# Diversification of *Wolbachia* Endosymbiont in the *Culex pipiens* Mosquito

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## Abstract

The α-proteobacteria *Wolbachia* are among the most common intracellular bacteria and have recently emerged as important drivers of arthropod biology. *Wolbachia* commonly act as reproductive parasites in arthropods by inducing cytoplasmic incompatibility (CI), a type of conditional sterility between hosts harboring incompatible infections. In this study, we examined the evolutionary histories of *Wolbachia* infections, known as *w*Pip, in the common house mosquito *Culex pipiens*, which exhibits the greatest variation in CI crossing patterns observed in any insect. We first investigated a panel of 20 *w*Pip strains for their genetic diversity through a multilocus scheme combining 13 *Wolbachia* genes. Because *Wolbachia* depend primarily on maternal transmission for spreading within arthropod populations, we also studied the variability in the coinherited *Cx. pipiens* mitochondria. In total, we identified 14 *w*Pip haplotypes, which all share a monophyletic origin and clearly cluster into five distinct *w*Pip groups. The diversity of *Cx. pipiens* mitochondria was extremely reduced, which is likely a consequence of cytoplasmic hitchhiking driven by a unique and recent *Wolbachia* invasion. Phylogenetic evidence indicates that *w*Pip infections and mitochondrial DNA have codiverged through stable cotransmission within the cytoplasm and shows that a rapid diversification of *w*Pip has occurred. The observed pattern demonstrates that a considerable degree of *Wolbachia* diversity can evolve within a single host species over short evolutionary periods. In addition, multiple signatures of recombination were found in most *w*Pip genomic regions, leading us to conclude that the mosaic nature of *w*Pip genomes may play a key role in their evolution.

**Key words:** endosymbiosis, *Wolbachia*, mitochondrial hitchhiking.

Research article

## Introduction

Arthropods are commonly infected by maternally transmitted endosymbionts. Although some endosymbionts confer direct benefits to their hosts by providing anabolic functions or resistance to pathogens (Haine 2008; Moran et al. 2008), others are associated with alterations of host reproduction (Werren et al. 2008; Engelstadter and Hurst 2009). These symbionts represent reproductive parasites that include diverse unrelated bacteria, among which the alpha-proteobacteria *Wolbachia* are the most widespread (Duron et al. 2008; Hilgenboecker et al. 2008). In some host species, the successful spread of *Wolbachia* is achieved by biasing the host's sex ratio toward the production of females (the transmitting sex) through the induction of parthenogenesis, feminization, or male-killing. More commonly, *Wolbachia* are able to induce a form of conditional sterility, termed cytoplasmic incompatibility (CI), between infected males and uninfected females or females infected by incompatible strains (Werren et al. 2008; Engelstadter and Hurst 2009). Such manipulations enable *Wolbachia* to spread through arthropod populations and may drive arthropod evolution through their effects on host phenotypes (Moran et al. 2008; Werren et al. 2008; Engelstadter and Hurst 2009).

The dynamics of *Wolbachia* infections within the common house mosquito, *Culex pipiens* complex, remain poorly understood. The most common members of the complex are the subspecies *Cx. p. quinquefasciatus* (Say) and *Cx. p. pipiens* (L.), representing the southern and northern mosquito populations, which are ubiquitous in tropical and temperate regions, respectively (Barr 1982). Members of the *Cx. pipiens* complex exhibit the greatest variation of CI crossing types observed in arthropods thus far (Laven 1967; O'Neill and Paterson 1992; Guillemaud et al. 1997; Duron et al. 2006; Atyame et al. 2011). However, an early genotyping approach using the *ftsZ* gene failed to reveal any polymorphism between incompatible *Wolbachia* strains (Guillemaud et al. 1997). Further analyses used sequences of published complete *Wolbachia* genomes to characterize polymorphic molecular markers. Genomes of *Wolbachia* strains infecting arthropod are scattered with mobile genetic elements (MGEs), such as prophages and transposable elements, which can represent more than 20% of genome content (Wu et al. 2004; Klasson et al. 2008, 2009; Salzberg et al. 2009). Additionally, they contain an unusual high number of genes encoding proteins with ankyrin (ANK) motifs, which possibly mediate specific protein–protein interactions (Sinkins et al. 2005; Duron et al. 2007; Walker et al. 2007). We further developed genotyping approaches using ANK and MGE markers and used them to identify more than 100 genetically distinct *Wolbachia* strains (referred to as *w*Pip strains) in natural populations of *Cx. pipiens* (Duron et al. 2005, 2006, 2007, forthcoming; Atyame et al. 2011).

**Table 1.** Description of *Culex pipiens* Lines and *w*Pip Strains.

| Mosquito Line | Abbreviation | *Wolbachia* Strain | *Culex pipiens* Subspecies | Origin | Year of Collection | References |
|---|---|---|---|---|---|---|
| Pel | Pel | *w*Pip(Pel) | *quinquefasciatus* | Sri Lanka | 1984 | Klasson et al. (2008) |
| JHB | JHB | *w*Pip(JHB) | *quinquefasciatus* | South Africa | 2001 | Salzberg et al. (2009) |
| Manille-A | Ma-A | *w*Pip(Ma-A) | *quinquefasciatus* | Philippines | 2003 | Duron et al. (2006) |
| Manille-B | Ma-B | *w*Pip(Ma-B) | *quinquefasciatus* | Philippines | 2003 | Duron et al. (2006) |
| Kara-C | Ka-C | *w*Pip(Ka-C) | *quinquefasciatus* | China | 2003 | Duron et al. (2006) |
| MaClo | Mc | *w*Pip(Mc) | *quinquefasciatus* | California | 1984 | Duron et al. (2005) |
| Slab | Sl | *w*Pip(Sl) | *quinquefasciatus* | California | 1950 | Duron et al. (2005) |
| Cotonou-A | Cot-A | *w*Pip(Cot-A) | *quinquefasciatus* | Benin | 2005 | This study |
| Cotonou-B | Cot-B | *w*Pip(Cot-B) | *quinquefasciatus* | Benin | 2005 | This study |
| Australie | Au | *w*Pip(Au) | Hybrid (*quinquefasciatus*/*pipiens*) | Australia | 2004 | Duron et al. (2006) |
| El Palmar-A | Ep-A | *w*Pip(Ep-A) | *pipiens* | Spain | 2005 | Duron et al. (2007) |
| El Palmar-B | Ep-B | *w*Pip(Ep-B) | *pipiens* | Spain | 2005 | Duron et al. (2007) |
| LaVar | Lv | *w*Pip(Lv) | *pipiens* | France | 2003 | Duron et al. (2005) |
| Bifa-A | Bf-A | *w*Pip(Bf-A) | *pipiens* | France | 2002 | Duron et al. (2006) |
| Bifa-B | Bf-B | *w*Pip(Bf-B) | *pipiens* | France | 2002 | Duron et al. (2006) |
| Kol | Ko | *w*Pip(Ko) | *pipiens* | Crete | 2002 | Duron et al. (2006) |
| Keo-A | Ke-A | *w*Pip(Ke-A) | *pipiens* | Cyprus | 2003 | Duron et al. (2006) |
| Keo-B | Ke-B | *w*Pip(Ke-B) | *pipiens* | Cyprus | 2003 | Duron et al. (2006) |
| Tunis | Tn | *w*Pip(Tn) | *pipiens* | Tunisia | 1992 | Duron et al. (2005) |
| Istanbul | Is | *w*Pip(Is) | *pipiens* | Turkey | 2003 | Duron et al. (2005) |

In this study, we characterized the evolutionary history of *Wolbachia* infections in the *Cx. pipiens* complex by examining the association between the *w*Pip strains and *Cx. pipiens* mitochondrial DNA (mtDNA) variation. The predominant mode of *Wolbachia* transmission within a species is vertical via the egg cytoplasm (Werren et al. 2008; Engelstadter and Hurst 2009). Because *Wolbachia* and mitochondrial genomes are cotransmitted, and therefore, in linkage disequilibrium (LD), the spread of *Wolbachia* will strongly affects a host's mtDNA diversity through indirect selection (review in Hurst and Jiggins 2005). However, exceptions to strict vertical transmission have been found; in some cases, *Wolbachia* are also transferred through horizontal transmission both within and among different host species, although the mechanisms of transfer are not well understood (Ahrens and Shoemaker 2005; Baldo et al. 2008; Raychoudhury et al. 2009). Consequently, the wide distribution of *Wolbachia* among arthropods is generally assumed to result from complex interactions between vertical and horizontal modes of transmission, modulated by their capacity to alter host reproduction.

Here, we analyzed *w*Pip variability and the associated mtDNA diversity in 20 *Cx. pipiens* lines encompassing unidirectionally and bidirectionally incompatible strains that originated from different geographic areas. Our results showed that the *w*Pip strains form a monophyletic clade of closely related bacteria and that *Cx. pipiens* harbors a low level of mitochondrial variability, which is a probable consequence of a recent *Wolbachia* invasion through cytoplasmic hitchhiking. Investigation of *w*Pip sequences revealed extensive recombination between *w*Pip strains, although multiple infections within a single mosquito were never detected using our markers. However, a congruence between *w*Pip and mtDNA phylogenies was shown, demonstrating that *Wolbachia* mainly use maternal inheritance to spread through *Cx. pipiens* populations. The evolutionary implications of horizontal transfers and the question of whether the *Cx. pipiens*–*Wolbachia* association is a unique case or a representative example is discussed.

## Materials and Methods

### Mosquito Collection

Twenty *Cx. pipiens* lines from a broad geographical range were examined (table 1). This collection encompassed the two main *Cx. pipiens* subspecies, *Cx. p. pipiens* and *Cx. p. quinquefasciatus*, which are naturally infected with compatible and incompatible *w*Pip strains (for more details, see Duron et al. 2006, 2007). The study also included the two lines for which the *w*Pip genome has been sequenced, *w*Pip(Pel) (GenBank AM999887; Klasson et al. 2008) and *w*Pip(JHB) (ABZA01000000; Salzberg et al. 2009).

### *Wolbachia* Markers

The *w*Pip strains were first genotyped for the five housekeeping genes developed for the *Wolbachia* multilocus strain typing (MLST) methodology, *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA* (Baldo et al. 2006) and the *Wolbachia* surface protein gene *wsp* (Braig et al. 1998). The MLST system is classically used to characterize the eight supergroups (A–I) currently recognized within the *Wolbachia* genus (Lo et al. 2007; Ros et al. 2009). The polymorphism of seven additional genes was also examined: the DNA mismatch repair protein gene *MutL* (one copy in the *w*Pip(Pel) genome), 3 ANK genes, *ank2* (one copy), *pk1* (3 identical copies), and *pk2* (2 identical copies), and 3 phage genes, the methylase gene *GP12* (4 identical copies), the putative secreted protein gene *GP15* (also known as *VrlC*; one copy), and the regulatory protein gene *RepA* (one copy). None of these genes was amplified from *Wolbachia*-free *Cx. pipiens* lines, which confirmed their *Wolbachia* origin. A total of 13 *Wolbachia* genes, encompassing 19 distinct loci with a wide distribution along the *w*Pip(Pel) chromosome, were

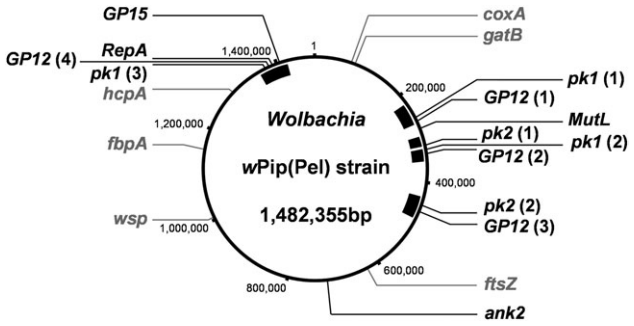


**Fig. 1.** Map of the *w*Pip(Pel) genome showing the position of the thirteen genes examined. Number in brackets (1–4) indicates identical copies of a given gene located in different positions along the chromosome. Black boxes indicate the locations of prophage regions; the MLST and *wsp* genes are reported in gray.

examined (fig. 1, supplementary table S1, Supplementary Material online).

## *Culex pipiens* Mitochondrial Markers

The complete mitochondrial genome of the Pel line (15,587 bp) was obtained through Blast searches of the database of *w*Pip(Pel) contig DNA sequences from the Wellcome Trust-Sanger Institute Web site (http://www.sanger.ac.uk/Projects/W_pipientis/) using the mitochondrial genome of *Aedes albopictus* (GenBank AY072044) as a probe. A similar approach to obtain the mitochondrial sequences of the JHB line from the VectorBase Web site (http://www.vectorbase.org/) showed several divergent mitochondrial sequences, some of which exhibited low-read coverage, making the JHB sequences unreliable for further analysis.

A set of primers (supplementary table S2, Supplementary Material online) was designed from the Pel sequences and further used to obtain the complete mitochondrial genomes (with the exception of the A + T-rich region) of additional *Cx. pipiens* lines. Specific primers were also used to amplify a 613-bp fragment from the NADH dehydrogenase subunit 2 (*ND2*) gene, a 1,132-bp fragment from the NADH dehydrogenase subunit 5 (*ND5*) gene, and an 852-bp fragment from the cytochrome b (*cytb*) gene from all the investigated *Cx. pipiens* lines (supplementary table S2, Supplementary Material online).

## PCR Amplification and Sequencing

DNA was extracted from individual mosquitoes using a cetyltrimethylammonium bromide (CTAB) protocol (Rogers and Bendich 1988). Amplification conditions were 3 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s (58 °C for *MutL*), and 72 °C for 1–1.5 min depending on the fragment size. Amplified fragments were run in agarose gel (1.5%) electrophoresis. The QIAquick gel extraction kit (QIAGEN, Valencia, CA) was then used to purify the polymerase chain reaction (PCR) products. Sequences were obtained directly for purified products using an ABI Prism 3130 sequencer with the BigDye Terminator Kit (Applied Biosystems). The sequences have been deposited in the GenBank database (accession numbers in supplementary tables S1 and S2, Supplementary Material online).

## Sequence Analyses

Sequence alignments were carried out using ClustalW (Thompson et al. 2002) and corrected using MEGA (Tamura et al. 2007). The GBLOCKS program (Castresana 2000) with default parameters was used to remove poorly aligned positions and to obtain nonambiguous sequence alignments. The number of variable sites, nucleotide diversity ($\pi$), G + C content, and the ratios of nonsynonymous versus synonymous substitutions ($K_a/K_s$) were computed using DNASP (Librado and Rozas 2009). Nonrandom associations between each pair of loci were estimated through the measure of allele LD using the $D'$ statistic (Lewontin 1964; Hedrick 1987). The exact test procedure implemented in GENEPOP (Raymond and Rousset 1995) was further used to test LD significance. Statistical analyses for intragenic recombination were performed with the Sawyer's test implemented in GENECONV (Sawyer 1999). A Bonferroni adjustment correction for multiple testing was applied (Hochberg 1988).

Annotation of the *Cx. pipiens* mitochondrial genome was based on alignments with mitochondrial sequences from *Ae. albopictus* (AY072044), *Ae. aegypti* (EU352212), *Anopheles gambiae* (L20934), *An. funestus* (DQ146364), and *An. quadrimaculatus* (L04272).

## Tree Reconstruction

Phylogenetic relationships were evaluated for *Wolbachia* and *Cx. pipiens* mitochondrial sequences. The best-fitting models of sequence evolution for each data set were determined using the Akaike information criterion with Modeltest v3.7 (Posada and Crandall 1998). The selected model was the general time reversible model, with gamma distributed among site rate variation (GTR + G) for both *Wolbachia* and mitochondrial sequence data sets. Bayesian inferences (BIs) were used to reconstruct phylogenies using MrBayes v 3.1.2 (Ronquist and Huelsenbeck 2003). Two independent replicates of four Metropolis-coupled Monte Carlo Markov chains were run for 2,000,000 generations with Model parameters and trees sampled every 200 generations. Bayesian posterior probabilities were obtained from the 50% majority-rule consensus of the sampled trees after discarding the initial burn-in period. The resulting phylogenetic trees were visualized and edited in MEGA (Tamura et al. 2007).

*Wolbachia* genes were also analyzed within a phylogenetic network framework to account for potentially conflicting signals due to recombination (Fitch 1997). A phylogenetic network was constructed based on uncorrected *P* distances using the Neighbor-net method (Bryant and Moulton 2004) implemented in SPLITSTREE (Huson and Bryant 2006). Neighbor-net is a distance-based method to construct a network as a generalization of all possible phylogenetic trees that can be reconstructed from conflicting signals in the data.

## Assessing the Maximum Age of Mitochondrial Sweep

We used the mitochondria data to infer the maximum age of the *Wolbachia* infection in *Cx. pipiens* following the

**Table 2.** Allelic Profiles of the Seven Polymorphic *w*Pip Genes in 20 *w*Pip Strains.

| Strain | Gene | | | | | | | Haplotype |
|---|---|---|---|---|---|---|---|---|
| | *MutL* | *ank2* | *pk1* | *pk2* | *GP12* | *GP15* | *RepA* | |
| *w*Pip(Pel) | a | a | a | a | a | a | a | A |
| *w*Pip(Cot-A) | a | a | a | a | a | a | a | |
| *w*Pip(Cot-B) | a | a | a | a | a | a | a | |
| *w*Pip(Ko) | a | a | a | a | a | a | a | |
| *w*Pip(Tn) | a | a | a | a | a | a | a | |
| *w*Pip(Ma-B) | a | a | a | a | a | a | a | |
| *w*Pip(JHB) | a | a | a | a | a | - | a | B |
| *w*Pip(Ep-A) | a | a | a | d | a | a | a | C |
| *w*Pip(Ep-B) | a | a | a | d | a | a | a | |
| *w*Pip(Bf-A) | a | a | a | a | a | a | b | D |
| *w*Pip(Lv) | b | e | c | a | b | b | a | E |
| *w*Pip(Au) | d | e | c | a | b | f | a | F |
| *w*Pip(Ke-A) | c | e | c | a | d | e | a | G |
| *w*Pip(Ke-B) | c | e | c | a | e | e | a | H |
| *w*Pip(Sl) | e | b | b | b | b | c | b | I |
| *w*Pip(Bf-B) | e | b | b | c | b | c | b | J |
| *w*Pip(Mc) | b | b | b | b | b | c | b | K |
| *w*Pip(Is) | c | c | d | a | c | d | a | L |
| *w*Pip(Ka-C) | f | d | e | a | f | g | a | M |
| *w*Pip(Ma-A) | f | d | e | a | g | g | a | N |

NOTE.—Letters A–N represent the 14 *w*Pip haplotypes. Dash indicates a gene deletion.

method of Rich et al. (1998). This method assumes that selection only occurs at the protein level and that DNA polymorphism in degenerate sites is neutral. We used 4-fold and 2-fold synonymous sites from protein-coding mtDNA sequences to assess the age of the sweep. The number of 2-fold and 4-fold synonymous sites in each coding region was computed with MEGA (Tamura et al. 2007), and a conservative Jukes–Cantor correction was applied for multiple hits.

## Results

### Monophyletic Origin of the *w*Pip Strains

The MLST and *wsp* genes did not exhibit sequence variation between the *w*Pip strains (eight strains were examined here, i.e., *w*Pip(Sl), *w*Pip(Tn), *w*Pip(Ko), *w*Pip(Lv), *w*Pip(Is), *w*Pip(Mc), *w*Pip(Pel), and *w*Pip(JHB)), establishing that these strains are very closely related. The *w*Pip MLST sequence data were compared with sequences from 18 other strains belonging to five distinct *Wolbachia* supergroups (A, B, D, F, and H). The phylogenetic tree obtained from the 2,079-bp concatenated MLST genes revealed that the *w*Pip strains form a robust monophyletic clade within the B supergroup, which is closely related to the *w*Bol1 strain present in the butterfly *Hypolimnas bolina* (identity >99.9%; supplementary fig. S1, Supplementary Material online).

### High Variability of *w*Pip Genomes

Seven of the examined *Wolbachia* genes were polymorphic among the *w*Pip genomes: the DNA mismatch protein gene *MutL*, 3 ANK genes *ank2*, *pk1*, and *pk2* and 3 phage genes *GP12*, *GP15*, and *RepA*. Analyses revealed considerable allelic variability among the 20 *w*Pip strains, with 2–8 alleles being found per gene (supplementary table S3, Supplementary Material online). This polymorphism was mainly due to nucleotide substitutions, insertions or deletions (indels); note that an insertion of the *Tr1* transposon (also known as ISWpi1; see Duron et al. 2005; Cordaux 2008) was observed within the *RepA* sequence of four *w*Pip strains. An additional source of variability arose from a *GP15* deletion in the *w*Pip(JHB) genome. A letter was attributed to each distinct allele of the seven genes, the combination of which identified 14 *w*Pip haplotypes among the 20 strains (table 2).

Although the prophage-related genes *pk1*, *pk2*, and *GP12* were found to be present in several copies in the *w*Pip(Pel) genome, divergent copies were never amplified from our *w*Pip strains: direct sequences of PCR products were easily readable and showed no overlapping peaks. This indicates that the different copies (if any) present in each *w*Pip strain examined here are identical, as observed in *w*Pip(Pel). It further shows that only mono-*w*Pip-infections (or multiinfections by closely related *w*Pip strains) are present within *Cx. pipiens* individuals.

### High Recombination in *w*Pip Genomes

Recombination, both within and between *Wolbachia* genes, can blur molecular signals and result in misleading observations related to strain relationships. For this reason, we checked the possibility of recombination among the seven polymorphic markers obtained here (*MutL*, *ank2*, *pk1*, *pk2*, *GP12*, *GP15*, and *RepA*). Pairwise tests for intergenic recombination revealed significant LD for *MutL*, *ank2*, *pk1*, *GP12*, and *GP15* (supplementary table S4, Supplementary Material online). Alleles at these five loci are not randomly associated and are stably cotransmitted within the *w*Pip chromosome. However, nonsignificant LD was found between *pk2* and *RepA* and between these two genes and the other five genes, showing that recombination has disrupted genome clonality by shuffling the *RepA* and *pk2* alleles among *w*Pip strains. Intragenic recombination was also detected for at least five genes by Sawyer's test (*MutL*, *pk1*, *pk2*, *GP12*, and *GP15*; supplementary table S3, Supplementary Material online). Intragenic recombination results in identical nucleotides or amino acid motifs in *w*Pip strains divergent at other loci, which are readily apparent through the examination of sequence alignments (supplementary fig. S2, Supplementary Material online).

### Gene Rearrangements in *w*Pip Genomes

Genome organization of *w*Pip strains was analyzed by comparing the locations of the 13 genes surveyed in this study in the *w*Pip(Pel) chromosome and in the five major *w*Pip(JHB) contigs presently available (supplementary fig. S3, Supplementary Material online). There are several rearrangements distinguishing these genomes, in which diverse genes have been inverted (e.g., *gatB*, *coxA*), translocated (*MutL*, *RepA*), duplicated, or deleted (three and one *pk1* copies are found in *w*Pip(Pel) and *w*Pip(JHB), respectively). Notably, rearrangements are not limited to

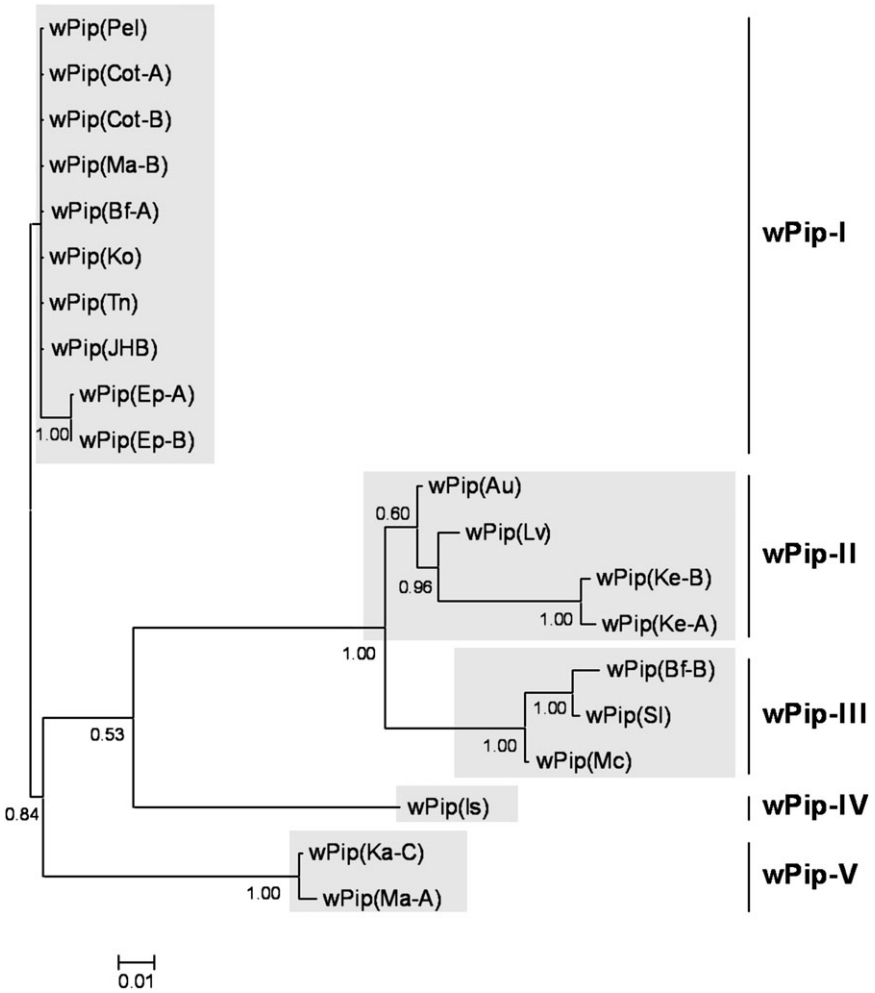


**Fig. 2.** Phylogenic tree of *w*Pip strains obtained from concatenated data set (*MutL*, *ank2*, *pk1*, *pk2*, *GP12*, *GP15*, and *RepA* sequences) by Bayesian analysis. Posterior probabilities obtained are shown at major nodes. The scale bar is in units of substitutions/site.

phage regions, which are prone to movements within and between genomes, but also affect housekeeping genes.

## Inference of *w*Pip Strain Relationships

Phylogenetic analyses of the 20 *w*Pip strains using the six *w*Pip genes, *MutL*, *ank2*, *pk1*, *pk2*, *GP12*, and *GP15*, revealed significant topological incongruence, as expected for a data set affected by recombination (supplementary fig. S4, Supplementary Material online). For instance, the *w*Pip(Sl), *w*Pip(Bf-B), and *w*Pip(Mc) strains are genetically similar for four markers (*ank2*, *pk1*, *GP12*, and *GP15*) but appear distantly related for two others markers (*MutL* and *pk2*).

To assess *w*Pip strain relationships, we performed phylogenetic analyses based on the concatenated sequences of the seven genes. The concatenated tree deduced from BI splits the *w*Pip clade into five groups (designated *w*Pip-I to *w*Pip-V; fig. 2). However, recombination can create artificial grouping of *w*Pip strains, and network analysis was thus conducted to visualize recombination effects, which were illustrated by multiple boxes (fig. 3). The evolutionary history of *w*Pip strains appears as a complex network with multiple pathways interconnecting strains, emphasizing the mosaic nature of *w*Pip genomes. Interestingly, despite recombination, the network analysis was congruent with the Bayesian tree in recovering the same five *w*Pip groups with strong bootstrap values.

A spatial structuring of *w*Pip diversity emerged when the geographic distribution of *w*Pip groups was examined, despite the limited number (19) of strains. The most common group, *w*Pip-I, is distributed widely from Asia to Europe (fig. 4), and all *w*Pip strains recently identified at La Réunion island (Indian Ocean) by Atyame et al. (2011) belong to that group. The *w*Pip-V group is only found in East Asia, and the *w*Pip-II and *w*Pip-III groups have an apparently discontinuous distribution, with strains being found in very distant geographic areas (e.g., the *w*Pip-II strains are from Australia and Europe).

## Low Mitochondrial Diversity in *Cx. pipiens*

*Culex pipiens* Pel mtDNA exhibits classical features found in other mosquito species that have been analyzed. It contains tightly packed genes with high A + T content (78.2%). There are 22 genes coding tRNAs, 2 coding ribosomal RNAs, 13 genes coding subunits of enzymes involved in oxidative phosphorylation, and finally, an A + T-rich noncoding region (supplementary fig. S5 and table S5,

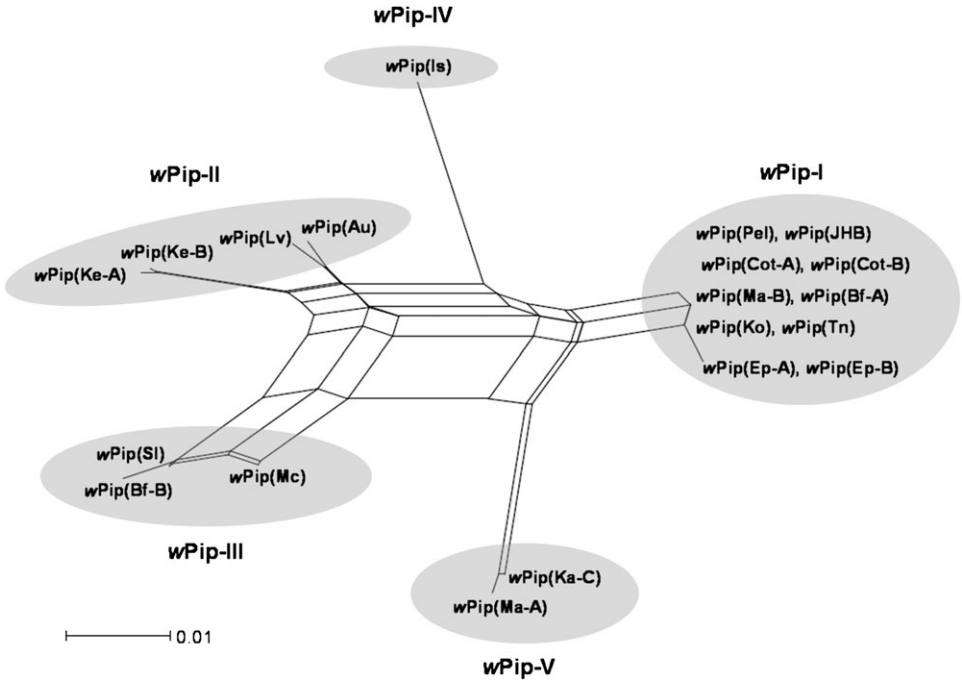


**Fig. 3.** Network analysis obtained from concatenated data set (*MutL*, *ank2*, *pk1*, *pk2*, *GP12*, *GP15*, and *RepA* sequences) using the Neighbor-net method. Each edge (or a set of parallel edges) corresponds to a split in the data set and has length equal to the weight of the split. Incompatible splits produced by recombination are represented by boxes in the network. Only bootstrap values for major grouping are indicated. The five *w*Pip groups (highlighted) are connected by multiple pathways resulting from recombination between *Wolbachia* genomes.

Supplementary Material online). The genes are arranged along the chromosome in a manner similar to that of other mosquito species (Beard et al. 1993; Mitchell et al. 1993; Krzywinski et al. 1997).

The complete *Cx. pipiens* mitochondrial genome (14,856 bp without the A + T-rich region) was sequenced from the lines Ko, Tn, Sl, and Is and compared with the Pel genome. Overall, the five mtDNA sequences displayed a very low variability, with only 36 variable nucleotidic positions being found (ca. 0.2%), and two sequences were strictly identical (lines Ko and Tn). Among the 13 protein-coding genes, five genes (*atp8*, *atp6*, *ND3*, *ND4L*, and *ND6*) showed no polymorphism, whereas *ND2*, *ND5*, and *cytb* were the most polymorphic (supplementary fig. S5, Supplementary Material online).

A likely explanation of the low mtDNA diversity in *Cx. pipiens* populations is that cytoplasmic hitchhiking has occurred during *Wolbachia* invasion, as suggested earlier by Guillemaud et al. (1997) and Rasgon et al. (2006). To confirm this hypothesis, we compared the nucleotide

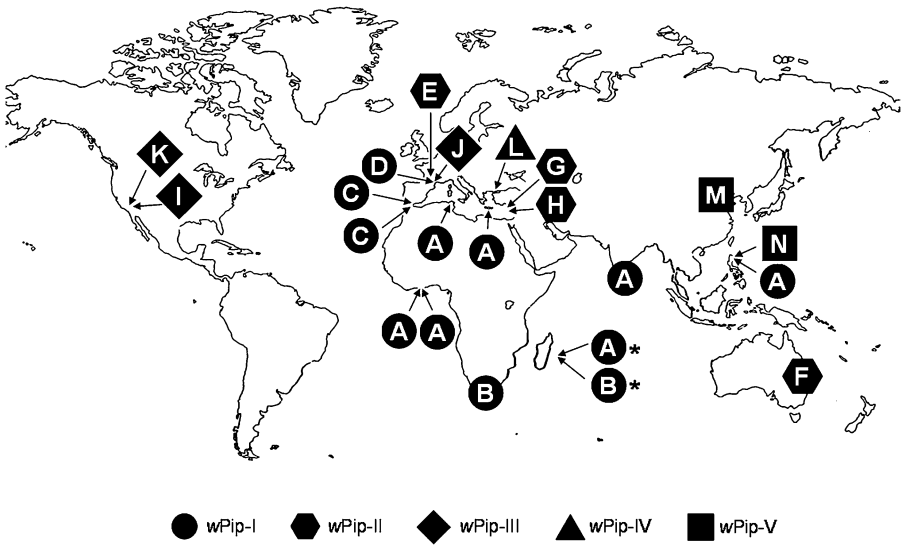


**Fig. 4.** Distribution of *w*Pip haplotypes and *w*Pip groups in *Culex pipiens* populations. Letters and symbols represent the *w*Pip haplotypes and *w*Pip groups, respectively. **w*Pip haplotypes recently described by Atyame et al. (2011).

**Table 3.** Nucleotide Diversity ($\pi$) of Mitochondrial Genes in 11 Mosquito Species (Culicidae).

| Taxon (subfamily, species) | $\pi$ (number of sequences) | | | | *Wolbachia* Infection (references) |
|---|---|---|---|---|---|
| | *cytb* | *ND4* | *COI* | *COII* | |
| Culicinae | | | | | |
| *Culex pipiens* | 0.0021 (19) | 0.0004 (14) | 0.0000 (24) | 0.0007 (8) | Yes (Hertig and Wolbach 1924) |
| *Cx. tarsalis* | — | 0.0116 (64) | — | — | No (Rasgon and Scott 2004) |
| *Culex* sp. | — | 0.0090 (10) | — | — | No (Rasgon et al. 2006) |
| *Aedes aegypti* | 0.0094 (16) | 0.0202 (46) | — | — | No (Kittayapong et al. 2000) |
| *Aedes albopictus* | 0.0043 (14) | — | 0.0039 (23) | — | Yes (O'Neill et al. 1992) |
| *Ae. caspius* | — | — | 0.0094 (7) | 0.0063 (21) | No (Ricci et al. 2002) |
| *Ae. vexans* | — | — | 0.0185 (7) | 0.0084 (7) | No (Kittayapong et al. 2000; Ricci et al. 2002) |
| Anophelinae | | | | | |
| *Anopheles aconitus* | — | — | 0.0053 (13) | 0.0066 (35) | No (Kittayapong et al. 2000) |
| *An. funestus* | 0.0066 (11) | — | — | — | No (Ricci et al. 2002) |
| *Anopheles gambiae* | — | — | 0.0053 (48) | — | No (Ricci et al. 2002) |
| *An. maculipennis* | — | — | 0.0063 (62) | — | No (Ricci et al. 2002) |

diversity per site ($\pi$) at four mitochondrial loci in the 11 Culicidae species for which the presence or absence of *Wolbachia* has been documented (table 3 and supplementary materials). Only two species, *Cx. pipiens* and *Ae. albopictus*, are known to be infected, whereas *Wolbachia* infection was never found in the nine other species. These two *Wolbachia*-infected species harbor significantly lower mtDNA diversity than the uninfected species (Wilcoxon test, $W = 10$, $P = 0.008$). For instance, the worldwide mtDNA diversity of *Cx. pipiens* is lower than the diversity observed in the North American populations of *Cx. tarsalis*, an uninfected species (Venkatesan et al. 2007). The low diversity of mtDNA observed in the *Cx. pipiens* and *Ae. albopictus* populations led us to conclude that *Wolbachia* is most likely the causative agent of mitochondrial sweeps in these taxa.

### Recent Mitochondrial Sweep in *Cx. pipiens* Complex

We then assessed the date of the mitochondrial sweep using the nucleotide divergence of 13 protein-coding mtDNA genes from the *Cx. pipiens* Is line and *Ae. albopictus* (GenBank AY072044). We estimated the substitution rate for these genes at 2-fold and 4-fold degenerate sites with a conservative Jukes–Cantor correction. The genera *Culex* and *Aedes* diverged approximately 172 to 226 Ma (Reidenbach et al. 2009). Using the most recent estimate (172 My), the mtDNA substitution rates (substitution/site/year) were estimated at $5.1 \times 10^{-8}$ and $19 \times 10^{-8}$ for the 2-fold and 4-fold degenerate sites, respectively, whereas, when using the oldest estimate (226 My), the substitution rates were $39 \times 10^{-9}$ and $15 \times 10^{-8}$. Among the mitochondrial genomes of five *Cx. pipiens* lines (Is, Sl, Tn, Ko, and Pel), we observed 13 and 10 nucleotide differences among the 2-fold ($n = 2{,}938$) and 4-fold ($n = 1{,}343$) synonymous sites, respectively. Thus, this dates the *Cx. pipiens* mitochondrial sweep between 12,000 and 16,000 years before present (95% confidence interval, if *Culex* and *Aedes* diverged 172 Ma) or between 16,000 and 21,000 (95% confidence interval, if the two genera diverged 226 Ma). It is possible that the date of the mitochondrial sweep is even more recent as it was assumed here that the substitution rates are constant, an assumption known to overestimate divergence times (Ho et al. 2005).

### Clear Codivergence of *w*Pip and *Cx. pipiens* Mitochondria

The codivergence of mitochondria and *w*Pip was assessed by studying the sequences of the three polymorphic *Cx. pipiens* mtDNA genes (*ND2*, *ND5* and *cytb*), encompassing 2,549 bp (16.4% of the whole mitochondrial genome).

Analysis of the *Cx. pipiens* mtDNA sequences among the 19 lines indicated the presence of 14 haplotypes (named *pi*1 to *pi*14), which differed overall at 22 variable nucleotide sites (supplementary table S6, Supplementary Material online). The mtDNA of the *Cx. pipiens* lines differed by only 1 to 9 nucleotides, confirming their very high homology (99.6–99.9%). Phylogenetic analyses revealed two main mitochondrial lineages (*pi*1 to *pi*5 and *pi*6 to *pi*14) with strong branching support (fig. 5*A*).

The concatenated mtDNA phylogeny and the *w*Pip phylogeny were congruent (fig. 5*A* and *B*). A significant association was found between mtDNA haplotypes and *w*Pip haplotypes (Fisher's exact test, $P = 3 \times 10^{-5}$) as well as *w*Pip groups ($P = 8 \times 10^{-4}$). This demonstrates that *w*Pip infections and mtDNA have codiverged through stable cotransmission within the cytoplasm in *Cx. pipiens* populations. Hence, the two main mitochondrial lineages parallel the *w*Pip divergence pattern and strongly confirm the *w*Pip phylogeny. Additionally, *Cx. pipiens* subspecies are not significantly associated with *w*Pip haplotypes ($P = 0.37$), *w*Pip groups ($P = 0.26$), or mtDNA haplotypes ($P = 0.10$). Thus, *Cx. pipiens* nuclear genomes have not codiverged with mitochondria and *w*Pip infections and exhibit a different evolutionary history.

## Discussion

Here, we examined 20 isolates of *Wolbachia* and their associated mitochondria within the *Cx. pipiens* complex. The combined use of *Wolbachia* and host mtDNA multilocus sequencing revealed the processes driving the evolution of *Wolbachia* infections in this mosquito and raised the

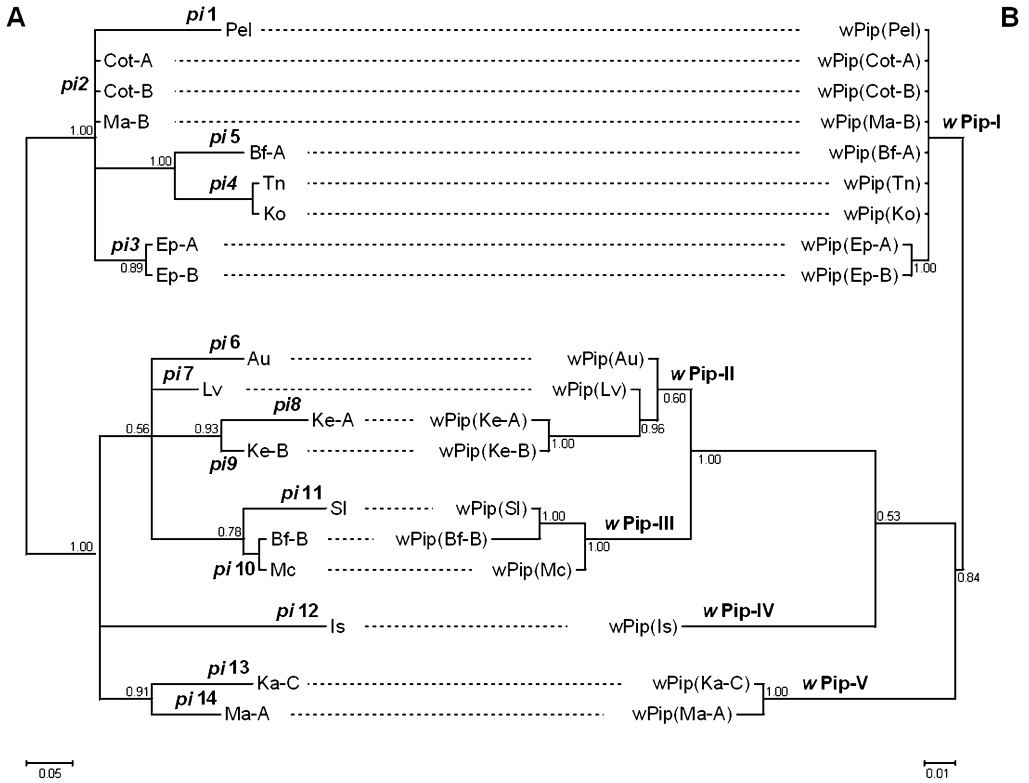


**Fig. 5.** Comparisons between phylogeny of *Culex pipiens* mitochondria and phylogeny of the *w*Pip strains. *A*, mitochondrial phylogeny constructed using BIs based on *ND2*, *ND5*, and *cytb* concatenated sequences. Names on branches indicate the mtDNA haplotypes (*pi*I to *pi*XIV). *B*, *w*Pip phylogeny obtained from concatenated data set (*MutL*, *ank2*, *pk1*, *pk2*, *GP12*, *GP15*, and *RepA* sequences). The five *w*Pip groups are reported. The scale bar is in units of substitutions/site.

question of their likeliness to occur in other *Wolbachia*–arthropod associations.

## *Wolbachia* and Mitochondrial Markers Reveal a Recent Diversification of *w*Pip Strains

The examined MLST and *wsp* markers showed that *w*Pip infections form a robust monophyletic clade within the B group of *Wolbachia*, confirming the results of Baldo et al. (2006). Although these markers are widely used to characterize the genetic diversity of *Wolbachia*, even within a host species (e.g., Dedeine et al. 2004; Baldo et al. 2006, 2008; Raychoudhury et al. 2009), they displayed no variation among the *w*Pip infections in *Cx. pipiens*, which shows that *w*Pip strains have a unique and recent evolutionary origin. As observed in other *Wolbachia*-infected species (Hurst and Jiggins 2005), mitochondrial diversity was low in populations of *Cx. pipiens* (and highly significantly lower than in non–*Wolbachia*-infected mosquitoes), suggesting that *Wolbachia* have affected mitochondrial polymorphism in this species through cytoplasmic hitchhiking. The observed polymorphism of mitochondrial protein-coding genes indicates that the mitochondrial sweep due to the spread of *Wolbachia* occurred within the last 21,000 years. This dating is within the range of values classically estimated for other *Wolbachia* host species of approximately $<$100,000 years, (Jiggins 2003; Keller et al. 2004; Duplouy et al. 2010), and the evolutionary pathway of *w*Pip in *Cx. pipiens* could be similar to other *Wolbachia*/arthropod associations.

Multilocus typing using seven *w*Pip polymorphic markers, including domains of the MGE and ANK genes, allowed the identification of 14 distinct *w*Pip haplotypes, which cluster into five distinct *w*Pip groups. This typing approach also established that the two published *w*Pip genomes, *w*Pip(Pel) and *w*Pip(JHB), are genetically very close to each other compared with strains belonging to other *w*Pip groups, in spite of their genomic differences (Salzberg et al. 2009). The variability of the investigated mitochondrial markers corroborates the inferences made from the *w*Pip markers; thus, in *Cx. pipiens*, different mitochondrial haplotypes may indicate that *w*Pip infections are different. Overall, the observed genetic diversity indicates that, after the spread of *Wolbachia*, diversification of *w*Pip and *Cx. pipiens* mitochondria occurred.

The diversity found for *w*Pip exhibits geographic variations. A remarkable degree of diversity was found in the Mediterranean area, where four of the five *w*Pip groups are found, whereas a reduced diversity was observed in other regions. The most common group, *w*Pip-I, has a wide distribution (Asia, Africa, and Europe) and was also recently reported at La Réunion Island (Indian Ocean) (Atyame et al. 2011). In contrast, some *w*Pip groups have a discontinuous distribution, as exemplified by the *w*Pip-II strains, which were found in Europe and in Australia. Such

a geographic pattern is likely to be a consequence of a recent worldwide expansion due to human activity (Raymond et al. 1991; Fonseca et al. 2004, 2006) or/and to selective advantages, possibly including CI selection. However, the 20 *w*Pip infections investigated in this study represent a restricted sampling, occasionally from old mosquito colonies, and further investigations are required to improve our knowledge of the spatial structure of the *w*Pip groups worldwide.

### *w*Pip Strains Are Independent of *Cx. pipiens* Subspecies

Strict vertical transmission must have favored the codivergence of *w*Pip and mtDNA within shared cytoplasm. However, there was no clear association between *Cx. pipiens* subspecies (nuclear diversity) and cytoplasmic diversity (i.e., *Wolbachia* and mtDNA): identical *w*Pip strains and identical mitochondrial haplotypes were found in the two subspecies, *Cx. p. pipiens* and *Cx. p. quinquefasciatus*. A likely explanation for this is that the transfer of cytoplasm between *Cx. pipiens* subspecies occurred through hybridization events, as observed in *Drosophila* species (Rousset and Solignac 1995; Ballard 2000) and in butterfly species (Jiggins 2003; Narita et al. 2006; Charlat et al. 2009). In *Cx. pipiens*, this hypothesis is well supported by the many reports of genetic introgression between the two subspecies in areas where they come into contact (Cornel et al. 2003; Fonseca et al. 2004). Hence, we can predict that DNA bar coding programs using mtDNA will fail to discriminate between *Cx. p. pipiens* and *Cx. p. quinquefasciatus*. Overall, these observations support the call of Hurst and Jiggins (2005) to not use mtDNA alone as a reliable means of taxa resolution.

### Intense Recombination Impacts the Structure of *w*Pip Genomes

The existence of extensive recombination among *w*Pip strains sheds light on the mechanisms shaping the evolution of *w*Pip genomes since recombination can influence the adaptive dynamics of *Wolbachia* by creating new alleles and thus allow the emergence of new phenotypes. Recombination between distant *Wolbachia* genomes has been previously documented (Jiggins et al. 2001; Bordenstein and Wernegreen 2004; Baldo et al. 2005; Gavotte et al. 2007), although in this study, we found recombination among very closely related *Wolbachia* genomes. Evidence of recombination was found at almost all the examined *w*Pip loci, WO-phage genes, as well as nonrelated phage loci. This shows that a high level of gene flow occurs among the *Wolbachia* genomes in *Cx. pipiens*. Hence, the *w*Pip strains do not form a set of clones in which evolution is independent but, rather, represent a large population of bacteria exchanging genetic information through lateral transfers. Although no instances of multiple infection were detected using our markers, we must assume that they occur, at least during a period long enough to allow recombination between strains.

Another consequence of recombination is that it can lead to misinterpretation of phylogenetic relationships between strains. However, despite the extensive recombination observed, the *w*Pip and mitochondrial phylogenies are congruent: recombinations have not disrupted our grasp of the evolutionary history of *w*Pip strains, probably because the contribution of recombinant regions in the phylogeny is weak compared with the diversity existing in nonrecombinant DNA fragments. Therefore, as suggested by Baldo et al. (2006), the use of a multilocus approach, rather than single-locus analysis, is required for a correct understanding of the evolutionary history of *Wolbachia* infections.

### The *Cx. pipiens*–*Wolbachia* Association, a Unique Case?

The high number of *w*Pip strains, which is still certainly underestimated, makes the *Cx. pipiens* system remarkable because lower diversity is usually reported in *Wolbachia* of other host species (e.g., Vavre et al. 1999; Mercot and Charlat 2004; Charlat et al. 2006; Arthofer et al. 2009). However, it is possible that genetic variations of *Wolbachia* in other host species could have been missed due to the methodology generally used to characterize these bacteria, as it is generally assumed that a single *Wolbachia* strain is present within a host species when the MLST or *wsp* markers are not variable. In *D. melanogaster*, a single strain, *w*Mel, was presumed to be present until Riegler et al. (2005) identified five distinct genotypes by examining transposon insertion sites and chromosomal inversions. More recent studies have reported different *Wolbachia* haplotypes solely on the basis of WO-phage genes in various host species, including crickets, beetles, and butterflies (Charlat et al. 2009; Chafee et al. 2010). Hence, the classical MLST system is well suited to characterize *Wolbachia* belonging to distinct clades, but specific species-typing systems based on markers with rapid sequence evolution need to be developed to investigate the *Wolbachia* diversity that probably exists in most associations.

Finally, the question remains of whether the *Cx. pipiens*–*Wolbachia* association is unique in term of its extremely large CI diversity (e.g., Laven 1967; O'Neill and Paterson 1992; Guillemaud et al. 1997; Duron et al. 2006). We have clearly demonstrated that the diversity of crossing types in this species is independent of nuclear backgrounds and relies solely on *w*Pip variability (Duron et al. 2006; Atyame et al. 2011). The reason that a similar CI system has not been reported in other *Wolbachia*-infected species remains a matter for speculation, but it is possible that the crossing studies conducted in *Cx. pipiens* have been more exhaustive than in any other species because of the intensive investigations that have been carried out for clarifying its systematics and studying the inheritance of morphological characters since the 1930s (e.g., Marshall and Staley 1937; Roubaud 1941; Laven 1958, 1967; Rozeboom 1958; Barr 1975; Narang and Seawright 1982; Irving-Bell 1983). As a result, the high variability of CI crossing types was investigated much earlier than the causative agent was identified by Yen and Barr (1971). Comparatively little work on the variability of the effects of *Wolbachia* infection has

been conducted in most arthropods, except in *Drosophila* species, such as *D. simulans*, where five distinct crossing types associated with distinct *Wolbachia* infections have thus far been identified (for review, see Mercot and Charlat 2004). Therefore, the possibility of the existence of variable reproductive phenotypes in other host species remains to be examined.

In conclusion, the use of multilocus typing combining *Wolbachia* and mitochondrial markers highlights the processes underlying the evolutionary dynamics of *w*Pip infections. The diversification inside the *w*Pip clade shows that a considerable amount of *Wolbachia* diversity can be generated within a single host species in a short period of time. Further investigations should examine the roles of recombination and MGE in the adaptive capacities of *Wolbachia*. In particular, this could explain rapid changes of interactions between *Wolbachia* and their hosts (Weeks et al. 2007; Echaubard et al. 2010) and play a key role in the evolution of phenotypes induced by *Wolbachia*. Finally, an important question now is to determine whether the *Cx. pipiens*–*Wolbachia* association is a unique case or, rather, a representative example.

## Supplementary Material

Supplementary figures S1–S5 and tables S1–S6 are available at *Molecular Biology and Evolution* online (http://www.mbe. oxfordjournals.org/).

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