Maternally inherited Wolbachia often manipulate the reproduction of arthropods to promote their transmission. In most species, Wolbachia exert a form of conditional sterility termed cytoplasmic incompatibility (CI), characterized by the death of embryos produced by the mating between individuals with incompatible Wolbachia infections. From a theoretical perspective, no stable coexistence of incompatible Wolbachia infections is expected within host populations and CI should induce the invasion of one strain or a set of compatible strains. In this study, we investigated this prediction on CI dynamics in natural populations of the common house mosquito Culex pipiens. We surveyed the Wolbachia diversity and the expression of CI in breeding sites of the south of France between 1990 and 2005. We found that geographically close C. pipiens populations harbor considerable Wolbachia diversity, which is stably maintained over 15 years. We also observed a very low frequency of infertile clutches within each sampled site. Meanwhile, mating choice experiments conducted in laboratory conditions showed that assortative mating does not occur. Overall, this suggests that a large set of compatible Wolbachia strains are always locally dominant within mosquito populations thus, fitting with the theoretical expectations on CI dynamics. Heredity (2011) 106, 986–993; doi:10.1038/hdy.2010.146; published online 1 December 2010

Keywords: symbiosis; arthropod; Wolbachia; cytoplasmic incompatibility

Introduction

Maternally inherited Wolbachia (α-Proteobacteria) are commonly found in arthropods (Duron et al., 2008; Hilgenboecker et al., 2008), often behaving as reproductive parasites by manipulating host reproduction to promote their own transmission (Engelstadter and Hurst, 2009; Werren et al., 2008). Commonly, Wolbachia exert a form of conditional sterility termed cytoplasmic incompatibility (CI), which causes a drastic reduction in the hatching rate (HR) of eggs produced by the mating between individuals with incompatible Wolbachia infections. Through CI, Wolbachia hamper the reproduction of uninfected females mated with infected males by killing their embryos, providing a reproductive advantage to infected females. When individuals are infected by different Wolbachia strains (here arbitrarily named w1 and w2), their crosses can be (i) compatible and produce viable offspring; (ii) incompatible in both directions and produce infertile eggs (a phenomenon called bidirectional CI) or (iii) incompatible in one direction only (unidirectional CI, for example, the cross w1 males with w2 females is incompatible, while the reciprocal cross, w2 males with w1 females, is compatible).

When incompatible Wolbachia strains are present within the same host population, they enter into competition through the expression of CI. Theoretically, the presence of incompatible Wolbachia strains within the same host population should lead to the rarefaction of one of the strains (Rousset et al., 1991; Dobson, 2003; Engelstadter and Telschow, 2009). The nature of CI is of major importance. In the case of bidirectional CI, all mating combinations between individuals infected by different Wolbachia strains being infertile, the most common Wolbachia strain will eliminate the rarest. In the case of unidirectional CI, only one mating combination is infertile (for example, w1 males with w2 females, providing a reproductive advantage to w1 females) and the strain inducing CI (here, w1) should invade. Aside from CI, the outcome of the competition will also be influenced by antagonist forces, such as an infection cost imposed on hosts and imperfect transmission of Wolbachia to the eggs, which can slow the spread of a Wolbachia strain. Taken together, these parameters determine an invasion threshold for CI, that is, an infection frequency below which a Wolbachia strain becomes extinct and above which it invades. If a Wolbachia strain exceeds the invasion threshold, it is expected to invade to reach a high frequency, possibly until fixation, resulting in host population largely dominated by one infection type (Engelstadter and Telschow, 2009).

One of the major models to study the dynamics of CI system is the Wolbachia infection found in the common house mosquito Culex pipiens and known as w1Pip. More than 99% of C. pipiens individuals are found to be infected by w1Pip within natural populations (Rasgon and Scott, 2003; Duron et al., 2005). Such high prevalence is well explained by the ability of w1Pip-infected males to induce complete CI with uninfected females, a near perfect maternal transmission of infection and a reduced effect on female fecundity (Rasgon and Scott, 2003;
Duron et al., 2006c). Furthermore, crossing experiments conducted over the past 70 years indicate that C. pipiens lines exhibit a high level of uni- and bidirectional CI (for example, Marshall and Staley, 1937; Geelhoedvitch, 1952; O’Neill and Paterson, 1992; Guillemaud et al., 1997), as best illustrated by Laven (1967c) who described 17 different crossing types worldwide. The wPip strains are so closely related that the multilocus sequence typing multi locus strain typing genes normally used to construct Wolbachia phylogeny are monomorphic and thus, not informative to discriminate between incompatible wPip strains (Guillemaud et al., 1997; Baldo et al., 2006). However, recent work has shown more than 60 wPip haplotypes in C. pipiens populations and confirmed that CI was associated with infection by genetically distinct wPip strains (Sinkins et al., 2005; Duron et al., 2006a,b). Hence, one would expect that incompatible wPip strains are present in natural populations of C. pipiens, and that competition between infections should occur.

In this study, we tested the prediction that incompatible Wolbachia infections cannot stably coexist sympatrically in a restricted study area in the south of France. The best approach to document the CI dynamics would have been to identify all the Wolbachia strains present within a host population, to determine their CI relationships and to further measure how Wolbachia diversity varies across years. Such methodology was used to demonstrate the invasion of one Wolbachia strain in uninfected populations of Drosophila simulans (Turelli and Hoffmann, 1991). However, this methodology is not possible in the French C. pipiens populations for several reasons. First, a high level of wPip genetic diversity exists locally in France where at least 5–10 strains per population were found through genotyping (Duron et al., 2006b). Second, the frequencies of incompatible wPip strains cannot be determined through genotyping of infections found in wild specimens, because no molecular marker correlated with CI properties has been identified to date in C. pipiens (Sanogo et al., 2005; Duron et al., 2006a, 2007a). As a result, it is not possible to predict the CI relationships between wPip strains from molecular data. Thus, massive crossing experiments would be needed to characterize the CI relationships between all the wPip strains, which would be too arduous. We, therefore, used another method to examine the CI dynamics in C. pipiens populations. This method assumes that, under panmixia, the co-occurrence of incompatible wPip strains within a host population should produce a substantial proportion of CI infertile matings, resulting in the production of infertile clutches. Quantifying the frequency of such infertile clutches can thus be used for a posteriori estimation of the frequency of incompatible wPip strains that coexist in C. pipiens field populations. To estimate the level of wPip genetic diversity, infections of wild C. pipiens specimens caught between 1990 and 2005 were genotyped. To quantify the frequency of incompatible crosses, egg rafts were collected in natural breeding sites. In parallel, crossing experiments were conducted between incompatible C. pipiens lines to characterize possible assortative mating (that is, preferential mating between individuals infected by compatible Wolbachia), a phenomenon which could reduce the production of infertile eggs when several incompatible wPip strains coexist.

Materials and methods

Screening of wPip infection

We delineated a restricted study area in the south of France (Figure 1). Mosquito larvae were randomly collected from four natural breeding sites (Figure 1): Ganges (sample A) in 1990 (n = 18) and 2001 (n = 20), Saint Bauzille de Putois (sample B) in 1990 (n = 20) and 2001 (n = 20), Maurin (sample C) in 2001 (n = 10) and Viols le Fort (sample D) in 2005 (n = 90). Mosquito larvae were reared in the laboratory until emergence for species identification and then stored in liquid nitrogen. DNA extraction was performed directly on the entire body of the hosts. Mosquito DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) protocol (Rogers and Bendich, 1988). Wolbachia polymorphism was analyzed through PCR amplifications of 11 mobile genetic elements (MGE): (i) the putatively active transposable element ISWpi1 (also called Tr1; Duron et al., 2005; Cordaux et al., 2008) and (ii) 10 WO prophage genes (that is, GP1b, GP2a, GP2b, GP2e, GP3a, GP3b, GP3c, GP3d, GP15a and GP15b, cf. Duron et al., 2006b). These MGE are known to be maternally inherited and inserted within the Wolbachia genome, which allows their use as wPip markers (Duron et al., 2005, 2006b).

The PCRs were carried out following previously published protocols using specific primers for each marker (Duron et al., 2005, 2006b). The PCRs were run for 30 cycles (94 C for 30 s, 52 C for 30 s and 72 C for 1–1 min 30 s) and the products were electrophoresed in a 1.5% agarose gel. Controls of DNA from C. pipiens laboratory strains served as a template for positive control and were included in each PCR plate. DNA quality was controlled by amplifying the C. pipiens acetylcholinesterase ace-2 gene, as described in Weill et al. (2000).

Collection of eggs

Eggs were sampled during summer in 14 C. pipiens natural breeding sites (Figure 1), which were highly polluted habitats. Morphological examination and molecular genotyping (Wolbachia and Culex markers) of randomly sampled larvae (n > 50 per sampling site) confirmed C. pipiens to be the only mosquito species present.
present. Eggs were collected as follows. Gravid C. pipiens females stick their eggs together to form a raft of 100–300 eggs on the surface of stagnant water. The egg rafts (each being produced by one female) were carefully removed with a paintbrush from the surface of stagnant water, placed separately in 24-well plates and brought to the laboratory for hatching. Larvae hatch within 36–48 h after oviposition at 25°C. The HR was evaluated over more than 72 h after collection under a binocular microscope. HR was used to characterize the nature of the parental mating, with the following arbitrary scale: (1) fertile if HR ≥75%, (2) intermediate if 25% ≤ HR <75% and (3) infertile if HR ≤ 25%. Fertilization of non-hatching egg rafts was checked by observing embryo development: egg rafts from non-inseminated C. pipiens females show an absence of embryo development whereas a high level of embryo development is found in incompatible egg rafts (Duron and Weill, 2006).

Mating experiments
Three C. pipiens laboratory lines infected by incompatible Wolbachia strains were used to investigate mating behavior. The Bifa-A and Bifa-B lines were isolated from the same larval collection in a breeding site from Ganges (south of France) in 2002; the Istanbul line was isolated from a sample collected in Turkey in 2003 (Duron et al., 2006a). Crossing relationships between the three lines have been previously characterized: Bifa-A and Bifa-B showed unidirectional CI (the cross Bifa-A male × Bifa-B female is incompatible while the cross Bifa-B male × Bifa-A female is compatible). The Istanbul line showed bidirectional CI with both Bifa-A and Bifa-B (Duron et al., 2006a). The CI occurring between these lines is complete, that is, less than 1% of eggs hatch in incompatible crosses. Thus, it is easy to distinguish egg rafts produced from compatible or incompatible crosses (90–100% or <1% HR, respectively).

Mating preferences were measured in cages (70 × 70 × 70 cm) where 100 males and 100 females from one line were placed with an equivalent number of males and females from an incompatible line. All individuals were 1-day-old and virgin. Each cage contained honey placed on top of moistened paper towel as source of food. The trials were performed at 25°C under a 12h light/12h dark cycle. Females were blood-fed 5 days after their introduction into the cage and allowed to oviposit on a water cup. Egg rafts were individually collected every day during the 5 days following blood feeding and scored for hatching during 72h. Fertilization of the egg rafts was checked as described above. Egg rafts from non-inseminated females were discarded.

Statistical analysis
We tested for the structure of wPip genetic diversity between mosquito populations by calculating an unbiased estimate of the $P$-value of a Fisher's exact test on a $R \times C$ contingency table (Raymond and Rousset, 1995a). Population differentiation was measured using a statistical analysis of variance through the $F_{ST}$ estimator (Weir and Cockerham, 1984). $F_{ST}$ values range classically from 0 to 1 and high $F_{ST}$ classically implies a considerable degree of differentiation among populations. A Bonferroni’s adjustment correction for multiple testing was applied, based on the number of comparisons. Calculations were made using GENEPOP version 3.4 (Raymond and Rousset, 1995b).

Results
High wPip genetic diversity within populations
We assayed for the presence and the variability of Wolbachia in 178 field-caught C. pipiens mosquitoes from four locations sampled in 1990, 2001 and 2005. All the specimens were found infected by wPip, and each was further characterized by the presence/absence patterns for 11 MGEs PCR products. Combination of the 11 MGE typings revealed the existence of 37 distinct wPip strains (Table 1). Nomenclature of wPip was defined according to the list previously published (Duron et al., 2006b). Only three wPip strains (wPip5, wPip8 and wPip66) were found in more than 10% of individuals. The wPip8 strain was the most frequent, present in all populations, but its prevalence never exceeded 40% of the population level. The other 34 wPip strains were rare, each being found in less than 10 hosts (prevalence < 5.6%), but taken together represented 48.4% of the infections. Hence, most of the wPip diversity is represented by rare strains. Note that our method first underestimates the overall wPip genetic diversity, as using more markers would certainly have revealed more distinct strains. Second, we did not take into account allelic diversity previously shown for certain markers (Duron et al., 2006b). Third, we did not consider variation in the number or variability of MGE copies in their insertion sites, as observed for the transposable element ISWp1 (Duron et al., 2005). Overall, this indicates that much Wolbachia diversity, certainly more than we described here, exists in French C. pipiens populations.

We found 11 wPip strains on average per population, 6–21 strains coexisting in each population (Table 1). The wPip polymorphism was not uniformly distributed between the six populations. Significant differentiation of the wPip distribution occurred if all populations were considered together (global $F_{ST} = 0.032; P < 10^{-4}$), and some pair comparisons between populations also showed significant differences (Table 2). Notably, the distribution of the wPip diversity varied significantly between the three locations examined in 2001 ($F_{ST} = 0.096; P < 10^{-4}$), although no significant differentiation was found between the two locations examined in 1990 ($F_{ST} = 0.096; P = 0.08$). Thus, it is clear that similar C. pipiens populations can harbor different wPip strains at the same time, which indicates that the wPip diversity is geographically structured.

We further examined whether the wPip polymorphism varied over time within two locations (Ganges and Saint Bauzille de Putois). No significant differentiation was detected between 1990 and 2001 and no local Wolbachia replacement was found, which suggests that the wPip distribution is locally stable over long periods. It should be noted that most of the polymorphism consisted of rare strains, resulting in a small sample size within each class of individuals, giving a low significance to statistical tests, which could not reveal subtle variations. However, the number of wPip strains per location (weighted by sample size) did not vary significantly between years (Kruskal–Wallis test, df = 2, $P = 0.15$): 21 strains were found in 1990, 19 in 2001 and 21 in 2005. We further
examined the dynamics of each MGE separately, as this approach might reveal the spread of one marker, which could have a role in CI. However, the MGE distribution is actually constant over time. For instance, the three most common strains, \( \text{wPip5}, \text{wPip8} \) and \( \text{wPip66} \), all sharing a set of eight markers, were present at intermediate frequency (\( \sim 50\% \)) since 1990. This confirms that the overall \( \text{wPip} \) diversity is maintained for over 15 years, and that sweep events, if any, do not erode the global \( \text{wPip} \) diversity in \( C. \text{pipiens} \) populations.

Very low incidence of CI mating in natural breeding sites

Mosquito eggs were collected from natural oviposition sites during the summers of 1984, 1987 and 2005 in 14 locations of the south of France (Figure 1). Samples collected in the same location at different dates were considered as representing distinct populations. A total of 2988 egg rafts were collected in 16 \( C. \text{pipiens} \) samples (Table 3), and the number of egg rafts per sample varied from 45 to 1163 (between 100 and 150 egg rafts were collected in most samples). Most egg rafts (2950, that is, 98.7\%) were found to be fertile, whereas only 12 (0.4\%) and 26 (0.9\%) appeared intermediate and infertile, respectively. We observed embryonic development in the 26 infertile egg rafts (each harboring embryos in 20–80\% of the eggs), which established that they were laid by mated females rather than by non-inseminated females. It should also be noted that insecticide toxicity,
parasitism or environmental damage can also affect egg hatching, so that this frequency of infertile egg rafts is a conservative maximum incidence of CI egg rafts produced in the field.

Infertile egg rafts were uncommon in all the breeding sites, ranging from 0 to 2.2% (Table 3). In general, the frequencies of fertile, intermediate and sterile egg rafts did not vary significantly between all the populations ($\chi^2 = 27.2$, df = 30, $P = 0.61$). There is no significant difference between the nine populations sampled in 1987 ($\chi^2 = 14.7$, df = 16, $P = 0.55$) or between the six samples of 2005 ($\chi^2 = 7.7$, df = 10, $P = 0.66$). Two locations were sampled in 1987 and 2005 but no significant year effect was found (Fisher's exact test on 2 x 3 contingency table, $P = 0.20$ and 0.14 in Ganges and Viols le Fort, respectively).

No evidence of assortative mating

To test for potential mating preference, cages containing an equal number of individuals from two C. pipiens lines infected by incompatible Wolbachia strains were set up. Two types of trials were studied (Table 4): unidirectional CI (Bifa-A x Bifa-B, with the cross Bifa-A male x Bifa-B female incompatible) and bidirectional CI (Bifa-A x Istanbul and Bifa-B x Istanbul). A total of four cages were set up, including two replicates of the Bifa-A x Bifa-B trial. Assuming random mating, 25 and 50% of incompatible egg rafts are expected in the cases of unidirectional and bidirectional CI, respectively. For each trial, no significant deviation from the random mating hypothesis was found (exact binomial test, all $P > 0.14$; Table 4).

Local predominance of compatible wPip strains

Assuming random mating, we attempted to estimate the theoretical prevalence of incompatible wPip strains in C. pipiens natural populations. Two types of Wolbachia infection ($w_1$ and $w_2$) displaying either unidirectional CI or bidirectional CI were considered. The frequency of egg rafts from CI mating ($E_{CI}$) depends on the frequency $f_{w1}$ and $f_{w2}$ of the $w_1$ and $w_2$ strains, respectively. $E_{CI}$ varies according to the nature of the CI, being twice as high with bidirectional CI than with unidirectional CI. $E_{CI}$ is determined in the case of unidirectional CI by:

$$E_{CI} = f_{w1} \times f_{w2}$$

and in the case of bidirectional CI by:

$$E_{CI} = 2f_{w1} \times f_{w2}$$

The magnitude of the difference between $f_{w1}$ and $f_{w2}$ noted $\delta_{w1w2}$, was used as an indicator of infection diversity and is thus determined by:

$$\delta_{w1w2} = |f_{w1} - f_{w2}|$$

In Figure 2a, $E_{CI}$ is plotted as a function of $\delta_{w1w2}$. $E_{CI}$ cannot exceed 25% in the case of unidirectional CI, and 50% in the case of bidirectional CI. The larger values of $E_{CI}$ occur when the $w_1$ and $w_2$ infections reach similar diversity and is thus determined by:

$$\delta_{w1w2} = |f_{w1} - f_{w2}|$$

In Figure 2a, $E_{CI}$ is plotted as a function of $\delta_{w1w2}$. $E_{CI}$ cannot exceed 25% in the case of unidirectional CI, and 50% in the case of bidirectional CI. The larger values of $E_{CI}$ occur when the $w_1$ and $w_2$ infections reach similar diversity and is thus determined by:

$$\delta_{w1w2} = |f_{w1} - f_{w2}|$$

In Figure 2a, $E_{CI}$ is plotted as a function of $\delta_{w1w2}$. $E_{CI}$ cannot exceed 25% in the case of unidirectional CI, and 50% in the case of bidirectional CI. The larger values of $E_{CI}$ occur when the $w_1$ and $w_2$ infections reach similar frequency ($f_{w1} = f_{w2} = 0.5$), that is, when $\delta_{w1w2}$ is close to 0. Low values of $E_{CI}$ are obtained when one Wolbachia infection is largely dominant ($f_{w1} >> f_{w2}$ or $f_{w1} << f_{w2}$), that is, when $\delta_{w1w2}$ is close to 1 (Figure 2a).

The incidence of infertile egg rafts observed in the field was used to estimate the predicted hypothetical values

Table 3 Characteristics of the egg rafts collected in the C. pipiens samples

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Samples</th>
<th>Fertile</th>
<th>Intermediate</th>
<th>Infertile</th>
<th>$E_{CI}$ (95% c.i.)</th>
<th>$\delta_{w1w2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>G</td>
<td>1 45</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.000 (0.000–0.079)</td>
<td>1.00</td>
</tr>
<tr>
<td>1987</td>
<td>K</td>
<td>2 117</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.008 (0.000–0.046)</td>
<td>0.98</td>
</tr>
<tr>
<td>1987</td>
<td>L</td>
<td>3 118</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.000 (0.000–0.031)</td>
<td>1.00</td>
</tr>
<tr>
<td>1987</td>
<td>A</td>
<td>4 115</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0.009 (0.000–0.050)</td>
<td>0.98</td>
</tr>
<tr>
<td>1987</td>
<td>H</td>
<td>5 112</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.009 (0.000–0.048)</td>
<td>0.98</td>
</tr>
<tr>
<td>1987</td>
<td>B</td>
<td>6 131</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0.022 (0.004–0.064)</td>
<td>0.96</td>
</tr>
<tr>
<td>1987</td>
<td>F</td>
<td>7 1151</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>0.008 (0.000–0.015)</td>
<td>0.98</td>
</tr>
<tr>
<td>1987</td>
<td>D</td>
<td>8 113</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0.017 (0.002–0.061)</td>
<td>0.97</td>
</tr>
<tr>
<td>1987</td>
<td>M</td>
<td>9 67</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.015 (0.000–0.079)</td>
<td>0.97</td>
</tr>
<tr>
<td>1987</td>
<td>N</td>
<td>10 229</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0.026 (0.009–0.055)</td>
<td>0.97</td>
</tr>
<tr>
<td>2005</td>
<td>C</td>
<td>11 87</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.011 (0.000–0.062)</td>
<td>0.98</td>
</tr>
<tr>
<td>2005</td>
<td>J</td>
<td>12 83</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.000 (0.000–0.043)</td>
<td>1.00</td>
</tr>
<tr>
<td>2005</td>
<td>A</td>
<td>13 141</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.000 (0.000–0.026)</td>
<td>1.00</td>
</tr>
<tr>
<td>2005</td>
<td>E</td>
<td>14 142</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0.007 (0.000–0.038)</td>
<td>0.99</td>
</tr>
<tr>
<td>2005</td>
<td>D</td>
<td>15 238</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0.000 (0.000–0.015)</td>
<td>1.00</td>
</tr>
<tr>
<td>2005</td>
<td>I</td>
<td>16 61</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.000 (0.000–0.059)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Abbreviations: CI, cytoplasmic incompatibility; $\delta_{w1w2}$, magnitude of the frequency difference between $w_1$ ($f_{w1}$) and $w_2$ ($f_{w2}$) infections estimated assuming unidirectional CI (uni-CI) and bidirectional CI (bi-CI); $E_{CI}$, frequency of CI egg rafts (calculated using the number of fertile and infertile egg rafts—see text for more details); HR, number of egg rafts collected for each hatching rate category; 95% c.i., 95% confidence intervals of $E_{CI}$ estimated from the binomial distribution.

Table 4 Mating preferences in population cages

<table>
<thead>
<tr>
<th>Population cage</th>
<th>Number of collected egg rafts</th>
<th>Observed $E_{CI}$ (n)</th>
<th>Expected $E_{CI}$</th>
<th>P-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifa-A x Bifa-B (cage 1)</td>
<td>179</td>
<td>0.29 (51)</td>
<td>0.25$^b$</td>
<td>0.30</td>
</tr>
<tr>
<td>Bifa-A x Bifa-B (cage 2)</td>
<td>170</td>
<td>0.24 (40)</td>
<td>0.25$^b$</td>
<td>0.72</td>
</tr>
<tr>
<td>Bifa-A x Istanbul</td>
<td>138</td>
<td>0.44 (60)</td>
<td>0.50$^b$</td>
<td>0.15</td>
</tr>
<tr>
<td>Bifa-B x Istanbul</td>
<td>152</td>
<td>0.50 (76)</td>
<td>0.50$^b$</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Abbreviation: $E_{CI}$, frequency of CI egg rafts.

$^a$Expected $E_{CI}$ under the random mating hypothesis in the case of uni-CI and of bi-CI.

$^b$Exact binomial test.

$w_1$ and $w_2$, Wolbachia strains; $E_{CI}$, frequency of CI egg rafts.
for $\delta_{w1w2}$, assuming that two Wolbachia types are present. The interpretation of egg rafts with intermediate HR remains ambiguous, and they were excluded from the analysis. The analysis was conducted on 2950 fertile (99.1%) and 26 infertile (0.9%) egg rafts, that is, a total of 2976 independent matings. When all the samples were considered together, $\delta_{w1w2}$ estimations were 0.98 and 0.99 for unidirectional CI and bidirectional CI, respectively. When each sample was considered independently, $\delta_{w1w2}$ ranged from 0.95 to 1 for unidirectional CI and 0.97 to 1 for bidirectional CI (Table 3, Figure 2b). These values suggest that one $w$ Pip type (or one set of compatible $w$ Pip strains) is largely dominant within each C. pipiens population.

**Discussion**

The use of WO prophage elements and the transposon ISWpi1 revealed a high level of $w$ Pip genetic diversity in the south of France, where at least 37 $w$ Pip haplotypes were found. The contribution of MGE in the reproductive phenotypes exerted by Wolbachia is currently not clear, but WO prophage may affect the capacity of Wolbachia to induce CI in the Nasonia vitripennis wasp (Bordenstein et al., 2006) and might assist Wolbachia in host cell interactions (Kent and Bordenstein, 2010). Furthermore, MGE are prone to move largely between Wolbachia genomes, and this process could have major implications for functional and evolutionary interactions of Wolbachia with their hosts (Klasson et al., 2008, 2009), and possibly explain the rapid evolution of $w$ Pip-C. pipiens interactions (Echaubard et al., 2010). However, despite an impressive MGE diversity between the $w$ Pip strains found in France, we observed around 99% of fully fertile egg rafts in C. pipiens populations. Notably, in 2005, we found 21 distinct $w$ Pip genetic strains in Viols le Fort, but no incompatible egg rafts. Overall, these results establish that mating between C. pipiens infected by incompatible Wolbachia strains occurs rarely in natural populations from this region. This situation could be explained by (1) the high predominance of a set of $w$ Pip compatible strains within each breeding site or by (2) the occurrence of adaptive mechanism(s) preventing the expression of CI in the field.

With respect to the first hypothesis, the rarity of CI egg rafts suggests that one $w$ Pip CI type is largely dominant within each breeding site, infecting 97–100% of the individuals. In all, 6–21 $w$ Pip genetic strains were found to coexist within each population and it is, therefore, likely that most of the sympatric $w$ Pip strains were compatible. This is corroborated by results of previous crossing experiments which showed that most of the sympatric C. pipiens lines from the south of France are generally compatible (Magnin et al., 1987)—although CI between sympatric lines was reported once (Duron et al., 2006a). The local dominance of compatible $w$ Pip strains fits with the theoretical expectations on CI dynamics and suggests that field populations did not suffer from CI during our survey. In the only other investigation that tracked the occurrence of field CI egg rafts, Barr (1980) recorded a relatively high incidence of incompatible egg rafts (4 of 47, that is, 8.5%) in one Californian C. pipiens population. It is possible that this observation indicates that a $w$ Pip sweep occurred, as it was later confirmed that some mosquitoes from this population were incompatible. However, in the south of France, there is no evidence of a decrease of the overall $w$ Pip genetic diversity over 15 years, which could indicate potential infection sweeps.

The second factor that could explain the low CI rate observed in natural populations is that, incompatible $w$ Pip strains coexist locally but that their hosts do not express CI. In a few host species, such as D. simulans, mating involves older males who induce weaker CI (Hoffmann et al., 1990; Turelli and Hoffmann, 1995), an effect related to the lower Wolbachia density observed in old male testes (Bressac and Rousset, 1993;
Clark et al., 2002, 2003; Veneti et al., 2003). However, the interactions between wPip and C. p. pipiens show subtle differences: CI is expressed at the same intensity throughout the male lifespan, whether males are from laboratory lines or from natural populations (Rasgon and Scott, 2003; Duron et al., 2007b). Indeed, young and old wild C. p. males from Viols le Fort are both able to express complete CI (Duron et al., 2007b). This suggests that the absence of CI mating in C. p. natural populations is not because of a male age effect.

It is also likely that any adaptation suppressing the expression of CI should be selected because CI imposes a substantial cost to the hosts through sterile mating (Rousset et al., 1991). Among these mechanisms, those that reduce or suppress panmixia such as assortative mating may lead to the stable coexistence of incompatible Wolbachia. Our mating experiments disclosed no evidence that C. p. can discriminate between compatible and incompatible partners, corroborating similar results of previous laboratory investigations (Curtis and Adak, 1974; Curtis et al., 1982). We note that such an experimental approach would not be appropriate for females to avoid incompatible males in small-cage experiments. Furthermore, the behavior of C. p. lines could be altered under artificial selection because of long-term laboratory conditions. However, field release of incompatible C. p. males gave rise to high percentages of incompatible egg rafts (Laven, 1967a; Curtis et al., 1982). For instance, Laven (1967a) obtained 100% of incompatible egg rafts within a few weeks of release of incompatible males in natural breeding sites, suggesting that females in the field did not discriminate between compatible and incompatible males.

An alternative mechanism which could also suppress the CI expression could be that C. p. has selected genes restoring the compatibility; individuals infected by incompatible Wolbachia mate randomly but the expression of CI is suppressed by a restorer gene in the host. Although reported once (Sinkins et al., 2005), a large number of investigations failed to identify such a nuclear restorer gene, and suggest a predominantly cytoplasmic determinism of incompatibilities in C. p. (Ghelelo-vitch, 1952; Barr, 1966; Laven, 1967c, b; Raymond et al., 1986; Duron et al., 2006a; Walker et al., 2008).

The natural populations of C. p. harbor high levels of Wolbachia diversity, but most wPip strains coexisting in the same host population are compatible. However, it is likely that different populations harbor incompatible wPip strains and that geographical structuring of host populations prevents these strains from entering into contact. The evidence for this derives from a series of observations. First, CI was frequently observed between C. p. lines obtained from different sampling sites, even close together (~2 km), in the south of France (Raymond et al., 1986; Magini et al., 1987; Guillemaud et al., 1997). Second, the distribution of wPip genetic diversity varies between populations less than 10 km apart, suggesting that population structure has a key role in CI dynamics. Host species harboring distinct Wolbachia strains in different geographic regions were previously described but over large geographical areas (Mercot et al., 1995; Baudy et al., 2003; Keller et al., 2004). The structure of C. p. populations in restricted areas and how it mediates the regional coexistence of incompatible wPip infections still needs to be studied.

**Conflict of interest**

The authors declare no conflict of interest.

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