

Loss of *Wolbachia* infection during colonisation in the invasive Argentine ant *Linepithema humile*

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Wolbachia are maternally inherited bacteria, which are very common in arthropods and nematodes. *Wolbachia* infection may affect host reproduction through feminisation, parthenogenesis, male-killing, cytoplasmic incompatibility and increased fecundity. Previous studies showing discrepancies between the phylogenies of *Wolbachia* and its arthropod hosts indicate that infection is frequently lost, but the causes of symbiont extinction have so far remained elusive. Here, we report data showing that colonisation of new habitats is a possible mechanism leading to the loss of infection. The presence and prevalence of *Wolbachia* were studied in three native and eight introduced populations of the Argentine ant *Linepithema humile*. The screening shows that the symbiont is common in the three native *L. humile* populations

analysed. In contrast, *Wolbachia* was detected in only one of the introduced populations. The loss of infection associated with colonisation of new habitats may result from drift (founder effect) or altered selection pressures in the new habitat. Furthermore, a molecular phylogeny based on sequences of the *Wolbachia* *wsp* gene indicates that *L. humile* has been infected by a single strain. Horizontal transmission of the symbiont may be important in ants as suggested by the sequence similarity of strains in the three genera *Linepithema*, *Acromyrmex*, and *Solenopsis* native from South and Central America.

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Introduction

Wolbachia are maternally inherited bacterial symbionts of arthropods and nematodes (Werren, 1997; Stouthamer *et al.*, 1999). They are extraordinarily successful, being present in a large number of host species and attaining high prevalence within infected host populations. The spread of *Wolbachia* through the host population relies on selfish alterations imposed on the host's reproductive physiology. As *Wolbachia* is transmitted only by females, the symbiont's manipulation acts to increase the number of infected daughters produced by an infected female above the average production of daughters per female (Werren and O'Neill, 1997). This is achieved in one of two ways. Some *Wolbachia* strains increase the proportion of females among the surviving offspring of infected females by transforming males into functional daughters (feminisation), inducing parthenogenetic reproduction, or provoking the selective abortion of sons (male-killing) in species with sib competition. Alternatively, other strains reduce the reproductive success of uninfected females by a mechanism called cytoplasmic incompatibility (CI), which causes partial or complete infertility of matings between infected males and uninfected females (Rousset and Raymond, 1991; Werren, 1997).

Despite the capacity of *Wolbachia* to spread rapidly, the lifespan of infection within host populations appears to

be limited. This is suggested by strong discrepancies between the phylogenies of hosts and symbionts, indicating that the distribution of *Wolbachia* strains among host species is determined by frequent horizontal transmission and loss of infection rather than cospeciation between host and symbiont (Werren *et al.*, 1995; van Meer *et al.*, 1999). Contrary to the mechanisms promoting infection, the factors causing the extinction of *Wolbachia* within host populations are not well understood (Werren and Windsor, 2000). It has been suggested that infection might be lost when hosts colonise new distant habitats. Thus, native (Argentinean) populations of the fire ant *Solenopsis invicta* are commonly infected with *Wolbachia*, while introduced populations, established in the US in the early 20th century, lack the symbiont (Shoemaker *et al.*, 2000). It is not yet clear why the introduction of *S. invicta* has been associated with a loss of *Wolbachia* infection. The loss could have been due to a founder event, with all founding individuals being uninfected. Alternatively, the infection could have been lost in the early growth phase of the introduced population, either by drift (small population size) or selection (different environment or population dynamics compared to the source population) (Shoemaker *et al.*, 2000).

In order to assess the general significance of long-distance dispersal as a cause of *Wolbachia* loss, it is helpful to investigate other species that have been introduced into new habitats. An ideal system is the invasive Argentine ant *Linepithema humile*, a species known to carry *Wolbachia* (Jeyaprakash and Hoy, 2000). Like *S. invicta*, *L. humile* originates from South America. During the last century, it has been introduced

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worldwide (Passera, 1994). As for many other ant species (Ross and Keller, 1995), introduction events probably have been associated with a major shift in social organisation. Native populations of *L. humile* are multi-colonial and characterised by low densities and high levels of aggression between workers of different nests (Suarez *et al*, 1999). In contrast, introduced populations are ecologically dominant and unicolonial (Passera, 1994). Nests are socially connected in a dense network throughout the population, resulting in a complete lack of kin structure among nestmate workers and no aggression between workers of different nests of the same 'supercolony', even when these are separated by thousands of kilometres (Giraud *et al*, 2002).

To test the hypothesis that long-distance dispersal and colonisation might be associated with a loss of infection, we analysed *L. humile* samples from native populations in Argentina as well as introduced populations in Argentina, Chile, USA, Australia, Spain, and France. Furthermore, we isolated and sequenced the *wsp* gene to conduct a phylogenetic analysis that included *Wolbachia* strains isolated from other insects.

Materials and methods

Sampling

In this study, we used samples of *L. humile* workers collected in three native populations in Argentina as well as eight introduced populations. Two of these introduced populations are situated in Argentina, one in Chile, two in the US, one in Australia, one in Spain (Catalonian supercolony), and one in France (European supercolony). Samples were collected between 1997 and 2000 and DNA was extracted from specimens using standard protocols. All DNA extractions included in this study were known to contain DNA because they had been successfully used for analyses of ant nuclear markers. Information on the location of populations and sample sizes are given in Table 1.

Population screening

All populations were screened for the presence of *Wolbachia*. For each population, we analysed 20 workers sampled from as many different nests as possible (Table 1). This sample size is sufficient to detect infection (sample of at least one infected individual) with a probability of 95% even if infection frequency is as low as 0.15 (assuming a binomial distribution). In one population (Tucumán) only a limited number of individuals were available resulting in a smaller sample size.

The presence of *Wolbachia* in individual workers was detected by PCR amplification of the *Wolbachia* gene *wsp*. We used primers *wsp81F* and *wsp691R* (Zhou *et al*, 1998) with PCR conditions as proposed by the authors. Positive and negative controls were included in every PCR reaction. PCR products were run on 1.5% agarose gels, which were stained with SYBR Gold (Molecular Probes) before photographing.

The association between *Wolbachia* infection and the population type (native or introduced) was analysed using generalised linear models (GLM). Given the binary nature of the data (presence/absence of infection), we used a binomial error structure (logit link function) and χ^2 tests of significance. We first performed a global

analysis on the infection status (infected/uninfected) of all individual worker samples as a function of the independent variables 'population type' (native or introduced) and 'population nested in population type'. This analysis suffers from two potential problems. First, populations are represented by variable numbers of nests and variable numbers of individuals per nest. Second, some introduced populations might be derived from a single and same event of introduction and hence do not represent independent samples. We therefore repeated the analysis with a minimal data set, by using nests and not individuals as sampling units (scoring a nest as infected if at least one worker had been found positive) and including only those introduced populations that differ in the composition of ant mitochondrial genotypes and thus most probably represent independent events of introduction (V Vogel, JS Pedersen, L Keller, unpublished data). The populations included in the minimal data set were Tucumán, Corrientes, Buenos Aires, La Rioja 2, Chile, Hawaii, and Catalonia.

Local prevalence

To estimate the prevalence of *Wolbachia* in infected populations, we undertook a more detailed screening in the populations Corrientes and Buenos Aires. We analysed 10 workers from each of 23 (Corrientes) and 26 (Buenos Aires) nests. A GLM was used to detect differences in prevalence between populations and nests. The analysis included both populations, with the infection status of individual workers as dependent variable and the factors 'population' and 'nest nested within population' as independent variables. All statistical tests were performed with the statistical package R (Ihaka and Gentleman, 1996).

Sequencing

We sequenced the *Wolbachia* gene *wsp* from a small number of infected individuals to determine the identity of the symbiont strains found in the different populations. The sample included three individuals from Buenos Aires and one from each of the populations in Corrientes, Hawaii, and Tucumán. The *wsp* gene was amplified from these samples using the same methods as for screening. Amplified DNA was recovered from excised gel slices and cloned with the help of a TOPO TA cloning kit (Invitrogen). Plasmids were extracted with the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced using the ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For each host ant, one clone was sequenced on both strands. The forward and reverse sequences from same clones were aligned and corrected by hand to obtain an unambiguous sequence for each clone. A consensus sequence has been deposited on Genbank under accession number AF546756.

Phylogenetic analysis

A phylogenetic analysis was performed to assess the position of the *Wolbachia* detected in this study within the *Wolbachia* A-group as well as their phylogenetic relationship with *Wolbachia* strains detected in other ant species. The sequence matrix included the *wsp* sequences of the *Wolbachia* reference strains defining phylogenetic clades within the *Wolbachia* phylogeny (van Meer *et al*, 1999):

wMel (AF020063), wAlbA (AF020058), wMors (AF020079), wKue (AF071911), wRi (AF020070), wUni (AF020071), wHa (AF020073), wPap (AF020082), wAus (AF020077), and wDro (AF071910). Furthermore, we included *wsp* sequences from all A-group *Wolbachia* detected so far in ants, that is, *S. invicta* A (AF243435, Shoemaker et al, 2000), *S. richteri* (AF243435, Shoemaker et al, 2000), *Acromyrmex octospinosus* A1 (AF472557, van Borm et al, 2003), *A. echinator* A1 (AF472558, van Borm et al, 2003), *A. insinuator* A1 (AF47255, van Borm et al, 2003), *Formica exsecta* wFex2 (AY101197, Reuter and Keller, 2003), and *F. exsecta* wFex4 (AY101199, Reuter and Keller, 2003, identical to strain AF472557 from *F. truncorum*). Three additional *Wolbachia* strains from *F. exsecta*, wFex1, wFex3, and wFex5, were not included because their *wsp* genes are most likely recombinants (Reuter and Keller, 2003). The *wsp* sequence of the B-group *Wolbachia* strain wCon (AF020083, van Meer et al, 1999) was included in order to root the tree. Sequences were aligned with the help of ClustalX (Thompson et al, 1997) and alignments corrected by hand. Two regions (positions 73–99 and 214–249) could not be aligned with certainty and were excluded from the data set. This resulted in a final alignment of 450 nucleotides in length. Identical sequences were grouped into one taxon. A phylogenetic tree was constructed in PAUP* 4.0b10 (Swofford, 1998) using the neighbour-joining method with the Kimura two-parameter distance measure. Bootstrap analysis was performed with 1000 replicates.

Results

Population screening

Individuals infected with *Wolbachia* were detected in four populations (Table 1). While the three native populations of *L. humile* (Tucumán, Corrientes, and Buenos Aires) were found to be infected, *Wolbachia* was detected in only one of the eight introduced populations (Hawaii).

The GLM analyses showed a significant difference in prevalence between native and introduced populations both in the global analysis and the analysis of the minimal data set (Tables 2a and b, 'population type'). Within the native and introduced range populations also differed significantly (Tables 2a and b, 'population nested in population type'). This between-population

variance in infection is most likely due to the high prevalence found in the Tucumán population (where a single nest was analysed) compared to Buenos Aires and Corrientes, as well as due to the difference in infection between the population in Hawaii and those in the rest of the introduced range.

Local prevalence

In the two native populations of *L. humile* where several nests were analysed, the proportion of individuals infected across all nests were $0.27 \pm 0.16/0.3/0-0.5$ (mean \pm SD/median/range) in Corrientes and $0.33 \pm 0.22/0.3/0-0.8$ in Buenos Aires. The GLM analysis revealed that prevalence did not differ significantly between the two populations, but varied significantly between nests within populations (Table 3).

Sequencing and phylogeny

Within the six sequences of 608 nucleotides, only four sites were variable due to nucleotide substitutions in single sequences. All four nucleotide substitutions were silent (ie do not result in an altered amino-acid

Table 2 Results of GLM analyses of *Wolbachia* infection of individual worker samples in native and introduced populations of *L. humile*. (a) Global analysis of all worker samples. (b) Analysis of the minimal data set (see Materials and methods section for details)

Factor	df	P-value	Explained D (%)
<i>(a) Global analysis</i>			
Population type	1	<0.0001	40.3
Population within population type	9	0.015	13.4
<i>(b) Minimal data set</i>			
Population type	1	0.0002	16.2
Population within population type	5	0.035	14.4

The dependent variable was the infection status of workers (infected/uninfected) in the global analysis and the infection status of nests (infected/uninfected) in the analysis of the minimal data set. The table shows the independent variables entered in the analysis (Factor), the number of their degrees of freedom (df), and their significance calculated by a χ^2 test (P-value). Further, the percentage of the total deviance explained by the significant factors is given (Explained D).

Table 1 *L. humile* samples included in the screening for infection by *Wolbachia*

Name	Locality	Type	No. of nests	No. of individuals in total	No. of positive individuals (%)
Tucumán	Ticucho, Tucumán, Argentina	Native	1	11	9 (82%)
Corrientes	Arroyo Cuay Grande, Corrientes, Argentina	Native	20	20	4 (20%)
Buenos Aires	Otamendi, Buenos Aires, Argentina	Native	20	20	10 (50%)
La Rioja 1	Chuquis, La Rioja, Argentina	Introduced	20	20	0
La Rioja 2	Aminga, La Rioja, Argentina	Introduced	14	20	0
Chile	La Cruz, Santiago, Chile	Introduced	10	20	0
California	Davis, California, USA	Introduced	10	20	0
Hawaii	Haleakala, Hawaii, USA	Introduced	10	20	2 (10%) ^a
Melbourne	Bundoora, Melbourne, Australia	Introduced	20	20	0
Port Leucate	Port Leucate, France	Introduced	10	20	0
Catalonia	Catalonia, Spain	Introduced	4+1+1 ^b	20	0

^aFrom two different nests.

^bNests 4, 1, and 1, respectively, were sampled from three different localities within the Catalonian supercolony (Giraud et al, 2002).

Table 3 Results of the GLM analysis of *Wolbachia* infection in native populations Buenos Aires and Corrientes

Factor	df	P-value	Explained D
Population	1	0.14	—
Nest within population	47	<0.0001	16.2%

The dependent variable in the analysis was the infection status of workers (infected/uninfected). The table shows the independent variables entered in the analysis (Factor), the number of their degrees of freedom (df), and their significance calculated by a χ^2 test (P-value). Further, the percentage of the total deviance explained by the significant factors is given (Explained D).

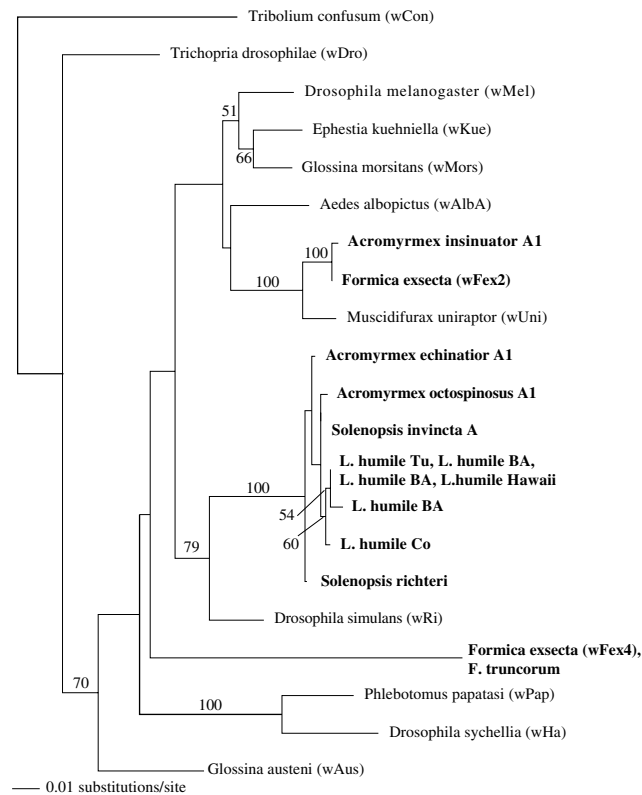


Figure 1 Phylogenetic tree of *Wolbachia* *wsp* sequences constructed with neighbour-joining and Kimura two-parameter distances. *Wolbachia* strains are designated by the name of their host species or strain names. Sequences from ant hosts are marked by bold face. Tu, BA, and Co indicate *L. humile* from populations in Tucumán, Buenos Aires, and Corrientes. Numbers next to nodes indicate the statistical support as the percentage of 1000 bootstrap replicates in which the taxa of a group were placed together in a monophyletic clade.

sequence), but it must be noted that the degree of variation observed is in the range of *Taq* polymerase error (Sanson *et al*, 2002). Owing to the similarity in *wsp* sequences, the *Wolbachia* lineages from *L. humile* form a monophyletic group in the *wsp* phylogeny (Figure 1). The phylogeny also reveals that the *Wolbachia* from *L. humile* cluster together with the symbiotic lineages identified in other ant species from South and Central America, *A. echinator*, *A. octospinosus*, *S. invicta*, and *S. richteri*. The eight *Wolbachia* lineages are genetically very similar and form a very strongly supported monophyletic group (Figure 1).

Discussion

Our study shows that the level of *Wolbachia* infection in the Argentine ant *L. humile* differs greatly between native and introduced populations. Whereas *Wolbachia* is common in ants from native South American populations, only one of the eight introduced populations is infected. This pattern of infection is identical to that found in the fire ant *S. invicta* (Shoemaker *et al*, 2000).

There are several possible mechanisms that could lead to the observed pattern of infection (Shoemaker *et al*, 2000). First, the difference in infection between the two population types could be independent of introduction events and arise because (i) native populations have been infected after the introduction into new habitats, (ii) infection occurs in introduced populations but remained undetected, and (iii) introduced populations originate from an uninfected native population not included in our sample. All three of these hypotheses are unlikely to apply. Since the three native populations analysed are separated by long distances (650–1050 km) covering most of the species' native range and because Argentine ants have been introduced worldwide less than 100 years ago, it is doubtful that *Wolbachia* would have spread in native populations only after the establishment of introduced populations. It is also unlikely that we failed to detect *Wolbachia* in introduced populations. Our screening provided a high probability of detecting *Wolbachia* infections even at relatively low prevalence, and certainly at a prevalence comparable to that observed in native populations (30%): with a sample size of 20 ants (as used in the study), the detection probability is higher than 0.999. Finally, it is improbable that the foundresses of all uninfected introduced populations stem from an uninfected native population. The invasive populations of *L. humile* outside South America most likely originate from the Buenos Aires region (V Vogel, JS Pedersen, L Keller, unpublished data; Tsutsui *et al*, 2001), which was found to be infected in the present study.

Given the absence of a convincing alternative explanation, it seems most likely that the loss of *Wolbachia* infection is associated with the introduction of the ants into novel habitats. This conclusion is further corroborated by the difference in infection rate between the introduced populations from the US, Australia, and Europe and its putative source, the native population of Buenos Aires. The loss of *Wolbachia* infection during introduction could have occurred via one of three processes. First, *Wolbachia* could have been eliminated through drift during introduction if all founder queens were uninfected. Although possible, this scenario is highly unlikely. European populations of *L. humile* originate from at least six to 12 foundress queens (Giraud *et al*, 2002). Assuming that the prevalence in native populations is 30%, the probability that all foundresses of introduced populations were uninfected ranges from 1.4% (12 foundresses) to 12% (six foundresses). These estimates decrease considerably if we take into account the fact that there have probably been several independent events of introduction (Tsutsui *et al*, 2001; Giraud *et al*, 2002). With only two events of introduction of six or 12 queens each, the probability of all foundresses being uninfected drops to 1.4% (six foundresses per introduction) and 0.02% (12 foundresses). Alternatively, *Wolbachia* may have been lost by drift after the introduction into

new habitats. This would be particularly likely if *Wolbachia* caused CI. CI *Wolbachia* are likely to be lost by selection if their frequency drops below a threshold frequency determined by the rate of vertical transmission and the effect of infection on host fertility (Caspari and Watson, 1959; Hoffmann and Turelli, 1997). Strong drift during and after introduction might have reduced infection frequency in novel populations below this threshold and thus have led to a loss of the symbiont. Finally, *Wolbachia* might have been lost in introduced populations because infection is counter-selected in the new habitat, even at frequencies that represent a stable equilibrium in native conditions. It is possible that the parameters governing infection dynamics (eg the rate of horizontal transmission and the impact of infection on host fitness) differ between native and introduced population because of environmental and ecological differences. Of interest in this context is the fact that introduced populations have a different social structure than native populations. In particular, introduced populations have gone through a prolonged phase of exponential growth and the social structure has changed from multicoloniality to unicoloniality with the population forming a dense network of interconnected nests (see Introduction). Such changes may play a significant role in the dynamics of propagation of *Wolbachia* and be involved in the loss of infection in introduced populations.

Our analysis of the *Wolbachia* gene *wsp* revealed strong sequence similarity between the genes from bacteria found in different populations of *L. humile* and in four other ant species from South and Central America, *A. octospinosus*, *A. echinator*, *S. invicta*, and *S. richteri* (Figure 1). The observed sequence similarity can have different causes. The first is that hosts have inherited their *Wolbachia* from a common ancestor carrying an infection. This is a likely explanation for the similarity between *Wolbachia* sequences of different *L. humile* populations. Second, sequence similarity can arise due to horizontal transmission of bacteria between hosts. Horizontal transmission could explain why closely related *Wolbachia* occur in the distantly related ant genera *Acromyrmex*, *Solenopsis*, and *Linepithema*. The vector of horizontal transmission could have been parasites or parasitoids specialised on ants (van Borm et al, 2003). Finally, it should be noted that sequence similarity might also stem from homologous recombination between *Wolbachia* lineages. Recombinant *Wolbachia* strains have been recently described (Werren and Bartos, 2001; Reuter and Keller, 2003), showing that genetic exchange is possible between intracellular symbionts. In order to determine whether recombination has played a role in generating the sequence similarity described here, more genes should be sequenced from *Wolbachia* in *Acromyrmex*, *Solenopsis*, and *Linepithema*. This would allow to determine whether sequence similarity is restricted to certain regions of the *Wolbachia* genome, as predicted if recombination has taken place.

In conclusion, this study demonstrates that the introduction of the Argentine ant has been frequently accompanied by the loss of *Wolbachia* infection. The most likely explanation is that the loss resulted from a change in the parameters governing infection dynamics possibly because of environmental differences between the native and introduced range and/or a shift in social organisa-

tion that was associated with the introduction. Whatever the causes responsible for the loss of *Wolbachia* in the introduced range, this study indicates that long-distance dispersal and colonisation of new habitats can affect the dynamics of infection by *Wolbachia* and ultimately cause the loss of infection. Finally, the finding that several ant genera of South and Central American harbour very similar stains of *Wolbachia* indicates that horizontal transmission of *Wolbachia* may be common in ants.

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