Effects of *Wolbachia* on mtDNA variation in two fire ant species

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Abstract

Wolbachia **are endosymbiotic bacteria that infect arthropods. As they are maternally transmitted, the spread of *Wolbachia* variants within host populations may affect host mtDNA evolution. We sequenced a portion of the mitochondrial cytochrome oxidase I gene from numerous individuals of two *Wolbachia*-infected fire ant species, *Solenopsis invicta* and *S. richteri*, to determine how these bacteria influence patterns of mtDNA variation. As predicted, there was a strong association between *Wolbachia* strain and host mtDNA lineage within and between these fire ant species. However, there was no consistent association between the presence of *Wolbachia* and a reduction in mtDNA diversity. Moreover, patterns of mtDNA variation within *Wolbachia*-infected populations did not differ consistently from neutral expectations, despite our prediction that strong positive selection acting on *Wolbachia* influences the evolutionary dynamics of other cytoplasmic genomes. Specifically, while values of Tajima's D consistently were less than zero for all six samples of fire ants harbouring *Wolbachia*, MacDonald–Kreitman tests suggested that the patterns of variation were different from those expected under neutrality in only two of the samples. We conclude that these neutrality tests do not unambiguously reveal a clear effect of *Wolbachia* infection on patterns of mtDNA variation and substitution in fire ants. Finally, consistent with an earlier study, our data revealed the presence of two divergent mtDNA haplotype lineages and *Wolbachia* strains within *S. invicta*. Recognition of these two lineages has important consequences for interpreting patterns of mtDNA evolution and genetic differentiation between conspecific social forms of this species.**

Keywords: fire ants, mtDNA, population structure, *Solenopsis invicta*, *Solenopsis richteri*, *Wolbachia*

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Introduction

Use of variable genetic markers in population and evolutionary genetics is important for understanding the roles of organismal attributes (e.g. vagility, mating preferences, habitat continuity), historical processes (e.g. bottlenecks, range expansions), and direct and indirect selective forces in shaping patterns of genetic variation in natural populations (Avise 1994, 1995). Variation in animal mitochondrial DNA (mtDNA) is used extensively in such studies because this genome possesses many ideal properties for discerning the forces of evolution, including maternal inheritance, lack of recombination and relatively

high rates of evolution (Avise 1994). Thus, mtDNA data often prove useful in assessing genetic structure, gene flow or phylogenetic relationships among populations or closely-related species when other types of traditional, nuclear markers fail (Avise 1994). mtDNA data can also complement data from nuclear genes to provide unique insights into mating patterns and gene flow (Shoemaker & Ross 1996).

While mtDNA analyses clearly have many useful applications, recent studies suggest that patterns of mtDNA variation may sometimes be misleading in reflecting evolutionary history. For example, low intraspecific mtDNA variation is often interpreted as resulting from some demographic event affecting females (i.e. recent bottleneck, low effective population size) or from a recent selective sweep of a favoured mtDNA variant (Johnstone & Hurst 1996),

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and lack of mtDNA differentiation among populations is typically interpreted as reflecting persistent gene flow. Yet these patterns may also result from mtDNA sweeps associated with the spread of a selectively-favoured, maternally-inherited microorganism (Johnstone & Hurst 1996). One group of microorganisms known to affect mtDNA dynamics in this way are bacteria in the genus *Wolbachia* (Ballard & Kreitman 1994; Rand *et al.* 1994; Ballard *et al.* 1996; Johnstone *et al.* 1996; Rigaud *et al.* 1999; Shoemaker *et al.* 1999; Ballard 2000). These endosymbiotic bacteria infect a wide variety of arthropods and filarial nematodes (Werren & O'Neill 1997), with recent surveys suggesting that *Wolbachia* commonly infect 17–22% (Werren *et al.* 1995; West *et al.* 1998; Werren & Windsor 2000), and possibly up to 76% (Jeyaprakash & Hoy 2000), of all insects.

Wolbachia enhance their transmission by a variety of mechanisms, including cytoplasmic incompatibility, thelytokous parthenogenesis, feminization of genetic males and male-killing (Breeuwer *et al.* 1992; O'Neill *et al.* 1992; Beard *et al.* 1993; Stouthamer *et al.* 1993; Sinkins *et al.* 1995a,b; Hurst *et al.* 1999). Regardless of the specific mechanism by which they manipulate host reproduction, their spread entails a concomitant spread of all maternally-inherited organelles, including mitochondria, found in the initially infected female (Turelli & Hoffmann 1991, 1995; Solignac *et al.* 1994; Turelli 1994; Hoffmann *et al.* 1998). Thus, one particular mitochondrial DNA haplotype (and its mutational derivatives) will sweep through a population, via hitchhiking, in association with the selectively-driven sweep of *Wolbachia* (Caspari *et al.* 1959; Fine 1978; Turelli *et al.* 1991, 1995; Prout 1994; Turelli 1994). With the possible exception of *Wolbachia* that induce parthenogenesis, patterns of variation at nuclear genes should be largely unaffected by the presence of these bacteria in a population.

Given the association between this endosymbiont and host cytoplasmic organelles, *Wolbachia* may be expected to dramatically affect host mtDNA genome evolution. For example, assuming the bacteria are transmitted mostly vertically, an infected population should have lower mtDNA diversity than an uninfected population. Additionally, if sequential replacement of *Wolbachia* strains and associated cytoplasmic elements occurs as predicted (Prout 1994; Turelli 1994; Hurst & McVean 1996), patterns of nucleotide substitution may differ between infected and uninfected lineages. In particular, because each *Wolbachia* sweep reduces the effective population size of mtDNA to one, an infected population may experience a relatively high rate of substitution of slightly deleterious mutations, leading to an accelerated rate of mtDNA evolution (Shoemaker *et al.* 2003). Finally, *Wolbachia* may affect levels of mtDNA differentiation. For instance, *Wolbachia* sweeps can result in minimal mtDNA differentiation between populations even in the virtual absence of gene flow, because even

very small amounts of interbreeding can lead to transmission of *Wolbachia* between populations and parallel sweeps of mtDNA variants due to the reproductive advantage to infected females (Caspari & Watson 1959; Turelli 1994; Turelli *et al.* 1995). On the other hand, the spread of *Wolbachia* through a single population may inflate between-population mtDNA differentiation if the bacteria are unable to invade neighbouring populations. Therefore, it is important to consider not only organismal attributes, historical processes and potential for selection when interpreting patterns of mtDNA variation, but also the possible effects of cytoplasmic associates such as *Wolbachia* (Johnstone *et al.* 1996).

In this paper, we present data on patterns of mtDNA variation in native populations of the fire ants *Solenopsis invicta* and *S. richteri*. Both species harbour *Wolbachia* in their native South American ranges (Shoemaker *et al.* 2000). In the case of *S. invicta*, it appears that only one of two previously studied geographical populations has high infection frequencies. Thus, comparing patterns of mtDNA variation between these two populations may reveal the effects of *Wolbachia* on host mtDNA evolution. Our sequence data from a portion of the cytochrome oxidase I (COI) gene in numerous individuals of both species suggest that *Wolbachia* probably do affect host mtDNA evolution, although in complex and sometimes unanticipated ways. Moreover, these data offer new insights into the patterns and causes of genetic differentiation between conspecific forms of these ants that display alternative colony social organizations.

Materials and methods

Collection and identification of ants

Samples representing both the monogyne (single queen per nest) and polygyne (multiple queens per nest) social forms of native *S. invicta* were collected from two geographical populations located near the cities of Corrientes and Formosa in northeastern Argentina (for sample sizes, see Table 1). These geographical populations are separated by a distance of about 160 km. Distinguishing between the two social forms in *S. invicta* and other fire ant species is important because the two forms differ in many important reproductive traits, and female-mediated gene flow between them is thought to be limited (Ross *et al.* 1993, 1997; Shoemaker *et al.* 1996). The colonies from which material was collected have been the subject of previous extensive genetic studies, for which specimens were confirmed as *S. invicta* and nests were carefully classified as to social form (Ross *et al.* 1993; 1996a,b, 1997; Ross 1997; Shoemaker *et al.* 2000).

The native ranges of *S. invicta* and *S. richteri* in South America are parapatric, with a narrow zone of overlap located just north of the city of Rosario in central Argentina.

Table 1 Haplotype and nucleotide diversity for mtDNA COI sequences in *Solenopsis invicta* and *S. richteri*

	N	Number of haplotypes	Haplotype diversity	Number of variable sites (S)	π	SD (π)	θ (S)	SD (θ)
S. invicta								
Corrientes M	29	10	0.574	57	0.0213	0.0050	0.0187	0.0062
Corrientes M, C group	23	4	0.320	3	0.0004	0.0002	0.0011	0.0007
Corrientes M, non-C group	6	5	0.933	24	0.0102	0.0053	0.0134	0.0067
Corrientes M, C group (W+)	19	4	0.380	3	0.0005	0.0002	0.0011	0.0007
Corrientes M, non-C group (W+)	6	5	0.933	24	0.0102	0.0053	0.0134	0.0067
Corrientes P	22	13	0.931	49	0.0294	0.0026	0.0180	0.0064
Corrientes P, C Group	9	4	0.694	3	0.0011	0.0003	0.0014	0.0009
Corrientes P, non-C group	13	9	0.936	12	0.0030	0.0005	0.0052	0.0024
Corrientes P, C group (W+)	7	4	0.810	3	0.0013	0.0003	0.0016	0.0011
Corrientes P, non-C group (W+)	6	4	0.800	4	0.0018	0.0006	0.0024	0.0015
Formosa M	21	11	0.886	28	0.0134	0.0016	0.0102	0.0038
Formosa P	17	11	0.941	48	0.0159	0.0043	0.0182	0.0068
S. richteri								
M	9	4	0.806	3	0.0011	0.0003	0.0014	0.0010
P	9	6	0.833	10	0.0031	0.0009	0.0048	0.0024

M and P refer to monogyne and polygyne social forms, respectively. C and non-C refer to the two major haplotype groups within *S. invicta* (see text). W + indicates *Wolbachia*-infected individuals. *N* is the number of individuals studied (one per nest).

Nine colonies of each social form of *S. richteri*, identified to species by J. C. Trager, were sampled from a site near this city. The social form of each sampled colony was determined by examining the genotypes of 12 nestmate workers at seven polymorphic allozyme loci; genotype distributions inconsistent with the workers being full sisters are diagnostic for polygyne colonies (Ross *et al.* 1988, 1993, 1999; Ross 1992).

Only a single specimen per colony was used as the source material in this study, because nestmate fire ants are usually matrilineal relatives (e.g. Ross *et al.* 1997).

Sequencing of Wolbachia *strains and mtDNA*

Total DNA was isolated from each ant using the Puregene® DNA isolation kit (Ross *et al.* 1997). We previously screened the DNA from each ant for the presence of *Wolbachia* by means of polymerase chain reaction (PCR) using the primers *wsp*81F and *wsp*691R (Zhou *et al.* 1998; Shoemaker *et al.* 2000). These primers amplify a 575–625 bp portion of a highly variable gene encoding the bacterial surface protein (Braig *et al.* 1998; Zhou *et al.* 1998). We extended these earlier analyses by sequencing this same *Wolbachia* gene region for an expanded set of individuals representing *S. invicta* and *S. richteri*. For sequencing, *Wolbachia* DNA was PCR-amplified in 50 μL volumes, with the PCR reaction components and thermal cycling conditions as described in Shoemaker *et al.* (2000). *Wsp* PCR amplicons were cleaned for sequencing using Agencourt® magnetic beads,

and the purified products were used directly in standard fluorescent cycle-sequencing PCR reactions (ABI Prism® Big Dye™ terminator chemistry).

A 785 bp portion of the COI gene from the mtDNA genome was sequenced from the infected individuals of *S. invicta* and *S. richteri* described above, additional uninfected individuals of *S. invicta* and *S. richteri*, and a single individual of *S. electra*. The PCR reaction mixes, sequences of external PCR and sequencing primers, and thermal cycling conditions, are described elsewhere (Shoemaker *et al.* 2000). MtDNA amplicons were purified and sequenced in the same manner as the *wsp* amplicons.

Phylogenetic analyses of mtDNA

MtDNA sequences were aligned manually and phylogenetic trees were constructed using neighbour-joining (NJ) and maximum parsimony (MP) methods, as implemented in PAUP* 4.0b10 (Swofford 1999). The program MODELTEST was used to determine the appropriate model of sequence evolution (Posada & Crandall 1998). Based on these results, we used the HKY85 distance measure to construct the NJ tree using *S. electra* as the outgroup. Morphological analyses place *S. electra* as a basal member of the *S. saevissima* species group, to which *S. invicta* and *S. richteri* belong (Trager 1991). Bootstrap support values were generated from 10 000 replicates (HKY85 model). MP trees were constructed using the heuristic search option (1000 random addition searches), with tree bisection and reconnection (TBR) and branch swapping; other settings

were the PAUP* defaults. The resulting trees again were rooted using *S. electra* as the outgroup. Bootstrap values were generated using a heuristic search algorithm (500 bootstrap replicates with 10 random addition searches per replicate) with TBR and branch swapping.

Analyses of molecular evolution

Sequence data for the COI gene were used in our analyses of mtDNA molecular evolution. As a previous study employing restriction fragment length polymorphism (RFLP) analyses of mtDNA (Shoemaker *et al.* 2000), as well as the current study, showed that mtDNA haplotypes within *S. invicta* form two distinct lineages, we conducted all of our analyses in two ways: by combining all individuals of each social form within each geographical population without regard to haplotype lineage, and by separating such individuals by lineage (designated as the 'C' and 'non-C' haplotype groups). Haplotype diversity, nucleotide diversity (π), defined as the average number of pairwise nucleotide differences per site (Nei & Li 1979; Tajima 1983; Nei 1987), and θ (= $2N_f u$, where N_f is the effective population size of females and u is the mutation rate), which is based on the number of segregating sites (Watterson 1975; Nei 1987), were calculated for species, geographical populations, social forms and haplotype groups using the program DnaSP (Rozas & Rozas 1999). For *S. invicta* from Corrientes, we also calculated these measures considering only *Wolbachia*-infected individuals. The net number of nucleotide substitutions per site between groups (sequence divergence, Da) was estimated using equation 10.21 of Nei (1987).

Values of Tajima's *D* statistic were calculated to test the neutral mutation hypothesis. Tajima's *D* is based on differences between two estimates of θ, namely, π and θ_W (Watterson's θ or the number of segregating sites within a population) (Watterson 1975; Nei *et al.* 1979; Tajima 1983, 1989; Nei 1987). Under neutrality, estimates of Tajima's *D* are expected to equal zero. Positive *D* values indicate an excess of intermediate-frequency variants, whereas negative values indicate an excess of rare variants, as can result from a recent population bottleneck (hitchhiking event) or processes such as background selection. Tajima's *D* statistic was calculated for the same groups as were the diversity statistics using the program DnaSP (Rozas & Rozas 1999).

We also calculated Fay and Wu's *H* statistic (Fay & Wu 2000), which compares the occurrence of intermediate-frequency and high-frequency derived haplotypes. Under neutrality, *H* is expected to be zero, whereas negative values indicate an excess of high-frequency derived variants, such as may occur with hitchhiking. We calculated *H* using a program provided by J. Fay, with *S. electra* designated as the outgroup for inferring the ancestral and derived states of polymorphic sites in *S. invicta* and *S. richteri*. The recom-

bination rate was assumed to equal zero and the probability of backmutation was calculated as $d/3$, where d equals the average number of fixed differences at synonymous sites between *S. electra* and a given ingroup (Fay & Wu 2000).

Finally, we used the MacDonald–Kreitman (M–K) test as implemented in DnaSP as a test for departure from neutrality (McDonald & Kreitman 1991). This test compares the ratio of the number of nonsynonymous to synonymous polymorphisms (K_A/K_S) within species to the ratio of the number of nonsynonymous to synonymous differences between species (McDonald *et al.* 1991). These ratios are expected to be the same under neutrality, whereas a significant difference between them suggests departures from neutral evolution (McDonald *et al.* 1991).

Analyses of population genetic structure

Estimates of mtDNA differentiation and gene flow among groups were calculated using N_{ST} of Lynch & Crease (1990) and F_{ST} of Hudson *et al.* (1992). N_{ST}, the nucleotide-level analogue of Wright's F_{ST} (Wright 1951), gives the ratio of the average genetic distance between genes within different populations to that for genes in the metapopulation as a whole. F_{ST} of Hudson *et al.* (1992) is essentially the same as N_{ST}, except no Jukes–Cantor correction is performed (Hudson *et al.* 1992).

The extent of mtDNA differentiation between geographical populations of *S. invicta*, as well as between social forms within populations of both *S. invicta* and *S. richteri*, was further analysed using the AMOVA (analysis of molecular variance) approach implemented in the program ARLEQUIN (Excoffier *et al.* 1992; Schneider *et al.* 2000). AMOVA subdivides the total haplotype variance into hierarchical components and calculates ϕ indices, which are the molecular equivalents of Wright's *F* statistics (Wright 1951). These indices were calculated both with and without taking sequence divergence between haplotypes into account. Divergence between haplotypes was defined as the Euclidean metric equalling the square of the nucleotide differences between them. Significance levels of estimated ϕ indices and associated variance components were assessed by using permutation tests (1000 permutations of the original data set) to calculate the probability of obtaining more extreme values by chance.

Results

Analyses of wsp and mtDNA sequences

All *wsp* sequences from native *S. invicta* were found to be identical to either of two previously-described sequences that correspond to two distinct *Wolbachia* strains infecting *S. invicta* (Shoemaker *et al.* 2000). All *wsp* sequences from native *S. richteri* correspond to a third, unique strain of
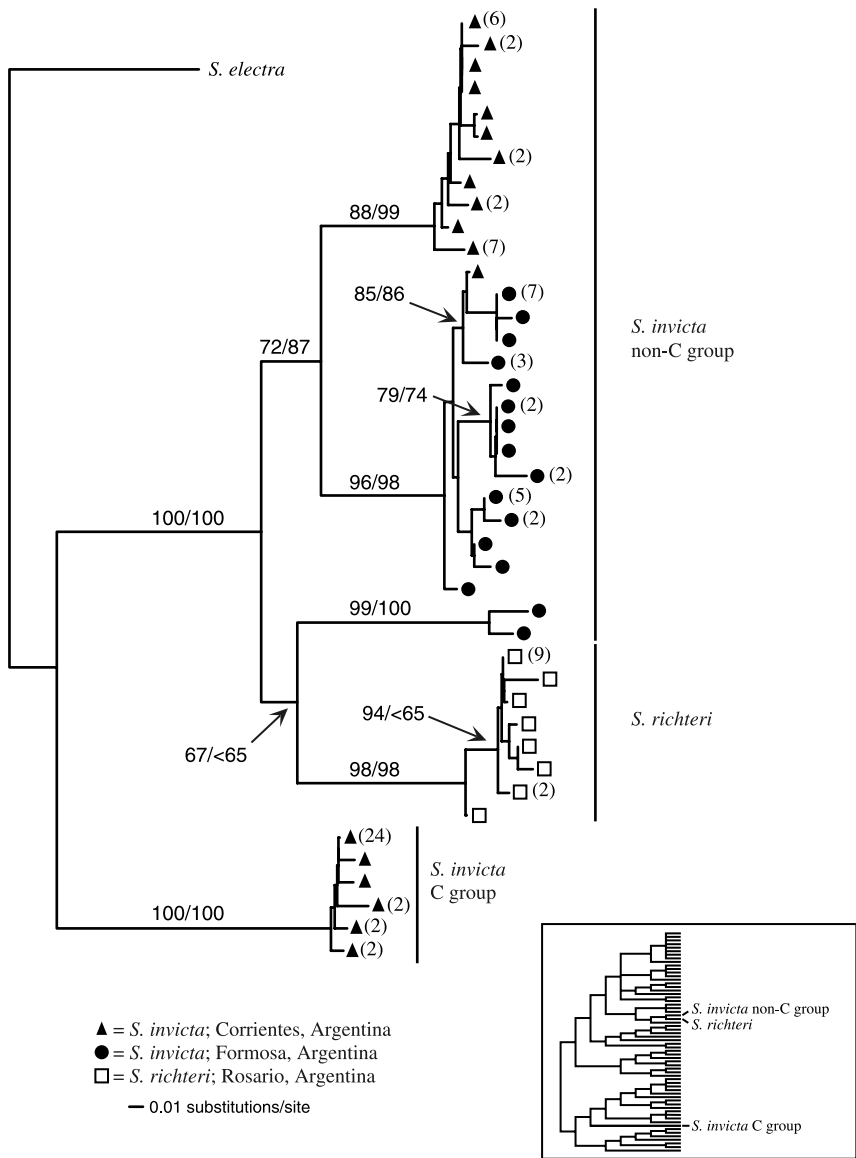
Fig. 1 Neighbour-joining (NJ) tree for mtDNA haplotypes of *Solenopsis invicta* and *S. richteri* based on sequences from a portion of the cytochrome oxidase I (COI) gene. Bootstrap support values greater than 65% are shown as percentages above branches for each node (NJ/MP). Numbers beside each haplotype indicate how many individuals possess it if greater than one. Inset: Phylogenetic positions of the *Wolbachia* strains infecting *S. invicta* and *S. richteri* with respect to other insect-infecting *Wolbachia* (from Shoemaker *et al.* 2000). The *Wolbachia* strains infecting the non-C group of *S. invicta* and *S. richteri* belong to the A subgroup of *Wolbachia*, whereas the strain infecting C group *S. invicta* belongs to the B subgroup of *Wolbachia* (Zhou *et al.* 1998).

Wolbachia that is identical to one described previously from this same host (Shoemaker *et al.* 2000). The phylogenetic positions of these three strains with respect to other insect-infecting *Wolbachia* are depicted in the inset of Fig. 1 (see Shoemaker *et al.* 2000).

The entire mtDNA data set consists of 785 bp sequenced from 108 individuals, including *S. electra*. A total of 107 bp are variable (118 total mutations), of which 82 are informative for MP analyses. There are no indels. Of the 118 total mutations, 106 are silent mutations and 12 are replacement mutations. Transitions occur almost 10 times more often than transversions. The base composition of the mtDNA sequences is highly AT-biased (average of 69.35% AT), consistent with previous studies of mtDNA sequences from other insects, including Hymenoptera (Clary & Wolstenholme 1985; Simon *et al.* 1994; Dowton & Austin 1995).

A large number of unique sequence haplotypes was found in native *S. invicta* (34) and *S. richteri* (eight) (see Table 1 for haplotype numbers within each population and social form; GenBank sequence accession numbers AY249092–AY249134). The NJ tree showing the evolutionary relationships of these haplotypes is presented in Fig. 1. MP analysis resulted in a total of 192 most parsimonious trees (consistency index = 0.683, retention index = 0.929), but the differences among these trees were essentially limited to rearrangements within each of the well-supported major clades of *S. invicta* and *S. richteri* recovered in the NJ tree. Consistent with earlier results based on RFLP analyses (Ross *et al.* 1997), two highly distinct haplotype lineages differing at about the 5% sequence divergence level occur within *S. invicta* (see also Table 2). Remarkably, one of these lineages (non-C group) shares a more recent

Table 2 Net number of mtDNA nucleotide substitutions per site (sequence divergence) between groups, Da (with Jukes and Cantor correction; above diagonal), and standard deviation of Da (below diagonal). M and P refer to monogyne and polygyne social forms, respectively. C and non-C refer to the two major haplotype groups within *S. invicta*. Values for comparisons between the major haplotype groups of *S. invicta* are shown in bold. Values for interspecific comparisons are shaded. Dashes indicate missing values where sequence divergence was not calculated because comparisons were inappropriate

	Corrientes M	Corrientes M, C group	Corrientes M, non-C group	Corrientes P	Corrientes P, C group	Corrientes P, non-C group	Formosa M	Formosa P	*S. richteri* M	*S. richteri* P	*S. electra*
S. invicta											
Corrientes M	———	—	—	0.0067	—	—	0.0322	0.0323	0.0428	0.0376	0.0811
Corrientes M, C group	—	———	**0.0335**	—	<0.0001	**0.0523**	**0.0485**	**0.0473**	0.0549	0.0497	0.0863
Corrientes M, non-C group	—	**0.0120**	———	—	**0.0380**	0.0003	0.0058	0.0096	0.0297	0.0246	0.0908
Corrientes P	0.0056	—	—	———	—	—	0.0153	0.0184	0.0300	0.0247	0.0852
Corrientes P, C group	—	0.0026	**0.0143**	—	———	**0.0557**	**0.0526**	**0.0533**	0.0593	0.0542	0.0906
Corrientes P, non-C group	—	**0.0056**	0.0076	—	**0.0072**	———	0.0125	0.0181	0.0356	0.0301	0.1051
Formosa M	0.0061	**0.0055**	0.0071	0.0053	**0.0061**	0.0034	———	0.0005	0.0310	0.0262	0.0971
Formosa P	0.0063	**0.0060**	0.0078	0.0052	**0.0071**	0.0037	0.0031	———	0.0294	0.0256	0.0949
S. richteri											
M	0.0058	0.0062	0.0086	0.0050	0.0090	0.0047	0.0039	0.0048	———	<0.0001	0.1030
P	0.0055	0.0058	0.0080	0.0046	0.0084	0.0042	0.0036	0.0044	0.0007	———	0.0952
S. electra	0.0167	0.0183	0.0366	0.0211	0.0286	0.0284	0.0222	0.0244	0.0326	0.0304	———

common ancestor with haplotypes of *S. richteri* than with the *S. invicta* haplotypes constituting the other lineage (C group). Bootstrap support for the monophyly of *S. richteri* plus *S. invicta* non-C haplotypes was 100% using both NJ and MP, strongly supporting the idea that *S. invicta* haplotypes are paraphyletic with respect to those of *S. richteri*. Notably, *S. invicta* carrying C group haplotypes were found only at the Corrientes locality, whereas individuals with non-C haplotypes were present in both geographical populations studied. All haplotypes from *S. richteri* form a well-supported monophyletic group.

These mtDNA sequence data are entirely concordant with the *wsp* sequence data. All *S. invicta* with the C group haplotypes shared an identical *Wolbachia* strain, which differs substantially from the *Wolbachia* strain shared by all *S. invicta* with non-C haplotypes. Furthermore, the *wsp* sequence of the non-C group of *S. invicta* differs by only a single base substitution from the *wsp* sequence possessed by all *S. richteri*. Thus, *Wolbachia* from non-C group *S. invicta* and from *S. richteri* share a more recent common ancestor than either does with *Wolbachia* from the *S. invicta* C group (Fig. 1). While it is likely that the two *Wolbachia* strains invaded these ants independently, with the invasion of one strain possibly predating the divergence between *S. invicta* and *S. richteri*, the complete concordance of the two genomes suggests that they are coinherited vertically and that horizontal transfer of *Wolbachia* is rare or absent.

Analyses of molecular evolution

Haplotype and nucleotide diversity estimates calculated from the mtDNA sequence data for various groups of *S. invicta* and *S. richteri* are presented in Table 1. Haplotype diversity generally is quite high for the different groups, reflecting the numerous unique haplotypes discovered in our limited samples of each group. Nucleotide diversity (π) for each social form of *S. invicta* from each region spans a substantial range, from 0.013 to 0.029. However, the highest values, for *S. invicta* from Corrientes, drop to 0.01 or less when the haplotypes are separated into the two major lineages. Consistent with the prediction of reduced mtDNA variation in a *Wolbachia*-infected population, estimates of nucleotide diversity are more than 10-fold lower for the C haplotype group of *S. invicta* from Corrientes, where infection frequencies are high, than for either *S. invicta* population from Formosa, where *Wolbachia* is absent or present at very low frequencies. On the other hand, nucleotide diversity estimates were essentially identical between the monogyne non-C haplotype group from Corrientes, which also has relatively high infection frequencies, and the uninfected Formosa populations. Nucleotide diversity estimates in the *Wolbachia*-infected *S. richteri* populations are comparable to the estimates for the

C haplotype group of *S. invicta* from Corrientes but substantially lower than the diversity estimates for the Corrientes non-C group and the Formosa populations of *S. invicta*. Patterns for θ estimates generally parallel those for nucleotide diversity, as do patterns of haplotype diversity in *S. invicta*.

While mtDNA nucleotide diversity in *S. invicta* generally is relatively low within each haplotype group of each social form, sequence divergence between the two haplotype groups is high (0.034–0.056; Table 2), comparable with the typical pairwise sequence divergence between *S. richteri* and any given group of *S. invicta* (0.025–0.060). This suggests that the split between the lineages is relatively old and may predate the origin of *S. invicta*. Moreover, if gene flow between the haplotype groups is limited as a result of bidirectional cytoplasmic incompatibility associated with their divergent *Wolbachia* strains, then these haplotype lineages constitute evolutionarily-independent entities. Sequence divergence between the outgroup species, *S. electra*, and populations of the other species is typically twofold higher than the largest divergence within *S. invicta* or between *S. invicta* and *S. richteri*.

Results of the tests of neutral evolution are presented in Table 3. Estimates of Tajima's *D* statistic were generally negative for all populations containing a significant number of *Wolbachia*-infected ants, and the negative estimate obtained for the non-C haplotype group of monogyne *S. invicta* from Corrientes was statistically significant. The two exceptional, positive *D* values for infected ants, for the Corrientes monogyne and polygyne samples considered as groups, become negative (but not significantly so) when estimated separately for the two haplotype lineages in each group. The only other instance in which *D* was greater than zero is for the Formosa monogyne sample of *S. invicta*, which lacks *Wolbachia*. In aggregate, these results may be taken to support the general prediction that recent selective sweeps associated with the invasion of *Wolbachia* have left their footprint in the form of excess rare variants in fire ant mtDNA.

An excess of rare variants and the negative *D* values associated with it can result not only from a recent hitchhiking event but also from processes such as background selection or changes in population size. In contrast, the *H* statistic of Fay and Wu is more sensitive to hitchhiking (as well as demographic events and population structure) but apparently is insensitive to background selection (Fay & Wu 2000). We obtained significant, negative estimates of *H* only for monogyne *S. invicta* from Corrientes (pooled haplotype groups) and polygyne *S. invicta* from Formosa. These negative values of *H* indicate an excess of high-frequency derived variants in these two populations, which is consistent with an ongoing hitchhiking event (or, possibly, a recent demographic event or hidden population structure). In the former case, *H* is not significantly

Table 3 Tests for departure from neutrality for mtDNA sequence variation in *Solenopsis invicta* and *S. richteri*

	N	Tajima's D	Fay and Wu's H	M–K test P-value†
S. invicta				
Corrientes M	29	0.306	**−14.33***	0.207
Corrientes M, C group	23	−1.483	0.32	1.000
Corrientes M, non-C group	6	**−1.503***	−2.67	1.000
Corrientes M, C group (W+)	19	−1.422	1.19	1.000
Corrientes M, non-C group (W+)	6	**−1.503***	−2.67	1.000
Corrientes P	22	**2.123***	−3.24	0.678
Corrientes P, C Group	9	−0.936	0.67	1.000
Corrientes P, non-C group	13	−1.728	−1.83	**0.003***
Corrientes P, C group (W+)	7	−0.654	0.52	1.000
Corrientes P, non-C group (W+)	6	−1.295	−0.53	**0.012***
Formosa M	21	1.041	−6.10	0.586
Formosa P	17	−0.593	**−14.78***	0.499
S. richteri				
M	9	−0.936	1.72	0.112
M (W+)	3	0.000	0.67	0.077
M (W−)	6	−0.050	1.33	0.077
P	9	−1.633	−0.11	**< 0.001****
P (W+)	3	0.000	0.00	1.000
P (W−)	6	−1.072	2.13	**< 0.001****

M and P refer to monogyne and polygyne social forms, respectively. C and non-C refer to the two major haplotype groups within *S. invicta*. W+ and W− represent *Wolbachia*-infected and uninfected ants, respectively. N is the number of individuals studied (one per nest). Significant values of test statistics indicating departures from neutrality are shown in bold.
*$P < 0.05$, **$P < 0.01$.
†Test statistic calculated using Fisher's exact test.

different from zero when the C and non-C haplotype lineages are analysed separately. Thus, tests based on H statistics do not reveal the distinctive distribution of intermediate- and high-frequency derived variants regarded as the footprint of an ongoing selective sweep of *Wolbachia* with which the mtDNA is hitchhiking.

MacDonald–Kreitman tests gave significant results indicative of departures from neutrality only for the non-C haplotype group of polygyne *S. invicta* in Corrientes and for the polygyne social form of *S. richteri* (Table 3). In these two cases, we further subdivided groups according to infection status and performed additional M–K tests. These tests remained significant for infected ants in the case of *S. invicta* but not in the case of *S. richteri*. Application of the M–K tests thus does not unambiguously reveal a clear effect of *Wolbachia* infection on patterns of mtDNA variation and substitution in fire ants.

Results of analyses of between-population and between-form differentiation based on the mtDNA sequences are summarized in Tables 4 and 5. Gene flow estimates based on N_{ST} and F_{ST} values were essentially identical, so we report only F_{ST} and their derivative Nm values (Table 4). Estimates of F_{ST} between species and between geographical populations of *S. invicta* are uniformly high, with cor-

responding values of Nm generally well below 1.0. In contrast, estimates of F_{ST} between conspecific social forms within a geographical population are generally very low (high Nm), although in *S. invicta* this is true only if haplotype lineage is accounted for. Specifically, the estimated F_{ST} for the two social forms in Corrientes is 0.20 ($Nm = 2.00$) when the haplotype groups are pooled but drops by an order of magnitude when the groups are considered separately.

We also examined between-population and between-form differentiation using the AMOVA methods of Excoffier *et al.* (1992). Hierarchical estimates of ϕ statistics reveal significant differentiation between geographical populations of *S. invicta* as well as between the social forms within these populations (Table 5a). However, when we examine between-form differentiation within each population separately, only the two social forms from Corrientes are significantly differentiated (Table 5b). Even this between-form differentiation disappears when each haplotype lineage is considered separately, except for the single case of the C haplotype group when haplotype divergence is ignored. Estimates of ϕ_{ST} similarly do not indicate significant differentiation between the social forms of *S. richteri* (Table 5b).

Table 4 Estimates of genetic differentiation (F_{ST} above diagonal) and levels of maternal effective gene flow (Nm, below diagonal) between groups of native fire ants based on mtDNA sequences. M and P refer to monogyne and polygyne social forms, respectively. C and non-C refer to the two major haplotype groups within *S. invicta*. Values of Nm greater than 2.00, and their corresponding F_{ST} values, are shown in bold. Values for interspecific comparisons are shaded. Dashes indicate missing values where sequence divergence was not calculated because comparisons were inappropriate

| | *S. invicta* | | | | | | | | *S. richteri* | | *S. electra* |
	Corrientes M	Corrientes M, C group	Corrientes M, non-C group	Corrientes P	Corrientes P, C group	Corrientes P, non-C group	Formosa M	Formosa P	M	P	
S. invicta											
Corrientes M	------	–	–	0.2003	–	–	0.6363	0.6258	0.7852	0.7555	0.8771
Corrientes M, C group	–	------	0.6964	–	**0.0078**	0.9219	0.8304	0.8124	0.9427	0.9272	0.9684
Corrientes M, non-C group	–	0.22	------	–	0.7490	**0.0201**	0.2370	0.3230	0.7017	0.6438	0.8785
Corrientes P	**2.00**	–	–	------	–	–	0.4045	0.4424	0.6551	0.5971	0.8438
Corrientes P, C group	–	**63.86**	0.17	–	------	0.9631	0.8742	0.8573	0.9816	0.9614	0.9938
Corrientes P, non-C group	–	0.04	**24.38**	–	0.02	------	0.5919	0.6584	0.9439	0.9031	0.9850
Formosa M	0.29	0.10	1.61	0.74	0.07	0.34	------	**0.0317**	0.8050	0.7569	0.9309
Formosa P	0.30	0.12	1.05	0.63	0.08	0.26	**15.30**	------	0.7701	0.7331	0.9174
S. richteri											
M	0.14	0.03	0.21	0.26	0.01	0.03	0.12	0.15	------	**0.0116**	0.9945
P	0.16	0.04	0.28	0.34	0.02	0.05	0.16	0.18	**43.50**	------	0.9829
S. electra	0.07	0.02	0.07	0.09	0.00	0.01	0.04	0.04	0.00	0.01	------

Table 5 Estimates of genetic differentiation for *Solenopsis invicta* and *S. richteri* groups based on mtDNA sequences. (a) Values of hierarchical ϕ statistics for the levels of geographical population (Corrientes and Formosa) and social form (monogyne and polygyne) for *S. invicta*. (b) ϕ_{ST} values for differentiation between the social forms of *S. invicta* from two different geographical populations and between the social forms of *S. richteri* from a single site

(a)

Source of variation	d.f.	Sum of squares	Percentage of variation	ϕ statistics
Between populations	1	387.936	Va = 46.06	ϕ_{CT} = **0.461*** ($P < 0.001$)
		3.728	Va = 12.68	ϕ_{CT} = **0.127†** ($P < 0.001$)
Between social forms within populations	2	80.008	Vb = 8.48	ϕ_{SC} = **0.157*** ($P = 0.005$)
		1.932	Vb = 5.12	ϕ_{SC} = **0.059†** ($P = 0.005$)
Within social forms	86	671.712	Vc = 45.45*	
		34.984	Vc = 82.80†	

*Euclidean metric; †Equidistant metric.

(b)

	Percentage of variation		
	Between social forms	Within social forms	ϕ_{ST}
S. invicta			
Corrientes	20.41	79.59	**0.204*** ($P = 0.005$)
	9.35	90.65	**0.093†** ($P = 0.005$)
C haplotype group	0	100	−0.017* ($P = 0.332$)
	8.55	91.45	**0.086†** ($P = 0.047$)
non-C haplotype group	4.56	95.44	0.046* ($P = 0.264$)
			0.003† ($P = 0.356$)
Formosa	3.28	96.72	0.033* ($P = 0.133$)
			0.016† ($P = 0.198$)
S. richteri	0	100	−0.013* ($P = 0.394$)
			0.031† ($P = 0.475$)

*Euclidean metric; †Equidistant metric.
P indicates the probability that estimates of the ϕ statistics do not differ from zero (significantly positive estimates are shown in bold).

Discussion

Recent theory suggests that maternally-inherited microbes, such as bacteria in the genus *Wolbachia*, may affect host mtDNA genome evolution (Prout 1994; Turelli 1994; Hurst *et al.* 1996; Johnstone *et al.* 1996), and several empirical studies directly examining the effects of *Wolbachia* on mtDNA variation largely support these predictions (Turelli *et al.* 1991; Kambhampati *et al.* 1992, 1993; Hoffmann *et al.* 1994; Solignac *et al.* 1994; Guillemaud *et al.* 1997; Marcade *et al.* 1999; Rigaud *et al.* 1999; Shoemaker *et al.* 1999; Ballard 2000; Ballard *et al.* 2002). For example, most studies have demonstrated associations between particular *Wolbachia* strains and mtDNA haplotypes, as well as reduced mtDNA variation in infected individuals compared with uninfected individuals of the same or closely-related species. These findings are consistent with mainly vertical transmission of *Wolbachia* within populations or species and the occurrence of sweeps of *Wolbachia* through populations, such that all existing mtDNA haplotypes trace back to the single haplotype of the original infected female (Caspari *et al.* 1959; Fine 1978; Turelli *et al.* 1991, 1992).

In the current study, we sequenced a portion of the mtDNA genome from numerous individuals of the fire ant species *S. invicta* and *S. richteri*, both of which include populations harbouring *Wolbachia*. As predicted, there is a strong association between *Wolbachia* strain and host mtDNA lineage within and between these fire ant species. However, there is no consistent association between the presence of *Wolbachia* and reduced mtDNA diversity. Haplotype and nucleotide diversity were lower in some *Wolbachia*-infected populations (C haplotype group of *S. invicta*, *S. richteri*) than in uninfected populations (*S. invicta* from Formosa), as predicted. This reduced mtDNA diversity in the case of *S. invicta* is most likely the result of a *Wolbachia*-driven mtDNA sweep rather than some demographic process such as a population bottleneck, because levels of variation at several microsatellite loci are essentially identical between these infected and uninfected populations (Ross *et al.* 1997). In contrast to predictions, diversity estimates were essentially identical between another highly-infected *S. invicta* population (monogyne non-C haplotype group from Corrientes) and conspecific uninfected populations. This result is surprising because, in addition to the original reduction of mtDNA diversity resulting from the sweep of *Wolbachia*, these bacteria may further reduce mtDNA variation over the long term. The reason is that the effective population size of females is determined by the number of infected females rather than the total number of females in populations harbouring *Wolbachia*, since the cytoplasms of uninfected females are destined to be lost (Johnstone *et al.* 1996). If the equilibrium frequency of *Wolbachia* infection is less than 100%, which appears to be the case in *S. invicta*, the effective

population size of females is likely to be smaller after invasion by *Wolbachia* than before. On this basis alone, such infected populations should never achieve the same level of mtDNA variation present in the ancestral, uninfected population.

One possible explanation for the lack of reduced diversity in some infected *S. invicta* groups is that the *Wolbachia* sweep occurred sufficiently long ago [> $2N_f$ generations ago, where N_f is the effective population size of females (Avise 2000)] that the original reduction in nucleotide variation has been compensated for by accumulation of new mutations. This assumes that the equilibrium frequency of *Wolbachia* infection is high enough that the sustained reduction in female effective population size mentioned above is negligible. Alternatively, it is possible that *Wolbachia* only recently invaded, and are in the process of spreading through the *S. invicta* non-C lineage, in which case substantial variation may be present because of the presence of uninfected individuals. However, infection frequencies in the non-C groups (46–100%) were comparable with those in the C groups (78–83%), where variation is reduced, making tenuous the suggestion that *Wolbachia* are in the process of spreading only in the former group. Furthermore, the estimate of Fay and Wu's *H* statistic did not differ significantly from zero for the non-C group of either social form, which indicates that there is no signature of an ongoing selective sweep in the patterns of nucleotide substitution (see below).

Not only diversity but also patterns of mtDNA nucleotide substitution are expected to differ between infected and uninfected host lineages. Models of *Wolbachia* evolution predict sequential replacements of *Wolbachia* strains and associated cytoplasmic elements (Prout 1994; Turelli 1994; Hurst *et al*. 1996) and, because each *Wolbachia* sweep reduces the effective population size of mtDNA to one, infected populations should accumulate slightly deleterious mutations under the joint influences of weak purifying selection and strong drift. Our results were generally consistent with these predictions. Values of Tajima's *D* were consistently less than zero for all populations harbouring *Wolbachia* and in many cases, these values were significant. Negative values are expected for Tajima's *D* statistic following a recent selective sweep within a population. Estimates of *D* for the two social forms of *S. invicta* from the Formosa locality, where *Wolbachia* are rare or absent, were not significantly different from zero.

The Fay and Wu statistic (*H*) is a particularly sensitive indicator of genetic hitchhiking, as may occur at the mtDNA during a *Wolbachia* sweep. The fact that *H* did not differ significantly from zero in *S. invicta* with a 100% infection frequency (Corrientes non-C group) might be anticipated, since a *Wolbachia* sweep presumably occurred at some point in the remote past and its footprint may have been obscured by subsequent substitutions. However, estimates of *H* also did not differ from zero in populations with incomplete infections, a surprising result if *Wolbachia* is in the process of spreading through these populations. One explanation is that the infection frequencies are in fact at equilibrium, despite being less than 100%, and that the selective sweep also occurred some time ago in these populations (equilibrium frequencies of *Wolbachia* infection theoretically can range between 50% and 100%). The significant estimate of *H* for polygyne *S. invicta* from Formosa, which lacks *Wolbachia*, was also unexpected and may reflect either the presence of significant local genetic structure (Ross *et al*. 1997) or selection acting directly on a favoured mtDNA haplotype to drive it towards fixation in this population.

We also examined our data for departures from neutral expectations indicative of the influence of *Wolbachia* using the MacDonald–Kreitman (M–K) test. Under strict neutrality, the ratio of the number of nonsynonymous to synonymous polymorphisms (K_A/K_S) within a species is expected to be the same as the ratio of the number of nonsynonymous to synonymous differences between species (McDonald *et al*. 1991). Surprisingly, results of our M–K tests were significant in only a minority of groups harbouring *Wolbachia*. One possible explanation is that natural selection acting directly on the mtDNA masks the footprint of *Wolbachia*-induced positive selection (e.g. Weinrich & Rand 2000). In this scenario, negative selection on the mtDNA genome and *Wolbachia*-driven positive selection act in opposition to one another to create patterns of variation indistinguishable from those expected under neutrality. The general importance of such complex selective dynamics in shaping mtDNA sequence variation remains unknown, but two additional studies using neutrality tests to examine the effects of *Wolbachia* on mtDNA evolution are relevant. Ballard and colleagues (Ballard 2000; Ballard *et al*. 2002) sequenced the entire mtDNA genomes of 22 strains of *Drosophila simulans* and found an excess of nonsynonymous over synonymous substitutions within three different haplotype groups, a result consistent with *Wolbachia*-mediated sweeps. Also, results of neutrality tests on the infected species *Drosophila recens* were largely consistent with a recent *Wolbachia*-associated sweep in this fly (Shoemaker *et al*. 2003), although M–K tests did not reveal the expected pattern of excess between-species substitutions. Thus, only one of three studies using M–K tests has found significant effects of *Wolbachia* on host mtDNA variation, and this single example is a study based on a nonrandom sample of individuals (Ballard 2000). Clearly, nonneutral mtDNA evolution in populations harbouring *Wolbachia* remains a possibility despite nonsignificant results from the M–K procedure or other tests designed to detect departures from neutrality.

An important finding of our study is the presence of two divergent mtDNA haplotype lineages and *Wolbachia*

strains in *S. invicta* from the Corrientes population, with complete concordance between the two genomes with respect to assignment of individuals to each group. Phylogenetic analyses of each genome revealed that one of the two cytoplasmic lineages shares a more recent common ancestor with the lineage from *S. richteri* than the other *S. invicta* lineage. One hypothesis for the presence of these highly-divergent, paraphyletic cytoplasmic lineages within *S. invicta* is that individuals from the two clades represent two species that are morphologically indistinguishable (see Shoemaker *et al.* 2000 for discussion of other possible explanations, including interspecific hybridization). Preliminary analyses of sequence data from a number of related South American fire ant species suggest that such clear examples of mtDNA paraphyly are limited to *S. invicta* and just one other species, *S. saevissima*. These two species are exceptional within the *S. saevissima* species group in terms of their extensive geographical ranges (other species tend to be regional endemics) and the breadth of morphological variation each encompasses (Pitts 2002), consistent with the view that each of these nominal species comprises multiple cryptic species. Justification for distinguishing between these lineages in all of our analyses, regardless of whether or not they constitute different species, comes from the fact that each harbours a different strain of *Wolbachia*, leading to the expectation of continuing divergence as a result of bidirectional reproductive incompatibility.

A previous study of *Wolbachia* in fire ants showed that infection frequencies in *S. invicta* differ significantly between the Formosa and Corrientes populations, as well as between the two social forms within Corrientes (Shoemaker *et al.* 2000). These results are consistent with another study that used mitochondrial RFLPs along with nuclear DNA markers to show significant differentiation between the two regions and between the social forms within each region (Ross *et al.* 1997). These earlier data have been interpreted to suggest that substantial geographical and social barriers to gene flow exist in these ants. In the present study, we examined differentiation between sympatric social forms in *S. invicta* and *S. richteri*, as well as between geographical populations of *S. invicta*, using mtDNA sequence data. The only instance of significant differentiation between the two social forms in either species was for *S. invicta* from Corrientes. However, this differentiation almost completely disappeared when individuals from the two distinct haplotype lineages were examined separately, except in the case of the C haplotype group. These results suggest that the pronounced genetic differentiation reported previously between the social forms of *S. invicta* in South America may have been confounded by the unrecognized presence of these two divergent lineages.

On the other hand, the modest numbers of sequences available from each group for most of our analyses undoubtedly limit the power to detect significant interform differentiation. Indeed, slight but significant differentiation between sympatric social forms has been detected using both mtDNA and nuclear markers in Formosa (with larger sample sizes than here) as well as in two locations in the introduced U.S. range (Ross *et al.* 1997). Thus, while the sequence data show that the rather large mtDNA differentiation reported earlier in Corrientes is probably an artefact of the presence of two distinct lineages, some general barriers to interform gene flow nonetheless are likely to exist.

In general, analyses of gene flow based on patterns of mtDNA variation can be greatly confounded by the presence of *Wolbachia* (Johnstone *et al.* 1996). Restricted gene flow between populations allows their continual divergence via drift, but in the presence of *Wolbachia* even very small amounts of gene flow may quickly erode such divergence. Because of the reproductive advantage to infected females described earlier, migration of just a single infected female into an uninfected population can potentially result in the occurrence of a parallel selective sweep in the newly-invaded population, with the associated mtDNA genome also going to fixation (Caspari *et al.* 1959; Turelli 1994; Turelli *et al.* 1995). Thus, hitchhiking of the mtDNA associated with *Wolbachia* as it sweeps throughout populations may completely erode pre-existing mtDNA population differentiation, limiting the utility of this marker for inferring levels and patterns of gene flow. In our case, it is possible that the amount of historical and ongoing gene flow between the social forms of *S. invicta* is low, but that the lack of significant mtDNA differentiation in some instances is the result of recent parallel *Wolbachia* sweeps through sympatric populations of each form.

While there are uncertainties in our data regarding the extent of gene flow between sympatric social forms, gene flow between conspecific geographical populations of *S. invicta* is clearly severely limited, regardless of social form and independent of the presence of the two distinct haplotype lineages. This pronounced restriction on gene flow over relatively short distances is noteworthy because, as outlined above, even small amounts of gene flow can erode any pre-existing differentiation in the presence of *Wolbachia*. On the other hand, the spread of *Wolbachia* through a single population could potentially increase between-population differentiation if the bacteria are unable to successfully invade neighbouring populations. This could partly explain why the mtDNA differentiation between geographical populations of *S. invicta* is so pronounced, given that a series of sweeps of *Wolbachia* and associated mtDNA has probably occurred in Corrientes yet *Wolbachia* appear not to have swept through the nearby Formosa population.

Our findings illustrate the point that the effects of *Wolbachia* on the distribution of mtDNA variation in natural

populations can often be complex and sometimes unpredictable on the basis of current theory. Regardless of whether our interpretations of the causes of the patterns we report for native fire ant populations prove to be valid, it is clear that these microbes can have dramatic effects on the evolutionary dynamics of mtDNA variation in organisms that harbour them, including reduced haplotype variation, accelerated substitution rates and altered levels of divergence among populations. Given these dramatic potential effects, consideration must not only be given to how demography, historical processes and selection affect patterns of variation when using mtDNA as a marker for population genetic analyses, but the influence of cytoplasmic associates such as *Wolbachia* must also be taken into account (Johnstone & Hurst 1996; Ballard 2000; Ballard *et al.* 2002).

Acknowledgements

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