Wolbachia infections in native and introduced populations of fire ants (Solenopsis spp.)

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Wolbachia in S. invicta. These data are consistent with historical introgression of S. richteri cyttoplasmic elements into S. invicta populations, resulting in enhanced infection and mtDNA polymorphisms in S. invicta. Wolbachia may have significant fitness effects on these hosts (either directly or by cytoplasmic incompatibility) and therefore these microbes potentially could be used in biological control programmes to suppress introduced fire ant populations.

Keywords: biological control, fire ants, mtDNA, introgression, reproductive isolation, Solenopsis invicta, Wolbachia.

Introduction

Wolbachia are maternally transmitted alpha-proteobacteria that infect the various tissues of arthropods, and whose transmission is enhanced by a variety of mechanisms including cytoplasmic incompatibility, thelytokous parthenogenesis, feminization of genetic males, male-killing, and increased mating success of infected males via sperm competition (Beard et al., 1993; Breeuwer et al., 1992; Hurst et al., 1999; O'Neill et al., 1992; Sinkins et al., 1995a,b; Stouthamer et al., 1993; Wade & Chang, 1995; for recent reviews see Stouthamer et al., 1999; Werren & O'Neill, 1997). A survey of arthropods in Panama revealed Wolbachia infections in almost 17% of the species sampled (Werren et al., 1995a), and recent data suggest similar frequencies of Wolbachia infections in nearctic insects as well (Werren & Windsor, 2000). Furthermore, Wolbachia have been found in mites and filarial nematodes, showing that their distribution extends well beyond insects (Bandi et al., 1998; Breeuwer & Jacobs, 1996; Sironi et al., 1995). Extrapolation of these estimates of Wolbachia infections suggests that these microbes may be among the most abundant group of parasitic bacteria known, with well over one million species of insects alone infected by Wolbachia (Werren et al., 1995b).

The most commonly described phenotypic effect of Wolbachia is unidirectional cytoplasmic incompatibility (CI), which occurs when an uninfected female mates with an infected male (Breeuwer & Werren, 1990; Hoffmann & Turelli, 1997; Hoffmann et al., 1986). Such matings generally...
produce few or no progeny as the result of abortive karyogamy (Callaini et al., 1997; Lassy & Karr, 1996). Because the other possible types of matings yield normal progeny numbers, the net effect of CI is to reduce offspring production of uninfected females compared to infected females. This reproductive advantage to infected females results in the spread of Wolbachia through a population (Caspari & Watson, 1959; Fine, 1978; Turelli, 1994; Turelli & Hoffmann, 1991). As Wolbachia spreads, other maternally inherited genomes found in infected females, including mitochondrial, are expected to spread with it (Caspari & Watson, 1959; Fine, 1978; Turelli, 1994; Turelli & Hoffmann, 1991). Such mitochondrial genomes are currently limited to examples involving Drosophila (Hoffmann et al., 1994; Hoffmann & Turelli, 1997; Shoemaker et al., 1999; Solignac et al., 1994; Turelli & Hoffmann, 1991, 1995). This is somewhat surprising, given that the occurrence of Wolbachia sweeps through natural populations is a crucial argument in favour of using these microbes in biological control programmes (Beard et al., 1993; Sinkins et al., 1997).

The population dynamics of Wolbachia in Hymenoptera (such as fire ants) are additionally complicated because uninfected females that mate with Wolbachia-infected males may produce all-male broods rather than show complete reproductive failure (because of the male-haploid genetic system characteristic of the order), or lethality of embryos (Vavre et al., 2000). Furthermore, Wolbachia may induce thelytokous parthenogenesis rather than CI in Hymenoptera (Stouthamer, 1997; Stouthamer et al., 1993). Clearly, therefore, additional population studies of Wolbachia are needed, especially in Hymenoptera, in order to better understand the factors that affect their population dynamics within hosts, the effects of infections on the host population genetics, and the potential use of these microbes in biological control of pest populations.

We present the results of a survey for Wolbachia in both native and introduced populations of the fire ant Solenopsis invicta, a serious introduced pest in the USA, as well as in several closely related fire ant species in the S. saevissima and S. geminata species complexes. Our survey data reveal the presence of variable Wolbachia infections in native (South American) populations of S. invicta and S. richteri, but the complete absence of infections in introduced populations of these two species and their hybrids.

Results

Distribution of Wolbachia in fire ants

Wolbachia infections were found in six of the nine species surveyed from the S. saevissima complex in their native ranges in South America (Table 1). In four of these species, the infections occur at frequencies that are intermediate between zero (complete absence) and one (universal infection). We cannot rule out the possibility that Wolbachia infection also occurs at intermediate frequencies in S. megergates, because of the small sample size for this species. By the same logic, it is possible that the three ‘uninfected’ species (S. interrupta, S. quinquecuspis and S. electra) do carry Wolbachia at some frequency. In contrast to the widespread occurrence of Wolbachia in South American fire ants, none of the ants screened from the USA appeared to harbour Wolbachia (Table 1). This is true for populations of S. invicta and S. richteri, and their hybrids, that have been introduced into this country, as well as for the native species S. geminata, which is the member of a species complex which is different from the other ants in this study.

Different populations of native South American S. invicta vary dramatically in their infection status (Table 1). Wolbachia infections were found at intermediate frequencies in both the monogyne (M) and polygyne (P) social forms [The social forms of S. invicta and other fire ant species differ in many important reproductive traits (most notably, the number of reproductive queens per colony), and female-mediated gene flow between them is thought to be limited (Ross and Shoemaker 1993; Ross and Shoemaker 1997; Shoemaker and Ross 1996)).] sampled from Corrientes, Argentina, but were present at low frequency (6%) or were absent in the M and P social forms, respectively, from Formosa, Argentina. Frequencies of Wolbachia infections differ significantly between the two regions in Argentina from which native S. invicta were sampled (Corrientes and Formosa), as well as between the two social forms within the Corrientes collection locality. However, Wolbachia infection frequencies do not differ significantly between the two social forms of S. invicta in Formosa or between the two social forms of S. richteri in Santa Fe, Argentina.

Distribution of Wolbachia in different life stages and body regions

PCR analyses revealed that Wolbachia are distributed among all life stages and castes of S. invicta hosts that were examined, and within all three body regions of the adult queens (Fig. 1). These results are somewhat surprising for workers, given that the ovaries are vestigial in this caste of fire ants (Goetsch, 1953). However, our conclusion that Wolbachia are not confined to adult reproductive tissues is consistent with Dobson et al. (1999), who showed that in some hosts, Wolbachia are found in many tissues throughout the body.

Phylogeny of Wolbachia in S. invicta and S. richteri

Results of our phylogenetic analyses of the Wolbachia strains based on a portion of the wsp gene, a highly variable gene encoding the bacterial surface protein (Braig et al., 1998; Zhou et al., 1998), are shown in Fig. 2. These phylogenetic analyses are based on wsp sequences from S. invicta (sixteen individuals) and S. richteri (three individuals), as

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Table 1. Frequencies of Wolbachia infections in native and introduced populations of fire ants. 'Population' column indicates the province or state from which samples were obtained.

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>Native or introduced</th>
<th>Social form*</th>
<th>Frequency of Wolbachia</th>
<th>95% CI</th>
<th>n †</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. invicta</td>
<td>Georgia, USA</td>
<td>introduced</td>
<td>M</td>
<td>0.0</td>
<td>0.0–0.09</td>
<td>34</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Georgia, USA</td>
<td>introduced</td>
<td>P</td>
<td>0.0</td>
<td>0.0–0.10</td>
<td>31</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Mississippi, USA</td>
<td>introduced</td>
<td>?</td>
<td>0.0</td>
<td>0.0–0.14</td>
<td>20</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Corrientes, Arg.</td>
<td>native</td>
<td>M</td>
<td>0.86</td>
<td>0.75–0.97</td>
<td>36</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Corrientes, Arg.</td>
<td>native</td>
<td>P</td>
<td>0.56</td>
<td>0.42–0.70</td>
<td>43</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Formosa, Arg.</td>
<td>native</td>
<td>M</td>
<td>0.06</td>
<td>0.0–0.15</td>
<td>34</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Formosa, Arg.</td>
<td>native</td>
<td>P</td>
<td>0.0</td>
<td>0.0–0.09</td>
<td>34</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Santa Fe, Arg.</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>0.0</td>
<td>4</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Chaco, Arg.</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Santiago del Estero, Arg.</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>0.0</td>
<td>1</td>
</tr>
<tr>
<td>S. invicta</td>
<td>Mato Grosso, Brazil</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>0.0</td>
<td>1</td>
</tr>
<tr>
<td>S. richteri</td>
<td>Mississippi, USA</td>
<td>introduced</td>
<td>?</td>
<td>0.0</td>
<td>0.0–0.15</td>
<td>19</td>
</tr>
<tr>
<td>S. richteri</td>
<td>Santa Fe, Arg.</td>
<td>native</td>
<td>M</td>
<td>0.27</td>
<td>0.07–0.53</td>
<td>15</td>
</tr>
<tr>
<td>S. richteri</td>
<td>Santa Fe, Arg.</td>
<td>native</td>
<td>P</td>
<td>0.33</td>
<td>0.13–0.60</td>
<td>15</td>
</tr>
<tr>
<td>S. invicta/richteri hybrids‡</td>
<td>Mississippi, USA</td>
<td>introduced</td>
<td>?</td>
<td>0.0</td>
<td>0.0–0.18</td>
<td>15</td>
</tr>
<tr>
<td>S. macdonaghi</td>
<td>Corrientes, Arg.</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>0.0</td>
<td>2</td>
</tr>
<tr>
<td>S. interrupta</td>
<td>Santiago del Estero and Córdoba, Arg.</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>0.0</td>
<td>5</td>
</tr>
<tr>
<td>S. quinquacapsis</td>
<td>Santa Fe and Buenos Aires, Arg.</td>
<td>native</td>
<td>?</td>
<td>1.0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>S. saevissima</td>
<td>Minas Gerais, Brazil</td>
<td>native</td>
<td>?</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S. saevissima</td>
<td>Goias, Brazil</td>
<td>native</td>
<td>?</td>
<td>1.0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S. megergates</td>
<td>Parana, Brazil</td>
<td>native</td>
<td>?</td>
<td>1.0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S. Species ‘X’</td>
<td>Santa Fe and Buenos Aires, Arg.</td>
<td>native</td>
<td>?</td>
<td>0.75</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S. electra</td>
<td>Santiago del Estero, Arg.</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S. geminata</td>
<td>Florida, USA</td>
<td>native</td>
<td>?</td>
<td>0.0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

*M, monogyne form; P, polygyne form; ? , form unknown.
†Number of individuals (nests).
‡Solenopsis invicta and S. richteri form a large hybrid zone in the USA (see text).

Figure 1. Representative gel of wsp PCR products showing distribution of Wolbachia throughout the body of an adult fire ant. Bands migrating at 400 bp represent the PCR product of the nuclear gene EF-1α, and bands migrating at 600 bp represent the PCR product of wsp. (A) 100 bp DNA size ladder; (B–C) PCR assays of genomic DNA isolated from a single fire ant lacking Wolbachia infection (positive nuclear DNA controls); (D–F) PCR assays of genomic DNA isolated from head, thorax, and gaster (= abdomen), respectively, of a single infected virgin queen fire ant (note that both bands are present and strong using all three body parts as DNA sources); (G, H) negative controls (no genomic DNA).

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well as sequences from fifty-nine other Wolbachia strains from various hosts retrieved from GenBank. A total of sixty-four most parsimonious trees were found, with the tree in Fig. 2 representing the 50% majority rule consensus tree. Differences among the sixty-four most parsimonious trees mainly were confined to placement of taxa near the tips of the tree and to one large unresolved node (polytomy) within the group ‘A’ Wolbachia. The neighbour-joining tree (not shown) exhibited a topology very similar to the consensus parsimony tree. The topologies of our trees generally were consistent with those from previous studies (Vavre et al., 1999a; Werren et al., 1995b; Zhou et al., 1998).

We identified two distinct Wolbachia strains within S. invicta. One of the Wolbachia strains from S. invicta clustered with strains representing the A group, while the other fell within the B group. These data clearly indicate that two independent infections occurred within this species. Interestingly, all sixteen individuals of S. invicta examined carried only a single Wolbachia strain; that is, none had double infections, even though both A and B Wolbachia are found within both social forms in Corrientes. Furthermore, all of the wsp sequences from each Wolbachia group present in S. invicta were identical (six group A and ten group B sequences). Only one Wolbachia strain was identified in each of the three individuals of S. richteri examined, and the three wsp sequences were identical. Finally, our sequence data reveal that the Wolbachia A strain from S. invicta and the strain from S. richteri were nearly identical, differing by only a single synonymous substitution.

**MtDNA phylogenetic analyses**

MtDNA phylogenetic analyses were based on sequences from the COI and COII genes in a subset of the individuals

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**Figure 2.** Majority rule consensus parsimony tree (midpoint rooted) for Wolbachia strains based on sequences from the wsp gene. Wolbachia strains are identified by host species from which they were isolated. Wolbachia strains from S. invicta and S. richteri are indicated in bold. Wolbachia strain ‘A’ was found in six S. invicta individuals and three S. richteri individuals and strain ‘B’ was found in ten individuals of S. invicta. Consistency indices are shown above branches and bootstrap support values (500 replicates) below branches for each node. Bootstrap support values less than fifty are not indicated.
above (twelve *S. invicta* and two *S. richteri*), as well as from an individual of *S. electra*, which served as the outgroup taxon. A total of four most parsimonious trees were found, which differed only in the relationships of *S. invicta* haplotypes within one clade (Fig. 3). The neighbour-joining tree featured a virtually identical topology. Our results show that mtDNA haplotypes from *S. invicta* are paraphyletic, with one group of mtDNA haplotypes sharing a more recent ancestor with haplotypes from *S. richteri* than with other *S. invicta* haplotypes. This paraphyletic relationship was resolved with a high bootstrap support value in all four parsimony trees, as well as in the neighbour-joining tree.

Significantly, there was complete concordance between the particular Wolbachia strain carried by an individual and the haplotype clade in which its mtDNA was placed (Fig. 3). That is, the mtDNA from all individuals carrying Wolbachia strain B formed a monophyletic group that was distinct from the mtDNA of individuals carrying strain A, with the latter individuals representing both *S. invicta* and *S. richteri*. Thus, the paraphyly of mtDNA haplotypes in *S. invicta* apparently can be explained by some force that affects the entire cytoplasm rather than just this particular organelar genome.

**Discussion**

*Wolbachia* are currently of interest to a broad spectrum of biologists because of their widespread distribution, varied phenotypic effects on hosts, potential role in speciation, and
almost assuredly true for S. richteri. The inferred small populations are rare, and mostly are limited to Drosophila species of significant economic importance. Within natural populations of a social hymenopteran insect derived from a native population that completely lacked infections, whereas individuals representing these species into the USA. We briefly address each of these possibilities in turn. The absence of infections in introduced ants occurs because Wolbachia invaded native populations after the introduction of fire ants into the USA, given the levels of mtDNA variation within the two groups of native S. invicta infected by Wolbachia (A and B in Fig. 3). Because Wolbachia are maternally transmitted, as Wolbachia spreads through a population all other maternally inherited organelles, including mitochondria, 'hitchhike' along with it (Turelli, 1994; Turelli & Hoffmann, 1991, 1995). Thus, a Wolbachia-associated sweep of a given mtDNA haplotype eliminates all variation, and any sequence variation detected among the extant mtDNA haplotypes represents mutations that have arisen since the Wolbachia sweep (Caspari & Watson, 1959; Fine, 1978; Turelli, 1994; Turelli & Hoffmann, 1991). Given that S. invicta was introduced in the early twentieth century, the expected amount of mtDNA variation within these two groups is essentially zero (assuming a 2% sequence divergence of mtDNA per million years; Brower, 1994; Guillemaud et al., 1997; Venanzetti et al., 1993). The presence of any mtDNA variation among infected native ants would be surprising under this scenario. Our data on mtDNA variation suggest that Wolbachia have been present in S. invicta and S. richteri for a very
long period. We found several mtDNA haplotypes within each group of Wolbachia in S. invicta (see below) and, at least in the case of the four haplotypes sequenced from S. invicta group A, relatively high between-haplotype divergence. Estimates of \( n \), the average number of nucleotide differences per site, for haplotypes from groups A and B were 0.01795 \( (n = 4) \) and 0.00083 \( (n = 8) \), respectively. These estimates, although based on a small number of sequences, suggest that the two Wolbachia strains have been present within S. invicta for at least 40,000 years (and much longer than that in the case of group A: Brower, 1959; Caspari & Watson, 1959; Fine, 1978; Turelli, 1994). Thus, Wolbachia were most likely present in native S. invicta and S. richteri populations long before the introduction of these two species into the USA.

Our survey also revealed that Wolbachia infection frequencies differed significantly between the two geographical populations of native S. invicta (Formosa and Corrientes, Argentina), as well as between the two social forms in the Corrientes population. These data are concordant with previous results showing significant differentiation between the two regions and between the two social forms within a single region for both mitochondrial and nuclear DNA markers, indicating reduced levels of gene flow (Ross et al., 1997). Such concordant patterns of genetic structure recorded from the Wolbachia and mtDNA genomes are expected if the two genomes are inherited in a similar fashion, that is, only through females (no paternal leakage) and with no horizontal transfer (Caspari & Watson, 1959; Fine, 1978; Turelli, 1994).

The differences in Wolbachia infection frequencies that we detected between the two social forms may also stem from differences in the selective regimes acting on this microbe in each form. In this respect, the predicted effects of CI and parthenogenesis-inducing (PI) Wolbachia in S. invicta (production of males instead of females) parallel another interesting phenomenon observed in S. invicta that is associated with the genetic sex-determination system. Sex in fire ants, as in many other social Hymenoptera, is presumably determined by genotype at a single nuclear locus, with individuals heterozygous at this locus developing into females and individuals hemizygous or homozygous developing into males (Crozier, 1971; Ross & Fletcher, 1985, 1986; Ross et al., 1993). When a female mate with a male carrying an allele identical to one of hers at the sex locus, 50% of her diploid offspring will be hemizygous at this locus and develop into diploid males (Crozier, 1971; Ross & Fletcher, 1985; Ross et al., 1993). Such diploid-male-producing queens occur at a frequency of around 20% in newly mated polygyne (P) queens and monogyne (M) queens of S. invicta in the USA; however, such queens are never found heading mature M colonies, because the colonies they found invariably fail (Ross & Fletcher, 1985, 1986; Ross & Shoemaker, 1997; Ross et al., 1993).

differential mortality is caused by diploid-male-producing queens investing their limited resources in diploid males rather than in the workers that are crucial to early colony survival. Polygyne queens are not subject to this source of mortality because they do not attempt to found colonies independently but instead enter established nests to become egg layers (Ross & Fletcher, 1985, 1986; Ross & Shoemaker, 1997). Although we currently do not know what effects these microbes have on fire ants (see below), we predict a similar pattern of selection against uninfected M queens carrying CI or PI Wolbachia, because such queens would presumably produce only haploid males or diploid males, respectively, at the crucial colony-founding stage.

The predicted outcome of such strong selection in M queens compared to P queens is a faster sweep and higher equilibrium frequency of CI Wolbachia infections in the M form than the P form. This conclusion follows from the findings that the dynamics and equilibrium frequency of Wolbachia infections are mostly governed by the parameters outlined by Turelli (1994), namely: (i) \( F \), the fecundity of infected relative to uninfected females (ii) \( H \), the hatch rate of incompatible relative to compatible fertilizations, and (iii) \( \mu \), the fraction of uninfected ova produced by infected females (i.e. maternal transmission fidelity). The differences in social biology between the two forms discussed above imply that the reproductive advantage to infected females will be greater in the M social form (Caspari & Watson, 1959; Fine, 1978; Turelli, 1994). If maternal transmission of Wolbachia is incomplete (as suggested by preliminary laboratory studies; Shoemaker et al. unpublished results), then these P queens may contribute some uninfected females, as well as males, to the population every generation. The effects will be a slower sweep of Wolbachia in the P form (because \( H \), the hatch rate of incompatible relative to compatible fertilizations, will be greater in uninfected queens of the P form) as well as a lower equilibrium frequency of Wolbachia infections in the P form. This latter prediction is consistent with our survey data showing higher infection frequencies in the M form. Additionally, the persistence of incompatibly mated queens could impose a continuous fitness cost on polygyne colonies due to the production of male progeny (or lethality of incompatible eggs). Modelling is needed to determine more exactly the population dynamics of Wolbachia in monogyne and polygyne fire ant populations.

Wolbachia infections in fire ants

MtDNA and Wolbachia variation

There are three explanations for the paraphyly of S. invicta mtDNA haplotypes, namely: (i) individuals from the two mtDNA clades represent two species that are morphologically indistinguishable (cryptic species), (ii) there has been historical introgression between S. invicta and S. richteri, resulting in capture of cytoplasmic genomes, and (iii) S. richteri...
originated from within the *S. invicta* clade. Allozyme data generally support the morphology-based identification of *S. invicta* individuals in this study (Ross et al., 1997; Ross & Trager, 1990; Ross et al., 1998b); however, the allozyme data also reveal modest but statistically significant differentiation between the *S. invicta* of the two mtDNA clades in Corrientes (Shoemaker et al. unpublished data). This finding is consistent with the hypothesis that the ants identified as *S. invicta* from Corrientes actually represent cryptic species, and that these have been reproductively isolated for a sufficient period for significant nuclear genetic divergence to evolve. That sufficient time may have elapsed to allow such nuclear differentiation is suggested by the mtDNA divergence seen in these two groups of *S. invicta*. Total divergence estimates of θ, the average number of nucleotide differences per site, for all *S. invicta* haplotypes that we sampled is 0.027, suggesting a time to common ancestry greater than one million years. Furthermore, previous estimates of θ, the average pair-wise sequence divergence, for RFLP mtDNA haplotypes from a larger set of individuals from Corrientes are 0.034 and 0.041 (for the M and P forms, respectively). Future analyses involving additional nuclear DNA markers are necessary to resolve the issue of possible cryptic species within nominal *S. invicta* in South America.

The paraphyly of *S. invicta* mtDNA also could be explained by a hybridization event between *S. invicta* and *S. richteri*. Previous studies have shown that introduced populations of these two species form a large hybrid zone where they come into contact (Ross et al., 1987; Shoemaker et al., 1994). Although extensive hybridization apparently does not occur at present in South America, allozyme studies suggested at least some level of hybridization (Ross & Trager, 1990), and even very limited amounts of historical interspecific gene flow may be sufficient for complete capture of the cytoplasmic genomes when *Wolbachia* are involved. Indeed, even a single hybridization event followed by subsequent backcrossing to one species may introduce *Wolbachia* into a population previously lacking it. If infection frequencies subsequently drift high enough, then the selective advantage of carrying *Wolbachia* (i.e. causing CI) will be realized and the microbe will increase in frequency in the population (Hoffmann & Turelli, 1997; Turelli, 1994; Turelli & Hoffmann, 1995), carrying with it the ‘foreign’ mtDNA. The end result of such a selective sweep will be the capture of the cytoplasmic genomes of one species by another, with essentially no signal of hybridization in the nuclear genome (Rieseberg et al., 1991).

Finally, as mentioned before with respect to infection frequencies, the complete concordance of the mitochondrial and *Wolbachia* genomes in *S. invicta* suggests that the inheritance patterns of these genomes are similar (i.e. maternal inheritance), and that significant amounts of horizontal transfer or paternal leakage of *Wolbachia* do not occur.

**Phenotypic effects of *Wolbachia* on fire ants**

Possible phenotypic effects of *Wolbachia* on fire ants, some of which have been described recently in other arthropods, include: (i) cytoplasmic incompatibility (CI), (ii) parthenogenesis induction (PI) (Stouthamer et al., 1993), (iii) mutagenic interactions with the host, perhaps increasing host fitness (Vavre et al., 1999b), (iv) pathogenic relationships with the host (i.e. systemic infections; [Min & Benzer, 1988]), (v) no CI but the ability to rescue females from CI caused by other *Wolbachia* strains (Bourtzis et al., 1998; Meroz & Poinso, 1998), (vi) male killing (Hurst et al., 1999), or (vii) lack of any apparent effects, with the microbe behaving as a neutral cytoplasmic element (Clancy & Hoffman, 1996).

Choosing among these possibilities is speculative at present, but two points are worthy of mention. First, parthenogenesis induction can be ruled out, at least by the mechanism previously described (Stouthamer et al., 1993). Parthenogenesis in infected hymenopteran females carrying PI *Wolbachia* is caused by the process of gamete duplication, which typically results in a diploid female that is homozygous at every locus. However, in the case of fire ants, gamete duplication would result in a diploid male rather than a diploid female because of homozygosity at the sex-determination locus. We consistently observed *Wolbachia*-infected female progeny in our analyses, a finding inconsistent with gamete duplication. Second, inference of the phenotypic effects of *Wolbachia* is not aided by our phylogenetic analyses. This is because one *Wolbachia* strain from fire ants (B) is part of a clade containing strains that have diverse host effects (CI, PI, male killing), while the other (A) is not similar enough to previously described strains to infer its effects. It should be noted that the coexistence of different *Wolbachia* strains, such as those we found in *S. invicta*, is not predicted within panmictic host populations (Caspari & Watson, 1959; Turelli, 1994; but see Rouset et al. (1991) for a similar example in mosquitoes). One possible explanation for their coexistence is that the two strains have different phenotypic effects; for instance, one may induce CI while the other induces male-killing.

**Biological control implications**

*Wolbachia* may have significant fitness effects on fire ants, either directly or due to cytoplasmic incompatibility. The occurrence of *Wolbachia* at intermediate frequencies would impose a CI-induced fitness cost on populations due to incompatibilities between infected and uninfected individuals. Thus, these bacteria could reduce fire ant populations in North America, a noteworthy consideration given the significant pest status of fire ants and the desire to find new natural control agents (Lofgren, 1986; Lofgren et al., 1975; Patterson, 1994). The diminished genetic load on
the introduced pest populations, which are free of infection, may help explain the five- to tenfold increase in colony densities of this ant in the USA compared to its native range (Porter et al., 1992). Furthermore, a finding of any significant load on fire ants caused by Wolbachia (direct fitness effects of Wolbachia infection, CI coupled with imperfect transmission, or some other effect) would mean that there is considerable potential for successfully incorporating these microbes into an integrated pest management programme to suppress populations of introduced fire ants. Therefore, determination of the effects of Wolbachia on S. invicta is an important topic for future research.

**Experimental procedures**

**Collection and identification of ants**

Collection localities for all ants surveyed in the present study are shown in Table 1. Introduced fire ants representing S. invicta and S. richteri were collected in the spring of 1990 and fall of 1995. The majority of such S. invicta samples were from Monroe, Georgia, USA (see Ross and Shoemaker, 1997, Ross et al., 1999 and Shoemaker and Ross, 1996 for descriptions of this locality). A total of sixty-five colonies at this locality was used for the present study; thirty-four of these colonies were of the monogyne (M) social form and thirty-one were of the polygyne (P) form. Additional colonies of S. invicta of unidentified social form were collected from Vaiden, Mississippi, USA (four colonies), Morgan City, Mississippi (eight), and Durant, Mississippi (eight). Solenopsis richteri nests of unidentified social form were collected from Nixon, Mississippi (nine), Randolph, Mississippi (nine), and Banner, Mississippi (one). Finally, hybrid S. invicta/richteri colonies of unidentified social form were collected from Greenwood, Mississippi (one). Additional colonies of S. invicta (unknown social form) were sampled from Coffeeville, Mississippi (seven), Holcomb, Mississippi (one), and Yalobusha County, Mississippi (one). All of the above colonies previously have been confirmed as nests of either S. invicta, S. richteri or hybrids using at least three diagnostic allosyme markers (Shoemaker et al., 1996).

Native fire ants representing S. invicta of both social forms were collected in 1992 from two populations located near the cities of Rosario and Formosa in north-eastern Argentina. Over thirty colonies of each social form in each population were sampled (Table 1). These populations, which are separated by the Río Paraná (160 km), have been the subject of extensive previous genetic studies (Ross, 1997; Ross et al., 1993, 1996a,b, 1997), so we are confident that the classification of nests to both species and social form is correct (Ross et al., 1993). Ants from eleven additional colonies of S. invicta (unknown social form) were collected in the fall of 1988 and 1998 from the following locations: Santa Fe, Argentina (four colonies); Santiago del Estero, Argentina (one); Chaco, Argentina (three); Mato Grosso, Brazil (two) and Paraná, Brazil (one). Samples of native S. richteri of both social forms (fifteen colonies of each) were collected in 1992 from a population located near the city of Rosario in central Argentina. Social form of each colony was determined by examining the genotypes of twelve nestmate workers at seven polymorphic allosyme loci; genotype distributions inconsistent with the workers being full sisters are diagnostic of P colonies (Ross, 1992; Ross & Shoemaker, 1993; Ross et al., 1988, 1999).

**Screening for Wolbachia**

Total genomic DNA was isolated from each individual using the Puregene® DNA isolation kit, a simple method for isolating high molecular weight DNA (Ross & Shoemaker, 1997). Genomic DNA from each ant was screened for the presence of Wolbachia via the polymerase chain reaction using the primers Wsp81F and Wsp691R (Zhou et al., 1998). The wsp primers amplify a portion of a highly variable gene encoding the bacterial surface protein (Braig et al., 1996; Zhou et al., 1998). Details of the PCRs, PCR profiles, and electrophoresis of products are described in Shoemaker et al. (1999) and Zhou et al. (1998).

Wsp PCRs were performed in 15 μl volumes containing 13 μl of Platinum® PCR SuperMix (Gibco BRL): 0.18 μl of a 25 μl solution of each primer (Wsp81F and Wsp691R), and 1–3 μl of genomic DNA. Also included in each reaction were two control primers (0.12 μl of a 25 μl solution of each): EF1α-532F (5’-AGGCAAATGTCTTCTTGAAG-3’) and EF1α-610R (5’-GGGAGTGCCGAAAGTACAAG-3’). These primers amplify a 400 bp portion of one of the two copies of the nuclear gene EF1α (elongation factor). Inclusion of these two primers in every PCR reaction constitutes an important positive control for determining the infection status of individuals. The presence of the EF1α fragment and absence of the Wolbachia-specific fragment most likely reflects an absence of the bacteria rather than low quality or overly concentrated genomic DNA or an error associated with PCR setup.

On the other hand, when such control primers fail to work, one cannot confidently assert that the lack of a Wolbachia-specific PCR product results from an absence of the bacteria. Therefore, in cases where the EF1α fragments were absent, we serially diluted genomic DNA and performed PCR again.

Amplifications were carried out in a Perkin Elmer 9700 thermocycler programmed as follows: 1 min at 94 °C for one cycle; 30 s at 94 °C, 30 s at 60 °C (-2 °C per cycle) and 1 min at 72 °C for 10 cycles (touchdown PCR); 30 s at 94 °C, 30 s at 53 °C, and 1 min at 72 °C for twenty-five cycles; 5 min at 72 °C for one terminal cycle. Approximately 5 μl of each PCR reaction mixture was electrophoresed in 2% agarose gels. Gels were stained with ethidium bromide and bands visualized under UV illumination. Images of
stained gels were photographed and stored electronically using a gel photodocumentation system.

A single individual from each sampled fire ant colony was screened for Wolbachia to estimate population infection frequencies. A bootstrapping procedure was used to estimate the 95% confidence intervals (CIs) around nonzero frequency estimates for all samples with more than five individuals. Individuals were sampled randomly (with replacement) from the original data set for each bootstrap replicate. This procedure was repeated 1000 times, and the 95% CIs were found by eliminating the twenty five lowest and twenty five highest values from the ordered array of the 1000 estimates. In cases where infection frequencies were apparently zero and sample sizes were greater than five, 95% CIs were estimated from the binomial distribution as the interval zero to 1, where 1 represents the upper limit frequency of infection in the population in which Wolbachia infections may go undetected 95% or more of the time for a particular sample size. Samples with CIs that do not overlap are taken to have significantly different infection frequencies.

Distribution of Wolbachia within S. invicta

We conducted two analyses to determine the distribution of Wolbachia among the different life stages and castes of S. invicta, as well as among the different body regions of adult females. We screened for Wolbachia using the PCR assay described above using individuals of the following life stages and castes (two individuals each): fourth instar worker larvae; worker pupae; worker adults; queen pupae; adult virgin queens; male pupae; adult males. All of these individuals were taken from two field-collected monogyne colonies known to have Wolbachia-infected queens. To determine the distribution of Wolbachia throughout the body, we sectioned two adult virgin queens into three parts (head, thorax, gaster [= abdomen]), performed DNA isolations on each part, and screened for Wolbachia using our PCR assay described above.

Sequencing of Wolbachia strains and mtDNA

A portion of the wsp gene (~ 590 bp) was sequenced from a total of nineteen infected individuals (sixteen S. invicta and three S. richteri – see Fig. 2) using the primers Wsp 81F and Wsp 691R (Zhou et al., 1998). For sequencing, Wolbachia DNA was PCR-amplified as described above, except 50 μl volumes were used, no controlprimers were included, and the final extension at 72 °C was for 45 min rather than 5 min. PCR amplicons were gel-purified using Qiagen gel extraction spin columns, used in standard fluorescent cycle-sequencing PCR reactions (ABI Prism Big Dye terminator chemistry), and run on an automated ABI Prism 377 or 310 sequencer as described above. Sequences were aligned and phylogenetic trees were constructed using maximum parsimony and neighbour-joining methods, as implemented in PAUP* 4.0 (Swofford, 1999). Parsimony trees were constructed using the branch and bound search option, and the resulting trees were rooted using S. electra as an outgroup. Previous morphological analyses place this species in a different but closely related species subcomplex than the one to which S. invicta and S. richteri belong (Trager, 1991). Bootstrap values were generated using a heuristic search algorithm (1000 bootstrap replicates with ten random addition searches per replicate). The neighbour-joining tree was constructed using Jukes–Cantor distances and the resulting tree was midpoint rooted. Wsp sequences representing each Wolbachia strain have been deposited in GenBank (AF243435–AF243437).

A 910 bp portion of the mitochondrial (mtDNA) genome was sequenced from a subset of the individuals above (twelve S. invicta and two S. richteri), as well as from an additional individual S. electra (see Fig. 3). mtDNA PCR amplifications were carried out using the primer C1–J-2195 (COI-R): 5’-TTGATTTTTGTCATCCAGAGT3’; see Simon et al., 1994) and a primer we designed (DOS-COI-4: 5’-TAAGATGTTAATAGAAGATAG-3’; Ross & Shoemaker, 1997). These two primers amplify a portion of the mtDNA that includes regions of both the cytochrome oxidase I (COI) and cytochrome oxidase II (COII) genes. mtDNA PCRs were performed in 50 μl volumes containing 5 μl of 10× buffer (Gibco BRL), 3 μl of a 1.5×mixture solution of magnesium chloride, 0.6 μl of 10×mix solutions of dNTPs, 1.0 μl of 25 μM solutions of each primer, 1–3 μl of genomic DNA, and 0.2 μl (1 unit) of thermostable (Taq) DNA polymerase (Gibco BRL). Amplifications were carried out in a Perkin-Elmer 9700 thermocycler programmed as follows: 1 min at 94 °C for one cycle; 30 s at 94 °C, 1 min at 50 °C, and 2 min at 68 °C for thirty-five cycles; 5 min at 72 °C for one terminal cycle. The 910 bp mtDNA amplicons were gel-purified using Qiagen gel extraction spin columns, used in standard fluorescent cycle-sequencing PCR reactions (ABI Prism Big Dye terminator chemistry), and run on an automated ABI Prism 377 or 310 sequencer as described above. Sequences were aligned and phylogenetic trees were constructed using maximum parsimony and neighbour-joining methods, as implemented in PAUP* 4.0 (Swofford, 1999). Parsimony trees were constructed using the branch and bound search option, and the resulting trees were rooted using S. electra as an outgroup. Bootstrap values were generated using a heuristic search algorithm (1000 bootstrap replicates with ten random addition searches per replicate). The neighbour-joining tree was constructed using Jukes–Cantor distances and the resulting tree was rooted also using S. electra as an outgroup.

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References


