# High Levels of Multiple Wolbachia Infection and Recombination in the Ant *Formica exsecta*

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Wolbachia bacteria are intracellular symbionts of many arthropod species. Their spread through host populations is promoted by drastic alterations imposed on their hosts' reproductive physiology. In the present study, we analyzed the association between Wolbachia strains and host mitochondrial haplotypes in a Swiss population of the ant *Formica exsecta*. In this species, female dispersal is extremely limited and the mitochondrial haplotypes are strongly differentiated between and within subpopulations. Our study revealed exceptionally high levels of multiple infection, with all ants harboring four or five distinct Wolbachia strains. Four of these strains were present in all ants analyzed. A fifth strain was associated with only three of the five mitochondrial haplotypes. An analysis of the Wolbachia gene *wsp* further revealed an unexpected high rate of recombination, with three of the five Wolbachia strains appearing to have arisen by homologous recombination.

## Introduction

Wolbachia are probably the most successful bacterial symbionts in nature, estimated to be present in 20% to 75% of arthropod species (Jeyaprakash and Hoy 2000; Werren and Windsor 2000). Wolbachia belong to the alpha-proteobacterian group Rickettsiae and, like many of their relatives, live inside host cells. As cytoplasmic elements, their predominant mode of transmission is vertical. They are passed along with mitochondria from the mother to the offspring, and paternal transmission is probably very rare (Turelli, Hoffmann, and McKechnie 1992; Turelli and Hoffmann 1995). Although horizontal transfer appears to be very infrequent within host populations, it has occurred between host species. This is evidenced by the fact that the phylogeny of Wolbachia differs markedly from that of their hosts (O'Neill et al. 1992; Werren, Zhang, and Guo 1995; Cook and Butcher 1999) as well as by experiments demonstrating the transfer of symbionts between host individuals (Huigens et al. 2000).

Wolbachia are not only present in a large proportion of arthropod species but also attain a high prevalence within host populations. Their spread is promoted by alterations of the reproductive physiology of their host. Four different symbiont effects, called phenotypes, have been described in arthropods (Werren 1997). All increase infection frequency by assuring that the number of infected daughters produced by an infected female exceeds the average production of daughters per female. Three phenotypes increase the proportion of females among the offspring of infected females, either by transforming males into functional daughters (feminization), inducing parthenogenetic reproduction, or provoking the selective abortion of sons (male-killing). The latter phenotype is beneficial in species with sib competition because it increases survival of the daughters of infected females. The fourth phenotype, cytoplasmic incompatibility (CI), renders matings between infected males and uninfected females partly sterile. Incompatible matings promote the spread of Wolbachia infection because they lower the expected productivity of uninfected females as compared with infected females, the latter reproducing normally with both infected and uninfected males (Rousset and Raymond 1991; Werren 1997).

The aim of this study was to conduct a population genetic study of Wolbachia in a Swiss population of the ant *Formica exsecta* known to harbor Wolbachia (Keller et al. 2001). This ant population is of special interest with respect to Wolbachia infection because female dispersal, and hence gene flow of cytoplasmic genes, is extremely restricted. A microgeographic genetic study of mitochondrial haplotypes showed very high genetic differentiation between subpopulations ($\Phi_{st} = 0.72$) (Liautard and Keller 2001). In fact, most of the subpopulations contained a single haplotype, and in those showing polymorphism, the haplotypes were spatially clustered (isolation by distance).

In the present study, we identified the Wolbachia strains present in workers of *F. exsecta* and determined the association of the strains with host mitochondrial haplotypes. This analysis revealed high levels of multiple Wolbachia infection. We detected a total of five Wolbachia strains, four of which were present in all ants and a fifth that was exclusively associated with three out of five host mitochondrial haplotypes. This difference in infection between haplotypic lineages was independent of the subpopulation. An analysis of the *wsp* gene sequences of the Wolbachia strains also revealed an unexpected high rate of recombination, with three of the five strains most likely being the product of homologous recombination.

## Materials and Methods

We used DNA samples obtained for the study by Liautard and Keller (2001). Within the Swiss population of *F. exsecta*, workers had been collected in different nests from nine subpopulations, situated on isolated forest clearings. These subpopulations spread over about 25 square kilometers, and neighboring subpopulations are separated by 0.1 to 1 kilometer of unsuitable habitat (forest). The ant mitochondrial genotypes were determined by RFLP analyses of the mitochondrial *cytochrome b* and *NADH1* genes (Liautard and Keller 2001).

Key words: Wolbachia, social insects, *Formica exsecta*, population structure, homologous recombination, *wsp*.

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**Table 1**
**Presence/Absence of Wolbachia Strains As Detected by RFLP**

| Haplotype | Subpopulation[a] | Nest | wFex1[b] | wFex2[b] | wFex3[b] | wFex4[b] | wFex5[b] |
|---|---|---|---|---|---|---|---|
| **Hap1[c]** | **BA** | **2** | – | **X** | **X** | **X** | **X** |
| Hap1 | BA | 4 | – | X | X | X | X |
| Hap1 | BA | 10 | – | X | X | X | X |
| Hap1 | DU | 1 | – | X | X | X | X |
| Hap1 | DU | 5 | – | X | X | X | X |
| Hap1 | PJ | 1 | – | X | X | X | X |
| Hap1 | PJ | 6 | – | X | X | X | X |
| Hap1 | Chen | 8 | – | X | X | X | X |
| Hap1 | Chen | 74 | – | X | X | X | X |
| Hap1 | Chen | 213 | – | X | X | X | X |
| Hap2 | BB | 2 | X | X | X | X | X |
| Hap2 | BB | 6 | X | X | X | X | X |
| **Hap2[c]** | **BB** | **10** | **X** | **X** | **X** | **X** | **X** |
| Hap2 | PN | 1 | X | X | X | X | X |
| Hap2 | PN | 5 | X | X | X | X | X |
| Hap2 | PN | 8 | X | X | X | X | X |
| Hap2 | ROC | 3 | X | X | X | X | X |
| Hap2 | ROC | 7 | X | X | X | X | X |
| Hap2 | ROC | 10 | X | X | X | X | X |
| **Hap3[c]** | **PJ** | **3** | **–** | **X** | **X** | **X** | **X** |
| Hap3 | PJ | 4 | – | X | X | X | X |
| Hap3 | PJ | 7 | – | X | X | X | X |
| Hap3 | LO | 1 | – | X | X | X | X |
| Hap3 | LO | 2 | – | X | X | X | X |
| Hap3 | LO | 6 | – | X | X | X | X |
| **Hap4[c]** | **PN** | **1** | **X** | **X** | **X** | **X** | **X** |
| Hap4 | PN | 2 | X | X | X | X | X |
| **Hap6[c]** | **LC** | **1** | **X** | **X** | **X** | **X** | **X** |
| Hap6 | LC | 5 | X | X | X | X | X |
| Hap6 | LC | 8 | X | X | X | X | X |
| Hap6 | LO | 3 | X | X | X | X | X |
| Hap6 | LO | 4 | X | X | X | X | X |
| Hap6 | LO | 5 | X | X | X | X | X |
| Hap6 | PN | 9 | X | X | X | X | X |

NOTE.—The host individuals from the five haplotypes in indicated in bold face.
[a] See Liautard and Keller (2001) for more information.
[b] Wolbachia strains detected are indicated by X.
[c] Individuals used for sequencing of Wolbachia.

## Sequencing

We first identified the Wolbachia strains present by sequencing the *wsp* gene of Wolbachia harbored in ants with different mitochondrial haplotypes. Based on data from Liautard and Keller (2001), we chose one host individual for each of the five major haplotypes Hap1, Hap2, Hap3, Hap4, and Hap6 (table 1). Because of the possibility that different subpopulations had fixed different Wolbachia strains, we took care to choose ants from different subpopulations.

The Wolbachia gene *wsp* was amplified from the extractions of ant DNA using primers wsp81F and wsp691R (Braig et al. 1998) and following a protocol by Zhou, Rousset, and O'Neill (1998). The PCR product was run on 1.5% agarose gels. Individual bands were cut out of the gel and frozen at –20°C. DNA was recovered out of the gel slices and cloned using a TOPO TA Cloning Kit (Invitrogen). Plasmids were recovered out of positive clones with the Wizard Plus SV Minipreps DNA Purification System (Promega). Sequencing reactions were performed on one or both strands using the ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and run on an ABI 377 automated sequencer. Forward and reverse sequences from same clones were aligned and corrected by hand to obtain an unambiguous sequence for each clone. We sequenced a total of 65 clones (12 to 14 clones per host).

## Sequence Analysis

Sequences were aligned by eye in Se-Al (Rambaut 1996) while taking into account their amino acid translation. Analyses of sequence divergence were performed using PAUP* 4.0b8 (Swofford 1998). The putative recombinant sequences in our data set were subjected to a maximum-likelihood analysis developed by Holmes, Worobey, and Rambaut (1999) and implemented in the program LARD (Likelihood Analysis of Recombination in DNA, evolve.zoo.ox.ac.uk/software). The analysis allows the determination of the most probable recombination breakpoint within an alignment comprising one recombinant and two putative ''parental'' sequences. The most likely breakpoint can be identified as the one which maximizes the joint likelihood of two maximum-likelihood trees constructed independently on the sequence parts to its left and right. To assess whether the assumption of a recombination event explains the

sequence data significantly better than the null hypothesis of clonal evolution, a likelihood ratio test is performed, using the joint likelihood of the two independent trees left and right of the breakpoint and the likelihood of a single tree calculated on the entire alignment. The likelihood ratio is compared with a null distribution produced by applying the LARD analysis to sequences generated by Monte Carlo simulation of clonal evolution. For each of the tests, we generated 200 sets of sequences with Seq-Gen (Rambaut and Grassly 1997) (evolve.zoo.ox.ac.uk/software). The maximum-likelihood estimations of phylogenetic parameters needed for the test and generation of the null distribution were obtained by applying a HKY85 model (Hasegawa, Kishino, and Yano 1985) with gamma-distributed rate heterogeneity between sites to the ensemble of our sequences in PAUP* version 4.0b8 (Swofford 1998).

### RFLP Genotyping

RFLP analyses were used to determine whether the Wolbachia strains identified by sequencing were differentially associated with host mitochondrial haplotypes. For each of the haplotypes Hap1, Hap2, Hap3, Hap4, and Hap6, we analyzed ants from several nests from all subpopulations where that haplotype occurred (see table 1). The *wsp* gene was amplified with primers wsp81F and wsp691R and using a high-fidelity Taq polymerase (SuperTaq Plus, HT Biotechnology). PCR products were purified with Qiaquick spin columns (Qiagen) and digested with *Rsa*I and *Hin*dIII. These two restriction enzymes produced at least one specific band for each of the Wolbachia strains. Fragments were run on high-resolution Spreadex EL1200 gels (Elchrom Scientific). To exclude cross-contamination, clones of the sequenced Wolbachia strains were analyzed along with our samples.

## Results

We identified five Wolbachia strains designated wFex1 to wFex5. The *wsp* sequences can be accessed on GenBank under the numbers AY101196, AY101197, AY101198, AY101199, and AY101200, respectively. Each of the five sequences has been obtained independently from at least two host individuals. Blastn searches in GenBank identified members of the Wolbachia A group as the closest matches to all five wFex strains. These searches also revealed similarities between the *wsp* sequences of three strains with Wolbachia sequenced from other hosts. The *wsp* sequences of wFex1 and wFex4 were identical to those of Wolbachia sequenced from the tephritid fruit fly *Dacus destillatoria* (GenBank accession number AF295344) (Jamnongluk et al. 2000) and the ant *Formica truncorum* (GenBank accession number AF326978) (Wenseleers et al. 1998), respectively. The *wsp* sequence of wFex2 differed by only 3% of nucleotides from that of the Wolbachia strain wUni, identified in the wasp *Muscidifurax uniraptor* (GenBank accession number AF020071) (Zhou, Rousset, and O'Neill 1998). The strong similarity between the three wFex strains and Wolbachia in other host suggests that these strains have been transmitted horizontally between hosts.

For wFex3 and wFex5, no close matches were found, and the most similar sequences differed at 7% to 8% of nucleotides. The patterns of sequence similarity further suggest that wFex3 and wFex5 have arisen by homologous recombination between wFex2 and wFex4. The *wsp* sequences of both putative recombinant strains appear to be composed of two parts derived from each of the parental strains, with a breakpoint situated between nucleotide positions 294 and 348 (fig. 1). LARD analyses gave strong statistical support to the origin by recombination of wFex3 and wFex5. The log-likelihood ratios of the original sequences (wFex3: 98.9 and wFex4: 100.4) were much greater than those obtained from the simulated sequences (maxima 9.7 and 11.4, respectively). The null hypothesis of clonal evolution of strains wFex3 and wFex5 can therefore be rejected at a significance level of $P < 0.005$. In agreement with the pattern of sequence similarity (fig. 1), the LARD analyses identified nucleotide position 295 as the most probable recombination breakpoint in both wFex3 and wFex5.

Recombination might also be at the origin of wFex1 or wFex2. The 3′ parts of their *wsp* sequences differ by only 1% (three nucleotides), whereas they differ by 16% on the 5′ end (nucleotides 1 to 321). A significance test for this recombination event could not be performed because of the lack of a putative parental sequence for the 5′ end.

The RFLP analyses revealed high levels of multiple Wolbachia infection (table 1). All ants analyzed harbored the strains wFex2, wFex3, wFex4, and wFex5. Strain wFex1 was found in all ants with mitochondrial haplotypes Hap2, Hap4, and Hap6 but was invariably absent in ants with haplotypes Hap1 and Hap3. The tight association between the presence/absence of strain wFex1 and host mitochondrial haplotype was completely independent of the subpopulation. Ants sharing the same haplotype but sampled in different nests or subpopulations always harbored the same strains. By contrast, individuals from the same subpopulation but belonging to different haplotype groups (Hap1 and Hap3 versus Hap2, Hap4, and Hap6) always differed in whether or not they were infected with strain wFex1 (table 1).

## Discussion

Our study revealed high levels of multiple Wolbachia infection in the ant *F. exsecta*. All ants harbored four or five distinct strains. Whereas infections with two or three strains are quite common (e.g., Breeuwer et al. 1992; Merçot et al. 1995; Sinkins, Braig, and O'Neill 1995; Vavre et al. 1999; Kondo et al. 2002), levels of multiple infection comparable to those in *F. exsecta* have been reported so far from only three host species (*Doronomyrmex pacis*, six strains [Wenseleers 2001]; *Bactrocera ascita*, five strains [Jamnongluk et al. 2002]; and *Acromyrmex octospinosus*, four strains [van Borm et al. 2003]).

It is not well understood what factors are responsible for variations in the number of Wolbachia strains coinfecting host species. It has been proposed that some hosts might be less exposed to environmental curing and therefore harbor more strains because they lose infections at a lower rate (Jamnongluk et al. 2002).

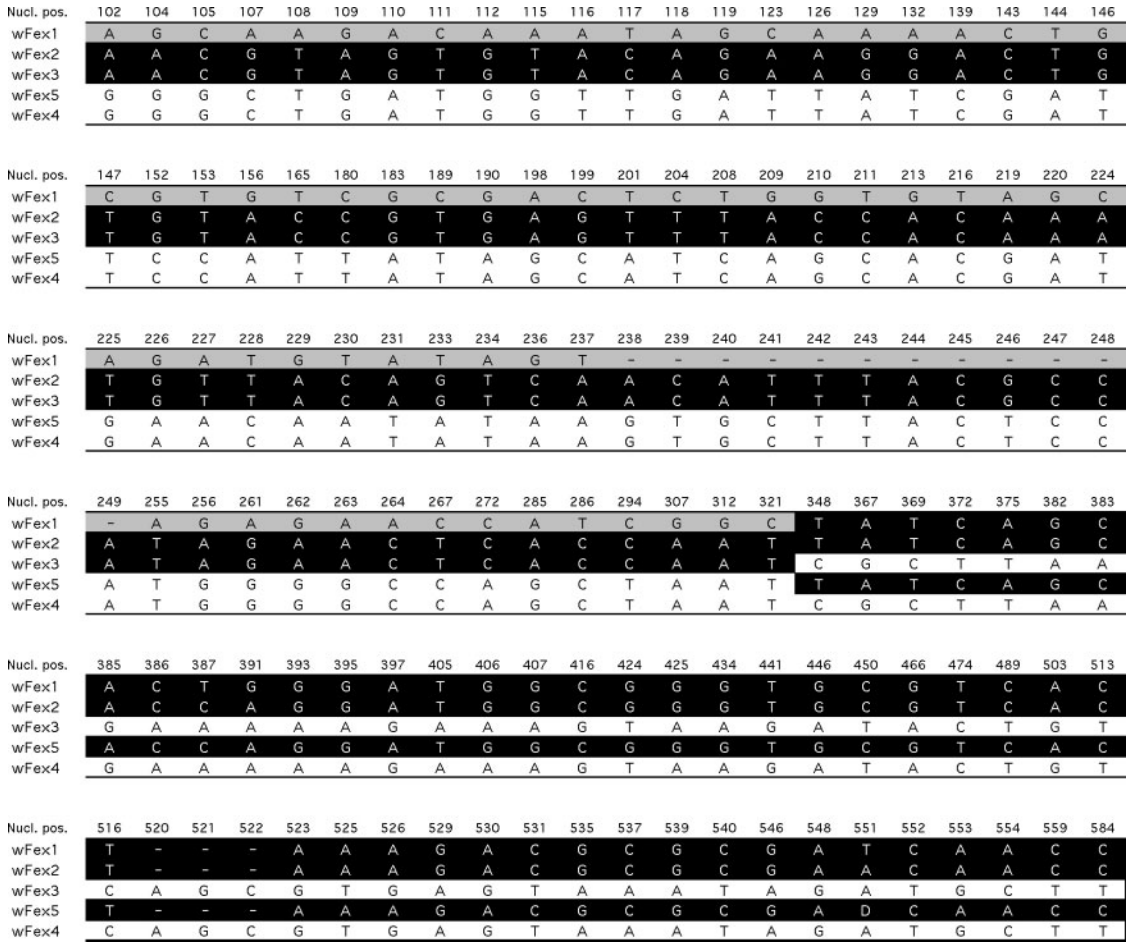


FIG. 1.—Sequence variation in the *wsp* gene of the five Wolbachia strains identified in *F. exsecta*. Position 1 is equivalent to position 386 in the original *wsp* sequence (Braig et al. 1998). Similar sequence stretches (≥1% nucleotide difference) appear with the same background.

Alternatively, some species might have high levels of multiple infection because they are particularly susceptible to acquiring new infections through horizontal transmission (Vavre et al. 1999; Jamnongluk et al. 2002; van Borm et al. 2003). Interesting is the fact that *F. exsecta* as well as two of the three other species with extreme levels of multiple infection (*A. octospinosus* and *D. pacis*) are social insects. Because of their reproductive division of labor, social insects tend to have small effective population sizes (Crozier 1979). This possibly facilitates the invasion of new strains, in particular that of CI-inducing Wolbachia, the frequency of which must exceed a critical level in order for infection to spread deterministically (Caspari and Watson 1959). Finally, our data suggests that high levels of multiple infection may also stem from recombination events. At least two out of the five wFex Wolbachia strains originated by recombination, and this event most likely occurred in *F. exsecta*. Thus, the level of multiple infection almost doubled without the invasion of additional strains.

The finding that two and possibly even three of the five wFex strains are the product of homologous recombination is surprising. Only very recently, recombination in Wolbachia had been inferred from sequence data (Jiggins et al. 2001), and a single recombinant *wsp* gene has been identified (Werren and Bartos 2001). The high incidence of recombination in *F. exsecta* raises the question of how new recombinant strains can spread through the host population? One possibility is random drift. However, recombinant strains are initially very rare, and drift should therefore allow only a tiny fraction of them to spread to high frequencies, implying that recombination occurs at rates much higher than what one would infer from the frequency of established recombinant strains. Alternatively, recombinants could spread deterministically if recombination provides benefits to the new strains. In CI-causing Wolbachia, recombination might create new incompatibility types, which could spread if associated with the two parental strains (Frank 1998). Alternatively, it has been proposed that recombination in *wsp* might be selected because the gene is thought to be involved in host-symbiont interactions (Jiggins et al. 2001; Werren and Bartos 2001). However, this hypothesis deserves further testing because although *wsp* appears to be under positive selection in parasitic Wolbachia strains (Jiggins, Hurst, and Yang 2002), recombination rates seem to be similar to those of the conserved cell cycle gene *ftsZ* (Jiggins 2002).

The RFLP analyses revealed that Wolbachia strains wFex2 to wFex5 were present in all ants independently of

their haplotype. wFex1, in contrast, was found only in hosts with mitochondrial haplotypes Hap2, Hap4, and Hap6 and was invariably absent in hosts with haplotypes Hap1 and Hap3. The clear association between host mitochondrial haplotypes and wFex1 indicates that horizontal transmission of symbionts is extremely rare or absent, at least between mitochondrial lineages. As a consequence, the fact that four Wolbachia strains are associated with all mitochondrial lineages is best explained by a diversification of host mitochondrial haplotypes after the fixation of Wolbachia strains wFex2 to wFex5.

The distribution of wFex1 is more difficult to explain. An infection polymorphism could arise if the strain did not cause a phenotypic effect or caused one that was neutral in the presence of one or several of the other strains. The spread of the strain may initially have occurred by hitchhiking on the invasion of one of the other strains (Giordano, O'Neill, and Robertson 1995). However, subsequent loss of the strain should have occurred randomly and independently of host mitochondrial lineage, hence failing to account for the observed association between wFex1 and host haplotype. Alternatively, wFex1 may cause a phenotype but selection on infection could be impeded by the strong population structure of the host. Selection on Wolbachia infection relies on competition between maternal lineages differing in infection status (Werren and O'Neill 1997). However, in species with extremely restricted female dispersal, such as *F. exsecta*, competition takes place almost exclusively between females of the same maternal lineage. Selection on Wolbachia infection should thus be less effective and infection polymorphisms, originating for example from founder events, could be maintained over long periods of time.

In conclusion, this study suggests that the infection dynamics of Wolbachia might be more complex than hitherto appreciated. In particular, the genetic structure of host populations might be a major determinant both of the probability of superinfection and the maintenance of infection polymorphism. In the future, detailed studies on the phenotypic effects of the wFex strains will hopefully allow elucidation of the evolutionary forces responsible for the unusual pattern of infection in *F. exsecta*.

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