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

D. DeWayne Shoemaker,[1] Kenneth G. Ross,[2] L. Keller,[3] E. L. Vargo[4] and John H. Werren[5]

[1]*Department of Biological Sciences, 3149 Wood Hall, 1903 West Michigan Avenue, Western Michigan University, Kalamazoo, MI 49008-5410, USA,* [2]*Department of Entomology, University of Georgia, Athens, GA 30602-2603, USA,* [3]*University of Lausanne, Institute of Ecology, Batiment de Biologie, 1015 Lausanne, Switzerland,* [4]*Department of Entomology, Campus Box 7613, North Carolina State University, Raleigh, NC, 27695-7613, USA,* [5]*Department of Biology, University of Rochester, Rochester, NY 14627-0211, USA*

Abstract

Wolbachia **are cytoplasmically inherited bacteria that induce a variety of effects with fitness consequences on host arthropods, including cytoplasmic incompatibility, parthenogenesis, male-killing and feminization. We report here the presence of** ***Wolbachia*** **in native South American populations of the fire ant** ***Solenopsis invicta*****, but the apparent absence of the bacteria in introduced populations of this pest species in the USA. The** ***Wolbachia*** **strains in native** ***S. invicta*** **are of two divergent types (A and B), and the frequency of infection varies dramatically between geographical regions and social forms of this host. Survey data reveal that** ***Wolbachia*** **also are found in other native fire ant species within the** ***Solenopsis saevissima*** **species complex from South America, including** ***S. richteri*****. This latter species also has been introduced in the USA, where it lacks** ***Wolbachia*****. Sequence data reveal complete phylogenetic concordance between mtDNA haplotype in** ***S. invicta*** **and** ***Wolbachia*** **infection type (A or B). In addition, the mtDNA and associated group A** ***Wolbachia*** **strain in** ***S. invicta*** **are more closely related to the mtDNA and** ***Wolbachia*** **strain found in** ***S. richteri*** **than they are to the mtDNA and associated group B** ***Wolbachia*** **in** ***S. invicta*****. These data are consistent with historical introgression of** ***S. richteri*** **cytoplasmic 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*****.**

Received 16 May 2000; accepted after revision 24 August 2000. Correspondence: D. DeWayne Shoemaker, Department of Biological Sciences, 3149 Wood Hall, 1903 West Michigan Avenue, Western Michigan University, Kalamazoo, MI 49008-5410, USA. Tel.: 616 387 2084; fax: 616 387 5609; e-mail: dewayne.shoemaker@wmich.edu

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 mitochondria, are expected to spread with it (Caspari & Watson, 1959). However, empirical tests of these theoretical predictions of the spread of *Wolbachia* and associated 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 embyros (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

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

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

*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).

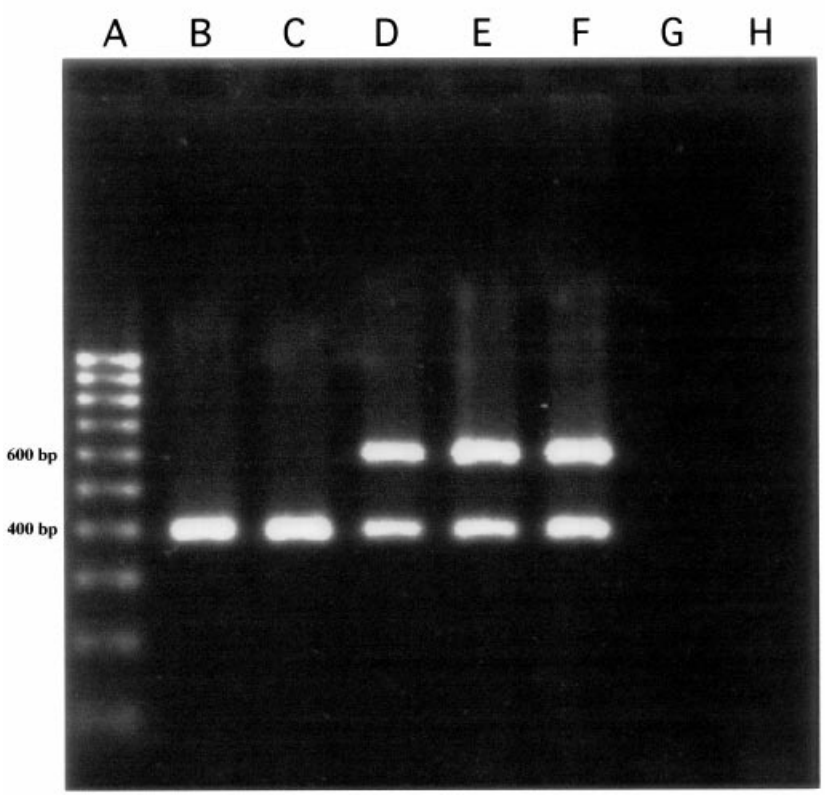

Figure 1. Representative gel of *wsp* PCR products showing distribution of *Wolbachia* throughout the body of an adult fire ant. Bands migrating at 400 bp represent the PCR product of the nuclear gene EF-1α, and bands migrating at 600 bp represent the PCR product of *wsp*. (A) 100 bp DNA size ladder; (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).

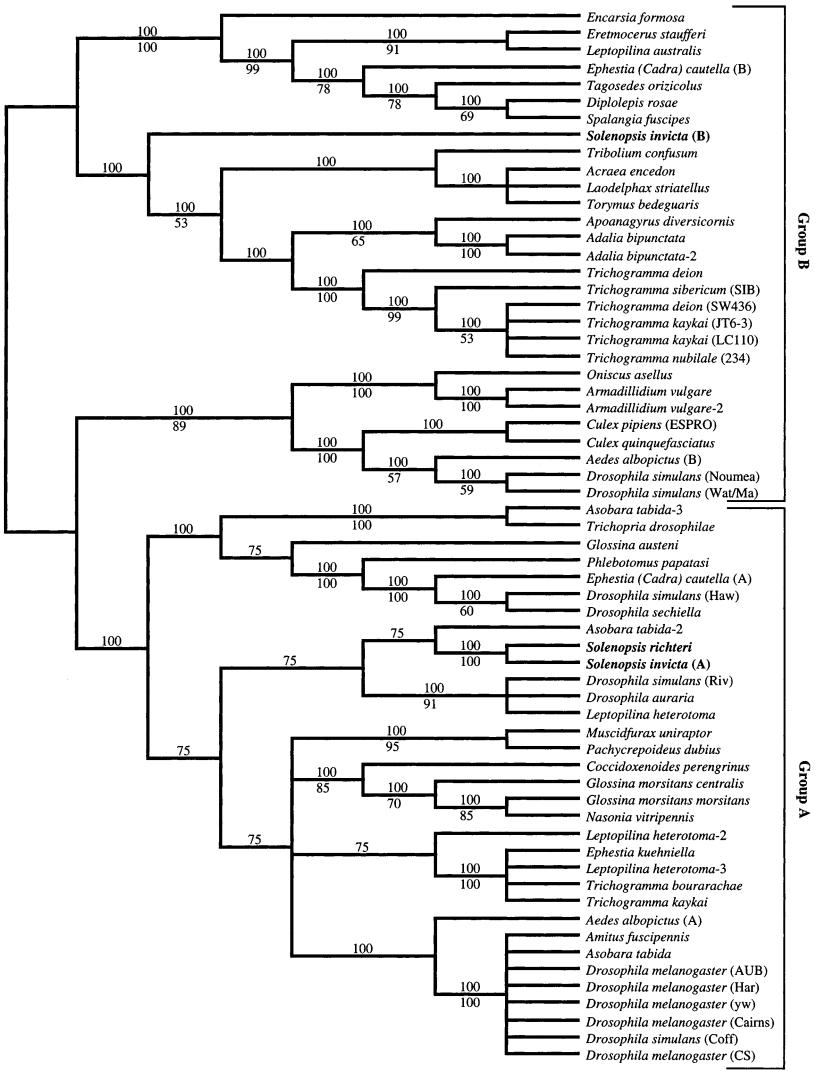

Figure 2. Majority rule consensus parsimony tree (midpoint rooted) for *Wolbachia* strains based on sequences from the *wsp* gene. *Wolbachia* strains are identified by host species from which they were isolated. *Wolbachia* strains from *S. invicta* and *S. richteri* are indicated in bold. *Wolbachia* 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.

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

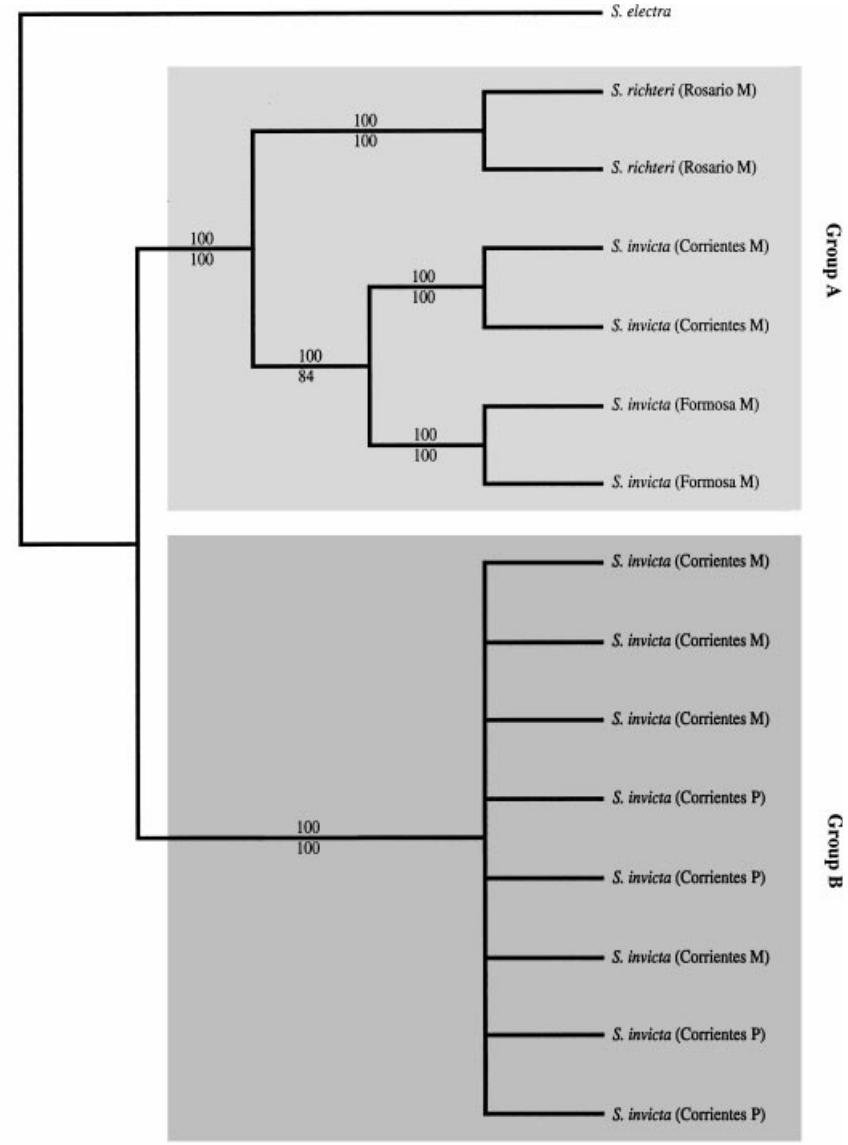

Figure 3. Majority rule consensus parsimony tree for mtDNA haplotypes based on sequences from the cytochrome oxidase I (COI) and cytochrome oxidase II (COII) genes. Consistency indices are shown above branches and bootstrap support values (500 replicates) below branches for each node. Bootstrap support 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 organellar 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

potential use in biological control programmes (Hoffmann & Turelli, 1997; Sinkins *et al*., 1997; Stouthamer, 1997; Werren, 1997, 1998; Werren & O'Neill, 1997 and references therein). However, detailed studies examining the frequencies of *Wolbachia* infections within and among natural populations are rare, and mostly are limited to *Drosophila* (Ballard *et al*., 1996; Hoffmann, 1988; Hoffmann *et al*., 1994, 1996, 1998, 1990; Shoemaker *et al*., 1999; Turelli & Hoffmann, 1991, 1995). This is surprising given the widespread interest in these microbes, the substantial amount of theory developed on their population dynamics, and the crucial need for such data in assessing the potential for using *Wolbachia* in biological control programmes (Sinkins *et al*., 1997; Turelli, 1994; Turelli & Hoffmann, 1999; Werren, 1997, 1998; Werren & O'Neill, 1997). In the present paper, we present the results of a survey for *Wolbachia* in native and introduced populations of the fire ant *S. invicta*, as well as in several other fire ant species. Our study represents the first survey of the frequencies of *Wolbachia* infections within natural populations of a social hymenopteran insect of significant economic importance.

Patterns of Wolbachia *infection in native and introduced fire ants*

Our survey data revealed that individuals from native populations of both *S. invicta* and *S. richteri* often harbour *Wolbachia* infections, whereas individuals representing both species from the USA, where they have been introduced, apparently lack *Wolbachia* (Table 1). Several possible explanations exist for these distinct patterns, including: (i) the absence of *Wolbachia* in all of the original foundresses introduced into the USA, (ii) the loss of *Wolbachia* infections in introduced fire ants due to drift or selection, (iii) the failure to detect *Wolbachia* in introduced ants (due to low frequency of infection) even though it is present, and (iv) the introduction and spread of *Wolbachia* in native *S. invicta* and *S. richteri* populations after introduction of these species into the USA. We briefly address each of these possibilities in turn.

The absence of *Wolbachia* in introduced fire ants may stem from the absence of these microbes in all of the original foundresses introduced into the USA. Previous genetic data clearly show that *S. invicta* experienced a severe population bottleneck associated with its introduction (Ross & Trager, 1990; Ross *et al*., 1993, 1996b), and the same is almost assuredly true for *S. richteri*. The inferred small number of foundresses introduced into the USA, coupled with the fact that many colonies of both *S. invicta* and *S. richteri* in South America lack *Wolbachia* (i.e. *Wolbachia* infections frequencies are variable within and between populations), suggests that this possibility is not unlikely. A related scenario holds that *Wolbachia* infections are absent in the USA because the original foundresses are derived from a native population that completely lacked *Wolbachia*. This is also not an unlikely scenario for *S. invicta*, especially given the fact that we did not detect *Wolbachia* infections in the three *S. invicta* colonies sampled from Brazil, the suspected source country of introduced ants of this species (Table 1).

It is also possible that some proportion of the original foundresses carried *Wolbachia*, but that the infections were subsequently lost due to drift and/or selection. Recent theory has shown that there is a threshold frequency that must be obtained for these microbes to increase in frequency and sweep to fixation or near-fixation (which varies depending on the fitness effects and maternal transmission rate), and that the equilibrium frequency is zero (i.e. loss of the bacteria) below this threshold (Turelli, 1994). Thus, if relatively few foundresses carried *Wolbachia*, the particular female lineages carrying these microbes may have been lost either through drift or selection associated with the fitness costs of carrying the bacteria. Consistent with this possibility, we find that one of the common mitochondrial haplotypes associated with *Wolbachia* in South America is common among fire ants in the USA (Shoemaker and Ross, unpublished data).

We consider it unlikely that *Wolbachia* are present in introduced fire ants but that we failed to detect them, given our large sample sizes (119 individuals of *S. invicta*, *S. richteri*, and their hybrids). Indeed, to go undetected, the frequencies of *Wolbachia* infections in the USA would have to be so low that the predicted stable equilibrium would likely be complete loss of the bacteria through drift or selection (Turelli, 1994).

We also consider it unlikely that the lack of *Wolbachia* in introduced fire 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 π, 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, 1994; Guillemaud *et al.*, 1997; Venanzetti *et al.*, 1993). 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 mates with a male carrying an allele identical to one of hers at the sex locus, 50% of her diploid offspring will be homozygous 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). This 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) μ, 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.

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*., 1996b); 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 π, 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 *d*, 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* that resulted in capture of the cytoplasmic genome of *S. richteri* by *S. invicta*. 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) mutualistic interactions with the host, perhaps increasing host fitness (Vavre *et al*., 1999b), (iv) pathogenic relationships with the host (i.e. systemic infections; (Min & Benzer, 1998)), (v) no CI but the ability to rescue females from CI caused by other *Wolbachia* strains (Bourtzis *et al*., 1998; Merçot & Poinsot, 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 Rousset *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 sampled from Greenwood, Mississippi (six), 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 allozyme 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 Corrientes 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 allozyme 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).

Samples of seven additional species of fire ants in the *S. saevissima* species complex were collected from their native South American ranges in 1988, 1992 and 1998 (one to seven nests per species). Species identifications were initially made in the field using a published morphological key and were subsequently confirmed in the laboratory using both morphological characters and allozyme markers that exhibit species-specific allele compositions (Ross *et al.*, 1993, 1997; Ross & Trager, 1990; Trager, 1991). Any nests for which species identifications disagreed between these two character sets were excluded, except for samples of Species 'X', a morphologically cryptic species that is distinguished from all the other species by the possession of unique alleles at two allozyme loci (Ross & Trager, 1990). The social form of colonies of these additional species is unknown. For further information on the collection localities and methods for assigning nests to species, see Table 1, Ross (1997), and Ross *et al.* (1993, 1996a,b, 1997).

Finally, three colonies of *S. geminata* of unknown social form were sampled in its native range from Tampa (one colony) and Tallahassee (two), Florida, USA. This fire ant is placed in a different species complex (*S. geminata* complex) than the other species we studied.

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 *Wsp*81F and *Wsp*691R (Zhou *et al.*, 1998). The *wsp* primers amplify a portion of a highly variable gene encoding the bacterial surface protein (Braig *et al.*, 1998; 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 μM solution of each primer (*Wsp*81f and *Wsp*691R), and 1–3 μl of genomic DNA. Also included in each reaction were two control primers (0.12 μl of a 25 μM solution of each): EF1α-532F (5′-AGGCAAATGTCTTATTGAAG-3′) and EF1α-610R (5′-GCGGG-TGCGAAGGTAACAAC-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 ($-^1/_2$ °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 f*, where f* 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 sexuals. 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 control primers 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 and used directly in standard fluorescent cycle-sequencing PCR reactions (ABI Prism Big Dye terminator chemistry). Sequencing reactions were cleaned using sephadex columns (Princeton Separations) and run on an automated ABI Prism 310 sequencer.

Sequences were aligned to a subset of previously published *Wolbachia* sequences from other insects and arthropods (fifty nine sequences found in GenBank, submitted by Braig *et al.* 1998; Hurst *et al.* 1999; Schulenburg *et al.* 2000; van Meer *et al.* 1999; Vavre *et al.* 1999a; Zhou *et al.* 1998). The sequence data were used to construct phylogenetic trees of *Wolbachia* strains using maximum parsimony and neighbour-joining methods, as implemented in PAUP* 4.0 (Swofford, 1999). The third, hypervariable region (positions 521–567) was excluded for these analyses (Vavre *et al.*, 1999a; Zhou *et al.*, 1998). Parsimony trees were constructed using the heuristic search option (1000 random additions) of PAUP* 4.0 and the resulting trees were midpoint rooted. Bootstrap values were generated using the heuristic search algorithm (500 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 representing *S. electra* (see Fig. 3). MtDNA PCR amplifications were carried out using the primer C1-J-2195 (COI-RLR; 5′-TTGATTTTTTGGTCATCCAGAAGT-3′; see Simon *et al.*, 1994) and a primer we designed (DDS-COII-4; 5′-TAAGATGGTTAATGAAGAGTAG-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-mM solution of magnesium chloride, 0.6 µl of 10 mM 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 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 was rooted also using *S. electra* as an outgroup.

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