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Species delimitation in native South American fire ants

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Abstract

The taxonomy of fire ants has been plagued by difficulties in recognizing species on the basis of morphological characters. We surveyed allozyme markers and sequences of the mtDNA *COI* gene in several closely related nominal species from two areas of sympatry in the native ranges to learn whether the morphology-based delimitation of these species is supported by genetic data. We found that *Solenopsis invicta* and *Solenopsis richteri*, pest species whose distinctiveness has been debated, appear to be fully reproductively isolated at both study sites. This isolation contrasts with the extensive hybridization occurring between them in the USA, where both have been introduced. We also found strong genetic differentiation consistent with barriers to gene flow between *Solenopsis quinquecuspis* and the other two species. However, several lines of evidence suggest that nuclear and mitochondrial genes of *S. invicta* and *S. richteri* are introgressing into *S. quinquecuspis*. The latter apparently is a recently derived member of the clade that includes all three species, suggesting that there has been insufficient time for its full development of intrinsic isolating mechanisms. Finally, our discovery of genetically distinct populations within both *S. invicta* and *S. richteri* suggests the presence of previously unrecognized (cryptic) species. Their existence, together with the difficulties in developing diagnostic morphological characters for described species, imply that the group is actively radiating species and that morphological divergence generally does not keep pace with the development of reproductive isolation and neutral genetic divergence in this process.

Keywords: fire ants, gene flow, introgression, reproductive isolation, *Solenopsis*, species delimitation

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Introduction

A fundamental task of population biologists and systematists is to delimit boundaries of species in taxa containing numerous phenotypically similar but apparently distinct populations. This essential task in revisionary taxonomic studies and phylogenetic analyses also figures prominently in a broader scientific context because species serve as fundamental units of analysis in diverse ecological, biogeographical, and macroevolutionary studies (e.g. Nelson & Platnick 1981; Wilson 1990; Hubbell 2001; Brooks & McLennan 2002; Bokma 2003; Scotland & Sanderson 2004; Sites & Marshall 2004). A potential source of bias in such analyses stems from the fact that species-rich taxa in phases of evolutionary radiation are especially susceptible to underresolving of species boundaries, so careful delineation of species in such taxa should be given high priority. Rigorous species delimitation in these problem taxa can also have practical implications — for example, in recognizing threatened evolutionary units meriting conservation efforts (Avise & Hamrick 1996; Kareiva & Levin 2002; Hey *et al*. 2003) or in identifying potential biological control agents for invasive pests of uncertain identity and provenance (Van Driesche & Bellows 1996).

Defining species boundaries in actively radiating clades also is of interest to evolutionary biologists concerned with speciation because these groups provide windows on different stages of species formation. Thus, data gathered to solve practical problems of species delimitation can be used as well to learn about mechanisms of speciation. The following are among the questions to be addressed in this context. (i) What are the relative dynamics of divergence of various character systems (morphological, behavioural, genetic) during speciation? (ii) How does divergence at these different systems map onto the development of reproductive

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isolation? (iii) What are the causes of intrinsic barriers to gene flow between newly diverged entities? (iv) Are incomplete barriers to gene flow uniformly porous or selectively permeable to certain genetic elements? (e.g. McMillan *et al*. 1997; Ritchie & Phillips 1998; Wu & Hollocher 1998; Rieseberg *et al*. 1999, 2004; Wu 2001; Machado *et al*. 2002; Tregenza 2002; Coyne & Orr 2004; Fukami *et al*. 2004).

The South American fire ants constitute a species-rich clade in the genus *Solenopsis* that have assumed considerable importance over recent decades. Two of the species, *Solenopsis invicta* and *Solenopsis richteri*, were inadvertently introduced into the USA early in the last century, where they have become significant pests (Lofgren 1986a, b). Because of the negative impacts of *S. invicta*, in particular, in the USA, a vast literature on the basic biology of this ant has been produced (Tschinkel 2005). The extensive research on *S. invicta* has in turn led to its emergence as a model for ecological and evolutionary studies. Although the South American fire ants have been the subjects of recent comprehensive taxonomic revisions (Trager 1991; Pitts 2002), two major unresolved issues persist with respect to the alpha-taxonomy of *S. invicta* and its close relatives.

The first is a concern over whether the boundaries of the recognized species actually mark reproductively isolated, evolutionarily independent lineages (de Queiroz 1998). Distinguishing species of South American fire ants has been a difficult task historically because of a paucity of reliable, discrete morphological characters that are constant within putative species yet differ among them (Creighton 1930; Wilson 1952; Buren 1972; Trager 1991). Although the development of new character systems from the immature stages and sexual forms has improved this situation (Pitts 2002), it is still the case that some nominal species are diagnosible by very few, often subtle or continuous characters that are not always invariant within species. These difficulties in developing diagnostic characters led Wilson (1952) to speculate that most of the observed fire ant diversity in South America represents geographical variation within a single widespread polytypic species, with hybridization of regional variants in areas of parapatry explaining the confusing character distributions. Further complicating this issue are the recent discoveries that hybridization does occur between species of both native and introduced fire ants in North America (e.g. Vander Meer *et al*. 1985; Shoemaker *et al*. 1996; Helms Cahan & Vinson 2003).

The second issue concerns the possibility that previously undetected (cryptic) species occur within *S. invicta* and, perhaps, other nominal fire ant species. This possibility was raised by earlier genetic data from native populations showing that (i) mitochondrial DNA (mtDNA) sequence haplotypes from northern Argentina populations of *S. invicta* form divergent clades, one of which is more closely related to *S. richteri* haplotypes than to other *S. invicta* clades (Shoemaker *et al*. 2003a); (ii) allozyme allele frequencies and mtDNA haplotype frequencies differ markedly between geographical populations of *S. invicta* in northern Argentina (Ross *et al*. 1997); and (iii) a cryptic species indistinguishable morphologically from *Solenopsis quinquecuspis* (a close relative of *S. invicta*) in central Argentina is recognizable by virtue of unique allozyme alleles (Ross & Trager 1990). These genetic data implicating strong differentiation within nominal species, coupled with the subtle and incomplete morphological distinctions clouding the alpha-taxonomy of the group, support the idea that the South American fire ant clade is a youthful group in an active phase of radiation (Creighton 1930; Ross & Trager 1990).

Given these unresolved issues concerning the delimitation of South American fire ant species, our goal was to use genetic markers of different classes (allozymes, mtDNA sequences) to learn if significant genetic discontinuities correspond to the morphological discontinuities by which the species are diagnosed. Delimitation of species boundaries requires at least an implicit concept of what constitutes a species, which has been a matter of longstanding debate (see Howard & Berlocher 1998; Dettman *et al*. 2003; Hey *et al*. 2003; Sites & Marshall 2004). That said, almost all species concepts acknowledge the importance of the evolution of more or less complete reproductive isolation between incipient sexually reproducing species at some early point in their divergence (de Queiroz 1998; Orr 2001; Wu 2001; Lee 2003). A reasonable approach to delimiting species with genetic data therefore is to determine the magnitude of genetic differentiation and inferred levels of gene flow between nominal species in sympatry in order to assess the development of intrinsic reproductive isolation (Sites & Marshall 2004; Irwin *et al*. 2005). Multiple markers of the nuclear and organellar genomes are most useful in this approach because barriers to gene flow between incipient species may develop in piecemeal fashion, affecting some genomes or genomic regions more rapidly than others (e.g. Rieseberg & Burke 2001; Wu 2001; Morjan & Rieseberg 2004). Moreover, multiple markers of different genomes can provide complementary evidence regarding the evolutionary independence of a lineage, another indicator of species status (de Queiroz 1998; Dettman *et al*. 2003).

Three nominal species of South American fire ants are the subject of this study — *S. invicta*, *S. richteri*, and *S. quinquecuspis*. These species were selected for several reasons. First, they are close relatives whose species status has been in doubt at some point over the last century; indeed, the taxonomic status of *S. invicta* and *S. richteri* has been the subject of particular controversy, in large part because they hybridize extensively in their introduced range in the USA (see Buren 1972; Vander Meer *et al*. 1985; Ross *et al*. 1987; Trager 1991). Also, the three focal species have native ranges that overlap, making it possible to assess the strength of intrinsic reproductive barriers between them. Finally, all are sufficiently common that suitable numbers of samples could be obtained

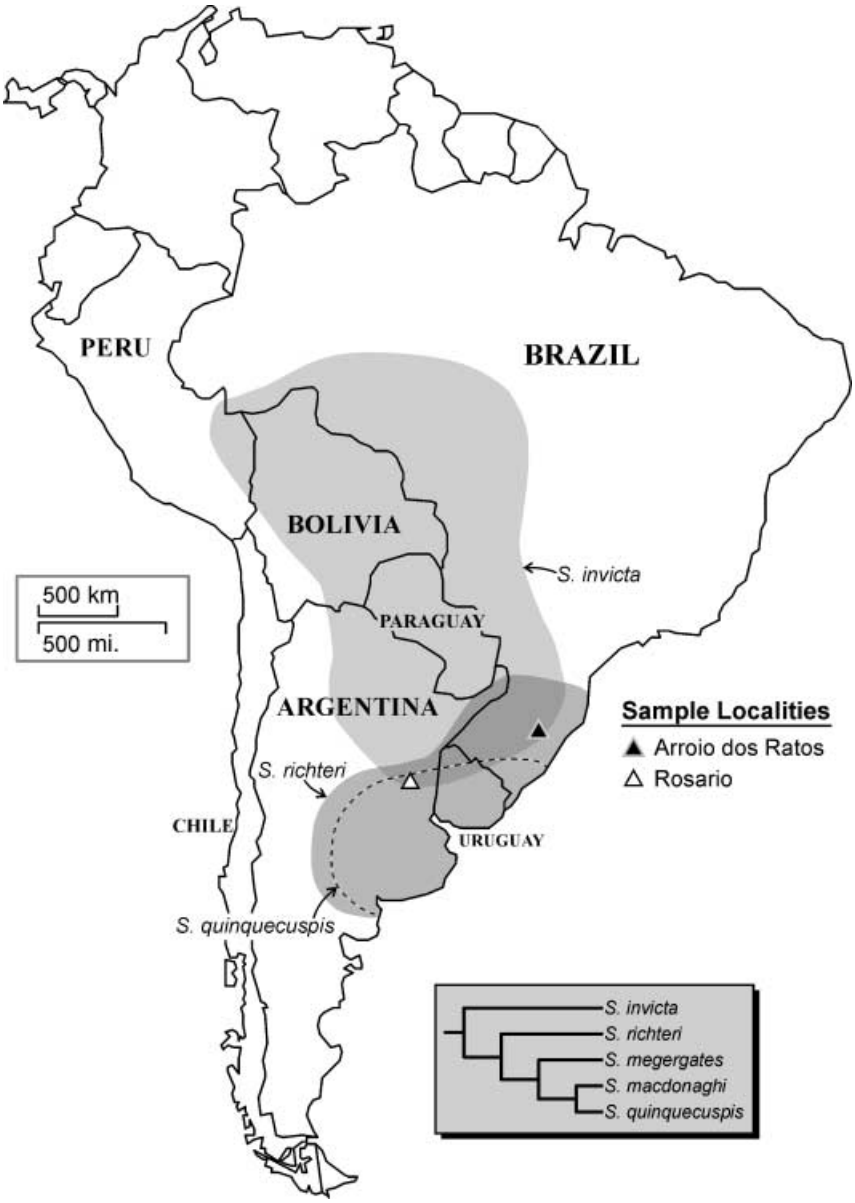


Fig. 1 Map of the native ranges of *Solenopsis invicta* (light shading), *Solenopsis richteri* (medium shading), and *Solenopsis quinquecuspis* (dashed outline), based on information in Buren *et al*. (1974), Trager (1991), and Pitts (2002). The area over which *S. invicta* and *S. richteri* occur in sympatry is indicated by the darkest shading. The range of *S. quinquecuspis* is thought to be contained entirely within that of *S. richteri*. Locations of the two sampling sites for the present study are indicated by the triangles; all three species were sampled at Rosario, whereas only *S. invicta* and *S. richteri* were sampled at Arroio dos Ratos. Inset: Tree obtained from maximum-parsimony analysis of morphological characters for the nominal species in the focal clade of the *Solenopsis saevissima* species-group (from Pitts *et al*. 2005). This tree was recovered using successive approximations weighting on six minimum-length trees.

from multiple sites to reliably estimate the extent of gene flow between regional conspecific populations as well as between species.

The present study builds on earlier work that addressed some similar issues. Ross & Trager (1990) examined the genetic distinctiveness of six mostly allopatric South American fire ant species. Evaluation of reproductive isolation in sympatric species pairs was hampered in this earlier study by the limited samples and low resolution of the available markers. Ross *et al*. (1997) studied regional differentiation within a single species (*S. invicta*) using data from a large number of diverse markers in a single pair of native populations from northern Argentina. The present research extends these earlier studies by analysing new samples of three fire ant species that occur in sympatry at two distant sites using informative nuclear and mitochondrial data. Our new sequence data for the mtDNA offer the advantage of yielding haplotype phylogenies of use in evaluating long-term reproductive isolation and genealogical exclusivity of populations.

Materials and methods

Sample collection

Solenopsis invicta, *Solenopsis richteri*, and *Solenopsis quinquecuspis* are members of a well-supported apical clade of the *Solenopsis saevissima* species group according to a recent morphology-based phylogenetic study (Pitts *et al*. 2005; see Fig. 1). Within this clade of five nominal species, *S. invicta*

and *S. richteri* arise from the most basal nodes, whereas *S. quinquecuspis* apparently originated most recently. *S. invicta* occurs over a large native range extending from southeastern Peru to central Argentina and southern Brazil; *S. richteri* and *S. quinquecuspis* are more localized, with native ranges extending from southernmost Brazil to east-central Argentina (Buren *et al*. 1974; Trager 1991; Pitts 2002). We collected samples of these species from two geographical localities separated by 880 km (Fig. 1); *S. invicta* and *S. richteri* occur together at the Arroio dos Ratos site in southern Brazil, whereas all three species co-occur at the Rosario site in east-central Argentina. The morphologically distinctive fire ant *Solenopsis megergates* also occurs at the Arroio dos Ratos site, but too few nests of this species were found there to warrant inclusion in this study. The three species included in this study are the only fire ants known to occur at the Rosario site (Pitts 2002). Adult winged queens were collected from each nest when available; otherwise, adult workers were collected (< 10% of samples). Study colonies were identified to species by J. P. Pitts based on 5–10 morphological characters of adult workers, winged queens, and males [diagnostic characters include facial colouration (workers, queens), the median ocellus (workers), and mandibular structure (workers) and colouration (males); see Pitts 2002]; no morphological intermediates or ambiguous specimens were discerned. From 33 to 44 nests of each nominal species were sampled at each locality (Appendix I).

Colony social organization (monogyny = single reproductive queen per nest, polygyny = multiple queens per nest) has been determined for all sampled colonies of *S. invicta* included in this study (Mescher *et al*. 2003), but not for *S. quinquecuspis* or *S. richteri*. Fewer than one-quarter of the *S. invicta* nests from Arroio dos Ratos and none from Rosario were determined to be polygyne. Because no differences in allele or haplotype distributions were evident between the two social forms of *S. invicta* from Arroio dos Ratos (data not shown), colony social organization was not considered subsequently in our analyses.

Genetic methods

The genotypes of one or two individuals per nest were determined at seven allozyme loci. These seven loci were chosen because they are expressed in adult females and have been shown to display significant levels of polymorphism within or between species of the focal clade; specifically, *Est-2* and *Gpi* often are diagnostic between species (Ross & Trager 1990; Shoemaker *et al*. 1996), while the remaining five loci and *Est-2* are variable within *S. invicta* (Ross *et al*. 1997). The locus *Pgm-1*, which was found to display important interspecific differences in the present study, has not been surveyed previously in native fire ants other than *S. invicta*. Electrophoresis and protein staining were conducted using methods from Shoemaker *et al*. (1992), with several standards representing known genotypes run on each gel to ensure proper identification of electromorphs. Mendelian inheritance of the common electromorphs at each locus has been demonstrated (Ross *et al*. 1988, 1997).

A 920-bp portion of the mitochondrial *COI* gene was sequenced for a single individual from most sampled nests (Appendix I). Sequences of the same region were obtained for single individuals of the fire ant species *Solenopsis electra* and *Solenopsis geminata* to serve as outgroups. *S. electra* belongs to the same species group as the study species but is rather distantly related to the apical clade containing them; *S. geminata* belongs to a different species group (Pitts *et al*. 2005). Polymerase chain reaction (PCR) mixes, sequences of external PCR and sequencing primers, and thermal cycling conditions are described in Shoemaker *et al*. (2000). Mitochondrial DNA amplicons were purified for sequencing using Agencourt magnetic beads, with the purified products used directly in standard fluorescent cycle-sequencing PCRs (ABI PRISM BigDye Terminator Chemistry, Applied Biosystems). Both strands of each mtDNA amplicon were sequenced. All sequences were edited using the program SEQUENCHER and aligned by eye using as references published fire ant sequences [*S. geminata* (GenBank Accession no. AY254476) and *S. invicta* (AY249093)].

Data analyses

The genotypes of one or two individuals per nest were used to estimate allele and genotype frequencies at the seven allozyme loci using the program GENEPOP (Raymond & Rousset 1995a). Nest mate genotypes are not independent because nest mates are related; however, our use of multiple nest mates is expected to have minimal influence on estimates of population genetic diversity and differentiation because of the large numbers of nests and few individuals per nest studied in each population (as demonstrated below for a subset of analyses). The identities of unique mtDNA sequence variants and their population frequencies were determined using the program ARLEQUIN (Schneider *et al*. 2000).

We estimated effective numbers of alleles at each allozyme locus using equation 16 in Nielsen *et al*. (2003). Gene diversity (expected heterozygosity; Nei 1987) averaged across all loci was estimated as a measure of nuclear genetic diversity using output from GENEPOP. For the mtDNA sequence data, haplotype diversity (gene diversity), which does not take sequence divergence among haplotypes into account, as well as nucleotide diversity, which does, were calculated as measures of within-population diversity of the mtDNA genome using ARLEQUIN (see Nei 1987). This program was used also to estimate the extent of mtDNA nucleotide divergence (d_A, average number of net substitutions per site) between pairs of populations (Nei 1987).

Allozyme genotype proportions were tested for conformity to proportions expected under Hardy–Weinberg equilibrium

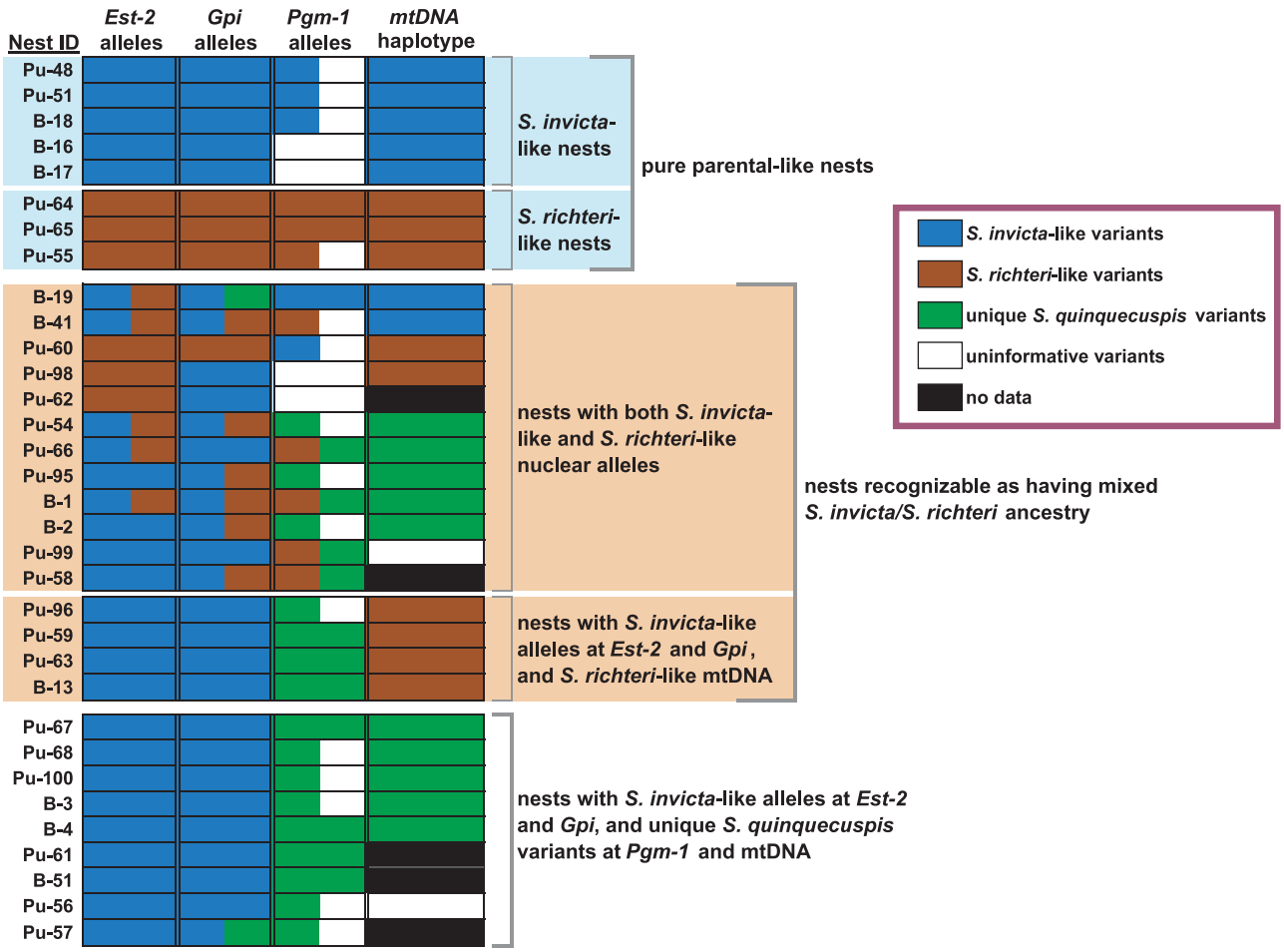


Fig. 2 Nuclear gene and mtDNA haplotype composition of 33 colonies identified as *Solenopsis quinquecuspis* on the basis of the morphology of colony members. The three allozyme loci summarized in the figure exhibit complete or pronounced allele frequency differences between *Solenopsis invicta* and *Solenopsis richteri*. An earlier study of *S. quinquecuspis* found that it is fixed for a *S. richteri*-like allele at *Est-2* and a *S. invicta*-like allele at *Gpi* (Ross & Trager 1990). The locus *Pgm-1* was not surveyed in that study.

(HWE) by calculating Fisher exact probabilities for each locus, then combining the probabilities across loci (Fisher method), using GENEPOP. Composite genotypic disequilibrium between pairs of polymorphic allozyme loci (Weir 1996) was evaluated using the same program. To ensure that the results were not influenced by sampling multiple genotypes per nest, we conducted these procedures on 10 separate data sets constructed by randomly resampling a single individual's genotype from each nest as well as on the full data set. Alpha levels were adjusted using the Bonferroni correction.

Cytonuclear disequilibrium between the mtDNA and three most informative allozyme loci was evaluated in *S. quinquecuspis* to reveal potential patterns of introgression. Composite nuclear genotypes were constructed for each nest for the loci *Est-2*, *Gpi*, and *Pgm-1* (see Fig. 2). For the first two loci, all alleles diagnostic for Rosario *S. invicta* were pooled into one class, whereas alleles diagnostic for Rosario *S. richteri* were pooled into another (Latta *et al*. 2001). [We note that a previous study of *S. quinquecuspis* from areas outside Rosario concluded that the species is fixed for the common *S. richteri* allele at *Est-2* and the common *S. invicta* allele at *Gpi* (Ross & Trager 1990)]. Alleles at *Pgm-1* were pooled into four classes: alleles diagnostic for *S. invicta*, alleles diagnostic for *S. richteri*, alleles diagnostic for *S. quinquecuspis*, and uninformative alleles. The mtDNA haplotypes were pooled in this same way. Normalized disequilibrium measures were estimated to quantify non-random associations between mtDNA haplotypes and allozyme alleles (allelic cytonuclear disequilibrium) or allozyme genotypes (genotypic cytonuclear disequilibrium) (Asmussen & Basten 1994, 1996). Monte Carlo approximations employed to test for general departures from random cytonuclear associations were followed by a series of exact tests to determine specific nonrandom associations (Basten & Asmussen 1997). The program CND was used for these analyses (Basten 2002).

Fisher exact probabilities of allele and haplotype frequency differentiation between paired populations were calculated for each gene using GENEPOP (Raymond & Rousset 1995b); exact probabilities also were calculated for all of the polymorphic allozyme loci combined (Fisher method of combining results). Alpha levels for judging the significance of these probabilities again were adjusted using the Bonferroni correction.

Estimates of F_{ST} between paired populations based on combined data for all seven allozyme loci were obtained with GENEPOP. Again, we also obtained F_{ST} estimates from each of the 10 resampled data sets with a single genotype per nest. Estimates of F_{ST} based on the mtDNA sequences were obtained with ARLEQUIN; these estimates incorporated information on the mutational divergence between haplotypes by using Euclidean squared distances between haplotype pairs (Excoffier *et al*. 1992). We also used the hierarchical analysis of molecular variance procedure in ARLEQUIN (Excoffier *et al*. 1992) to estimate F_{ST} simultaneously at two levels (geographical population, species) for both the nuclear and mtDNA data from *S. invicta* and *S. richteri*, the two species sampled at both localities.

Evolutionary relationships of the mtDNA sequences were inferred using neighbour-joining (NJ) and maximum-parsimony (MP) methods, as implemented in the program PAUP* (Swofford 1998). HKY85 distances between haplotypes were used for the NJ analysis and ties were broken randomly. To assess support for particular nodes in the NJ tree, a bootstrap analysis was performed with 10 000 data resamplings. For the MP analysis, gaps were treated as a fifth base, 200 random-addition-sequence replications were performed in the stepwise addition procedure to obtain starting trees, the tree-bisection–reconnection algorithm was used for branch swapping, and optimal trees discovered during the heuristic search were input into the branch swapping procedure. Node support was assessed by means of a bootstrap analysis featuring 1000 data resamplings with 10 random-addition-sequence replications per bootstrap replicate.

We estimated evolutionarily effective levels of gene flow between pairs of populations (N_em) in several ways. First, these values were calculated from the estimates of F_{ST} using the relationships $F_{ST} = 1/(4N_em + 1)$ for the allozymes and $F_{ST} = 1/(2N_em + 1)$ for the mtDNA (Slatkin 1987; Neigel 1997; Whitlock & McCauley 1999), with N_em in the latter instance representing gene flow mediated by queens. Because of concerns about inferring gene flow levels from F_{ST} values (e.g. Hedrick 1999; Whitlock & McCauley 1999), alternative methods of estimating N_em also were used. For the allozymes, the private alleles method was employed by using the average estimated frequencies of all private alleles in equation 3 of Slatkin (1985). The resulting N_em values were corrected for sample size by dividing the initial estimate by $N_{sam}/25$ (Slatkin 1985), where N_{sam} was the average number of nests sampled per population. For the mtDNA, the cladistic method of Slatkin & Maddison (1989) was employed by making use of the NJ tree of haplotype relationships. Individuals of different species or populations were assigned different states of a hypothetical multistate unordered character, and parsimony criteria were used to infer the minimum number of state changes consistent with the NJ tree. These state changes correspond to hypothetical gene flow events. Sample sizes were equalized between pairs of populations by randomly pruning individuals of the larger sample from the NJ tree (Slatkin & Maddison 1989). Joint possession of a single haplotype by *S. quinquecuspis* and another species was considered to represent a gene flow event. The calculated minimum numbers of gene flow events were used to estimate N_em based on the simulation results of Slatkin & Maddison (1989; interpolations from their Table 1).

Results

General results

Frequencies of the allozyme alleles and mtDNA sequence haplotypes are listed in Appendix I for each population. [An apparent cryptic species within nominal *Solenopsis invicta* from Arroio dos Ratos (see below) is considered as a separate population in the data summaries that follow.] From two (*Est-4*) to 10 (*Est-2*) alleles were resolved at each allozyme locus, with the effective number of alleles per polymorphic locus (n_e) ranging from 1.05 to 3.79 within each population. The mean n_e over all seven loci ranged from 1.25 to 1.71 in the different populations.

All mtDNA sequences were aligned readily by eye. A total of 45 unique haplotypes were found, with the number detected within each population ranging from 4 to 14. A total of 127 point substitutions distributed over 115 polymorphic sites (105 of which are parsimony informative), as well as a single 3-bp indel, distinguished these haplotypes. Sequence divergence between pairs of unique haplotypes, estimated as the proportion of different nucleotides, ranged from 0.11% to 6.04%, with an average sequence divergence of 2.99% between each pair of unique haplotypes. The average AT compositional bias among the 45 sequences was 69.8%, a level of bias typical of the same gene region in other native fire ant populations (Ross *et al*. 2003; Shoemaker *et al*. 2003a). The NJ tree depicting the evolutionary relationships of the mtDNA sequence haplotypes is presented as Fig. 3. The strict consensus of the numerous minimum-length MP trees recovered is fully compatible with this NJ tree.

We evaluated congruence of the allozyme and mtDNA data with respect to gauging the evolutionary divergence of native fire ant populations by plotting F_{ST} estimates from the two types of markers for all pairs of heterospecific populations from separate localities. The rationale for including

separate populations only is that they presumably are immune to recent introgression and therefore estimates should reflect divergence through evolutionary time in the absence of any homogenizing effects of gene flow. The plot of these data (Fig. 4, dark squares) indicates that divergence between species at the two genomes, as assessed from our markers, generally occurs in parallel.

Genetic divergence of sympatric fire ant species

An initial unexpected result from our data is that nine nests identified as *S. invicta* from the Arroio dos Ratos locality are likely to represent a previously unrecognized cryptic species. This putative cryptic species is fixed for an *Est-2* allele not found in other *S. invicta* at this site, and it possesses moderately common *G3pdh-2*, *Gpi*, and *Pgm-1* alleles that likewise are not found in co-occurring *S. invicta* (Appendix I). The cryptic species has four completely unique mtDNA haplotypes that form a fairly divergent monophyletic group to the exclusion of all other sequences (Fig. 3, Appendix I). [Sequences of the cryptic species also are distinct from those of *Solenopsis megergates* and *Solenopsis macdonaghi*, the remaining members of the focal clade (D. D. Shoemaker *et al*., unpublished).] Although individuals from these nests are not distinguishable from other *S. invicta* on the basis of their morphology (J. P. Pitts, personal communication), they almost certainly represent a completely reproductively isolated lineage that therefore is considered separately for all analyses.

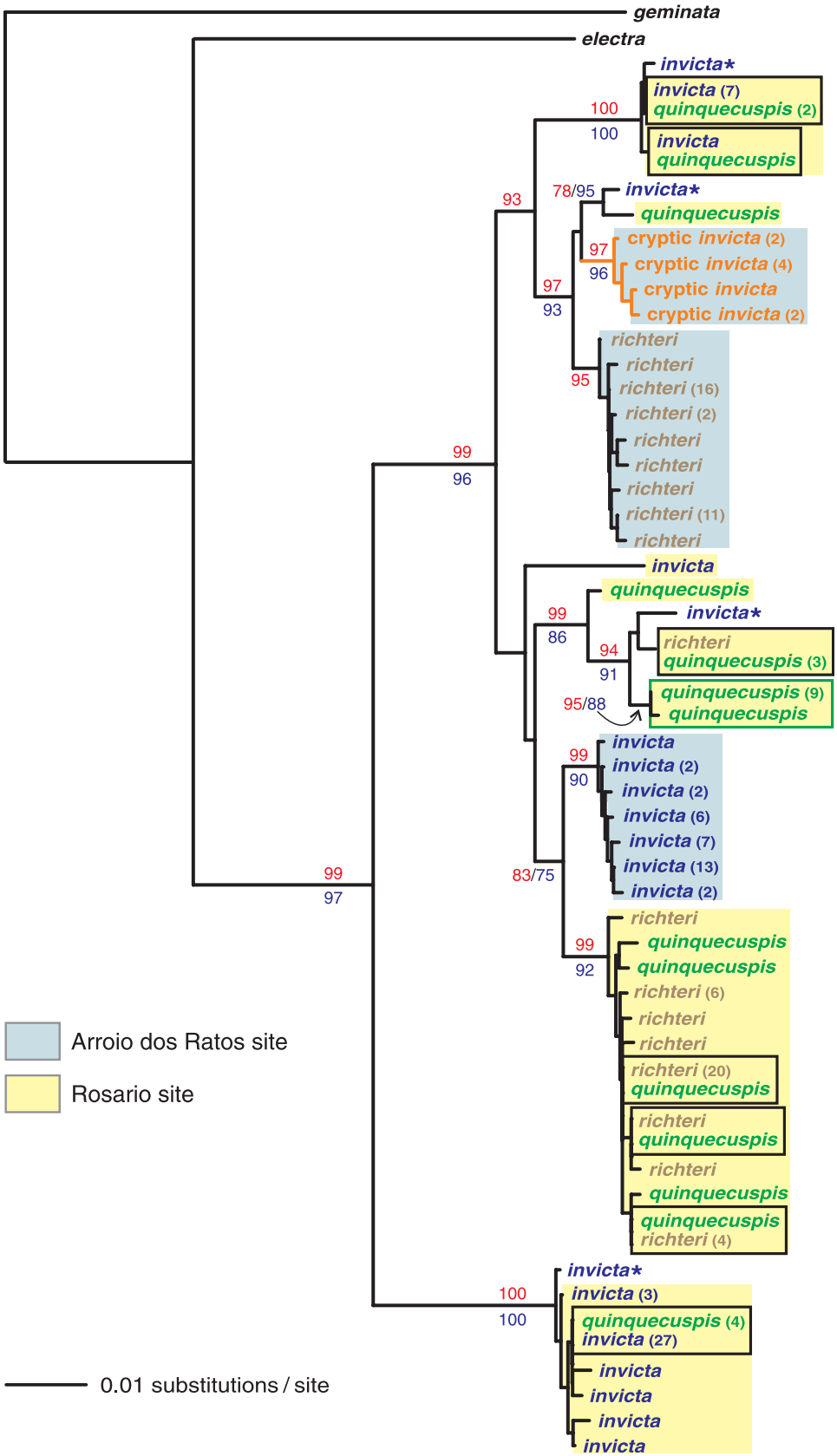
Turning to the issue of delimitation of the three focal fire ant species, the graphs in Fig. 5 depict allele proportions at the three most polymorphic allozyme loci and haplotype proportions for the mtDNA in co-occurring populations. It is evident from these graphs that such sympatric populations are strongly differentiated at both genomes. Considering first *S. invicta* and *Solenopsis richteri*, the two species are fixed for alternate alleles at *Gpi* and have virtually nonoverlapping sets of alleles at *Est-2* at both study sites. In addition, more or less pronounced allele frequency differences occur at one or more of the loci *Est-4*, *G3pdh-2*, and *Pgm-1* at each site (Fig. 5, Appendix I). Significantly, the two species share no mtDNA haplotypes at either site. Calculations of exact probabilities revealed that allele frequencies at each of the more polymorphic allozyme loci are highly significantly different between the two species at both sites, as are the mtDNA haplotype frequencies and joint frequencies across all allozyme loci (Fisher exact test, all $P < 0.001$). These data provide compelling evidence that *S. invicta* and *S. richteri* are fully reproductively isolated where they occur in sympatry in their native ranges.

The phylogenetic relationships of the mtDNA sequences provide further insights into the nature of genetic divergence between co-occurring *S. invicta* and *S. richteri* (Fig. 3). At Arroio dos Ratos, the haplotypes of the two species form reciprocally monophyletic groups that are relatively divergent from one another ($d_A = 2.5\%$), a pattern consistent with an absence of recent queen-mediated interspecific gene flow. At Rosario, the suites of haplotypes in each species do not form monophyletic groups. In *S. richteri*, this is due to the presence of a single divergent haplotype (disregarding the *Solenopsis quinquecuspis* haplotypes). In *S. invicta*, in contrast, this is due to the presence of three highly divergent haplotype clades (Fig. 3). The existence of similarly divergent, polyphyletic mtDNA lineages has been reported previously in *S. invicta* populations from northern Argentina (Ross *et al*. 1997; Shoemaker *et al*. 2000, 2003a). Despite the evident polyphyly of conspecific haplotypes at Rosario, all *S. richteri* sequences there apparently share a more recent common ancestor with one another than with any co-occurring *S. invicta* haplotype. Again, this pattern is consistent with a long-term absence of queen-mediated gene flow between these species at this site.

The issue of reproductive isolation between nominal species becomes more complicated when *S. quinquecuspis* is compared with the other two fire ant species where all three co-occur (Rosario). Pronounced, statistically significant allele frequency differences exist between *S. quinquecuspis* and each of the other species at virtually all of the polymorphic allozyme loci (see Fig. 5), with this species highly significantly differentiated from the others when these nuclear loci are considered in aggregate (Fisher exact test, all $P < 0.001$). Also, mtDNA haplotype frequencies of *S. quinquecuspis* are significantly different from those of the other species (both $P < 0.001$). Moreover, *S. quinquecuspis* possesses at moderate frequency a *Pgm-1* allele that is unique to it (allele *85*), and over half of the *S. quinquecuspis* individuals in the study possessed mtDNA haplotypes unique to this species (Fig. 5). These data suggest that there are significant barriers to gene flow between *S. quinquecuspis* and its sympatric congeners at Rosario.

On the other hand, both the nuclear and mitochondrial gene pools assigned to *S. quinquecuspis* give the appearance of having incorporated genes of the other two species. For instance, each of the common alleles at the loci *Est-2*, *Gpi*, and *Pgm-1* that are diagnostic for *S. invicta* or *S. richteri* in Rosario occur also in *S. quinquecuspis* (Fig. 5, Appendix I). This pattern is paralleled closely by the mtDNA, with half of the haplotypes in *S. quinquecuspis* shared with co-occurring *S. invicta* or *S. richteri* (typically, these are the more common variants in the latter). Even *S. quinquecuspis* haplotypes that are not shared with the other species are distributed throughout the mtDNA tree (Fig. 3), leading to the inference that they often are derived from haplotypes of the other two species rather than from other *S. quinquecuspis* haplotypes.

Inspection of the multilocus cytonuclear composition of morphologically identified *S. quinquecuspis* nests appears to confirm the chimeric nature of the gene pools of our study population (Fig. 2). Almost one-fourth of the nests have


geminata
electra
invicta*
invicta (7)
quinquecuspis (2)
invicta
quinquecuspis
100
100
93
78/95
invicta*
quinquecuspis
cryptic invicta (2)
cryptic invicta (4)
cryptic invicta
cryptic invicta (2)
97
96
97
93
richteri
richteri
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richteri (20)
quinquecuspis
richteri
quinquecuspis
richteri
quinquecuspis
quinquecuspis
richteri (4)
99
92
invicta*
invicta (3)
quinquecuspis (4)
invicta (27)
invicta
invicta
invicta
invicta
100
100
Arroio dos Ratos site
Rosario site
0.01 substitutions / site

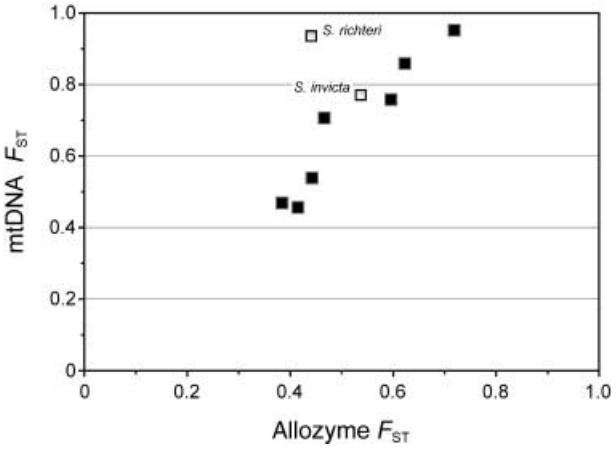

Fig. 4 Estimates of genetic divergence, as assessed by F_{ST}, between pairs of geographically separate populations of native fire ants based on seven allozyme loci and on mtDNA sequences. Values for populations of different species are shown by dark squares (data for the cryptic species within nominal *Solenopsis invicta* from Arroio dos Ratos are included); the correlation coefficient of 0.949 for this set of values is highly significant (permutation test with 200 random permutations of the original data matrices; $P < 0.005$). Values for conspecific populations of *S. invicta* and *Solenopsis richteri* are shown by light squares.

Table 1 Normalized allelic cytonuclear disequilibria for *Est-2*, *Gpi*, and *Pgm-1* in specimens identified morphologically as *Solenopsis quinquecuspis*. Open boxes highlight disequilibria showing positive associations of *Solenopsis invicta*-like or *Solenopsis richteri*-like nuclear alleles with conspecific mtDNA, whereas shaded boxes highlight disequilibria showing negative associations of these nuclear alleles with the mtDNA of the alternate species. Statistically significant disequilibrium measures are indicated by asterisks

	mtDNA haplotypes		
	S. invicta-like	*S. richteri*-like	*S. quinquecuspis*
Est-2 alleles			
S. invicta-like	0.467	–0.564*	0.440
S. richteri-like	–0.467	0.509*	–0.440
Gpi alleles			
S. invicta-like	0.654	–0.423*	0.169
S. richteri-like	–0.654	0.423*	–0.169
Pgm-1 alleles			
S. invicta-like	0.063*	–0.007	–0.004
S. richteri-like	–0.002	0.003	–0.002
S. quinquecuspis	–1.000*	0.030	0.525*

apparent pure heterospecific gene compositions at both the nuclear and mtDNA genomes, with some of these nests resembling *S. invicta* and others *S. richteri* (based on possession of diagnostic variants or, in the case of the mtDNA, their close mutational derivatives in the Rosario populations of these species). On the other hand, about half of the *S. quinquecuspis* nests show evidence of mixed ancestry, with the majority of these containing both *S. invicta*-like and *S. richteri*-like nuclear genes at *Est-2* and *Gpi*, and the rest containing *S. invicta* genes at these loci together with *S. richteri*-like mtDNA. The final one-fourth of nests contain only *S. invicta*-like genes at the loci *Est-2* and *Gpi* but bear unique variants diagnostic of *S. quinquecuspis* at *Pgm-1* (allele *85*) and, in most cases, the mtDNA (sister haplotypes *B-1* and *B-3*; Fig. 3). We note that only two colonies (Pu-62, Pu-98) would be considered pure *S. quinquecuspis* among all those identified morphologically as such based on the species-specific combination of alleles at *Est-2* and *Gpi* reported by Ross & Trager (1990). Nonetheless, over 60% of the colonies identified as *S. quinquecuspis* contain the diagnostic $Pgm\text{-}1^{85}$ allele, with the diagnostic haplotypes *B-1* and *B-3* occurring only in such nests (neither *Pgm-1* nor the mtDNA were studied by Ross & Trager 1990).

Formal analyses of associations between the nuclear and mtDNA variants in *S. quinquecuspis* revealed allelic cytonuclear disequilibria that were highly significant for *Est-2* and *Pgm-1* (both $P < 0.001$) and marginally nonsignificant for *Gpi* ($P = 0.10$). For all three loci, alleles characteristic of *S. invicta* or *S. richteri* tend to be associated with the mtDNA haplotypes of the same species, as indicated by positive disequilibria (Table 1, also Fig. 2). Conversely, nuclear alleles characteristic of each species are negatively associated with the mtDNA of the alternate species. These associations are statistically significant for the *S. richteri* haplotypes associated with *Est-2* and *Gpi* alleles, and for the positive association of the *S. invicta* haplotypes with the *S. invicta*-like *Pgm-1* allele (all $P < 0.02$). Finally, the single diagnostic nuclear allele for *S. quinquecuspis*, $Pgm\text{-}1^{85}$, is positively associated with the diagnostic mtDNA variants for *S. quinquecuspis* and negatively associated with *S. invicta*-like mtDNA variants. Significant genotypic cytonuclear disequilibria are in accord with the allelic disequilibria, with *S. richteri* haplotypes

Fig. 3 Neighbour-joining tree depicting relationships of mtDNA sequence haplotypes from three nominal South American fire ant species (*Solenopsis invicta*, *Solenopsis quinquecuspis*, and *Solenopsis richteri*). Included also are sequences from a presumed cryptic species identified as *S. invicta* from Arroio dos Ratos (orange branches). Sequences from *Solenopsis geminata* and *Solenopsis electra* serve as outgroups. The percentages of bootstrap replicates in which a node was recovered are shown for the neighbour-joining (red) and maximum-parsimony (blue) methods for all values of 75% or greater. Numbers of individual ants possessing a haplotype are indicated in parentheses when greater than one. Identical haplotypes shared by *S. quinquecuspis* and the other species are enclosed in black rectangles, while the diagnostic *S. quinquecuspis* haplotypes *B-1* and *B-3* are enclosed in a green rectangle. Four representative haplotypes of *S. invicta* described in a previous study of two populations from northern Argentina (Shoemaker *et al*. 2003a) are indicated by asterisks.

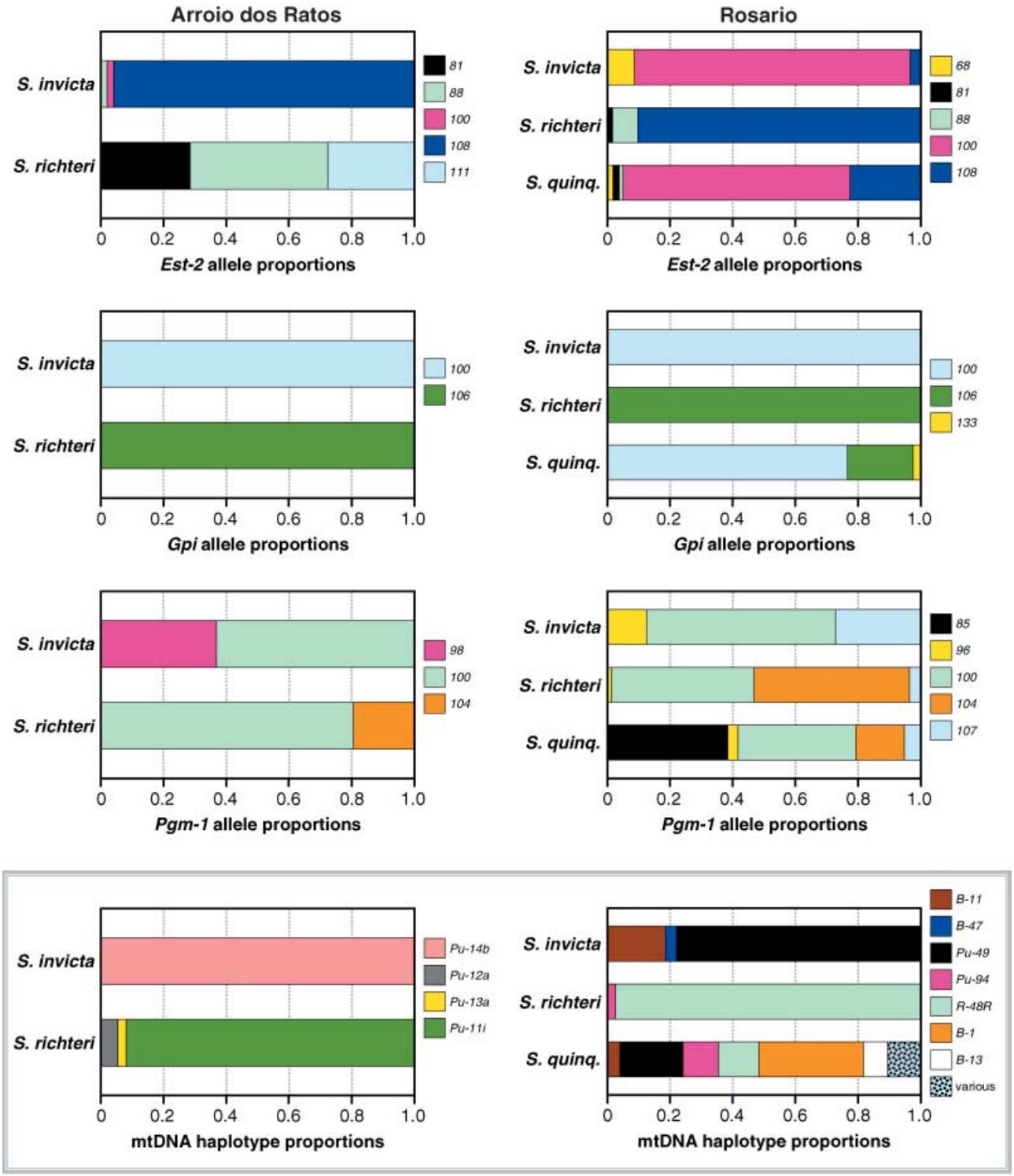

Fig. 5 Allozyme allele and mtDNA haplotype proportions in *Solenopsis invicta*, *Solenopsis richteri*, and *Solenopsis quinquecuspis* from two sampling sites. Rare allozyme alleles (those present at frequencies < 0.05 across all samples) and closely related mtDNA haplotypes (those distinguished by five or fewer nucleotide substitutions) are pooled; in these cases bins are designated by the code of a representative variant. The bin labelled 'various' for the mtDNA of *S. quinquecuspis* represents three sequences that are moderately diverged from each other and from all other haplotypes found in this study. Only the three most informative allozyme loci are depicted.

significantly associated with *S. richteri* genotypes at *Est-2* and *Gpi*, and *S. quinquecuspis* haplotypes associated with *S. quinquecuspis* genotypes at *Pgm-1*.

The evident composite nature of both the nuclear and mitochondrial gene pools of *S. quinquecuspis*, coupled with the observed patterns of cytonuclear disequilibria, suggest recurrent hybridization of both *S. invicta* and *S. richteri* with *S. quinquecuspis*. Indeed, the multilocus patterns suggest that morphologically defined *S. quinquecuspis* from Rosario includes a complex mixture of backcrossed and advanced generation hybrids, but with a large proportion of apparently introgressed nests bearing *Pgm-1* and mtDNA variants unique to *S. quinquecuspis*. One consequence of this presumed introgression from *S. invicta* and *S. richteri* is a lower level of differentiation between *S. quinquecuspis* and its sympatric congeners, as assessed by F_{ST} values, than is typically seen between co-occurring heterospecific populations (Table 2). Given the general absence in both *S. invicta* and *S. richteri* of nuclear and mtDNA variants that are common in *S. quinquecuspis* (Fig. 5), it does not appear that significant gene flow occurs in the opposite directions, that is, from *S. quinquecuspis* to the other species.

Table 2 Estimates of genetic divergence (F_{ST} values) between co-occurring pairs of populations of different fire ant species based on seven allozyme loci and on mtDNA sequences. Estimates involving *Solenopsis quinquecuspis* are highlighted in bold

	Allozymes	mtDNA
Arroio dos Ratos		
S. invicta/*S. richteri*	0.655	0.945
S. invicta/cryptic *S. invicta*	0.496	0.955
S. richteri/cryptic *S. invicta*	0.573	0.870
Rosario		
S. invicta/*S. richteri*	0.679	0.779
S. invicta/*S. quinquecuspis*	**0.131**	**0.490**
S. richteri/*S. quinquecuspis*	**0.502**	**0.443**

Genetic divergence of regional conspecific fire ant populations

We evaluated the genetic differentiation between the Arroio dos Ratos and Rosario populations of both *S. invicta* and *S. richteri* to assess levels of gene flow that occur over substantial geographical scales within each species. The extent of regional differentiation between conspecific populations of each species is striking (Fig. 5). Although conspecific populations share many of the same alleles at the allozyme loci, allele frequencies typically are highly significantly different at individual loci (Fisher exact test, $P < 0.001$ for seven of nine comparisons), and there are instances of high- or moderate-frequency alleles in one population being absent in the other conspecific population. Accordingly, conspecific populations of both species were found to be highly significantly differentiated when the allozyme loci were considered in aggregate (both $P < 0.001$). Regional differentiation is even more pronounced at the mtDNA, with no haplotypes shared between conspecific populations of either species. Remarkably, the haplotypes of each species from Arroio dos Ratos have closer affinities to various haplotypes of the alternate species from Rosario than they do to conspecific haplotypes from that region (Fig. 3). Thus, the regional populations within both *S. invicta* and *S. richteri* may not have been linked by gene flow of appreciable magnitude for some period. Indeed, the extent of nuclear and mtDNA differentiation observed between these regional conspecific populations is not unlike that observed between geographically separate populations of different nominal species (Fig. 4).

To further assess how regional genetic differentiation within these species compares to differentiation between them, we partitioned the total nuclear and mtDNA variation by estimating F_{ST} simultaneously at both levels using analysis of molecular variance (Excoffier *et al*. 1992). For the allozymes, less than 1% of the total variation at our markers is distributed between species, with about 32% distributed between regional populations within species (the remaining 67% resides within populations). For the mtDNA, the between-species variance component is negative, reflecting the fact that many haplotypes have higher sequence similarity to haplotypes of heterospecific ants than to haplotypes of conspecifics from the other site. If the between-species component is assumed to equal zero, then 84% of the total mtDNA variation resides between regional conspecific populations, with the remaining 16% distributed within populations. Thus, our data from both genomes indicate that the level of genetic differentiation between geographical populations within *S. invicta* and *S. richteri* is at least on a par with, and likely surpasses, the overall differentiation observed between these nominal species (also Fig. 4).

Effective gene flow levels between populations

The evolutionary significance of the genetic differentiation we measured can be judged by estimating effective levels of gene flow between populations (N_em) with respect to the set of markers we employed. For co-occurring *S. invicta* and *S. richteri*, the estimates of N_em obtained using different methods are well below 1.0 for the allozymes (which register biparental gene flow) and below 0.5 for the mtDNA (which registers maternal gene flow) at both study localities (Table 3). [The threshold value of 1.0 for a biparental marker represents the point where gene flow balances the effects of drift for neutral genes in a simple island model (Slatkin 1987). The threshold value of 0.5 for a maternal marker additionally assumes that gene flow is mediated equally by both sexes.] Similarly low values of gene flow are estimated

	Allozymes		mtDNA	
	F_{ST} method	Private alleles method	F_{ST} method	Cladistic method
Co-occurring heterospecific populations				
Arroio dos Ratos				
S. invicta/*S. richteri*	0.132	0.134	0.029	< 0.1
S. invicta/cryptic *S. invicta*	0.254	—*	0.023	< 0.1
S. richteri/cryptic *S. invicta*	0.186	—*	0.075	< 0.1
Rosario				
S. invicta/*S. richteri*	0.118	0.143	0.142	< 0.1
S. invicta/*S. quinquecuspis*	**1.655**	**2.980**	**0.520**	**0.562**
S. richteri/*S. quinquecuspis*	0.248	0.893	**0.630**	**2.871**
Regional conspecific populations				
S. invicta	0.235	0.400	0.144	< 0.1
S. richteri	0.344	0.571	0.034	< 0.1

Table 3 Estimates of effective levels of gene flow (N_em) between native fire ant populations based on seven allozyme loci and on mtDNA sequences. Estimates obtained using two different methods for each marker type are shown for co-occurring heterospecific populations at two localities (Arroio dos Ratos and Rosario) as well as for regional conspecific populations of *Solenopsis invicta* and *Solenopsis richteri* from these same localities. Allozyme-based (biparental) gene flow levels > 1.0 and mtDNA-based (maternal) gene flow levels > 0.5 are highlighted in bold

*Sample sizes for the cryptic species within nominal *S. invicta* from Arroio dos Ratos are insufficient to reliably calculate N_em using the private alleles method.

between each of these two species and the cryptic species with which they are sympatric in Arroio dos Ratos (Table 3). In contrast, levels of N_em between *S. quinquecuspis* and the other two species in Rosario exceed 0.5 for the mtDNA and exceed 1.0 for the allozymes in the case of *S. invicta*. For conspecific populations of *S. invicta* and *S. richteri*, values of N_em again are well below 1.0 for the allozymes and 0.5 for the mtDNA (Table 3). F_{ST} values for the 10 resampled data sets with a single genotype per nest are virtually identical to the values obtained using the full data set, as are the derivative N_em values.

Genetic disequilibrium and diversity within populations

Allozyme genotype proportions were found not to depart significantly from HWE proportions in *S. invicta* and *S. richteri* at each sampling site when probabilities were combined across loci for the complete data set (Fisher exact test, all $P > 0.05$). In contrast, four of the six polymorphic loci in *S. quinquecuspis* (including *Est-2*, *Gpi*, and *Pgm-1*) exhibited significant homozygote excesses, and the overall probability of departure across all six loci was highly significant ($P < 0.001$). Comparable results were obtained when the exact tests were conducted on the 10 resampled data sets with a single individual per nest (data not shown). Widespread excess homozygosity is expected under some scenarios of hybridization invoking under-dominance or homogamic matings (e.g. Kocher & Sage 1986; Szymura 1993; Harrison & Bogdanowicz 1997), so its presence in our *S. quinquecuspis* study population appears consistent with the hypothesis that this population experiences admixture of alleles from the other species.

Analyses of composite linkage disequilibrium between pairs of polymorphic allozyme loci revealed four significant patterns of genotypic association in the full data set, only one of which occurred outside of *S. quinquecuspis*. Genotypes at the two esterase loci (*Est-2* and *Est-4*) are not independent in *S. invicta* from Rosario, a result in keeping with the common linkage of esterase genes in some insects (Zhu *et al*. 1999; Bourguet *et al*. 2004). In *S. quinquecuspis*, genotypes are nonrandomly associated between each of the loci *Est-2*, *Gpi*, and *Pgm-1*. Specifically, there are excesses of most of the two-locus genotypes characteristic of *S. invicta* and *S. richteri* in Rosario, as well as an association of the diagnostic *S. quinquecuspis* genotype at *Pgm-1* (*85/85*) with the *S. invicta*-like genotypes at *Est-2* and *Gpi* (both *100/100*) (also Fig. 2). The former pattern again appears consistent with ongoing gene flow into the *S. quinquecuspis* study population from the other species (Barton & Clark 1990), whereas the latter pattern may reflect higher levels of nuclear introgression from *S. invicta* than *S. richteri*. The results for the full data set were confirmed for the 10 resampled data sets with a single genotype per nest (data not shown).

Genetic diversity at each genome within each study population, as assessed by several measures, is depicted graphically in Fig. 6. A conspicuous pattern is that *S. quinquecuspis* is an outlier with respect to the high diversity it possesses at both genomes. This pattern is expected if this population is unique in having been subjected to significant introgression of heterospecific genes following its origin.

Discussion

The primary objective of this study was to learn whether genetic discontinuities indicative of complete reproductive isolation occur between morphologically delimited species of South American fire ants where they co-occur. Genetic divergence between such nominal species is most usefully

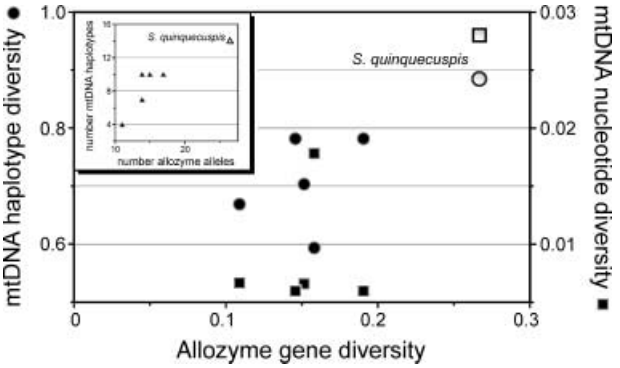

Fig. 6 Estimates of genetic diversity within populations of native fire ants based on seven allozyme loci and on mtDNA sequences. Diversity is assessed by two measures for the allozymes (gene diversity, number of alleles) and three measures for the mtDNA (haplotype diversity, nucleotide diversity, number of haplotypes). Values for *Solenopsis quinquecuspis* are indicated by light symbols and those for the other populations by dark symbols (data for the cryptic species within nominal *Solenopsis invicta* from Arroio dos Ratos are included).

evaluated when the extent of geographically based variation within the species also is known. From an operational perspective, formally recognized species should comprise the largest entities exhibiting free interbreeding among their component organisms (Lee 2003; also Morjan & Rieseberg 2004); that is, there should be regular gene flow among geographical populations. Thus, a secondary objective was to assess the magnitude of genetic differentiation between regional populations within nominal fire ant species. The motivation for both objectives stems from the historical difficulties in developing informative morphological characters for discerning fire ant species and the resulting instability in the alpha-taxonomy of the group, problems that in turn have led to uncertainties as to whether each nominal species corresponds to an evolutionarily independent entity and whether all such entities have been formally recognized by being given species taxon status (e.g. Hey *et al*. 2003).

Genetic differentiation of nominal species and the origins of reproductive isolation

We found that the fire ant species we studied generally are clearly distinguishable from one another by the joint use of allozyme data and mtDNA *COI* gene sequences. In particular, *Solenopsis invicta* and *Solenopsis richteri*, whose species distinctiveness has long been a matter of debate (Wilson 1952; Buren 1972; Ross *et al*. 1987), appear to be completely reproductively isolated in native areas of sympatry. As evidence of this, several unique variants at markers of both the nuclear and mtDNA genomes are fixed or virtually fixed in each species at both study localities. Moreover, relationships of the mtDNA sequences suggest that these presumably complete barriers to gene flow between co-occurring *S. invicta* and *S. richteri* populations have been stable over a substantial period. Thus, little doubt can remain that the entities to which these names refer at each study site are evolutionarily independent of the sympatric heterospecific entity and thus warrant species status.

The lack of consequential gene flow between *S. invicta* and *S. richteri* in their native ranges, anticipated in the more modest study of Ross & Trager (1990), stands in contrast to the extensive hybridization that occurs between them in their introduced ranges in the USA (Vander Meer *et al*. 1985; Ross *et al*. 1987; Shoemaker *et al*. 1996). Coupled with results suggesting only minor breakdown in hybrid fitness there (Ross & Robertson 1990; Shoemaker *et al*. 1996), our data may be taken to support earlier speculation that premating barriers normally act to block genetic exchange between the species and that these barriers were compromised once the ants were introduced into the new environment (Ross & Robertson 1990; Ross & Trager 1990). *S. invicta* and *S. richteri* are not sister species (see Fig. 1), implying that their ancestral lineages diverged before those of the other species in the apical clade to which they belong. Based on such information, Ross & Trager (1990) further speculated that intrinsic postmating barriers generally are poorly developed in the clade and that premating isolation plays a primary role in instigating the divergence of incipient fire ant species.

More recently, studies of the cytoplasmic endosymbiont *Wolbachia* have suggested that reproductive incompatibilities between fire ants from populations harbouring different bacterial strains may create barriers to gene flow that initiate speciation in the absence of intrinsic genomic incompatibilities (Shoemaker *et al*. 2000, 2003a). Depending on the form of cytoplasmic incompatibility displayed, matings between sexuals of different infection status could result in complete reproductive breakdown (postmating, prezygotic barrier) or in reductions of queen fertility that in turn drive the evolution of premating isolation (see Servedio 2001; Servedio & Noor 2003). The fact that the reproductive compatibility evident between *S. invicta* and *S. richteri* in the USA is associated with a lack of *Wolbachia* infections (Shoemaker *et al*. 2000) hints that the former mechanism could play a role in the isolation of these species where they occur in natural sympatry. If it does, interspecific matings may actually occur in native populations but fail to yield viable sexual progeny required for effective gene flow. Additional studies are needed to disentangle the proximate roles played by this symbiont and by any differences in timing of mating flights, site of mating, or sex pheromone chemistry in the initiation and subsequent enforcement of reproductive isolation in South American fire ants.

Our study also revealed significant genetic discontinuities between *Solenopsis quinquecuspis* and the other two species

where their native ranges overlap, indicative of at least partially developed reproductive isolation of *S. quinquecuspis*. Significantly different frequencies of variants at most allozyme loci and the mtDNA distinguish *S. quinquecuspis* from its congeners. Moreover, *S. quinquecuspis* possesses both a common allozyme allele (*Pgm-1*^{85}) and a common mtDNA clade (*B-1/B-3*) that we conclude are unique variants of this species. Indeed, over 60% of colonies identified as *S. quinquecuspis* on the basis of the morphology were found to bear these diagnostic variants.

Although our *S. quinquecuspis* study population appears at least partially reproductively isolated from sympatric *S. invicta* and *S. richteri*, it does share many allozyme and mtDNA variants in common with them, a pattern potentially explicable by at least two causes: incomplete sorting to reciprocal monophyly of variants present in the common ancestor of all three species (e.g. Avise 2000; Funk & Omland 2003; McCartney *et al*. 2003) or hybridization. The first explanation, appealing as it seems given the presumed recency of speciation in the group, appears unlikely. One reason is that the mtDNA haplotypes of *S. quinquecuspis* are deeply polyphyletic and widely scattered over the gene tree for the three species (Fig. 3), yet the haplotypes of *S. invicta* and *S. richteri* in Arroio dos Ratos are reciprocally monophyletic, suggesting ample time for complete sorting of ancestral mtDNA polymorphisms. Fixation of alternative *Gpi* alleles in *S. invicta* and *S. richteri* at each locality suggests the same for this nuclear marker, while possession of several diagnostic morphological traits by *S. quinquecuspis* implies fixation (complete sorting) of other nuclear genes. Significantly, variants shared between *S. quinquecuspis* and the other species occur at all of the polymorphic markers and they tend to be the most common variants in *S. invicta* and *S. richteri*. While this pattern is not necessarily expected under incomplete lineage sorting, it is predicted if recurrent hybridization with the other species has led to the introgression of these genes into *S. quinquecuspis*.

Other evidence for introgressive gene flow into *S. quinquecuspis* is its comparatively high genetic diversity (Fig. 6) and the fact that it was the only study population found to display widespread excess homozygosity, as may be expected following interspecific hybridization under some circumstances (e.g. Barton & Clark 1990; Harrison & Bogdanowicz 1997). Examination of the multilocus composition of *S. quinquecuspis* individuals and nests revealed apparent extensive recombination of the nuclear and mtDNA genes of *S. invicta* and *S. richteri* into the *S. quinquecuspis* genomic background. This recombination has not completely eroded parental patterns of linkage and cytonuclear disequilibrium, because co-occurring mtDNA and nuclear variants characteristic of each of the three species are present in excess in individuals identified as *S. quinquecuspis*. Random interbreeding is expected to rapidly break up these parental-like nuclear and cytonuclear associations (Asmussen *et al*. 1989; Barton & Clark 1990; Goodisman & Asmussen 1997; Goodisman *et al*. 1998), so that their persistence is consistent with ongoing introgression, positive assortative mating, selection against more highly admixed individuals, or any combination of these factors. It is noteworthy that the unique mtDNA haplotypes of *S. quinquecuspis* occur only in combination with its unique *Pgm-1* allele, because selection on, or mate choice by, *S. quinquecuspis*-like individuals presumably is required to maintain this association in the face of gene flow from the other species. The operation of either force makes it doubtful that *S. quinquecuspis* actually originated as a hybrid species, suggesting instead that introgression from *S. invicta* and *S. richteri* commenced after its initial divergence.

This hypothesized introgression into *S. quinquecuspis* apparently has not been sufficient to erase the unique morphological features of the species (most notably, the presence of a median ocellus in the major workers — Pitts 2002) nor to eliminate the marked nuclear and mtDNA gene frequency differences between it and its close relatives. Our estimates of effective levels of gene flow (N_em) into *S. quinquecuspis* obtained by different methods generally are somewhat greater than the threshold values of 1.0 for the allozymes and 0.5 for the mtDNA that theoretically mark a balance between the effects of gene flow and drift. Nonetheless, depending on the particular selective regimes acting on *S. quinquecuspis* (Nagylaki 1975), it would seem feasible for this ant to maintain its unique biological attributes and thus display a distinct evolutionary trajectory in the face of the moderate gene flow inferred at the Rosario site (Templeton 1989; Rieseberg & Burke 2001). Moreover, this introgression appears to be a local phenomenon, given the lack of evidence for it in an earlier study of *S. quinquecuspis* from other sites (Ross & Trager 1990). (The collection sites for this earlier study lie outside the range of *S. invicta* but within the range of *S. richteri*.) Evidence that reproductive isolation of *S. quinquecuspis* is moderately developed in some areas and effectively complete in others, taken with the existence of unique nuclear DNA, mtDNA, and morphological character states indicating its evolutionary distinctiveness, tentatively corroborates the delimitation of *S. quinquecuspis* as a species separate from both *S. invicta* and *S. richteri*. However, additional data from numerous newly developed genetic markers and morphological characters in samples collected throughout its distribution are required to further test this hypothesis.

Our finding that *S. quinquecuspis*, the most recently diverged species in the clade containing the three study species, apparently is more susceptible to introgression than *S. invicta* or *S. richteri*, the two basal species of the group, is noteworthy because it suggests there has been insufficient time for complete development of intrinsic isolating mechanisms in *S. quinquecuspis*. Nonetheless, retention of the species-specific morphology of *S. quinquecuspis* in the

presence of moderate introgression suggests that the genes underlying these quantitative traits are not distributed extensively across the nuclear genome and that they penetrate species boundaries less readily than the presumably neutral genetic variants (e.g. Crochet *et al*. 2003). Studies using many nuclear markers are necessary to clarify how much, and which parts, of the *S. invicta* and *S. richteri* genomes are free to introgress into *S. quinquecuspis*, and why hybridization involving *S. quinquecuspis* apparently is limited to only part of its range (where it co-occurs with *S. invicta*). Also, studies detailing the frequency and identity of *Wolbachia* infections in *S. quinquecuspis* and its sympatric congeners are needed to learn how such introgression can occur in the face of the potential reproductive barriers induced by this symbiont. Such information may clarify why *S. quinquecuspis* apparently has a more porous species boundary than other fire ants and thereby help illuminate the forces involved in species formation in the group (e.g. Coyne & Orr 2004).

Relevance of the genetic data to the alpha-taxonomy of fire ants

Our general finding that the morphologically delimited species we studied are genetically distinct parallels the earlier conclusions of Ross & Trager (1990). These authors surveyed allozyme loci in six mostly allopatric South American fire ant species (including *S. invicta*, *S. richteri*, and *S. quinquecuspis*) and found that each nominal species could be distinguished from the others by unique suites of alleles at one or more loci. Our studies thus jointly contribute to an emerging consensus that the fire ant species recognized in modern morphological taxonomic studies are reproductively isolated from, and evolutionarily independent of, other such nominal species. The proposal that most of the observed fire ant diversity in South America represents geographical variation within a single widespread polytypic species (Wilson 1952) clearly is no longer tenable.

Although the current alpha-taxonomy succeeds in the sense that recognized fire ant species generally seem to be reproductively and evolutionarily independent of one another, the existence of genetically distinct populations within nominal species boundaries suggests that the taxonomy has not fully captured the species-level diversity of these ants. The clearest example in our study is the nine nests from Arroio dos Ratos identified morphologically as *S. invicta* that possess divergent mtDNA haplotypes and a diagnostic *Est-2* allele. A similar example from the study of Ross & Trager (1990) comprised several nests identified as *S. quinquecuspis* that possessed completely unique alleles at three nuclear loci (including the *G3pdh-1* locus studied here). The pronounced nuclear and mtDNA differentiation we found between regional populations within both *S. invicta* and *S. richteri*, which rivals the extent of differentiation between separate heterospecific populations, further hints at the presence of reproductively isolated, evolutionarily independent populations within these nominal species. Again, an earlier parallel example can be found. Ross *et al*. (1997) surveyed numerous nuclear loci and the mtDNA in samples of *S. invicta* from northern Argentina and showed that strong differentiation occurs at both genomes between two populations separated by less than 200 km. For both *S. invicta* and *S. richteri*, observed divergences between regional sites may have arisen as an effect of isolation by distance, although divergence between some population pairs appears to have been facilitated by major landforms of biogeographical relevance (see Ross *et al*. 1997; Ahrens *et al*. 2005). This body of results implies a general underresolution in the morphological delimitation of *Solenopsis saevissima*-group fire ant species, consistent with the idea that speciation is an ongoing process in these ants. Moreover, the results emphasize that additional comprehensive surveys using diverse genetic markers and newly developed morphological characters are necessary to approach a complete understanding of the fundamental units of diversity and evolution in this taxonomically difficult group.

Our observation of widespread species-level mtDNA paraphyly and polyphyly in the native fire ants we studied confirms earlier reports of this in *S. invicta* (Shoemaker *et al*. 2000, 2003a). Discordance between mtDNA gene trees and species trees is common in studies such as ours that feature large numbers of samples collected widely across the ranges of closely related species (reviewed in Funk & Omland 2003). Possible causes of such discordance in fire ants include ongoing hybridization and the presence of unrecognized species (both proposed in the present study), incomplete lineage sorting, and trans-specific mtDNA capture following past hybridization (perhaps coupled with *Wolbachia*-mediated selective sweeps) (Shoemaker *et al*. 2000). Incomplete sorting may contribute to the paraphyly within nominal *S. invicta* if this geographically widespread form has produced multiple unrecognized daughter species through peripheral isolation (see Funk & Omland 2003; also Ahrens *et al*. 2005), an idea compatible with the basal position of *S. invicta* in the clade containing the study species (Pitts *et al*. 2005). More generally, *Wolbachia*-mediated mtDNA capture is an attractive explanation for mtDNA/nuclear discordance in native fire ants because of the widespread occurrence of the symbiont and the likely efficiency of the process following even rare instances of hybridization (Shoemaker *et al*. 2000, 2003b). Indeed, the deep divergence between some conspecific mtDNA clades may be explained by another predicted effect of infection, an accelerated substitution rate resulting from recurrent *Wolbachia* sweeps in geographical host populations linked by minimal gene flow (see Shoemaker *et al*. 2004 for full discussion).

Genetic vs. morphological divergence in fire ant speciation

The evident occurrence of cryptic species, as well as the difficulties in developing morphological characters to diagnose accepted species, hint that morphological divergence often is uncoupled from the emergence of reproductive isolation in fire ants (Ross & Trager 1990). Convergent reduction and simplification of the morphology associated with the universally complex social habits of these ants has been proposed to explain the relative uniformity of the worker caste (e.g. Trager 1991; Pitts 2002), the material on which most revisionary taxonomic work is based. However, recent analyses indicate that such uniformity across species extends to the male and female sexual castes as well (Pitts 2002). While the causes of the apparently conservative rates of morphological evolution in fire ants remain obscure, continued study of genetic differences among closely related but distinct evolutionary entities promises to yield fresh insights into the origins and consequences of barriers to gene flow and, in so doing, to provide additional clues to solve this puzzle.

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Ken Ross's research focuses on the evolutionary genetics of social Hymenoptera and other insects, with much of his work concerning native and introduced fire ant populations. Current projects on these ants include studies of the molecular systematics, large-scale population genetic structure, and the genetic basis of social organization. The main emphasis of DeWayne Shoemaker's research is the population and evolutionary genetics of various insects, focusing on fire ants and *Wolbachia*-insect interactions.

Appendix I

Frequencies of allozyme alleles and mtDNA sequence haplotypes in native South American fire ant populations

	Arroio dos Ratos, Brazil			Rosario, Argentina		
	S. invicta	cryptic *S. invicta*	*S. richteri*	*S. invicta*	*S. richteri*	*S. quinquecuspis*
Allozyme loci						
Aat-2	$N = 35$, $n = 70$	$N = 9$, $n = 18$	$N = 35$, $n = 70$	$N = 44$, $n = 88$	$N = 57$, $n = 84$	$N = 33$, $n = 66$
100	0	0	0	0.017	0.042	0.129
144	0.964	1.0	1.0	0.983	0.958	0.871
172	0.036	0	0	0	0	0
Est-2	$N = 35$, $n = 70$	$N = 9$, $n = 18$	$N = 35$, $n = 70$	$N = 44$, $n = 88$	$N = 57$, $n = 84$	$N = 33$, $n = 64$
58	0	0	0	0	0.012	0
68	0	0	0	0.017	0	0.008
76	0	0	0	0.057	0	0.016
81	0	0	0.014	0	0.012	0.023
83.5	0	0	0.271	0	0	0
88	0.014	0	0.436	0	0.048	0.008
97	0	0	0.007	0	0	0
100	0.014	0	0	0.898	0	0.734
108	0.971	0	0	0.023	0.929	0.211
111	0	1.0	0.271	0.006	0	0
Est-4	$N = 35$, $n = 65$	$N = 9$, $n = 16$	$N = 35$, $n = 68$	$N = 44$, $n = 87$	$N = 57$, $n = 84$	$N = 33$, $n = 66$
100	0.331	0.719	1.0	0.908	1.0	0.955
160	0.669	0.281	0	0.092	0	0.045
G3pdh-1	$N = 35$, $n = 68$	$N = 9$, $n = 18$	$N = 35$, $n = 68$	$N = 44$, $n = 87$	$N = 57$, $n = 84$	$N = 33$, $n = 66$
96	0	0	0.015	0	0	0
100	1.0	1.0	0.971	1.0	1.0	1.0
118	0	0	0.015	0	0	0
G3pdh-2	$N = 35$, $n = 66$	$N = 9$, $n = 17$	$N = 35$, $n = 70$	$N = 44$, $n = 87$	$N = 57$, $n = 84$	$N = 33$, $n = 65$
33	0	0	0	0	0	0.031
60	0.076	0	0	0	0	0
74	0.061	0	0	0	0	0.008
100	0.864	0.941	1.0	0.908	1.0	0.938
124	0	0	0	0.023	0	0.008
137	0	0.059	0	0.069	0	0.015
Gpi	$N = 35$, $n = 70$	$N = 9$, $n = 18$	$N = 35$, $n = 70$	$N = 44$, $n = 88$	$N = 57$, $n = 84$	$N = 33$, $n = 66$
79	0	0.111	0	0	0	0
88	0	0	0	0	0	0.008
100	1.0	0.889	0	1.0	0	0.773
106	0	0	1.0	0	1.0	0.205
133	0	0	0	0	0	0.015
Pgm-1	$N = 27$, $n = 27$	$N = 9$, $n = 18$	$N = 35$, $n = 66$	$N = 44$, $n = 88$	$N = 57$, $n = 84$	$N = 33$, $n = 66$
85	0	0	0	0	0	0.379
86	0	0	0	0	0	0.008
96	0	0.167	0	0.136	0.006	0.03
98	0.370	0	0	0	0	0
100	0.630	0.833	0.803	0.608	0.464	0.379
104	0	0	0.197	0	0.494	0.152
107	0	0	0	0.256	0.036	0.053
mtDNA	$N = 33$	$N = 9$	$N = 35$	$N = 43$	$N = 36$	$N = 28$
Pu-14b (AY499583)	0.394	0	0	0	0	0
Pu-14f (AY499585)	0.212	0	0	0	0	0
Pu-14j (AY499586)	0.182	0	0	0	0	0
Pu-14n (AY499587)	0.061	0	0	0	0	0
Pu-15a (AY499588)	0.061	0	0	0	0	0
Pu-14a (AY499582)	0.061	0	0	0	0	0
Pu-14c (AY499584)	0.030	0	0	0	0	0
Pu-11h (AY499580)	0	0.444	0	0	0	0
Pu-13t (AY499581)	0	0.222	0	0	0	0

Appendix I *Continued*

	Arroio dos Ratos, Brazil			Rosario, Argentina		
	S. invicta	cryptic *S. invicta*	*S. richteri*	*S. invicta*	*S. richteri*	*S. quinquecuspis*
Pu-15k (AY499590)	0	0.222	0	0	0	0
Pu-15b (AY499589)	0	0.111	0	0	0	0
Pu-11i (AY499607)	0	0	0.457	0	0	0
Pu-11c (AY499606)	0	0	0.314	0	0	0
Pu-12a (AY499610)	0	0	0.057	0	0	0
Pu-11a (AY499605)	0	0	0.029	0	0	0
Pu-11j (AY499608)	0	0	0.029	0	0	0
Pu-11n (AY499609)	0	0	0.029	0	0	0
Pu-13a (AY499611)	0	0	0.029	0	0	0
Pu-13c (AY499612)	0	0	0.029	0	0	0
Pu-13s (AY499613)	0	0	0.029	0	0	0
Pu-49 (AY499571)	0	0	0	0.628	0	0.143
B-11 (AY499575)	0	0	0	0.163	0	0.071
B-25 (AY499577)	0	0	0	0.070	0	0
Pu-52 (AY499572)	0	0	0	0.023	0	0
B-5 (AY499573)	0	0	0	0.023	0	0
B-7 (AY499574)	0	0	0	0.023	0	0
B-24 (AY499576)	0	0	0	0.023	0	0
B-47 (AY499578)	0	0	0	0.023	0	0
B-49 (AY499579)	0	0	0	0.023	0	0.036
R-48R (AY586448)	0	0	0	0	0.556	0.036
Pu-73 (AY585346)	0	0	0	0	0.167	0
Pu-74 (AY585347)	0	0	0	0	0.111	0.036
Pu-94 (AY499614)	0	0	0	0	0.028	0.107
R-3R (AY249134)	0	0	0	0	0.028	0
R-19R (AY249132)	0	0	0	0	0.028	0
R-34R (AY249130)	0	0	0	0	0.028	0
R-46R (AY586449)	0	0	0	0	0.028	0
R-59R (AY249128)	0	0	0	0	0.028	0.036
B-1 (AY499591)	0	0	0	0	0	0.321
B-3 (AY499592)	0	0	0	0	0	0.036
B-13 (AY499593)	0	0	0	0	0	0.036
Pu-56 (AY499598)	0	0	0	0	0	0.036
Pu-64 (AY499600)	0	0	0	0	0	0.036
Pu-98 (AY499603)	0	0	0	0	0	0.036
Pu-99 (AY499604)	0	0	0	0	0	0.036

N indicates the number of nests and *n* the number of individuals in each population for which genotypes or haplotypes were scored for each marker (these are identical for the mtDNA because a single individual per nest was sequenced). Designations of allozyme alleles refer to the relative electrophoretic mobilities of the protein products. Designations of the mtDNA sequence haplotypes refer to an exemplar colony in which the haplotype was found (GenBank Accession nos are in parentheses).