Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Phylogenetics and Evolution 38 (2006) 200–215
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular phylogeny of fire ants of the *Solenopsis saevissima* species-group based on mtDNA sequences

D. DeWayne Shoemaker [a,*], Michael E. Ahrens [a], Kenneth G. Ross [b]

[a] *Department of Entomology, University of Wisconsin-Madison, 643 Russell Labs, 1630 Linden Drive, Madison, WI 53706, USA*
[b] *Department of Entomology, University of Georgia, Athens, GA 30602-2603, USA*

Received 21 March 2005; revised 13 July 2005; accepted 26 July 2005
Available online 19 September 2005

Abstract

The systematics of South American fire ants (*Solenopsis saevissima* species-group) has been plagued by difficulties in recognizing species and their relationships on the basis of morphological characters. We surveyed mtDNA sequences from 623 individuals representing 13 described and undescribed species within the species-group and 18 individuals representing other major *Solenopsis* lineages to generate a phylogeny of the mitochondrial genome. Our analyses support the monophyly of the *S. saevissima* species-group, consistent with a single Neotropical origin and radiation of this important group of ants, as well as the monophyly of the socially polymorphic species within the group, consistent with a single origin of polygyny (multiple queens per colony) as a derived form of social organization. The mtDNA sequences of the inquiline social parasite *S. daguerrei* form a clade that appears to be distantly related to sequences from the several host species, consistent with the view that advanced social parasitism did not evolve via sympatric speciation of intraspecific parasites. An important general finding is that species-level polyphyly of the mtDNA appears to be the rule in this group of ants. The existence of multiple divergent mtDNA lineages within several nominal species (including the pest *S. invicta*) suggests that the pattern of widespread polyphyly often stems from morphological delimitation that overcircumscribes species. However, in two cases the mtDNA polyphyly likely results from recent interspecific hybridization. While resolving species boundaries and relationships is important for understanding general patterns of diversification of South American fire ants, these issues are of added importance because invasive fire ants are emerging as global pests and becoming important model organisms for evolutionary research.

Keywords: Fire ants; mtDNA; Introgression; Phylogeny; Reproductive isolation; *Solenopsis*

1. Introduction

Two fundamental tasks of systematic biologists are to delineate the boundaries of species and to infer the relationships among them (Sites and Marshall, 2003, 2004; Wiens and Penkrot, 2002). One common impediment to developing hypotheses of species relationships is a failure to distinguish intraspecific from interspecific variation, which confounds definitions of the entities that serve as terminals. Conversely, assessment of valid species boundaries often hinges on phylogenetic analyses of single genes, which yield gene genealogies. For these reasons, it is often the case that the tasks of delimiting species and inferring their relationships (or the relationships of the genes they contain) must proceed simultaneously in reciprocally illuminating fashion (Sites and Marshall, 2003, 2004). Such a dual approach can be particularly challenging in phylogenetically young, actively radiating taxa composed of phenotypically similar but genetically distinct entities that are little diverged and occasionally exchange genes. Yet an understanding of species

* Corresponding author. Fax: +1 608 262 3322.
E-mail addresses: dshoemak@entomology.wisc.edu (D.D. Shoemaker), meahrens@wisc.edu (M.E. Ahrens), kenross@uga.edu (K.G. Ross).

1055-7903/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2005.07.014

boundaries and relationships in these "problematic" taxa is of particular interest to evolutionary biologists, because these taxa are the most promising candidates for providing unique insights into the crucial early stages of the speciation process (Harrison, 1991; Hey et al., 2003; Machado et al., 2002; Wiens and Penkrot, 2002).

One important problematic taxon of insects for which such studies are warranted is the *Solenopsis saevissima* species-group of fire ants (Pitts, 2002; Trager, 1991). This group consists of 13 described species of fire ants and their social parasites, all of which are native to South America (Pitts, 2002). Two of the species, *S. invicta* and *S. richteri*, now also occur in the USA, following their separate, unintentional introductions early last century, and *S. invicta* recently has been introduced as well to islands of the West Indies (Davis et al., 2001) and several Pacific Rim countries (Morrison et al., 2004; Nattrass and Vanderwoude, 2001; Pascoe, 2001; Solley et al., 2002). All fire ants are considered agricultural and public health pests in areas they have recently invaded (Eubanks, 2001; Lofgren, 1986; Lofgren et al., 1975; Porter and Savignano, 1990; Solley et al., 2002; Vinson, 1994). As a result of the pest status of *S. invicta* in the USA, there has been an enormous amount of biological research that has led to the emergence of the species as an important model study organism (Lofgren, 1986; Lofgren et al., 1975; Ross, 1993; Ross and Keller, 1995; Ross et al., 1997, 1999, 1996; Tschinkel, 1993, 2005; Vinson, 1994).

Despite the impressive body of general knowledge of fire ant biology, the development of a stable α-taxonomy of the *S. saevissima* species-group has proven to be a difficult task, in large part because of a lack of informative morphological characters that are invariant within but fixed between putative species (but see Pitts, 2002). These difficulties in developing fully 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, a belief given some credence by convincing demonstrations that nominal species of both native and introduced fire ants can readily hybridize in North America (Cahan and Vinson, 2003; Ross et al., 1987; Shoemaker et al., 1996; Vander Meer et al., 1985).

Associated with and compounding the difficulties surrounding the α-taxonomy of fire ants, surprisingly little attention has been paid to developing well-supported hypotheses of the relationships among putative species in the *S. saevissima*-group. Indeed, only two studies exist that explicitly present such hypotheses. The first was based on allozyme data (Ross and Trager, 1990), which, while useful for discerning boundaries of the subset of species studied, were much less informative for inferring species relationships. As a result of the paucity of shared apomorphic alleles, different methods of analyses resulted in different trees and unresolved nodes were common.

More recently, Pitts et al. (2005) performed a cladistic analysis of all described species within the group based on 36 morphological characters from different castes and life stages. This extensive study represents a major advance in terms of stabilizing the α-taxonomy and providing a comprehensive phylogeny based on a cladistic approach, yet some important unresolved issues remain. First, the level of support for many clades was quite low, due in part to the rarity of phylogenetically informative morphological characters. Second, many of the suggested species relationships are at variance with those proposed on the basis of the allozyme data by Ross and Trager (1990) or inferred from a phylogenetic analysis of sequence variants of the nuclear gene *Gp-9* (Krieger and Ross, 2005). As one example, while the allozyme data support a sister-species relationship between *S. richteri* and *S. quinquecuspis*, the morphological data do not. As another example, the *Gp-9* sequence data suggest a close relationship of the social parasite *S. daguerrei* to the basal fire ant species *S. electra* and *S. pusillignis*, a relationship not supported by the morphology-based tree.

A final unresolved issue with respect to the phylogenetic study of Pitts et al. (2005) concerns the validity of the species serving as terminals. Two population genetic studies of several *S. saevissima*-group species in their native ranges strongly suggest that the current α-taxonomy based on morphological characters overestimates the breadth of species limits in some cases (Ross and Shoemaker, 2005; Ross and Trager, 1990). The allozyme study of Ross and Trager (1990) revealed a "cryptic" species morphologically indistinguishable from *S. quinquecuspis*, a close relative of *S. invicta.* More recently, Ross and Shoemaker (2005) obtained evidence from both allozyme and mtDNA markers for a genetically distinct, morphologically cryptic species within nominal *S. invicta,* and for highly divergent geographic populations within both this species and *S. richteri.* Although the existence of cryptic species within other nominal species remains to be determined, it is apparent from these studies that our current understanding of species boundaries in this group of fire ants remains incomplete.

In the present study, we generated mitochondrial DNA (mtDNA) sequence data from numerous individuals representing almost every described species, one undescribed species, and two suspected cryptic species within the *S. saevissima* species-group. We then inferred the evolutionary relationships of the sequence haplotypes in order to gain insights into the boundaries and relationships of these species. We targeted the mtDNA because this molecule possesses many properties ideally suited to gene phylogeny reconstruction in closely related, rapidly radiating species, including maternal inheritance, lack of recombination, and relatively high rates of evolution (Avise, 1986, 1991, 1994; but see Ballard and Whitlock, 2004). Our broad and intensive

sampling scheme allows robust assessment of patterns of intraspecific and interspecific variation, which in turn allows rigorous testing of hypotheses of species and higher-level monophyly (Funk and Omland, 2003). The mtDNA phylogeny that we generate also is used to test specific hypotheses of the monophyly of the *S. saevissima* species-group, the monophlyly of the socially polymorphic species within this group, and the relationship of the social parasite *S. daguerrei* to its free-living fire ant hosts. This first molecular study intended specifically to infer relationships among *S. saevissima*-group fire ants thus contributes to the construction of a sound evolutionary framework to underpin a variety of comparative physiological, behavioral, and ecological studies. From a more practical standpoint, a well founded α-taxonomy and phylogeny of these species is essential to the task of pinpointing source populations of the invasive species, which in turn is crucial for focusing research intended to foster development of alternative management practices.

2. Materials and methods

2.1. Collection and identification of ants

A recent re-classification by Pitts (2002) resulted in the placement of thirteen described species in the *Solenopsis saevissima* species-group. We obtained sequences from 623 individuals representing 10 of these 13 species as well as three undescribed species (see Table 1 and Fig. 1). The three described species for which we were unable to obtain specimens are *S. hostilis* (an inquiline social parasite), *S. pythia*, and *S. weyrauchi*. Preliminary species identifications made in the field using a published key were confirmed in most cases by Trager or Pitts using morphological characters (Pitts, 2002; Trager, 1991). In the case of the three undescribed species (referred to here as *Solenopsis* sp. "A," *Solenopsis* sp. "X," and *S. invicta* "cryptic" species), diagnostic morphological features or diagnostic allelic compositions at one or more allozyme loci were used for identification (Pitts, 2002; Ross and Shoemaker, 2005; Ross and Trager, 1990).

In addition to the *S. saevissima*-group species, we obtained exemplar sequences from several *Solenopsis* species in different species-groups and from two species in different myrmicine genera (all identified by Pitts). The additional *Solenopsis* species, which represent all presumed major fire ant lineages (Pitts, 2002; Trager, 1991), include: *S. amblychila* (Arizona, USA), *S. aurea* (Arizona, USA—two individuals), *S. geminata* (Florida, USA [AY254476], Espírito Santo and Pará, Brazil—three individuals), *S. nigella gensterblumi* (Mato Grosso do Sul, Brazil—four individuals), *S. substituta* (Paraíba and Piauí, Brazil—two individuals), *S. tridens* (Bahia and Maranhão, Brazil—two individuals), and *S. xyloni* (Arizona, USA). The additional myrmicine species are: *Monomorium pharaonis* (Arizona, USA—two individuals) and *Myrmica rubra* (AY280600). Sequences of these additional species were included to evaluate the monophyly of the *S. saevissima* species-group and to serve as outgroups for detailed analyses of species relationships within the group (*S. amblychila* and *S. geminata* only).

2.2. Sequencing of mtDNA

Total genomic DNA was isolated from single individuals using the Puregene DNA Isolation Kit (Gentra systems). A ~950 bp portion of the mitochondrial genome that includes regions of the cytochrome oxidase I (COI), $tRNA_{LEU}$, and cytochrome oxidase II (COII) genes was amplified via the polymerase chain reaction (PCR) using the two mtDNA primers C1-J-2195 (COI-RLR) and DDS-COII-4 (see Ahrens et al., 2005 for primer sequences). PCR conditions, thermal cycling profiles, and sequencing reaction protocols were identical to those reported in Ahrens et al. (2005). For a subset of the individuals outside of the *S. saevissima* species-group, our standard methods failed to reliably amplify the ~950 bp mtDNA fragment. Therefore, we substituted a newly designed primer (JerryGarcia-C1; 5′-GGGAAT TAGAATTTTGAAGAG-3′) for DDS-COII-4 to amplify a 785 bp fragment from these samples that includes a portion of the COI gene only. PCR conditions, thermal cycling profiles, and sequencing reaction protocols were identical to those for the other samples. All mtDNA amplicons were sequenced in both directions.

2.3. Phylogenetic analyses of mtDNA sequences

All sequences were edited using the program Sequencher (Gene Codes) and aligned by eye using as references published fire ant sequences obtained from GenBank (*S. geminata* [AY254476] and *S. invicta* [AY249093]).

The number and frequency of unique haplotypes were determined using ARLEQUIN ver. 2.000 (Schneider et al., 2000). This program also was used to calculate nucleotide diversity (π), defined as the average number of pairwise differences per site between sequences, and sequence divergence (Da), defined as the net number of nucleotide substitutions per site between groups of haplotypes. The latter statistic was estimated for haplotypes from different species and, in some cases, for haplotypes from different clades within species (Nei, 1987; Nei and Li, 1979; Tajima, 1983).

We performed phylogenetic analyses on two mtDNA sequence datasets. The first set of analyses was conducted on a dataset containing 24 exemplar sequences from species within the *S. saevissima* species-group (underlined haplotypes in Table 1) as well as all 21

Table 1
Geographic locations of samples of *S. saevissima*-group species included in this study (city, state/province, country)

Species	Sample location	Sample location code	*N*	Haplotype	GenBank Accession No.
Solenopsis sp. "A"	Santa Cecília, Santa Catarina, Brazil	17	2	Alti1	AY950704
S. daguerrei	Guará, Paraná, Brazil	17	1	Dag1	AY950705
	Planalto, Paraná, Brazil	19	1	Dag2	AY950706
	Resistencia, Chaco, Argentina	10	1	Dag2	AY950706
	Corrientes, Corrientes, Argentina	10	1	Dag3	AY950722
	Junin, Buenos Aires, Argentina	7	2	Dag4	AY950707
S. electra	Villa San Martín, Santiago del Estero, Argentina	2	1	Elec1	AY249092
S. interrupta	Villa San Martín, Santiago del Estero, Argentina	2	1	Int1	AY950723
	Santiago del Estero, Santiago del Estero, Argentina	1	1	Int1	AY950723
	Villa Ojo de Agua, Santiago del Estero, Argentina	3	2	Int2	AY950724
	Villa del Totoral, Córdoba, Argentina	4	1	Int3	AY950725
	Villa San Martín, Santiago del Estero, Argentina	2	1	Int4	AY950726
S. invicta	Corrientes, Corrientes, Argentina	10	5	H1	AY499575
	Roldan, Santa Fe, Argentina	6	2	H1	AY499575
	Rosario, Santa Fe, Argentina	6	6	H1	AY499575
	Corrientes, Corrientes, Argentina	10	2	H2	AY249094
	Corrientes, Corrientes, Argentina	10	1	H3	AY249095
	Corrientes, Corrientes, Argentina	10	1	H4	AY249096
	Corrientes, Corrientes, Argentina	10	52	H5	AY249097
	Rosario, Santa Fe, Argentina	6	3	H5	AY249097
	Corrientes, Corrientes, Argentina	10	2	H6	AY249098
	Corrientes, Corrientes, Argentina	10	1	H7	AY499571
	Roldan, Santa Fe, Argentina	6	9	H7	AY499571
	Rosario, Santa Fe, Argentina	6	18	H7	AY499571
	Corrientes, Corrientes, Argentina	10	2	H8	AY249100
	Corrientes, Corrientes, Argentina	10	1	H9	AY249101
	Corrientes, Corrientes, Argentina	10	1	H10	AY249102
	Corrientes, Corrientes, Argentina	10	2	H11	AY249103
	Corrientes, Corrientes, Argentina	10	1	H12	AY249104
	Corrientes, Corrientes, Argentina	10	2	H13	AY249105
	Corrientes, Corrientes, Argentina	10	2	H14	AY249106
	Corrientes, Corrientes, Argentina	10	1	H15	AY249107
	Corrientes, Corrientes, Argentina	10	1	H16	AY249108
	Corrientes, Corrientes, Argentina	10	1	H17	AY249109
	Formosa, Formosa, Argentina	14	3	H18	AY249110
	Formosa, Formosa, Argentina	14	1	H19	AY249111
	Formosa, Formosa, Argentina	14	7	H20	AY249112
	Formosa, Formosa, Argentina	14	5	H21	AY249113
	Formosa, Formosa, Argentina	14	2	H22	AY249114
	Formosa, Formosa, Argentina	14	1	H23	AY249115
	Formosa, Formosa, Argentina	14	1	H24	AY249116
	Formosa, Formosa, Argentina	14	2	H25	AY249117
	Formosa, Formosa, Argentina	14	1	H26	AY249118
	Formosa, Formosa, Argentina	14	1	H27	AY249119
	Formosa, Formosa, Argentina	14	1	H28	AY249120
	Formosa, Formosa, Argentina	14	1	H29	AY249121
	Formosa, Formosa, Argentina	14	2	H30	AY249122
	Formosa, Formosa, Argentina	14	1	H31	AY249123
	Formosa, Formosa, Argentina	14	2	H32	AY249124
	Formosa, Formosa, Argentina	14	2	H33	AY249125
	Formosa, Formosa, Argentina	14	1	H34	AY249126
	Formosa, Formosa, Argentina	14	2	H35	
	Formosa, Formosa, Argentina	14	4	H36	
	Ceu Azul, Paraná, Brazil	18	33	H37	
	Ceu Azul, Paraná, Brazil	18	7	H38	
	Ceu Azul, Paraná, Brazil	18	5	H39	
	Ceu Azul, Paraná, Brazil	18	8	H40	
	Ceu Azul, Paraná, Brazil	18	4	H41	
	Ceu Azul, Paraná, Brazil	18	2	H42	

(*continued on next page*)

Table 1(*contined*)

Species	Sample location	Sample location code	*N*	Haplotype	GenBank Accession No.
S. invicta	Ceu Azul, Paraná, Brazil	18	3	H43	
	Pedra Preta, Mato Grosso, Brazil	29	46	H44	
	Pontes E Lacerda, Mato Grosso, Brazil	23	23	H45	
	Campo Grande, Mato Grosso do Sul, Brazil	28	5	H46	
	Campo Grande, Mato Grosso do Sul, Brazil	28	3	H47	
	Campo Grande, Mato Grosso do Sul, Brazil	28	2	H48	
	Sao Gabriele do Oeste, Mato Grosso do Sul, Brazil	27	49	H48	
	Coxim, Mato Grosso do Sul, Brazil	26	1	H48	
	Campo Grande, Mato Grosso do Sul, Brazil	28	14	H49	
	Sao Gabriele do Oeste, Mato Grosso do Sul, Brazil	27	2	H49	
	Campo Grande, Mato Grosso do Sul, Brazil	28	5	H50	
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	3	H51	
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	2	H52	
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	3	H53	
	Corrientes, Corrientes, Argentina	10	1	H54	AY950749
	Corrientes, Corrientes, Argentina	10	1	H55	AY950750
	Resistencia, Chaco, Argentina	10	1	H55	AY950750
	Ceu Azul, Paraná, Brazil	18	1	H56	AY950751
	Ceu Azul, Paraná, Brazil	18	1	H57	AY950717
	Ceu Azul, Paraná Brazil	18	1	H58	AY950752
	Ceu Azul, Paraná, Brazil	18	1	H59	AY950710
	Pedra Preta, Mato Grosso, Brazil	29	1	H60	AY950718
	Pedra Preta, Mato Grosso, Brazil	29	1	H61	AY950753
	Pontes E Lacerda, Mato Grosso, Brazil	23	1	H62	AY950754
	Pontes E Lacerda, Mato Grosso, Brazil	23	1	H63	AY950755
	Pontes E Lacerda, Mato Grosso, Brazil	23	1	H64	AY950719
	Pontes E Lacerda, Mato Grosso, Brazil	23	1	H65	AY950756
	Pontes E Lacerda, Mato Grosso, Brazil	23	1	H66	AY950757
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	1	H67	AY950758
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	1	H68	AY950759
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	2	H69	AY499582
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	13	H70	AY499583
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	7	H71	AY499585
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	6	H72	AY499586
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	2	H73	AY499587
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	2	H74	AY499588
	Roldan, Santa Fe, Argentina	6	1	H75	AY499572
	Roldan, Santa Fe, Argentina	6	1	H76	AY499573
	Roldan, Santa Fe, Argentina	6	1	H77	AY499574
	Roldan, Santa Fe, Argentina	6	1	H78	AY499576
	Rosario, Santa Fe, Argentina	6	1	H79	AY499578
	Rosario, Santa Fe, Argentina	6	1	H80	AY499579
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	H81	AY499584
	Corrientes, Corrientes, Argentina	10	2	H82	AY950760
	Corrientes, Corrientes, Argentina	10	1	H83	AY950761
	Corrientes, Corrientes, Argentina	10	1	H84	AY950762
	Santiago del Estero, Santiago del Estero, Argentina	1	2	H85	AY950763
	Santiago del Estero, Santiago del Estero, Argentina	1	1	H87	AY950764
	Curitiba, Paraná, Brazil	17	1	H88	AY950708
	Rio Negro, Paraná, Brazil	17	1	H88	AY950708
	Comodoro, Mato Grosso, Brazil	24	1	H89	AY950765
	Posadas, Missiones, Argentina	15	1	H90	AY950720
	Cáceres, Mato Grosso, Brazil	25	2	H91	AY950766
	Coxim, Mato Grosso do Sul, Brazil	26	1	H92	AY950767
	Corumba, Mato Grosso do Sul, Brazil	22	1	H93	AY950768
S. invicta "cryptic" sp.	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	2	Cryp1	AY499581
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	1	Cryp1	AY499581
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	2	Cryp2	AY499589
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	1	Cryp2	AY499589
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	2	Cryp3	AY499590
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	8	Cryp3	AY499590
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	4	Cryp4	AY499580
	Rinco dos Cabrais, Rio Grande do Sul, Brazil	16	4	Cryp4	AY499580

Table 1(*contined*)

Species	Sample location	Sample location code	*N*	Haplotype	GenBank Accession No.
S. macdonaghi	San Luis del Palmar, Corrientes, Argentina	12	1	Mac1	AY950769
	Itaquirai, Mato Grosso do Sul, Brazil	21	1	Mac2	AY950770
	Itatí, Corrientes, Argentina	13	1	Mac3	AY950771
	Corrientes, Corrientes, Argentina	10	1	Mac3	AY950771
	Empedrado, Corrientes, Argentina	11	1	Mac3	AY950771
S. megergates	Palmeira, Paraná, Brazil	17	1	Meg1	AY950709
	Palmeira, Paraná, Brazil	17	2	Meg2	AY950772
	Curitiba, Paraná, Brazil	17	1	Meg3	AY950773
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Meg4 (= Cryp2)	AY499589
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Meg5	AY950721
S. pusillignis	Corumbá, Mato Grosso do Sul, Brazil	22	1	Pus1	AY950774
	Corumbá, Mato Grosso do Sul, Brazil	22	1	Pus2	AY950775
S. quinquecuspis	Rosario, Santa Fe, Argentina	6	9	Quin1	AY499571
	Rosario, Santa Fe, Argentina	6	1	Quin2	AY499592
	Rosario, Santa Fe, Argentina	6	1	Quin3	AY499593
	Pergamino, Santa Fe, Argentina	7	1	Quin3	AY499593
	Rosario, Santa Fe, Argentina	6	1	Quin4	AY499598
	Rosario, Santa Fe, Argentina	6	1	Quin5	AY499600
	Rosario, Santa Fe, Argentina	6	1	Quin6	AY499603
	Rosario, Santa Fe, Argentina	6	1	Quin7 (= H85)	AY499604
	Villa Constitución, Santa Fe, Argentina	7	2	Quin8	AY950776
	Santa Fe, Santa Fe, Argentina	9	1	Quin8	AY950776
	Santa Fe, Santa Fe, Argentina	9	4	Quin9	
	Santa Fe, Santa Fe, Argentina	9	1	Quin10	
	Rosario, Santa Fe, Argentina	6	4	Quin11 (= H7)	AY499594
	Rosario, Santa Fe, Argentina	6	3	Quin12 (= rich4)	AY499599
	Rosario, Santa Fe, Argentina	6	2	Quin13 (= Hl)	AY499595
	Rosario, Santa Fe, Argentina	6	1	Quin14 (= H80)	AY499596
	Rosario, Santa Fe, Argentina	6	1	Quin15 (= richl)	AY499597
	Rosario, Santa Fe, Argentina	6	1	Quin16 (= rich5)	AY499601
	Rosario, Santa Fe, Argentina	6	1	Quin17 (= rich3)	AY499602
S.richteri	Rosario, Santa Fe, Argentina	6	20	Rich1	AY586448
	Rosario, Santa Fe, Argentina	6	6	Rich2	AY585346
	Rosario, Santa Fe, Argentina	6	4	Rich3	AY585347
	Rosario, Santa Fe, Argentina	6	1	Rich4	AY499614
	Rosario, Santa Fe, Argentina	6	1	Rich5	AY249128
	Rosario, Santa Fe, Argentina	6	1	Rich6	AY249134
	Rosario, Santa Fe, Argentina	6	1	Rich7	AY249132
	Rosario, Santa Fe, Argentina	6	1	Rich8	AY586449
	Rosario, Santa Fe, Argentina	6	1	Rich9	AY249130
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	16	Rich10	AY499607
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	11	Rich11	AY499606
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	2	Rich12	AY499610
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Rich13	AY499605
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Rich14	AY499608
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Rich15	AY499609
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Rich16	AY499611
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Rich17	AY499612
	Arroio dos Ratos, Rio Grande do Sul, Brazil	16	1	Rich18	AY499613
S. saevissima	Belo Horizonte, Minas Gerais, Brazil	32	2	Saev1	AY950779
	Maurilandia, Goias, Brazil	31	1	Saev2	AY950780
	Cascavel, Paraná, Brazil	20	1	Saev3	AY950712
	Nazaré, Bahia, Brazil	34	1	Saev4	AY950781
	Fortaleza, Ceará, Brazil	35	1	Saev5	AY950713
	Zé Doca, Maranhão, Brazil	36	1	Saev6	AY950782
	Capanema, Pará, Brazil	38	1	Saev7	AY950714
	Ulianopolis, Pará, Brazil	37	1	Saev8	AY950715
	Posse, Goiás, Brazil	33	1	Saev9	AY950716
	Aqua Clara, Mato Grosso do Sul, Brazil	30	1	Saev10	AY950783
	Rio Negro, Paraná, Brazil	17	1	Saev11	AY950711

(*continued on next page*)

Table 1(*contined*)

Species	Sample location	Sample location code	*N*	Haplotype	GenBank Accession No.
S. sp. "X"	Cañada de Gomez, Santa Fe, Argentina	5	2	Sol-X1	AY950784
	Rosario, Santa Fe, Argentina	6	1	Sol-X1	AY950784
	Arrecifes, Buenos Aires, Argentina	7	1	Sol-X1	AY950784
	Florencia Varela (Buenos Aires), Buenos Aires, Argentina	8	1	Sol-X1	AY950784

Sample location codes are those used in Fig. 1. *N* represents the number of individuals (one per nest) sampled from each location. mtDNA haplotypes used in the initial set of phylogenetic analyses undertaken to test the monophyly of the species-group are underlined (see text).

unique sequences from ants representing the other taxa. The *S. saevissima*-group exemplars were chosen to encompass the diversity within and among the major clades detected in the complete set of sequences (below). The goal of these initial analyses was to evaluate the monophyly of the *S. saevissima*-group sequences, which was accomplished by running separate analyses using the following taxa as outgroups: *Myrmica rubra* only; *Monomorium pharaonis* only; *M. rubra* + *M. pharaonis*; *M. rubra* + *M. pharaonis* + *S. nigella gensterblumi*; and *S. nigella gensterblumi* only. *Solenopsis nigella gensterblumi* is a morphologically atypical fire ant bearing a sequence at the nuclear gene *Gp-9* that is basal to those of all other fire ants (Krieger and Ross, 2005).

For the second set of detailed analyses of all 623 sequences from the *S. saevissima*-group, the *S. geminata* reference sequence and a new sequence generated from *S. amblychila* were specified as outgroups. This decision was made based on our finding from the first set of analyses that one or both sequences may fall in a clade that is sister to all the *S. saevissima*-group sequences (see also Pitts et al., 2005). We note that use of sequences from other *Solenopsis* species classified outside the *S. saevissima*-group as alternative outgroups did not affect the important features of the topology of the recovered trees.

MODELTEST (Posada and Crandall, 1998) was used to determine appropriate models of sequence evolution by statistically comparing successively nested models that become more parameter-rich. We also used MODELTEST to calculate the proportion of invariant sites and estimate a gamma shape value, which describes the rate of substitution at variable sites.

We used neighbor-joining (NJ), maximum parsimony (MP; first set of analyses only), and Bayesian inference, as implemented in the programs PAUP* 4.08b (Swofford, 1999) and MrBayes 3 (Ronquist and Huelsenbeck, 2003), to generate phylogenetic trees of the mtDNA haplotypes. Only non-redundant sequences (selected with ARLEQUIN) were used. The NJ tree was constructed using HKY85 + I + Γ distances (determined by MODELTEST to have the lowest likelihood ratio for both data sets; I = 0.59 and 0.61 and Γ = 0.85 and 0.71 for the first and second set, respectively), with the additional constraint that ties were broken randomly. All branches of zero length were collapsed during searches. Bootstrap support values for each node within the tree were calculated by performing 10,000 data resamplings.

MP trees were constructed using the heuristic search option (1000 random addition searches) and TBR branch swapping, with other settings the defaults in PAUP*. Bootstrap values were generated using a heuristic search algorithm (500 bootstrap replicates with 10 random addition searches per replicate) and TBR branch swapping.

Bayesian analyses were used to generate posterior probability values for presumptive clades (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). No a priori assumptions about the topology of the tree were made and all METROPOLIS-coupled MCMC searches were provided with a uniform prior based upon the values obtained from MODELTEST (see above). We ran the program MrBayes four separate times for each data set and compared corresponding posterior probability distributions to explore the parameter space and ensure thorough mixing. Each search was run for four million generations with four chains started from a random tree; every 100th tree was sampled to obtain the 40,001 sample points. The number of generations required for sample parameter values to reach stationarity (burn-in length) was set after determining by inspection when the log-likelihood values reached an asymptote. The posterior probability of each node (the percentage of run samples in which the node was recovered) was calculated using the trees visited by the Markov chains after burn-in samples were discarded.

3. Results

The first set of phylogenetic analyses on the data set containing mtDNA sequences from a diversity of *Solenopsis* species and other myrmicine ants was conducted to test the monophyly of the *S. saevissima* species-group (Pitts, 2002). The data matrix consisted of a 785 bp fragment of the COI gene from 55 individuals, 24 of which represent *S. saevissima*-group species. All sequences were aligned readily by eye. Regardless of the choice of outgroup(s) for rooting the trees, support values for an exclusive clade containing all of the *S. saevissima*-group sequences always exceeded 98, 85, and 65% for the NJ, MP, and Bayesian inference methods, respectively. These

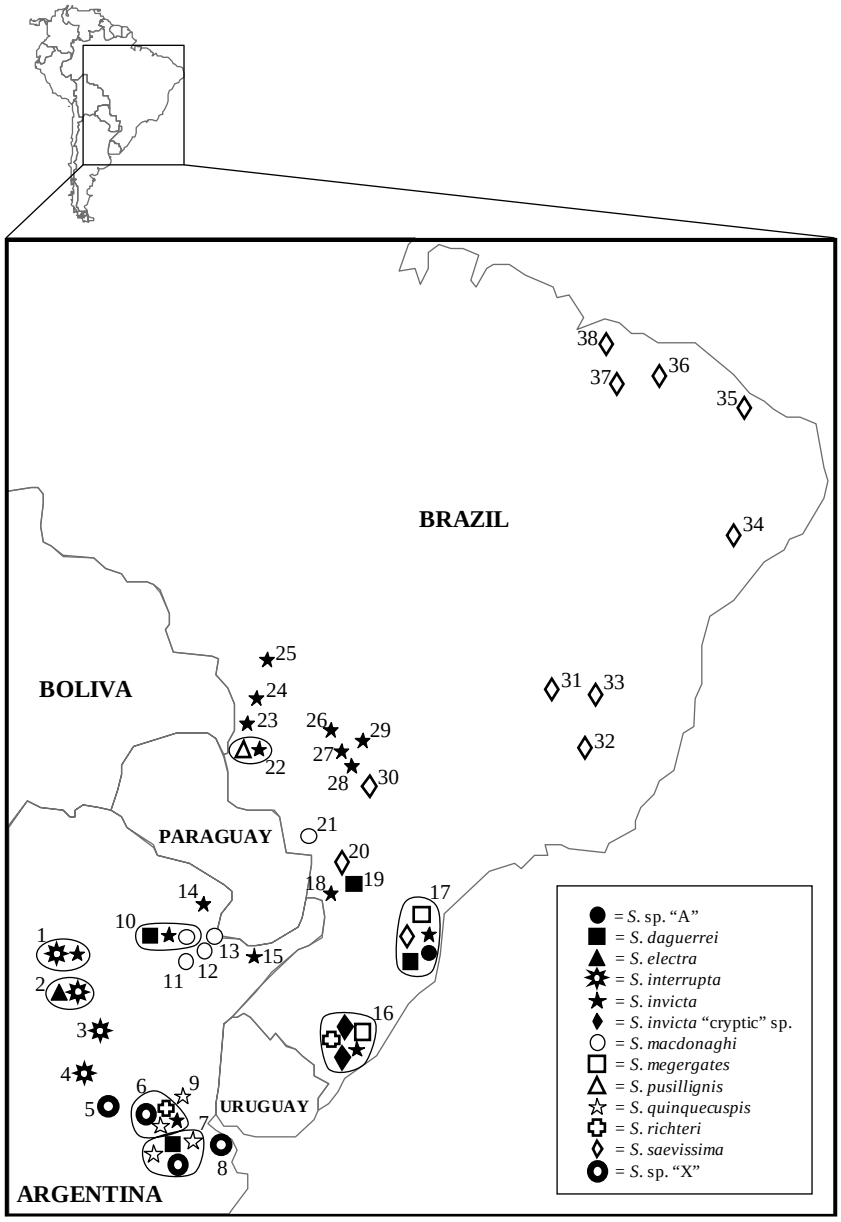

Fig. 1. Geographic locations of samples of *Solenopsis saevissima*-group species collected in South America for mtDNA sequencing in present study. Numeric codes refer to location names given in Table 1.

analyses thus provide reasonable support for the monophyly of the *S. saevissima* species-group.

The second set of analyses, performed on the data set including all 623 sequences from 13 *S. saevissima*-group species, was intended to examine the monophyly and relationships of these species. The data matrix consisted of a 910 bp portion of the mtDNA genome; bases 1–785 represent a portion of COI (the same fragment as above), bases 786–852 represent the intervening $tRNA_{LEU}$, and bases 853–910 represent a portion of COII. Only two indels were found: a 3 bp deletion corresponds to positions 24–26 (resulting in deletion of a single amino acid from COI) in haplotypes quin5 and rich16 from *S. quinquecuspis* and *S. richteri*, respectively, and a 1 bp deletion corresponds to position 838 (within $tRNA_{LEU}$) in the *S. geminata* and *S. amblychila* (outgroup) haplotypes. Of the total 910 bp (characters), 264 were variable, and 216 of these were parsimony informative. There was a substantial AT bias across the entire region sequenced (average of 72.0% AT), consistent with the bias found in other studies of insect mtDNA (Clary and Wolstenholme, 1985; Crozier and Crozier, 1993; Jermiin and Crozier, 1994). The estimated transition/transversion ratio was 6.5, again a value consistent with the ratios found for the COI gene in other insects (Gleason et al., 1997; Moriyama and Powell, 1997; Simon et al., 1994).

We detected a total of 156 unique mtDNA sequences (haplotypes). Only nine of these haplotypes were shared by more than one nominal or undescribed fire ant species, with the remainder exclusive to single species. Eight of these cases involved haplotype sharing between *S. quinquecuspis* and either *S. invicta* or *S. richteri*, a finding interpreted by Ross and Shoemaker (2005) as evidence for historical introgression of heterospecific mtDNA into *S. quinquecuspis* where the ranges overlap. The last case involves an mtDNA haplotype found in one individual of *S. megergates* and two individuals of the undescribed *S. invicta* "cryptic" species (Table 1).

Estimates of mtDNA nucleotide diversity (π) within each nominal and undescribed species were quite variable, ranging from zero to 0.081. The highest values were obtained for the five species *S. invicta*, *S. megergates*, *S. quinquecuspis*, *S. richteri*, and *S. saevissima* (0.017–0.081). These values, which in some cases equal or exceed the total nucleotide diversity estimated for the entire species-group ($\pi = 0.040$), reflect the presence of divergent, non-monophyletic haplotype lineages within each of these nominal species (below).

Nucleotide diversity and pairwise sequence divergence (*D*a) values estimated separately for the major lineages within each of these five species often are on a par with values for the remaining nominal species. Considering *S. invicta*, the study species for which the largest number of sequences are available and for which the overall estimate of π was 0.036, estimates for each major haplotype clade ranged from 0.001 to 0.012, values that span the range of estimates for most species. Estimates of *D*a between the different *S. invicta* clades ranged from 1.6 to 5.1%. These estimates are similar to or higher than divergence estimates between most species within the *Solenopsis saevissima* species-group, which ranged from 0.14% (*S. richteri* and *S. megergates*) to 10.3% (*Solenopsis* sp. "A" and *S. richteri*).

mtDNA gene trees reconstructed for the *Solenopsis saevissima* species-group using the Bayesian and NJ methods displayed virtually identical topologies (Fig. 2). Five major features of the estimated trees are evident.

1. The basal clade of sequences from the *S. saevissima*-group species comprises a subset of sequences from four different species, *Solenopsis* sp. "A," *S. invicta*, *S. megergates*, and *S. saevissima*. The assignment of haplotypes to this clade results in deep mtDNA polyphyly for three of the species (not *Solenopsis* sp. "A"), because conspecifics bear distantly related haplotypes (Fig. 2A). While species-level polyphyly of the mtDNA appears to be common in our data set (below), this basal clade is noteworthy because preliminary sequence data from portions of two nuclear genes similarly show that individuals from the seven sampled nests form a single divergent monophyletic group (D.D.S. and K.G.R., unpublished data). These seven nests were located in a relatively restricted area of southeastern Brazil (locations 17 and 20 in Fig. 1), and our preliminary species identifications of these ants in the field were ambiguous in every case. We hypothesize that these individuals represent a single species (presumably *Solenopsis* sp. "A," since all haplotypes from this undescribed species fall within this clade), and that most were simply misidentified on the basis of their morphology. Alternatively, they may represent a cluster of several closely related, previously unrecognized species. While the species-level sequence polyphyly resulting from recognition of this clade might be argued to reflect incomplete lineage sorting, this explanation seems implausible given the extreme divergence of this clade from all others combined with the similarity of the constituent haplotypes.

2. Among the remaining mtDNA sequences, those from individuals representing the three nominal species *S. daguerrei*, *S. electra*, and *S. pusillignis* (together with a subset of sequences from *S. saevissima*) form a well-supported monophyletic group that is sister to a large clade with the other sequences (Fig. 2A). Within the former clade, the mtDNA sequences of *S. daguerrei* are monophyletic and sister to all sequences of the other three species.

3. Within the large remaining group of sequences are three clades, two of which are quite divergent (Fig. 2A). One divergent basal clade consists only of *S. saevissima* sequences, while the other divergent clade, consisting of all the *S. interrupta* sequences plus a distinctive *S. saevissima* sequence, is sister to the clade of remaining sequences discussed below. While the sequences of *S. interrupta* are monophyletic, those of *S. saevissima* are deeply polyphyletic.

4. Eleven well-supported clades comprising the remaining sequences together constitute the single larger clade designated as the "socially polymorphic clade" in Fig. 2. Nominal species names are used to identify these 11 clades in Fig. 2B (i.e., *S. macdonaghi*, *S. invicta* clades 1–7, *S. richteri* [plus *S. megergates*], *S. richteri* [plus *S. quinquecuspis*], and *S. invicta* "cryptic" species). However, with the exception of the *S. macdonaghi* clade and *S. invicta* clades 1 and 7, each clade contains sequences from more than one nominal species. As examples, *S. invicta* clade 2 includes mostly haplotypes from *S. invicta* but also a single haplotype from *S. quinquecuspis*, while *S. invicta* clade 6 comprises haplotypes of these two species plus *S. richteri*. The recovery with strong support of the socially polymorphic clade is of special interest because the group contains sequences from all the *S. saevissima*-group species that are known to display polymorphism in their colony social organization (Krieger and Ross, 2002, 2005). Specifically, the species whose sequences appear in this clade exhibit variation in colony queen number such that colonies have either a single reproductive queen (monogyny) or multiple queens (polygyny).

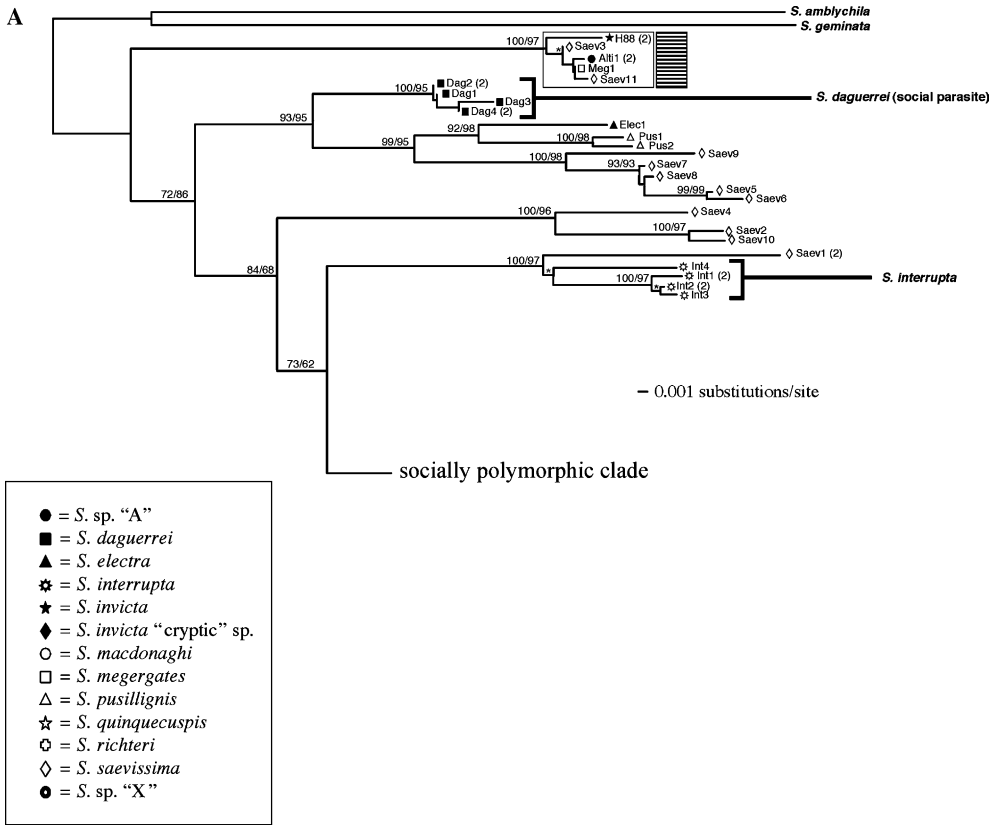

Fig. 2. Bayesian trees showing relationships of mtDNA sequences from *Solenopsis saevissima*-group species. The tree depicting the basal nodes is shown in (A), whereas the large "socially polymorphic clade" is shown in detail in (B) (see text). Numbers on branches represent NJ bootstrap support values followed by Bayesian posterior probability values (only values greater than 70% are shown); asterisks (∗) indicate additional nodes with bootstrap and posterior probability values greater than 70%. Symbols at terminals refer to nominal species from which sequences were obtained; haplotype codes are as in Table 1. Numbers of individuals possessing each haplotype are given in parentheses when greater than one. In (A) a basal lineage with haplotypes of several disparate species is indicated by a stippled vertical bar. In (B) the seven major haplotype clades within *S. invicta* are indicated by black vertical bars, while the two major clades within *S. richteri* are indicated by checkered vertical bars. Geographic areas from which most haplotypes of each major clade in the latter two species originated are indicated. Haplotypes depicted with white lettering occur within clades consisting mostly of sequences from a different nominal species (see text).

5. As evident from point 4, mtDNA sequences from most of the nominal species in the socially polymorphic clade are not monophyletic but instead fall into two or more divergent, well-supported clades. These widespread patterns of polyphyly are particularly evident for *S. invicta*, *S. megergates*, *S. quinquecuspis*, and *S. richteri* (paralleling the pronounced polyphyly of *S. saevissima* noted above).

Considering only sequences from the extensively sampled *S. invicta*, seven well-supported mtDNA lineages exist (Fig. 2b). The divergence among these lineages (1.6–5.1%) often is as great or greater than the divergence between sequences from *S. invicta* and other socially polymorphic species (0.4–5.5%) or between heterospecific sequences from this group when *S. invicta* is excluded (0.1–5.4%). A strong geographic component to this profound mtDNA divergence in nominal *S. invicta* is indicated by the fact that the great majority of sequences in a given clade derive from a single region of the vast species range (see Table 1 and Fig. 2B). This conclusion is reinforced by comparison of estimates of ϕ_{ST}, a metric of population genetic differentiation that accounts for presumed evolutionary divergence between haplotypes, with estimates of F_{ST}, a metric that does not consider haplotype relationships (Excoffier et al., 1992; Schneider et al., 2000). The estimate of $\phi_{ST} = 0.783$ is substantially greater than the estimate of $F_{ST} = 0.499$ (calculated for our sample locations from which more than 10 individuals were sequenced; Ahrens et al., 2005). Thus, there is considerably less genetic divergence among haplotypes within a population than among haplotypes from different populations, as expected with strong phylogeographic structure (Goropashnaya et al., 2004; Pons and Petit, 1996).

Two well-supported haplotype clades also exist in *S. richteri*, and the divergence between them is high (3.1%). A geographical component to the mtDNA variation within this nominal species is even more pronounced than in *S. invicta*. Specifically, all sequences of *S. richteri* collected from location 6 belong to one of the clades

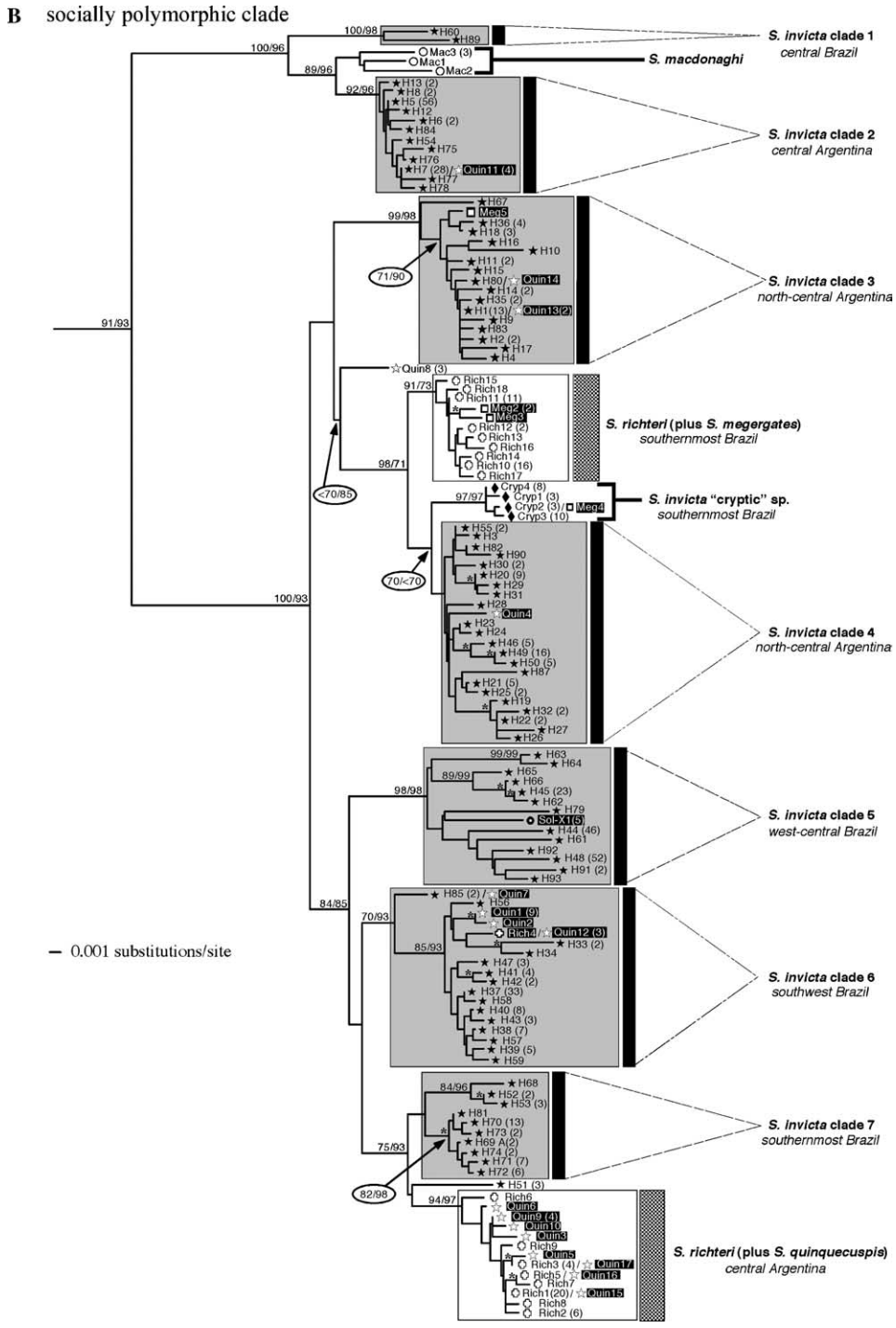

Fig. 2. (*continued*)

(*S. richteri* (plus *S. quinquecuspis*)), whereas all sequences collected from location 16 fall into the other clade (*S. richteri* (plus *S. megergates*)). This complete geographic coherence of sequences composing the divergent lineages, coupled with the very pronounced allele frequency differences discovered between the two localities at two allozyme loci (Ross and Shoemaker, 2005), suggests that nominal *S. richteri* comprises two evolutionarily independent, genetically distinct entities that are indistinguishable on the basis of morphological characters.

4. Discussion

We constructed a phylogeny for mtDNA sequences from most of the described as well as several undescribed

species of fire ants in the *S. saevissima* species-group. The primary goals of our study were to assess whether the inferred mtDNA phylogeny supported the current morphology-based delimitation of species within the *S. saevissima* species-group and whether the inferred species relationships are concordant with a recently generated hypothesis of relationships based on morphological characters (Pitts et al., 2005). Ancillary goals of our study were to use the mtDNA phylogeny to: (1) test the hypothesis of monophyly of the *S. saevissima* species-group, (2) test the hypothesis of monophyly of the socially polymorphic species within the species-group, and (3) infer the relationship of *S. daguerrei*, an inquiline social parasite, to its free-living fire ant host species.

4.1. Using the mtDNA tree to test phylogenetic hypotheses in the S. saevissima species-group

The monophyly of the *S. saevissima* species-group has proven difficult to establish in recent studies using characters from the morphology and the nuclear gene *Gp-9* (Krieger and Ross, 2005; Pitts et al., 2005). In contrast, we were able to confirm it with reasonable support using a variety of relevant outgroups and tree-building methods. This result implies a single Neotropical center of origin and subsequent radiation for this ecologically and economically important group of fire ant species, although its relationship to the biologically similar North American fire ants will require broader taxon sampling to resolve (also Pitts, 2002).

The mtDNA data strongly support the monophyly of the socially polymorphic species within the species-group, in agreement with both the morphological and *Gp-9* data. This clade contains the only South American fire ants species known to exhibit two contrasting forms of colony social organization, monogyny (single egg-laying queen per colony) and polygyny (multiple queens per colony), so its monophyly can be taken to support the view that polygyny arose only once in the South American fire ants as a derived form of social organization (Krieger and Ross, 2002).

Finally, the mtDNA tree helps clarify the relationship of *S. daguerrei*, an obligate social parasite that has lost the worker caste, to its free-living host species, and in so doing aids in testing a major hypothesis concerning the evolution of social parasitism. Emery's rule predicts that ant social parasites generally are sister species (strict version of the rule) or otherwise closely related (loose version of the rule) to the species they parasitize (Emery, 1909). The prediction stems from the belief that these parasitic species evolve from facultative intraspecific social parasites, presumably in sympatry with their hosts (Savolainen and Vepsäläinen, 2003). *Solenopsis daguerrei* attacks several fire ant species in the *S. saevissima* species-group, including *S. invicta*, *S. macdonaghi*, *S. quinquecuspis*, *S. richteri*, and *S. saevissima* (Calcaterra et al., 2000; and references therein). The mtDNA phylogeny is concordant with the morphological and *Gp-9* phylogenies in suggesting that *S. daguerrei* is a relatively basal taxon in the species-group that is quite distantly related to all its known hosts (Krieger and Ross, 2005; Pitts et al., 2005). Thus, the strict version of Emery's rule can be rejected. However, the loose version cannot, because the mtDNA sequences of this parasite are part of a larger clade that is sister to the clade containing the hosts (see also Pitts et al., 2005). Importantly, the sequences of the six *S. daguerrei* specimens collected over a vast geographic area (Fig. 1) are monophyletic, clearly supporting a single origin of social parasitism in this lineage. Although we cannot rule out the possibility that *S. daguerrei* co-speciated with its hosts, which would imply that nominal *S. daguerrei* in fact comprises multiple cryptic species, each of which is host specific, we consider this scenario unlikely in view of the low sequence divergence among *S. daguerrei* haplotypes relative to other species in the group (Fig. 2).

4.2. Species-level polyphyly of mtDNA sequences is widespread, yet species boundaries do not appear leaky

The mtDNA sequences of most nominal fire ant species are polyphyletic (Fig. 2). This is particularly evident for nominal *S. invicta*, the most intensively sampled species in our study, where seven well-supported clades were found. Although polyphyly of *S. invicta* haplotypes has been reported previously (Shoemaker et al., 2003; Shoemaker et al., 2000), both its scope and the deep divergence among many of the clades revealed in this most comprehensive study are nonetheless surprising. The extent of mtDNA polyphyly throughout the species-group in many respects renders one primary goal of our study, direct comparison of the mtDNA tree with the morphology-based phylogeny, futile. Clearly, the two trees will be discordant given the enforced monophyly of the terminals (nominal species) in the morphological tree.

While mtDNA genomes generally possess many properties ideally suited to phylogenetic studies of closely related species (Avise, 1986, 1991, 1994; but see Ballard and Whitlock, 2004), it is expected that mtDNA gene trees often will not completely parallel species phylogenies. Indeed, a recent review of the literature by Funk and Omland (2003) revealed that species-level polyphyly of mtDNA sequences is widespread in animals. Reasons given include flawed α-taxonomies, inadequate information with which to construct morphological or mtDNA trees, incomplete lineage sorting of mtDNA variants, cytoplasmic introgression, and unrecognized mtDNA gene paralogy (e.g., nuclear pseudogenes derived from mtDNA genes). These authors also highlight the importance of appropriate sampling schemes capable of detecting polyphyletic relationships

among mtDNA haplotypes, with such schemes featuring large numbers of individuals collected widely over the ranges of closely related species (Funk and Omland, 2003). Only with such sampling is it possible to rigorously test hypotheses of species-level monophyly.

Despite the obvious discordance between our mtDNA phylogeny and the morphology-based species phylogeny of fire ants, the mtDNA data set does offer support for the legitimacy of the α-taxonomy in one general respect. The species boundaries erected for the group in the current taxonomy are in most cases discernible in the mtDNA phylogeny, and there is little evidence that such proposed boundaries are leaky (with the exceptions of *S. quinquecuspis* and *S. megergates*—see below). Said another way, the two data sets are compatible in suggesting that the delineated entities (nominal species) generally are evolutionarily independent of one another, with little or no gene flow currently occurring between them. This conclusion is supported by our findings that, with the exceptions noted above, haplotypes of nominal species generally are highly divergent from those of other nominal species, haplotypes are not shared among nominal species, and haplotype clades are exclusive to single species.

Although this conclusion may appear to conflict with the observed widespread mtDNA polyphyly, we suggest that such polyphyly stems largely from a previously underappreciated deficiency of the α-taxonomy in which morphological circumscription often overestimates the breadth of species entities. That is, polyphyly of mtDNA sequences in these ants appears to result in most cases from the lumping of multiple morphologically indistinguishable entities that represent genetically and evolutionarily independent lineages (cryptic species). Thus, while the current α-taxonomy succeeds in somewhat coarsely demarcating reproductively and evolutionarily independent entities, it apparently fails to fully capture the species-level diversity in the *S. saevissima*-species group. This conclusion is not surprising in view of the historical difficulties in developing autapomorphic morphological characters to diagnose fire ant species, a problem that presumably can be ascribed to the recency of radiation in and morphological conservatism of the group.

Our conclusion that morphological delimitation currently overestimates fire ant species breadth assumes that the mtDNA gene tree we obtained faithfully tracks the development of reproductive isolation and patterns of population ancestry for species of the *S. saevissima* species-group, a perhaps risky assumption given the several potential causes of mtDNA/morphology discordance and the absence of supporting data from multiple nuclear gene phylogenies. However, data from other studies employing nuclear markers corroborate this conclusion by confirming the existence of at least two cryptic fire ant species. Ross and Trager (1990) demonstrated the presence of a cryptic species (designated *Solenopsis* sp. "X") that is sympatric with and morphologically indistinguishable from *S. quinquecuspis*, but which shares no alleles with it at three allozyme loci. Moreover, autapomorphic alleles of *S.* sp. "X" at the *Gr* locus distinguish it not only from *S. quinquecuspis* but from all other surveyed fire ant species (Ross and Trager, 1990). Two specimens of *S.* sp. "X" have been found to possess a unique *Gp-9* sequence (Krieger and Ross, 2005), although phylogenetic analyses failed to clearly resolve its relationship to the remaining *S. saevissima*-group *Gp-9* sequences. In the present study, we sequenced the mtDNA of five individuals (from as many nests) of *S.* sp. "X" collected across its known range. The five identical sequences are unique and, perhaps surprisingly, are nested within a well-supported haplotype clade otherwise composed of *S. invicta* sequences (clade 5; Fig. 2B). The combination of data from these three genetic sources leaves little doubt that *S.* sp. "X" is a genuine cryptic species.

Ross and Shoemaker (2005) recently identified a second cryptic fire ant species, in this case morphologically indistinguishable from nominal *S. invicta*. This species (designated *S. invicta* "cryptic" sp.) shares no alleles with other nominal *S. invicta* with which it is sympatric at the *Est-2* allozyme locus, and there are strong allele frequency differences between the two types at two other allozyme loci. We included 24 samples (nests) of this cryptic species in the present study. Its mtDNA sequences constitute a well supported clade that is the sister group to *S. invicta* clade 4 and excludes the sequences of all other nominal *S. invicta* (see Fig. 2B).

Aside from these two cases, the combined allozyme and mtDNA data of Ross and Shoemaker (2005) clearly hint at the existence of additional cryptic species within both *S. invicta* and *S. richteri*. These data revealed that geographically widely separated (800 km) conspecific populations are as distinct from one another at both genomes as are heterospecific populations of several nominal fire ant species. This finding, coupled with our expanded data set confirming multiple highly divergent, well-supported clades within *S. invicta* and *S. richteri*, means that multiple evolutionarily independent lineages that may eventually warrant recognition as distinct species likely exist in both taxa.

Although an insufficiently resolved α-taxonomy can be invoked to explain most of the extensive mtDNA polyphyly in the *S. saevissima* species-group, some portion of it is likely to result from past interspecific hybridization followed by mtDNA introgression. Such cytoplasmic genome capture is expected to be more common than nuclear introgression because mtDNA genes are less constrained by linkage to underdominant nuclear genes (Funk and Omland, 2003; Harrison, 1990), and the nuclear genes involved in species-specific phenotypes may themselves be under direct purifying selection.

In insects in which *Wolbachia* infection is common, such as fire ants, mtDNA introgression may be especially likely, even with infrequent hybridization, because it can be driven by *Wolbachia*-induced selective sweeps of cytoplasmic genomes (Shoemaker et al., 2003). It appears that most such hypothetical hybridization events between *S. saevissima*-group species would have been ancient, because instances of haplotype sharing among species are rare and most clades contain haplotypes exclusive to a single nominal species.

Obvious exceptions to these two generalizations are *S. quinquecuspis* and *S. megergates*. Both species harbor highly divergent haplotypes, one or more of which are identical to haplotypes found in another nominal species (Fig. 2B). This pattern suggests recent or ongoing hybridization and mtDNA introgression, a hypothesis well supported in *S. quinquecuspis* by evidence that it shares many nuclear alleles with both *S. invicta* and *S. richteri* in an area where all three co-occur (Ross and Shoemaker, 2005). Important additional evidence for recent hybridization comes from the close correspondence between the mtDNA variants and the *Wolbachia* strains possessed by individuals of *S. quinquecuspis* and *S. megergates* (D.D.S., unpublished data). For example, *S. megergates* individuals with mtDNA haplotypes Meg2 and Meg3 are infected with *Wolbachia* strain wSrichA2, the *Wolbachia* present in *S. richteri* individuals bearing what may be the sister haplotype to Meg2 + Meg3, Rich11 (Fig. 2B). Also, the single *S. quinquecuspis* with mtDNA haplotype Quin17 harbors a *Wolbachia* strain identical to that in *S. richteri* individuals with haplotype Rich3, which is identical to Quin17 (Fig. 2B). Finally, several *S. quinquecuspis* individuals with mtDNA haplotype Quin13 are infected with *Wolbachia* strain wSinvictaA, the strain present in *S. invicta* carrying a haplotype identical to Quin13, H1 (Fig. 2B). This strong mtDNA/*Wolbachia* association constitutes compelling evidence that cytoplasmic capture may be driven by ongoing selective sweeps of *Wolbachia* of foreign origin through *S. quinquecuspis* and *S. megergates*. We consider an alternative possibility that individuals of these latter species simply have been misclassified unlikely in light of the fact that both are readily diagnosable by a number of autapomorphic morphological characters (Pitts, 2002) and given the extensive nuclear data supporting introgression of *S. richteri* and *S. invicta* alleles into *S. quinquecuspis* (Ross and Shoemaker, 2005).

Additional possible evidence for the involvement of *Wolbachia* in fostering mtDNA polyphyly in South American fire ants is the remarkably deep divergence between mtDNA clades within the nominal species *S. invicta*, *S. richteri*, and *S. saevissima*. Such divergence may be the footprint of *Wolbachia*-driven increases in mtDNA substitution rates associated with recurrent *Wolbachia* sweeps in infected host lineages (see Shoemaker et al., 2004 for full discussion). One requirement of this scenario is that the separate clades within species correspond to different lineages or populations connected by minimal migration, because even modest gene flow can erode divergence among clades. For both *S. invicta* and *S. richteri*, extensive genetic data from previous studies suggest that this requirement often is met (Ahrens et al., 2005; Ross et al., 1997; Ross and Shoemaker, 2005; Ross and Trager, 1990).

Finally, consideration of the geographic dimension of mtDNA variation in the *S. saevissima* species-group may provide some insights into the history of divergence in the group. Strong phylogeographic structure clearly exists within the nominal species *S. invicta* and *S. richteri*. This geographic clustering of closely related haplotypes is evident from the fact that the estimate of ϕ_{ST} exceeds that of F_{ST} by 50% in *S. invicta* (Ahrens et al., 2005), and the fact that there is a complete geographic partitioning of the two divergent haplotype clades found in *S. richteri*. A reasonable working hypothesis explaining these patterns is that current regional populations of each of these nominal species are derived from refugia or largely isolated areas of past endemism from which range expansion has occurred (see also Ahrens et al., 2005). The issue of phylogeographic structure is more complex for nominal *S. saevissima*, a third widely distributed nominal species for which we have substantial samples, even after eliminating from consideration the specimens from southeastern Brazil with sequences closely allied to *Solenopsis* sp. "A." Phylogeographic structure seems to characterize *S. saevissima* from the northeastern seaboard of Brazil, given that the four haplotypes recovered from localities 35–38 form a monophyletic group (haplotypes Saev5 through Saev8; see Figs. 1 and 2A). On the other hand, each of the haplotypes Saev1, Saev2, and Saev9 recovered from the east-central Brazilian localities 31–33 reside in three separate, deeply divergent clades. These data are consistent with a hypothesis that the ants comprising nominal *S. saevissima* have long occupied the central part of their range but have only relatively recently colonized the northeastern part. The concordance between within-species mtDNA clades and geography lends further support to our assertion that morphological delimitation currently overestimates fire ant species breadth, because neither hybridization nor incomplete lineage sorting of mtDNA variants is expected to generate such patterns (Wiens and Penkrot, 2002).

Although more or less pronounced phylogeographic structure is evident within several of the nominal species, there appears to be minimal association between geography and haplotype ancestry within the *S. saevissima* species-group as a whole. For example, the haplotypes of *S.* sp. "X," which occurs in east-central Argentina, are not closely related to any haplotypes from the morphologically indistinguishable *S. quinquecuspis* with which it is

sympatric, but rather are embedded in an *S. invicta* clade limited almost exclusively to west-central Brazil. Also, the haplotypes of *S. invicta* "cryptic" sp., which is found only in southernmost Brazil, form a sister clade to *S. invicta* clade 4, which is restricted mostly to specimens from north-central Argentina. The haplotypes of *S. pusillignis* and *S. electra* form a well supported exclusive clade, and these appear to be sister species also on the basis of their morphology (Pitts et al., 2005) and *Gp-9* sequences (Krieger and Ross, 2005), yet their known ranges are separated by a gap of some 500 km (see Pitts, 2002). Finally, *S. invicta* and *S. richteri* co-occur at two widely separated sampling locations, but the haplotypes of each species are quite remotely related to the haplotypes of the other species from the same site (compare the *S. richteri* (plus *S. megergates*) clade with *S. invicta* clade 7, both of which are from southernmost Brazil, and the *S. richteri* (plus *S. quinquecuspis*) clade with *S. invicta* clade 2, both of which are from central Argentina; also Ross and Shoemaker, 2005). The striking dissociation of geographic proximity with mtDNA affinity supports our contention that hybridization had not been pervasive following radiation of the species. Moreover, this pattern hints at a complex history of species formation within the species-group. If supported by nuclear genetic data, such patterns would suggest that speciation typically did not occur sympatrically or parapatrically, but that long-distance founder events or contraction of ranges to isolated refugia may have been important demographic events involved in diversification in the group.

Acknowledgments

We thank Laurent Keller, Mark Mescher, and James Pitts for help collecting ants, and Dietrich Gotzek for helpful comments on the manuscript. DNA sequencing was done at the Biotechnology Center of the University of Wisconsin. This study was supported by grants from the College of Agriculture and Life Sciences at the University of Wisconsin, the United States Department of Agriculture NRICGP, and the US National Science Foundation to D.D.S. and K.G.R.

References

Ahrens, M., Ross, K.G., Shoemaker, D.D., 2005. Phylogeographic structure of the fire ant *Solenopsis invicta* in its native South American range: Roles of natural barriers and habitat connectivity. Evolution (in press).

Avise, J.C., 1986. Mitochondrial DNA and the evolutionary genetics of higher animals. Philosophical Transactions of the Royal Society of London B 312, 325–342.

Avise, J.C., 1991. Ten unorthodox perspectives on evolution prompted by comparative population genetic findings on mitochondrial DNA. Annual Review of Genetics 25, 45–69.

Avise, J.C., 1994. Molecular Markers, Natural History, and Evolution. Chapman and Hall, New York, NY.

Ballard, J.W.O., Whitlock, M.C., 2004. The incomplete natural history of mitochondria. Molecular Ecology 13, 729–744.

Cahan, S.H., Vinson, S.B., 2003. Reproductive division of labor between hybrid and nonhybrid offspring in a fire ant hybrid zone. Evolution 57, 1562–1570.

Calcaterra, L.A., Briano, J.A., Williams, D.F., 2000. New host for the parasitic ant *Solenopsis daguerrei* (Hymenoptera: Formicidae) in argentina. Florida Entomologist 83, 363–365.

Clary, D., Wolstenholme, D., 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. Journal of Molecular Evolution 22, 252–271.

Crozier, R.H., Crozier, Y.C., 1993. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. Genetics 133, 97–117.

Davis, L.R., Vander Meer, R.K., Porter, S.D., 2001. Red imported fire ants expand their range across the west indies. Florida Entomologist 84, 735–736.

Emery, C., 1909. Über den ursprung der dulotischen, parasitischen und myrmekophilen ameisen. Biologische Centralblatt 29, 352–362.

Eubanks, M.D., 2001. Estimates of the direct and indirect effects of red imported fire ants on biological control in field crops. Biological Control 21, 35–43.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Funk, D.J., Omland, K.E., 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annual Review of Ecology Evolution and Systematics 34, 397–423.

Gleason, J.M., Caccone, A., Moriyama, E.N., White, K.P., Powell, J.R., 1997. Mitochondrial DNA phylogenies for the *Drosophila obscura* group. Evolution 51, 433–440.

Goropashnaya, A.V., Fedorov, V.B., Seifert, B., Pamilo, P., 2004. Limited phylogeographical structure across eurasia in two red wood ant species *Formica pratensis* and *F-lugubris* (Hymenoptera, Formicidae). Molecular Ecology 13, 1849–1858.

Harrison, R.G., 1990. Hybrid zones: Windows on evolutionary process. Oxford Surveys in Evolutionary Biology 7, 69–128.

Harrison, R.G., 1991. Molecular changes at speciation. Annual Review of Ecology and Systematics 22, 281–308.

Hey, J., Waples, R.S., Arnold, M.L., Butlin, R.K., Harrison, R.G., 2003. Understanding and confronting species uncertainty in biology and conservation. Trends in Ecology & Evolution 18, 597–603.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Evolution—Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.

Jermiin, L.S., Crozier, R.H., 1994. The cytochrome b region in the mitochondrial DNA of the ant tetraponera rufoniger: sequence divergence in hymenoptera may be associated with nucleotide content. Journal of Molecular Evolution 38, 282–294.

Krieger, M.J.B., Ross, K.G., 2002. Identification of a major gene regulating complex social behavior. Science 295, 328–332.

Krieger, M.J.B., Ross, K.G., 2005. Molecular evolutionary analyses of the odorant-binding protein gene Gp-9 in fire ants and other *Solenopsis* species. Molecular Biology and Evolution (in press).

Lofgren, C.S., 1986. History of imported fire ants in the united states. In: Lofgren, C.S., VanderMeer, R.K. (Eds.), Fire Ants and Leaf Cutting Ants: Biology and Management. Westview Press, Boulder, CO, pp. 36–49.

Lofgren, C.S., Banks, W.A., Glancey, B.M., 1975. Biology and control of imported fire ants. Annual Review of Entomology 20, 1–30.

Machado, C.A., Kliman, R.M., Markert, J.A., Hey, J., 2002. Inferring the history of speciation from multilocus DNA sequence data: the

case of *Drosophila pseudoobscura* and close relatives. Molecular Biology and Evolution 19, 472–488.
Moriyama, E.N., Powell, J.R., 1997. Synonymous substitution rates in *Drosophila*: mitochondrial versus nuclear genes. Journal of Molecular Evolution 45, 378–391.
Morrison, L.W., Porter, S.D., Daniels, E., Korzhukin, M.D., 2004. Potential global range expansion of the invasive fire ant, *Solenopsis invicta*. Biological Invasions 6, 183–191.
Nattrass, R., Vanderwoude, C., 2001. A preliminary investigation of the ecological effects of red imported fire ants (*Solenopsis invicta*) in brisbane. Ecological Management and Restoration 2, 220–223.
Nei, M., 1987. Molecular evolutionary genetics. Columbia University Press, New York, NY.
Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonuclease. Proceedings of the National Academy of Sciences of the United States of America 76, 5269–5273.
Pascoe, A., 2001. Turning up the heat on fire ants. Biosecurity 32, 6.
Pitts, J.P., 2002. A cladistic analysis of the *Solenopsis saevissima* species-group (Hymenoptera: Formicidae). Ph.D., University of Georgia.
Pitts, J.P., McHugh, J.V., Ross, K.G., 2005. Cladistic analysis of the fire ants of the *Solenopsis saevissima* species-group (Hymenoptera: Formicidae). Zoologica Scripta 34, 493–505.
Pons, O., Petit, R.J., 1996. Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144, 1237–1245.
Porter, S.D., Savignano, D.A., 1990. Invasion of polygyne fire ants decimates native ants and disrupts arthropod community. Ecology 7, 2095–2106.
Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 19, 817–818.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Ross, K.G., 1993. The breeding system of the fire ant *Solenopsis invicta*: effects on colony genetic structure. American Naturalist 141, 554–576.
Ross, K.G., Keller, L., 1995. Ecology and evolution of social organization: insights from fire ants and other highly eusocial insects. Annual Review of Ecology and Systematics 26, 631–656.
Ross, K.G., Krieger, M.J.B., Shoemaker, D.D., Vargo, E.L., Keller, L., 1997. Hierarchical analysis of genetic structure in native fire ant populations: results from three classes of molecular markers. Genetics 147, 643–655.
Ross, K.G., Shoemaker, D.D., 2005. Species delimitation in native South American fire ants. Molecular Ecology (in press).
Ross, K.G., Shoemaker, D.D., Krieger, M.J.B., DeHeer, C.J., Keller, L., 1999. Assessing genetic structure with multiple classes of markers: a case study involving the introduced fire ant *Solenopsis invicta*. Molecular Biology and Evolution 16, 525–543.
Ross, K.G., Trager, J.C., 1990. Systematics and population genetics of fire ants (*Solenopsis saevissima* complex) from argentina. Evolution 44, 2113–2134.
Ross, K.G., Vandermeer, R.K., Fletcher, D.J.C., Vargo, E.L., 1987. Biochemical phenotypic and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). Evolution 41, 280–293.
Ross, K.G., Vargo, E.L., Keller, L., 1996. Social evolution in a new environment: the case of introduced fire ants. Proceedings of the National Academy of Sciences of the United States of America 93, 3021–3025.
Savolainen, R., Vepsäläinen, K., 2003. Sympatric speciation through intraspecific social parasitism. Proceedings of the National Academy of Sciences of the United States of America 100, 7169–7174.
Schneider, S., Roessli, D. Excoffier, L. 2000. Arlequin ver. 2.000: A Software for Population Genetic Data Analysis. University of Geneva, Switzerland: Genetics Biometry Laboratory.
Shoemaker, D.D., Dyer, K.A., Ahrens, M., McAbee, K., Jaenike, J., 2004. Decreased diversity but increased substitution rate in host mtDNA as a consequence of *Wolbachia* endosymbiont infection. Genetics 168, 2049–2058.
Shoemaker, D.D., Keller, G., Ross, K.G., 2003. Effects of *Wolbachia* on mtDNA variation in two fire ant species. Molecular Ecology 12, 1757–1771.
Shoemaker, D.D., Ross, K.G., Arnold, M.L., 1996. Genetic structure and evolution of a fire ant hybrid zone. Evolution 50, 1958–1976.
Shoemaker, D.D., Ross, K.G., Keller, L., Vargo, E.L., Werren, J.H., 2000. *Wolbachia* infections in native and introduced populations of fire ants (*Solenopsis* spp.). Insect Molecular Biology 9, 661–673.
Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of Entomological Society of America 81, 651–701.
Sites, J.W., Marshall, J.C., 2003. Delimiting species: a Renaissance issue in systematic biology. Trends in Ecology & Evolution 18, 462–470.
Sites, J.W., Marshall, J.C., 2004. Operational criteria for delimiting species. Annual Review of Ecology and Systematics 35, 199–227.
Solley, G.O., Vanderwoude, C., Knight, G.K., 2002. Anaphylaxis due to red imported fire ant sting. Medical Journal of Australia 176, 521–523.
Swofford, D.L., 1999. PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods), version 4.0. Sunderland, MA: Sinauer Associates.
Tajima, F., 1983. Evolutionary relationship of DNA sequences in finite populations. Genetics 105, 437–460.
Trager, J.C., 1991. A revision of the fire ants, *Solenopsis geminata* group (Hymenoptera: Formicidae: Myrmicinae). Journal of the New York Entomological Society 99, 141–198.
Tschinkel, W.R., 1993. Sociometry and sociogenesis of colonies of the fire ant *Solenopsis invicta* during one annual cycle. Ecological Monographs 63, 425–457.
Tschinkel, W.R., 2005. The Fire Ants. Harvard University Press, Cambridge, MA.
Vander Meer, R.K., Lofgren, C.S., Alvarez, F.M., 1985. Biochemical evidence for hybridization in fire ants. Florida Entomologist 68, 501–506.
Vinson, S.B., 1994. Impact and invasion of *Solenopsis invicta* (Buren) on native food webs. In: Williams, D.F. (Ed.), Exotic Ants: Biology, Impact, and Control of Introduced Species. Westview Press, Boulder, CO, pp. 293–308.
Wiens, J.J., Penkrot, T.A., 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Systematic Biology 51, 69–91.
Wilson, E.O., 1952. O complexo *Solenopsis saevissima* na America do Sul (Hymenoptera: Formicidae). Memorias do Instituto Oswaldo Cruz 50, 49–59.