygyne population possesses a subset of the nuclear alleles found in the neighboring monogyne populations (appendix b). Moreover, the sole polygyne mtDNA haplotype is embedded in the clade composed of all monogyne haplotypes (Figure 3), suggesting that it previously occurred in monogyne populations or that it still occurs there but was not sampled. Finally, results of our simulation models indicate that the particular patterns of variation observed in the polygyne form at all three classes of markers we employed can be explained by assuming that this population originated via a founder event from the neighboring monogyne form within the past 100 years.

A loss of genetic variation has been invoked previously to explain another radical shift in colony social organization in an ant, and the proximate genetic mechanisms hypothesized to be involved may pertain also to the inception of polygyny in *S. geminata*. The Argentine ant, *Linepithema humile*, exhibits well-developed nestmate discrimination that functions to maintain distinct colony boundaries in its native South American range. In areas where it has been introduced, such as the western United States and southern Europe, such nestmate discrimination is all but absent, leading to the development of geographically extensive "supercolonies" in which individual ants mix freely among physically distinct nests (Holway *et al.* 1998; Tsutsui and Case 2001; Giraud *et al.* 2002). Diminished nestmate recognition and ensuing supercolony organization in the introduced ranges coincide with losses of microsatellite allele diversity stemming from colonization of these areas (Tsutsui *et al.* 2000; Giraud *et al.* 2002). These observations have led to the suggestion that losses of alleles encoding individual chemical recognition cues, caused by the founder events and/or by subsequent frequency-dependent selection (Tsutsui *et al.* 2000, 2003; Giraud *et al.* 2002), have compromised the nestmate discrimination abilities of Argentine ant workers in their introduced ranges, thereby inducing the transition to supercoloniality. In the case of *S. geminata*, loss of genetically based chemical recognition cues in the ancestral polygyne population may similarly have eroded the ability of workers to distinguish nestmates from non-nestmates as well as to recognize individual queens, leading to a breakdown of colony boundaries (high between-nest relatedness) and a contingent runaway process of acceptance of multiple related and unrelated egg-laying queens in each nest (see also Crozier and Pamilo 1996, p. 144; van der Hammen *et al.* 2002). Weakly developed nestmate discrimination and permeable colony boundaries are common correlates of polygyny in ants (Bourke and Franks 1995).

Reduced genetic variation in the polygyne form of *S. geminata* is the only factor we could identify that corresponds with this alternative social organization and that therefore constitutes a plausible causal agent in the social transition from monogyny. *Gp-9* sequences apparently are identical between the forms, indicating that changes in the recognition capabilities of workers associated with amino acid replacements in GP-9 protein did not elicit the switch to polygyny. Inbreeding cannot be considered a contributory factor to the divergence between the forms, nor apparently can habitat specialization, given that colonies of both forms were collected alongside one another at one site in Alachua County. Wolbachia infections, which potentially could explain both the loss of mtDNA variation in the polygyne form [via selective sweeps and hitchhiking (Turelli *et al.* 1992)] and reproductive isolation between the forms [via reproductive incompatibility between populations harboring alternate strains (Werren 1998)], were not detected in either form and so also cannot be implicated in the social transition. Finally, there is no evidence of introgression from related fire ant species into the *S. geminata* populations of either form that we studied. This is in contrast to the widespread introgression of *S. xyloni* genes into *S. geminata* in central Texas (Hung and Vinson 1977; Trager 1991), where such hybridization appears to be inextricably linked to the adoption of polygyne social organization (Helms Cahan and Vinson 2003).

We conclude from this study that polygyny evolved via a different route in *S. geminata* than in fire ant species in the *S. richteri* clade, although both evolutionary scenarios invoke changes in the molecular components of the chemoreception systems functioning in individual recognition of conspecifics. On the one hand, evolution of polygyny via nonsynonymous substitutions in the presumed odorant-binding protein gene *Gp-9*, characteristic of the *S. richteri*-clade species, seems to involve a change in the pheromone-signal transduction component, with polygyne workers that bear the derived *b*-like alleles thought to exhibit altered recognition capabilities compared to other workers. On the other hand, evolution of polygyny via loss of alleles at loci encoding recognition cues seems to involve a reduction in the diversity of chemical labels necessary for the proper functioning of a discrimination system that serves in both maintaining colony integrity and regulating queen number. Additional polygyne populations of *S. geminata* occurring elsewhere in its vast range should be studied to determine whether *Gp-9* sequence variation ever corresponds with polygyny in the manner we initially hypothesized or if reductions in effective population size are consistently evident in the history of these polygyne populations (*e.g.*, van der Hammen *et al.* 2002). Also, other odorant-binding protein genes, which may often occur in multigene clusters in insect genomes (Galindo and Smith 2001; Hekmat-Scafe *et al.* 2002), should be examined for variation that corresponds with social organization in this species. Detailed molecular examination of both the transduction and the cue diversity components in chemoreception systems of appropriate social insects promises to yield new insights into the genetic foundations of the transition from monogyny to polygyny and, in so doing, may shed light on the evolution of other complex social adaptations.

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APPENDIX A: COMPUTER SIMULATIONS OF A FOUNDER EVENT PRODUCING THE ANCESTRAL POLYGYNE *S. GEMINATA* POPULATION

Methods: Computer simulations were carried out to model the effects on allele/haplotype counts of a founder event in which the ancestral polygyne population was derived from the local monogyne form. The models simulated the founding of an ancestral polygyne population by mated queens from the local monogyne source population, using the observed allele frequencies to specify genotypes of the founder queens and their mates. Subsequent generations of descendant queens mated with males produced in the newly founded population or with males immigrating from the local monogyne population. The models assumed Hardy-Weinberg genotype proportions at the nuclear markers (as observed in our genetic data) and random mating with respect to genotype/haplotype. We varied several model parameters in the different simulation runs, including numbers of founder queens, population growth rates, extent of gene flow from the monogyne form, and the presence of mutation at the microsatellites. Allele/haplotype counts were tracked through time as the simulated populations grew.

Simulations were started by randomly picking *N* mated

queens ($N = 1$–30) from the monogyne source population as founders. In each generation, the number of breeding queens was allowed to increase by a set rate until the population reached the carrying capacity of 150 queens. Six different growth rates were used: 5% ($N_t = N_{t-1} + 0.05 \cdot N_{t-1}$), 10% ($N_t = N_{t-1} + 0.1 \cdot N_{t-1}$), 20% ($N_t = N_{t-1} + 0.2 \cdot N_{t-1}$), 50% ($N_t = N_{t-1} + 0.5 \cdot N_{t-1}$), 100% ($N_t = 2 \cdot N_{t-1}$), and exponential ($N_t = N_{t-1}^2$). Once a population reached the carrying capacity, the simulations were continued for 50 additional generations while holding population size constant. Some models incorporated male-mediated gene flow from the monogyne source population, which was set at 2.5, 5, or 20% (the proportions of breeding queens mating with immigrant monogyne males in each generation). Our mtDNA data suggest that queens do not mediate gene flow from the monogyne form to the polygyne form (see text), so this potential source of added genetic variation was not considered. Finally, some models included mutation of the microsatellite alleles. We set the mutation rate at $\mu = 10^{-4}$ (Crozier *et al.* 1999; Zhang and Hewitt 2003) and assumed an infinite allele model of mutation for simplicity. We conducted 200 simulation runs for each unique combination of parameter values.

The numbers of alleles/haplotypes at each marker gene were counted in each generation of the bottlenecked population in each simulation run and compared with the actual counts obtained from the polygyne study population. Simulation results were considered compatible with the observed data when the following counts were obtained concurrently: a single mtDNA haplotype; one allele at the allozyme loci *Acon-5*, *Fdp-2*, *Gam*, and *Gpi*; at least one allele at the allozyme loci *Est-6* and *Odh*; and at least 6, 14, and 5 alleles at the microsatellite loci *Sol-11*, *Sol-42*, and *Sol-49*, respectively.

Results: One goal in simulating a founder event underlying the origin of the polygyne study population was to account for the negligible diversity at the allozymes and mtDNA in combination with the considerable diversity at the microsatellites that we observed in this population. In general, simulated allele/haplotype counts at the allozymes and mtDNA were compatible with the observed data in the early generations following the bottleneck, whereas simulated allele counts at the microsatellites tended to be compatible with observation in later generations. Thus, simulation results that accurately portrayed the overall patterns of polymorphism observed in the polygyne population were obtained for only a relatively narrow window of generations and range of parameter values (*e.g.*, Figure A1).

The most important factors affecting the outcomes of the simulations were the number of founder queens, extent of gene flow, and population growth rate. Only simulations with intermediate numbers of founders (7–15) yielded results compatible with the observed data for all three classes of markers (Figure A1). Similarly, only simulations with intermediate levels of gene flow from the monogyne form (2.5–5%) resulted in compatible allele counts when mutation of the microsatellites was not considered. Even with mutation, the absence of gene flow or high gene flow (20%) rarely produced compatible simulation results. Finally, only the three slowest population growth rates (5, 10, and 20%) produced allele/haplotype counts fully compatible with the observed counts across all markers.

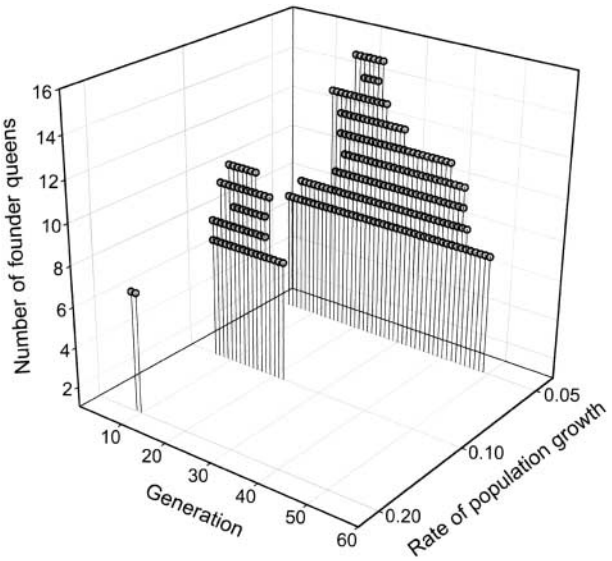

Figure A1.—Conditions under which allele/haplotype counts in simulated bottlenecked monogyne *S. geminata* populations were compatible with actual counts in the polygyne study population at all marker genes. Each point shows the parameter values that yielded compatible counts for one or more simulation runs for each generation in which these compatible counts occurred (the founder event occurred at generation zero). In this particular example, gene flow from the monogyne source population was held constant at 2.5% and no mutation of microsatellite alleles was incorporated.

In general, increased population growth rates narrowed the range of generations for which compatible simulation results were obtained (Figure A1). Similarly, increased growth rates reduced the ranges of gene flow rates and numbers of founders yielding compatible results. For instance, at 20% growth, only simulations with a single parameter setting for gene flow (2.5%) and founder number (7) produced compatible results, and these were obtained for only two generations (9 and 10). In contrast, at 5% growth, compatible results were obtained with two gene flow rates (2.5 and 5%) and with variable founder numbers (7–15 founders in runs with 2.5% gene flow), and results compatible with observation occurred for up to 45 generations.

Incorporating mutation at the microsatellite loci had little effect on the ranges of parameter values that yielded simulation data compatible with the observed data. The only conspicuous exception was that, at 10% population growth, gene flow from the monogyne form was not required when mutation was invoked [however,

compatible data were produced for only a few early generations (2–6) under these conditions]. Even though microsatellite mutation had negligible effects on the ranges of compatible parameter values, it did significantly increase the probabilities of obtaining compatible results. Over all simulations with at least some compatible outcomes, the frequency of obtaining such results when assuming microsatellite mutation significantly exceeded the frequency without this assumption (paired *t*-test: $t = 11.84$, d.f. = 14, $P < 0.0001$). Nonetheless, microsatellite mutation need not be invoked for the patterns of genetic variation observed in the polygyne study population to be explained as the result of a founder event.

Simulation results generally were compatible with observation for only relatively few generations following the bottleneck, with such results typically appearing by generation 10 and disappearing by generation 50 (Figure A1). Assuming that the polygyne study population did indeed originate via a founder event from the local monogyne form, it would appear from our simulations that this event occurred at some point within the past century.

APPENDIX B

Allele and haplotype frequencies in the two social forms of *S. geminata*

Gene and allele (haplotype)	Monogyne form: All nests	Monogyne form: Alachua County	Polygyne form
	Allozymes		
Acoh-5			
100	0.688	0.712	0
95.7	0.312	0.288	1.0
Est-6			
167	0.406	0.438	0.015
100	0.594	0.563	0.985
Fbp-2			
116	0.925	0.946	1.0
100	0.075	0.054	0
Gpi			
100	0.741	0.671	1.0
65	0.259	0.329	0
Hexa			
100	0.867	0.964	1.0
91.7	0.133	0.036	0
Odh			
164	0.630	0.737	0.998
100	0.370	0.263	0.002
	Microsatellites		
Sol-11			
143	0	0	0.032
145	0.028	0	0.078
147	0.019	0	0
151	0.157	0.171	0.074
153	0.167	0.079	0.532
155	0.315	0.382	0.138
157	0.157	0.184	0.145
159	0.037	0.026	0
161	0.009	0.013	0
163	0.065	0.092	0
165	0.037	0.039	0
167	0.009	0.013	0

(*continued*)

APPENDIX B

(Continued)

Gene and allele (haplotype)	Monogyne form: All nests	Monogyne form: Alachua County	Polygyne form
Sol-42			
155	0	0	0.024
157	0.028	0.039	0.006
159	0.028	0.039	0
161	0.028	0.026	0.518
163	0.037	0	0.038
165	0.046	0.053	0.015
167	0.130	0.118	0.083
169	0.083	0.105	0.086
171	0.093	0.066	0.026
173	0.130	0.132	0.047
175	0.111	0.132	0.056
177	0.028	0	0.012
179	0.093	0.079	0
181	0.074	0.092	0.003
183	0.046	0.053	0.006
185	0.028	0.039	0.081
187	0.019	0.026	0
Sol-49			
152	0	0	0.003
156	0.074	0.079	0
158	0.546	0.513	0.915
160	0.241	0.263	0.056
162	0.111	0.132	0.018
164	0.028	0.013	0.009
	COI (mtDNA)		
H1	0	0	1.0
H2	0.037	0.053	0
H3	0.148	0.211	0
H4	0.074	0.105	0
H5	0.037	0.053	0
H6	0.037	0.053	0
H7	0.111	0.105	0
H8	0.037	0.053	0
H9	0.074	0.105	0
H10	0.185	0.105	0
H11	0.037	0.053	0
H12	0.037	0.053	0
H13	0.037	0.053	0
H14	0.111	0	0
H15	0.037	0	0

Sample sizes (numbers of nests sampled) are: all monogyne nests, 27; Alachua County monogyne nests, 19; polygyne nests, 17. Allele designations for the allozymes indicate relative band mobilities for homozygotes, whereas designations for the microsatellites indicate the lengths of the amplification products.