ORIGINAL ARTICLE

Many compatible *Wolbachia* strains coexist within natural populations of *Culex pipiens* mosquito

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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 of 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.
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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 *w*Pip. More than 99% of *C. pipiens* individuals are found to be infected by *w*Pip within natural populations (Rasgon and Scott, 2003; Duron *et al.*, 2005). Such high prevalence is well explained by the ability of *w*Pip-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; Ghelelovitch, 1952; O'Neill and Paterson, 1992; Guillemaud *et al.*, 1997), as best illustrated by Laven (1967c) who described 17 different crossing types worldwide. The *w*Pip 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 *w*Pip strains (Guillemaud *et al.*, 1997; Baldo *et al.*, 2006). However, recent work has shown more than 60 *w*Pip haplotypes in *C. pipiens* populations and confirmed that CI was associated with infection by genetically distinct *w*Pip strains (Sinkins *et al.*, 2005; Duron *et al.*, 2006a, b). Hence, one would expect that incompatible *w*Pip 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 *w*Pip 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 *w*Pip infections 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 *w*Pip strains from molecular data. Thus, massive crossing experiments would be needed to characterize the CI relationships between all the *w*Pip 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 *w*Pip 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 *w*Pip strains that coexist in *C. pipiens* field populations. To estimate the level of *w*Pip 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 *w*Pip strains coexist.

Materials and methods

Screening of *w*Pip 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 *w*Pip 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

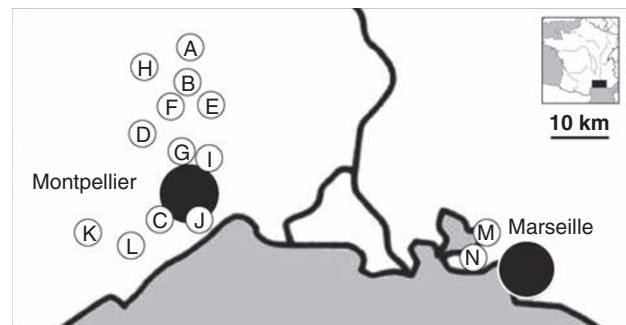

Figure 1 Sample site locations in the south of France. Letters designate the *Culex pipiens* natural breeding sites sampled in this study: A, Ganges; B, Saint Bauzille de Putois; C, Maurin; D, Viols le Fort; E, Notre Dame de Londres; F, Saint Martin de Londres; G, Saint Gelly du Fesc; H, Brissac; I, Prades le Lez; J, Lattes; K, Villeveyrac; L, Poussan; M, Rognac; N, Château les Martigues.

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 12 h light/12 h 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 72 h. 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 *w*Pip genetic diversity between mosquito populations by calculating an un-biased estimate of the *P*-value of a Fisher's exact test on a R × 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 *w*Pip 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 *w*Pip, 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 *w*Pip strains (Table 1). Nomenclature of *w*Pip was defined according to the list previously published (Duron *et al.*, 2006b). Only three *w*Pip strains (*w*Pip5, *w*Pip8 and *w*Pip66) were found in more than 10% of individuals. The *w*Pip8 strain was the most frequent, present in all populations, but its prevalence never exceeded 40% at the population level. The other 34 *w*Pip 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 *w*Pip diversity is represented by rare strains. Note that our method first underestimates the overall *w*Pip 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 ISWpi1 (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 *w*Pip strains on average per population, 6–21 strains coexisting in each population (Table 1). The *w*Pip polymorphism was not uniformly distributed between the six populations. Significant differentiation of the *w*Pip distribution occurred if all populations were considered together (global $F_{ST} = 0.032$; $P < 10^{-5}$), and some pair comparisons between populations also showed significant differences (Table 2). Notably, the distribution of the *w*Pip diversity varied significantly between the three locations examined in 2001 ($F_{ST} = 0.096$; $P < 10^{-5}$), 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 *w*Pip strains at the same time, which indicates that the *w*Pip diversity is geographically structured.

We further examined whether the *w*Pip 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 *w*Pip 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 *w*Pip 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

Table 1 Typing and distribution of the *w*Pip strains found in *Culex pipiens* populations from the south of France

Wolbachia strains	Molecular markers											*Culex pipiens* populations (n = 178)						
	ISWpi1	GP 1b	GP 2a	GP 2b	GP 2e	GP 3a	GP 3b	GP 3c	GP 3d	GP 15a	GP 15b	A90 (n=18)	B90 (n=20)	A01 (n=20)	B01 (n=20)	C01 (n=10)	D05 (n=90)	n
*w*Pip2	—	+	+	—	+	+	—	+	—	—	+		3				2	5
*w*Pip4	+	+	+	—	+	—	—	+	—	—	+		1			2	1	4
*w*Pip5	—	+	+	—	+	—	—	+	—	—	+	2		6	3		9	20
*w*Pip6	—	+	+	—	+	—	—	+	—	—	—		1	1			2	4
*w*Pip7	+	—	+	—	+	—	—	+	—	—	+	1				1		2
*w*Pip8	—	—	+	—	+	—	—	+	—	—	+	3	5	4	1	4	27	44
*w*Pip23	—	—	+	—	—	—	—	—	—	—	—					1		1
*w*Pip36	+	—	+	—	+	+	—	+	—	—	+	1					2	3
*w*Pip37	+	—	+	—	+	—	—	—	—	—	+		1				2	3
*w*Pip38	—	—	+	—	—	—	—	—	—	—	+	1					2	3
*w*Pip39	—	+	—	—	+	—	—	+	—	—	+	2	1	2				5
*w*Pip40	—	+	—	—	+	—	—	+	—	—	—		1				3	4
*w*Pip42	—	—	+	—	—	—	—	+	—	—	—		1			1		2
*w*Pip43	—	—	+	—	+	—	—	+	—	—	—	1					4	5
*w*Pip44	—	+	—	—	—	—	—	—	—	—	+					1		1
*w*Pip45	—	+	—	—	—	—	—	—	—	—	—		1					1
*w*Pip46	—	—	—	—	+	—	—	+	+	—	+				4			4
*w*Pip47	+	+	+	—	+	+	—	+	+	—	+				1			1
*w*Pip48	—	+	+	—	—	—	—	—	—	—	—		1					1
*w*Pip49	+	+	+	—	—	—	—	—	—	—	—		1					1
*w*Pip54	—	—	—	—	—	—	—	+	—	—	+						1	1
*w*Pip61	—	+	+	—	+	—	—	—	+	—	—		1					1
*w*Pip62	—	—	—	—	—	—	—	—	—	—	+		1					1
*w*Pip63	—	+	—	—	—	—	—	+	—	—	+	1	1					2
*w*Pip66	+	+	+	—	+	+	—	+	—	—	+	2	4		7		15	28
*w*Pip67	—	—	+	—	—	—	—	+	—	—	+	2					5	7
*w*Pip68	—	—	—	+	—	—	—	+	—	—	+	2					6	8
*w*Pip69	—	—	+	—	+	+	—	+	—	—	+						2	2
*w*Pip70	—	—	+	—	+	—	—	+	+	—	+						2	2
*w*Pip71	—	+	+	—	—	—	—	—	—	—	—	1					1	2
*w*Pip72	—	+	+	—	—	+	—	—	—	—	+						1	1
*w*Pip73	+	+	—	—	+	+	—	+	—	—	+	1					1	2
*w*Pip74	—	+	+	—	+	—	—	—	—	—	+		1				1	2
*w*Pip75	+	+	—	—	—	+	—	+	—	—	+						1	1
*w*Pip111	+	+	+	—	—	+	—	+	—	—	+		2					2
*w*Pip114	+	+	—	—	+	+	—	+	+	—	+				1			1
*w*Pip115	—	+	+	—	—	—	—	+	—	—	+	1						1

Each *w*Pip strain is here defined by the presence (+)/absence (−) pattern of 11 mobile genetic elements (the transposable element *Tr1* and 10 WO phage genes).

Table 2 Pair-wise F_{ST} measurements and tests of significance of the *w*Pip distribution between *Culex pipiens* populations

Populations	A90	B90	A01	B01	C01	D05
A90		0.081	0.111	0.011	0.045	0.244
B90	0.008		0.057	0.020	0.835	0.006
A01	0.016	0.059		0.000[a]	0.031	0.003[a]
B01	0.036	0.048	0.09		0.002[a]	0.000[a]
C01	0.037	0.007	0.06	0.143		0.005
D05	0.001	0.001	0.04	0.063	0.024	

The upper half shows probabilities based on the null hypothesis of random distribution of *w*Pip diversity between populations. The lower half shows F_{ST} measurements.
[a]The null hypothesis is rejected taking into account a Bonferroni's adjustment for 15 comparisons.

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, *w*Pip5, *w*Pip8 and *w*Pip66, all sharing a set of eight markers, were present at intermediate frequency (~50%) since 1990. This confirms that the overall *w*Pip diversity is maintained for over 15 years, and that sweep events, if any, do not erode the global *w*Pip diversity in *C. 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. 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,

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

Year	Location	Samples	HR			E_{CI} (95% c.i.)	δ_{w1w2}	
			Fertile	Intermediate	Infertile		uni-CI	bi-CI
1984	G	1	45	0	0	0.000 (0.000–0.079)	1.00	1.00
1987	K	2	117	0	1	0.008 (0.000–0.046)	0.98	0.99
	L	3	118	0	0	0.000 (0.000–0.031)	1.00	1.00
	A	4	115	1	1	0.009 (0.000–0.050)	0.98	0.99
	H	5	112	0	1	0.009 (0.000–0.048)	0.98	0.99
	B	6	131	1	3	0.022 (0.004–0.064)	0.96	0.98
	F	7	1151	3	9	0.008 (0.004–0.015)	0.98	0.99
	D	8	113	1	2	0.017 (0.002–0.061)	0.97	0.98
	M	9	67	0	1	0.015 (0.000–0.079)	0.97	0.98
	N	10	229	2	6	0.026 (0.009–0.055)	0.95	0.97
2005	C	11	87	0	1	0.011 (0.000–0.062)	0.98	0.99
	J	12	83	1	0	0.000 (0.000–0.043)	1.00	1.00
	A	13	141	0	0	0.000 (0.000–0.026)	1.00	1.00
	E	14	142	1	1	0.007 (0.000–0.038)	0.99	0.99
	D	15	238	2	0	0.000 (0.000–0.015)	1.00	1.00
	I	16	61	0	0	0.000 (0.000–0.059)	1.00	1.00

Abbreviations: CI, cytoplasmic incompatibility; δ_{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

Population cage	Number of collected egg rafts	Observed E_{CI}(n)	Expected E_{CI} [a]	P-value[d]
Bifa-A × Bifa-B (cage 1)	179	0.29 (51)	0.25[b]	0.30
Bifa-A × Bifa-B (cage 2)	170	0.24 (40)	0.25[b]	0.72
Bifa-A × Istanbul	138	0.44 (60)	0.50[c]	0.15
Bifa-B × Istanbul	152	0.50 (76)	0.50[c]	0.99

Abbreviation: E_{CI}, frequency of CI egg rafts.
[a]Expected E_{CI} under the random mating hypothesis in the case of
[b]Unidirectional CI and of [c]bidirectional CI.
[d]Exact binomial test.

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 2×3 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 × Bifa-B, with the cross Bifa-A male × Bifa-B female incompatible) and bidirectional

CI (Bifa-A × Istanbul and Bifa-B × Istanbul). A total of four cages were set up, including two replicates of the Bifa-A × 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 *w*Pip strains
Assuming random mating, we attempted to estimate the theoretical prevalence of incompatible *w*Pip 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:

$$E_{CI} = f_{w1} \times f_{w2} \tag{1}$$

and in the case of bidirectional CI by:

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

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

$$\delta_{w1w2} = |f_{w1} - f_{w2}| \tag{3}$$

In Figure 2a, E_{CI} is plotted as a function of δ_{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 frequencies ($f_{w1} = f_{w2} = 0.5$), that is, when δ_{w1w2} is close to 0. Low values of E_{CI} are obtained when one *Wolbachia* infection is largely dominant ($f_{w1} \gg f_{w2}$ or $f_{w1} \ll f_{w2}$), that is, when δ_{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
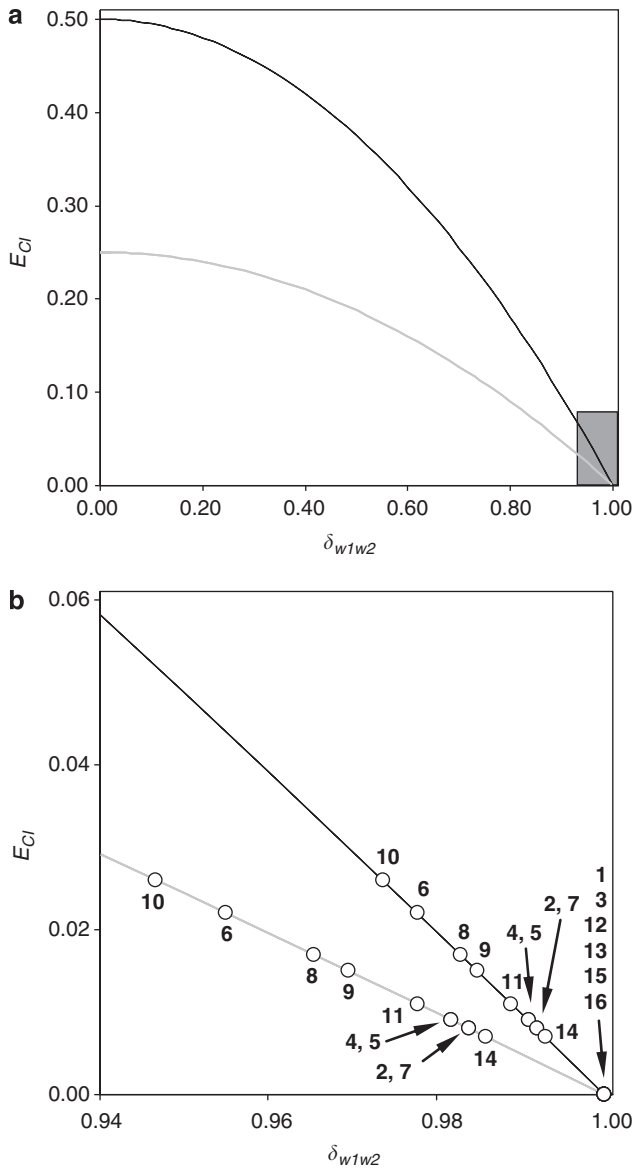
Figure 2 Frequency of CI egg rafts (E_{CI}) as a function of the magnitude of the frequency difference between two incompatible *Wolbachia* infections (δ_{w1w2}). Points on the chart denote the observed E_{CI} values in each *C. pipiens* population (identified by sample numbers, cf Table 3). Gray line, unidirectional CI; black line, bidirectional CI. In (**a**), the gray rectangle denotes the relative range of E_{CI} variations observed in *C. pipiens* populations, as depicted on a larger scale in (**b**).

for δ_{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, δ_{w1w2} estimations were 0.98 and 0.99 for unidirectional CI and bidirectional CI, respectively. When each sample was considered independently, δ_{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 *w*Pip and *C. 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. pipiens* 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. pipiens* 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. pipiens* 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. pipiens* lines could be altered under artificial selection because of long-term laboratory conditions. However, field release of incompatible *C. pipiens* 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 incompatibles males.

An alternative mechanism which could also suppress the CI expression could be that *C. pipiens* 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. pipiens* (Ghelelovitch, 1952; Barr, 1966; Laven, 1967c,b; Raymond *et al.*, 1986; Duron *et al.*, 2006a; Walker *et al.*, 2008).

The natural populations of *C. pipiens* harbor high levels of *Wolbachia* diversity, but most *w*Pip strains coexisting in the same host population are compatible. However, it is likely that different populations harbor incompatible *w*Pip 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. pipiens* lines obtained from different sampling sites, even close together (∼2 km), in the south of France (Raymond *et al.*, 1986; Magnin *et al.*, 1987; Guillemaud *et al.*, 1997). Second, the distribution of *w*Pip 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; Baudry *et al.*, 2003; Keller *et al.*, 2004). The structure of *C. pipiens* populations in restricted areas and how it mediates the regional coexistence of incompatible *w*Pip infections still needs to be studied.

Conflict of interest

The authors declare no conflict of interest.

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