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Tracking factors modulating cytoplasmic incompatibilities in the mosquito *Culex pipiens*

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Abstract

***Wolbachia* are maternally inherited endosymbiotic bacteria that infect many arthropod species and may induce cytoplasmic incompatibility (CI), resulting in abortive embryonic development. One *Wolbachia* host, *Culex pipiens* complex mosquitoes, displays high levels of variability in both CI crossing types (cytotypes) and DNA markers. We report here an analysis of 14 mosquito strains, containing 13 *Wolbachia* variants, and with 13 different cytotypes. Cytotypes were *Wolbachia*-dependent, as antibiotic treatment rendered all strains tested compatible. Cytotype distributions were independent of geographical distance between sampling sites and host subspecies, suggesting that *Wolbachia* does not promote a reproductive isolation depending on these parameters. Backcross analysis demonstrated a mild restoring effect of the nuclear genome, indicating that CI is mostly cytoplasmically determined for some crosses. No correlation was found between the phenotypic and genotypic variability of 16 WO prophage and transposon markers, except for the WO prophage *Gp15* gene, which encodes a protein similar to a bacterial virulence factor. However, *Gp15* is partially correlated with CI expression, suggesting that it could be just linked to a CI gene.**

Keywords: bacteriophage WO, *Culex pipiens*, cytoplasmic incompatibility, mobile genetic elements, nuclear restorer, *Wolbachia*

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Introduction

Wolbachia are maternally inherited bacteria, widespread in arthropods and filarial nematodes. The successful spread of *Wolbachia* in arthropods has been attributed to its ability to alter host reproduction to its own advantage (Rousset & Raymond 1991), by feminization, male-killing, thelytokous parthenogenesis and, most commonly, cytoplasmic incompatibility (CI) (for reviews see, e.g. Werren 1997; Stouthamer *et al*. 1999; Stevens *et al*. 2001). *Wolbachia*-induced CI results in the death of embryos generated by the mating of infected males with uninfected females or with females infected with an incompatible *Wolbachia* strain. CI is generally interpreted as resulting from two bacterial components: a *mod* function (for modification) that affects sperm and induces embryo death, and a *resc* function (for rescue) provided by the *Wolbachia* present in the egg, which restores male and female chromosome compatibilities in compatible crosses (Werren 1997). Thus, in a mixed population, *Wolbachia* that induce CI have a selective advantage, leading to their spread, even if maternal transmission is partial and infection decreases fecundity (Turelli 1994). Indeed, studies of the worldwide prevalence of infection in the mosquito *Culex pipiens* have shown that *Wolbachia* (termed *w*Pip in *C. pipiens*) is fixed in natural populations (Rasgon & Scott 2003; Duron *et al*. 2005), although a few uninfected populations were identified on the border of the host distribution area (Cornel *et al*. 2003).

Crosses between *C. pipiens* mosquitoes of various origins have revealed a high frequency of uni- or bidirectional incompatibilities, contrasting with the low frequency of CI observed in other insects (Laven 1967; Magnin *et al*. 1987; O'Neill & Paterson 1992; Guillemaud *et al*. 1997). In addition, Laven (1967) presented evidence for the geographical separation of cytotypes (i.e. crossing types). *Wolbachia*-induced

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CI may reduce gene flow between populations and has therefore been suggested as a possible mechanism of reproductive host isolation (Bordenstein 2003; Hurst & Schilthuizen 1998; Werren 1998), leading to speciation events within the *C. pipiens* complex (Laven 1967; Werren 1998). The *C. pipiens* complex demonstrates an array of behavioural, morphological and physiological characters reflecting an assemblage of closely related taxa with a worldwide distribution. Two subspecies are presently recognized in the complex: *C. p. quinquefasciatus* and *C. p. pipiens* are southern and northern house mosquitoes, ubiquitous in tropical and temperate regions, respectively. In addition, two coexisting *Culex* forms — *molestus* and *pipiens* — are currently distinguished in temperate areas and correspond to behavioural, physiological and genetical entities (Fonseca *et al*. 2004). The *pipiens* form would be a bird-dependent anautogeneous mosquito (a blood meal is required for batching eggs) that diapauses during winter and need open space to mate (eurygamy). The *molestus* form would be adapted to environments associated with human activity (i.e. mammal-dependence, autogeny, lack of diapause and stenogamy) (Vinogradova 2000).

The presence of different *w*Pip variants is one possible explanation for the complex pattern of CI in *C. pipiens*. However, *w*Pip polymorphism was long considered absent, as no mutation was found in genes described as polymorphic in *Wolbachia* from other host species (Rouxrjxsset *et al*. 1992; Guillemaud *et al*. 1997; Zhou *et al*. 1998). Recently published genome sequences have shown that the *Wolbachia* strains infecting arthropods are unusual in that they contain large numbers of repetitive and mobile genetic elements (Wu *et al*. 2004; Salzberg *et al*. 2005). Among these, the phage WO has showed to be either lysogenic and integrated into the *Wolbachia* chromosome, or lytic and free in the cytoplasm (Masui *et al*. 2000, 2001). It has been suggested that such elements could have a significant effect on genome organization and host reproduction, and might increase the rate of evolution (Brownlie & O'Neill 2005). Consistent with this hypothesis, WO prophage sequences are widespread in reproductive *Wolbachia* parasites (Bordenstein & Wernegreen 2004; Wu *et al*. 2004) but absent from the mutualistic *Wolbachia* lineage (Foster *et al*. 2005). Searches for *w*Pip polymorphism then focused on prophages and transposable elements, which turned out to be highly polymorphic (Sanogo & Dobson 2004; Duron *et al*. 2005; 2006a; Sinkins *et al*. 2005). *Tr1*, an apparently functional transposable element that can be used to discriminate between *w*Pip groups (Duron *et al*. 2005), is homologous to the IS5 transposon, which was used to discriminate between *Wolbachia* variants in *Drosophila melanogaster* that were previously considered identical (Riegler *et al*. 2005).

WO prophage sequences, present in multiple copies dispersed throughout the *w*Pip genome (Sanogo & Dobson 2004; Duron *et al*. 2006a), display particularly high levels of polymorphism, making it possible to identify more than 60 variants in a moderate number of *C. pipiens* strains and field populations (Duron *et al*. 2006a). We investigated the factors involved in CI by analysing 14 mosquito strains infected with different *w*Pip variants for interstrain incompatibilities. We investigated the effects of host subspecies, geographical distribution, *Wolbachia* variability and host nuclear genome on CI expression.

Materials and methods

Mosquito strains

Eleven laboratory strains of *Culex pipiens* complex mosquitoes, established from field-collected larvae, were studied. The countries of origin, years of collection and bibliographic references for each strain are indicated in Table S1, Supplementary material. Geographical distances (from 0 to 17 000 km) between sampling sites were measured using the MAPSOURCE WORLDMAP 3.01 software (Garmin).

Strains were reared in 65 dm^3 screen cages kept in a single room at 22 to 25 °C, under a 12-h light/12-h dark cycle. Larvae were fed with a mixture of shrimp powder and rabbit pellets. Adult mosquitoes were fed honey solution.

We carried out polymerase chain reaction (PCR) for rDNA ITS1 sequences to assign mosquitoes to the *C. pipiens* complex (Miller *et al*. 1996; Severini *et al*. 1996). The taxonomic status of the host was next determined by subspecies-specific restriction digestions of two independent genes, *ace-2* and *ace-1*. The acetylcholinesterase *ace-2* gene discriminates between *quinquefasciatus* and *pipiens/molestus* (Bourguet *et al*. 1998). A PCR/RFLP (restriction fragment length polymorphism) diagnosis was developed for the acetylcholinesterase *ace-1* gene using sequences published by Weill *et al*. (2003). A 526-bp *ace-1* fragment was amplified using primers CxEx3dir (5′-CGA CTC GGA CCC ACT CGT) and CxEx3rev (5′-GTT CTG ATC AAA CAG CCC CGC). A *BsrB*I digestion of the PCR product allowed discrimination between *quinquefasciatus* and *pipiens/molestus*. *BsrB*I cuts twice the *quinquefasciatus ace-1* fragment (positions +135 and +276) and only once the *pipiens/molestus ace-1* fragment (+135). A rapid assay is not yet available to distinguish *pipiens* from *molestus*, but a protocol using eight microsatellite loci exists (Fonseca *et al*. 2004). However, we choose to discriminate these forms basing on ecological criteria, i.e. above-ground origin and anautogeny (*pipiens*) vs. underground origin and autogeny (*molestus*) (Vinogradova 2000).

Wolbachia polymorphism was addressed by analysing: (i) the transposable element *Tr1*, which allow the discrimination of *Wolbachia* variants differing by sequence polymorphism, presence or absence pattern, or insertion site (Duron *et al*. 2005), and (ii) the presence or absence pattern

of 15 WO prophage gene products (Gps) present in multicopy dispersed in the *w*Pip genome (Duron *et al*. 2006a). Ten to 15 females from each strain have been analysed. DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) protocol (Rogers & Bendich 1988). PCR products were sequenced directly, using the Big Dye Terminator kit, and analysed on an ABI PRISM 310 sequencer. Control DNAs from positive and negative strains were included in each PCR.

Uninfected strains were created artificially by antibiotic treatment on at least 10 000 larvae per strain, using a modification of the technique described by Portaro & Barr (1975). Larvae were reared for three generations in a solution containing tetracycline hydrochloride (TC) at concentrations of 10^{-4}, 2×10^{-4} and 4×10^{-4} M for the first, second and third generation, respectively. The effective loss of *Wolbachia* was monitored by amplification of the *wsp* gene, using the specific primers wolpipdir and wolpiprev (Berticat *et al*. 2002). *Wolbachia*-free strains are referred hereafter as TC-treated. TC-treated strains were reared for at least two generations in the absence of tetracycline before being used in experiments, to prevent any possible side-effects of the treatment and to restore the overall bacterial flora (except for vertically transmitted bacteria).

Crossing experiments

Strains were reared for at least 10 generations before crossing experiments, to optimize mating and blood feeding. For each cross, 20–50 females and an equivalent number of males were mated. Ageing effects were reduced by using 2- to 5-day-old adults. Females were allowed to feed on blood after 6 days of mating. Egg rafts were collected daily and the egg-hatching rate (HR) was quantified by counting under a binocular microscope to determine the CI status of each cross. If an egg raft produced no larvae, the spermathecae of the corresponding females were checked for insemination. Egg rafts from noninseminated females were excluded from the comparison. Incompatible crosses were repeated at least twice and the results pooled for analysis. Backcrosses were performed up to the F_7 generation, leading to a replacement estimated of over 97% of the maternal nuclear genome by the paternal nuclear genome, with retention of the maternal cytoplasm. For each generation, 100–200 females from the previous generation were crossed with an equal number of males.

Statistical analysis

We used Mantel's test (Mantel 1967) to assess the relationship between CI pattern and geographical distance or host subspecies. CI data were transformed into a half-matrix, in which crosses were coded according to their level of compatibility. Statistical analyses were performed using GENEPOP freeware (Raymond & Rousset 1995). We used 50 000 randomly permutated matrices to generate statistical distributions, which were used to estimate the probability of obtaining an association between two matrices.

Correlation between genetic polymorphism and CI was investigated using linear models. Each cross was characterized by a combination of qualitative variables corresponding to the *Wolbachia* markers of males and females. We restricted the analysis to two-variable tests, including additive and independent effects. Models were fitted to data, and the significance of the various terms was tested, beginning with the higher-order terms, by means of χ^2 tests (Crawley 1993). Nonsignificant terms ($P > 0.05$) were removed. This process gave a minimal model. Multiple testing was carried out, applying Bonferroni's correction. Calculations were done with R freeware (R Development Core Team 2004).

Results

Identification of Wolbachia *variants and cytotypes of 14 strains*

We examined the variability of *Wolbachia* in 11 mosquito strains, using the *Tr1* (Duron *et al*. 2005) and 15 WO phage DNA markers (Duron *et al*. 2006a). All strains except Kol and Tunis were found to be infected with genetically different *Wolbachia* strains (Table 1). The Bifa, Keo, Kara and Manille strains each contained two or three subpopulations infected with different *Wolbachia* strains and were subcloned to generate the corresponding A, B and C substrains. Fourteen mosquito strains harbouring 13 distinct *Wolbachia* variants were thus established and their cytotypes examined.

We carried out 196 crosses, which were classified (Table 2) as compatible [hatching rate (HR) ≥ 70%], intermediate (70% > HR ≥ 30%) or incompatible (HR < 30%). This classification is based on the bimodal distribution of HR. For interstrain crosses, we obtained 151 crosses with HRs were upper 80% (i.e. compatible crosses), 43 with HRs under 15% (i.e. incompatible crosses) and only two HRs around 50% (i.e. intermediate crosses: ♀ BifaB × ♂ Kol and ♀ Keo-B × ♂ Manille-B). No incompatibility was observed between mosquitoes of the same strain. Hatching heterogeneity — the production of both compatible and incompatible egg rafts from a single cross — was never observed. Pairwise comparison of the 26-cross patterns (crosses with each of the other strains, in both directions) of the 14 strains led to the identification of 13 distinct cytotypes (only Kara-C and Manille-A were identical). Each strain displayed at least one unidirectional incompatible cross (2.8 ± 2.0 mean incompatible crosses). Istanbul was unusual in displaying a high frequency of incompatibility (11.0 ± 1.8 mean incompatible crosses), and this incompatibility was bidirectional

Table 1 Genetic characterization of the strains. *w*Pip groups were determined on the basis of *Tr1* sequences. Gp is the WO gene product. WO allelic sequences are identified by numbers, according to the nomenclature of Duron *et al*. (2006a). Bars indicate the absence of a PCR product. When several *Wolbachia* variants were identified in a mosquito strain, we distinguished between them by adding a letter suffix to the strain name. *, the Istanbul *Gp15* gene was efficiently amplified but differed from *Gp15*a and b (Duron *et al*. 2006)

Strains	Host subspecies	*w*Pip groups	GP1b	GP2a	GP2b	GP2e	GP3a	GP3b	GP3c	GP3d	Gp7d	Gp9a	Gp9b	Gp15a	Gp15b	Gp24a	Gp24b
La Var	*pipiens*	*w*Pip3	1	1	—	1	—	—	2	—	1	1	—	—	2	1	—
Bifa-A	*pipiens*	*w*Pip1	—	1	1	1	2	1	—	1	1	1	1	1	—	1	1
Bifa-B	*pipiens*	*w*Pip1	—	1	—	1	2	—	1	—	—	—	—	—	1	—	—
Kol	*pipiens*	*w*Pip3	—	2	1	1	2	1	—	1	1	1	1	1	—	1	1
Kéo-A	*pipiens*	*w*Pip2-B	—	—	2	1	2	—	3	1	—	1	1	—	3	1	—
Kéo-B	*pipiens*	*w*Pip2-B	—	—	2	1	—	—	3	1	—	1	1	—	3	1	—
Tunis	*molestus*	*w*Pip3	—	2	1	1	2	1	—	1	1	1	1	1	—	1	1
Istanbul	*molestus*	*w*Pip3	1	—	1	1	4	1	3	3	1	1	1	—*	—*	1	—
Aus	*pipiens/quinquefasciatus*	*w*Pip3	—	1	—	2	—	—	2	—	1	1	—	—	1	1	2
Slab	*quinquefasciatus*	*w*Pip1/4	1	1	—	1	2	—	1	—	1	1	—	—	1	1	2
MaClo	*quinquefasciatus*	*w*Pip4	1	1	—	—	3	1	2	—	1	1	—	—	1	1	2
Kara-C	*quinquefasciatus*	*w*Pip3	1	—	—	—	—	—	—	2	—	1	—	2	—	1	—
Manille-A	*quinquefasciatus*	*w*Pip3	—	1	—	—	—	—	—	2	1	1	1	2	—	1	3
Manille-B	*quinquefasciatus*	*w*Pip3	1	1	1	1	5	1	—	—	1	1	1	1	—	—	1

Table 2 Incompatibilities between *Culex pipiens* strains. Each cross was characterized by determining hatching rate (HR), indicating CI level. HR was classified into three categories: 1, HR ≥ 70% (compatible); 2, 70% > HR ≥ 30% (intermediate); 3, HR < 30% (incompatible). Uni- and bidirectional incompatible crosses are shaded in grey, clear and dark, respectively. The number of egg rafts collected is specified in brackets

Female strain	Male strain													
	LaVar	Bifa-A	Bifa-B	Kol	Keo-A	Keo-B	Tunis	Istanbul	Aus	Slab	MaClo	Kara-C	Manille-A	Manille-B
LaVar	1 (8)	1 (10)	**3 (17)**	1 (17)	1 (19)	1 (13)	**3 (21)**	**3 (26)**	1 (20)	1 (8)	1 (10)	1 (4)	1 (9)	**3 (7)**
Bifa-A	1 (21)	1 (14)	1 (18)	1 (21)	1 (5)	1 (26)	1 (16)	**3 (15)**	1 (35)	1 (12)	1 (14)	1 (20)	1 (28)	1 (11)
Bifa-B	1 (18)	**3 (31)**	1 (13)	**2 (12)**	1 (17)	**3 (28)**	1 (17)	**3 (27)**	1 (12)	1 (15)	1 (9)	1 (25)	1 (25)	**3 (18)**
Kol	1 (19)	1 (11)	1(11)	1 (18)	1 (13)	1 (13)	1 (15)	**3 (50)**	1 (12)	1 (30)	1 (24)	1 (7)	1 (22)	1 (8)
Keo-A	1 (15)	1 (15)	1 (11)	1 (17)	1 (13)	1 (15)	1 (6)	**3 (15)**	1 (3)	1 (5)	1 (9)	1 (23)	1 (22)	1 (10)
Keo-B	1 (14)	1 (9)	1 (11)	1 (9)	1 (8)	1 (8)	1 (17)	**3 (10)**	1 (21)	1 (12)	1 (23)	1 (7)	1 (24)	**2 (15)**
Tunis	1 (35)	1 (27)	1 (20)	1 (33)	1 (15)	1 (17)	1 (55)	**3 (35)**	1 (13)	1 (66)	1 (54)	1 (24)	1 (20)	1 (14)
Istanbul	**3 (40)**	**3 (5)**	**3 (11)**	**3 (26)**	**3 (12)**	1 (5)	**3 (30)**	1 (26)	1 (11)	**3 (34)**	1 (31)	**3 (7)**	**3 (5)**	**3 (6)**
Aus	1 (23)	1 (5)	**3 (14)**	1 (11)	1 (11)	1 (18)	**3 (10)**	**3 (7)**	1 (6)	1 (12)	1 (9)	1 (7)	1 (18)	**3 (11)**
Slab	**3 (30)**	1 (18)	**3 (27)**	1 (39)	1 (14)	1 (19)	**3 (31)**	1 (33)	**3 (34)**	1 (14)	**3 (99)**	1 (23)	1 (26)	**3 (19)**
MaClo	1 (36)	**3 (14)**	1 (11)	1 (9)	1 (17)	1 (9)	1 (19)	**3 (53)**	1 (9)	1 (43)	1 (44)	1 (4)	1 (16)	**3 (14)**
Kara-C	1 (9)	1 (10)	1 (11)	1 (9)	1 (6)	**3 (23)**	1 (11)	**3 (7)**	1 (13)	1 (14)	1 (20)	1 (6)	1 (8)	1 (14)
Manille-A	1 (9)	1 (13)	1 (6)	1 (6)	1 (12)	**3 (17)**	1 (5)	**3 (11)**	1 (13)	1 (4)	1 (8)	1 (6)	1 (27)	1 (12)
Manille-B	1 (8)	1 (9)	1 (8)	1 (8)	1 (6)	1 (10)	1 (6)	**3 (8)**	1 (11)	1 (6)	1 (12)	1 (11)	1 (6)	1 (7)

in most cases (67%). The A and B substrains of the Bifa, Keo and Manille populations were mutually compatible, with the exception of crosses between Bifa-A males and Bifa-B females. Thus, CI may even occur within populations.

If we considered female compatibility (*resc* ability) only, the 14 strains formed eight distinct groups. For example, females from the Bifa-A, Kol, Keo-A, Tunis and Manille-B strains behaved identically. The reciprocal analysis of males (*mod* ability) identified nine distinct groups. However, the situation in *Culex pipiens* is complex based on the separate analysis of *resc* and *mod* components, as exemplified by the full compatibility observed between Slab and Keo-A or Slab and Istanbul strains, whereas Keo-A and Istanbul were bidirectionally incompatible.

Wolbachia *is responsible for incompatibilities*

We obtained five *Wolbachia*-cured strains from LaVar, Tunis, Istanbul, Slab and MaClo strains, and compared their CI patterns with those of their infected counterparts,

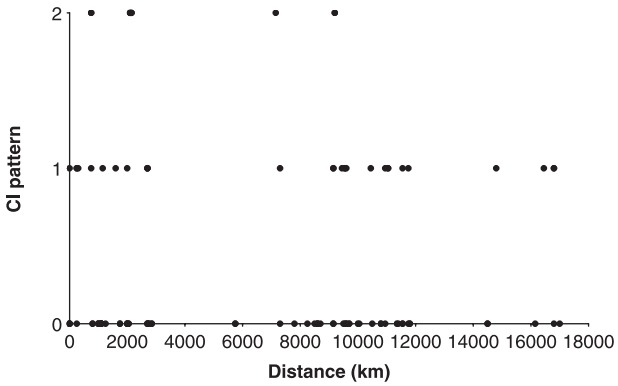

Fig. 1 Occurrence of CI and geographical distances between sampling sites. Each point represents an interstrain cross. The numbers 0, 1 and 2 correspond to compatibility, uni- and bidirectional incompatibility, respectively.

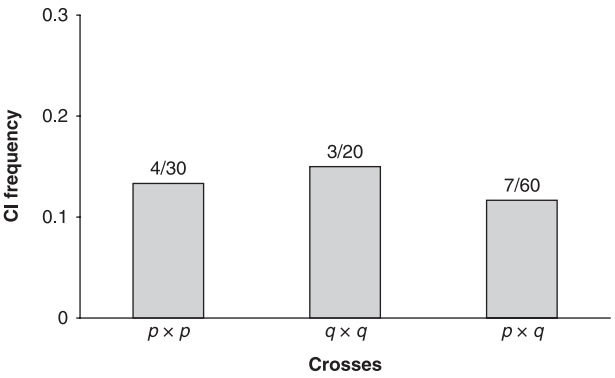

Fig. 2 CI frequency in crosses between the *Culex pipiens* subspecies. The first number corresponds to occurrence of incompatible crosses, and the second, to the total number of interstrain crosses. *p*, *pipiens*; *q*, *quinquefasciatus*.

to demonstrate that the observed incompatibilities resulted from *Wolbachia* infection. Like infected strains, uninfected strains showed no self-incompatibility (data not shown), confirming that *Wolbachia* is not required for fertility in *C. pipiens*. Uninfected females crossed with males from the parental infected strain or from other infected strains gave no offspring (HRs < 1% for 13 cross combinations), showing that all the *w*Pip variants tested (i.e. from LaVar, Kol, Keo-A, Keo-B, Tunis, Istanbul, Slab and MaClo strains) induced incompatibility with *Wolbachia*-free cytoplasms. Conversely, infected males displaying incompatibility with infected females (HR_s < 20%) were found to be fully compatible with these females when uninfected (HR_s > 80% for 15 cross combinations). *Wolbachia* infection is therefore required for incompatibility.

CI does not correlate with geographical distance or host subspecies

The strains analysed here originated from distant regions of the world (five Mediterranean countries, Australia, China, Philippines and California; see Table S1, Supplementary material) and from different subspecies (*C. pipiens pipiens*, *molestus* and *quinquefasciatus*, see Table 1). ITS genotyping of the strains provides evidence for their belonging to *C. pipiens* complex. Subspecies-specific markers demonstrate that the strains have a pure *pipiens*/*molestus* or *quinquefasciatus* origin except for the Aus strain that displayed *quinquefasciatus* and *pipiens*/*molestus* alleles.

We therefore investigated whether CI pattern depended on geographical origin or subspecies. CI frequencies did not vary significantly with geographical distance between strain sampling sites (Fig. 1; Mantel two-sided test; P = 0.96). There was therefore no evidence of geographical isolation of cytotypes. We found also no correlation between CI frequency and the *quinquefasciatus* and *pipiens* subspecies (Fig. 2; Mantel two-sided test, P = 0.80). For clarity, the Aus strain (hybrid) was excluded from the analysis as the *molestus* strains (i.e. Tunis and Istambul). Thus, the frequency of incompatible crosses did not differ significantly within and between these two subspecies. This finding is not consistent with the hypothesis that *Wolbachia*-dependent CI drives the isolation of *C. pipiens quinquefasciatus* and *C. p. pipiens* subspecies.

Influence of nuclear factors

We investigated whether factors other than *Wolbachia* influence CI, by carrying out backcross experiments to construct the Is(*w*KB), KB(*w*Is) and Tu(*w*Sl) strains, in which the Istanbul, Keo-B, and Tunis nuclear genomes were introgressed into the Keo-B, Istanbul and Slab cytoplasms, respectively. For all crosses tested, Is(*w*KB), KB(*w*Is) and Tu(*w*Sl) males gave hatching rates similar to those of the Keo-B, Istanbul and Slab strains, respectively (Mann–Whitney two-sided test; all $P > 0.15$) (Tables 3 and 4). For females, Is(*w*KB) and KB(*w*Is) behaved similarly to Keo-B and Istanbul, respectively (Mann–Whitney two-sided test; all $P > 0.40$), whereas hatching rate was significantly higher for Tu(*w*Sl) than for Slab (27.8% vs. 4.3%, respectively; Mann–Whitney two-sided test; $P < 10^{-2}$) (Tables 3 and 4), providing evidence of a very small, but direct host effect. Thus, the host nuclear genome may influence the expression of CI. However, this effect may be restricted to females and may therefore concern only the *resc* function.

CI is partially correlated with WO phage Gp15 *variants*

We then compared CI patterns with polymorphism data (nucleic acid or amino acid sequences). No correlation with CI was observed for Tr1 and 14 of the 15 WO markers, tested alone or in pairwise combinations. We did not expect

Table 3 Effect of host genome on CI expression in Keo-B and Istanbul strains. Is(*w*KB) corresponds to Keo-B cytoplasm introgressed in the Istanbul nuclear genome, and KB(*w*Is) corresponds to the reverse backcross. For each cross, mean hatching rate (HR) ± standard deviation and number of egg rafts collected (*n*) are reported. Bars indicate that the cross was not performed

	Male strain							
	Keo-B		Istanbul		Is(*w*KB)		KB(*w*Is)	
Female strain	HR	*n*	HR	*n*	HR	*n*	HR	*n*
Keo-B	0.850 ± 0.007	24	0.000 ± 0.000	11	0.902 ± 0.009	23	0.000 ± 0.000	14
Istanbul	0.920 ± 0.057	10	0.899 ± 0.098	10	—	—	—	—
Is(*w*KB)	0.951 ± 0.094	18	0.000 ± 0.000	3	0.890 ± 0.028	15	0.000 ± 0.001	4
KB(*w*Is)	0.901 ± 0.048	7	0.877 ± 0.031	10	0.879 ± 0.042	18	0.962 ± 0.032	8
LaVar	0.900 ± 0.074	18	0.000 ± 0.000	19	0.829 ± 0.058	10	0.001 ± 0.002	9
Keo-A	0.889 ± 0.034	18	0.000 ± 0.000	20	0.934 ± 0.027	12	—	—
Tunis	0.901 ± 0.044	16	0.000 ± 0.000	7	0.861 ± 0.011	14	—	—
MaClo	0.850 ± 0.014	11	0.000 ± 0.000	17	0.907 ± 0.059	6	—	—

Table 4 Effect of host genome on CI expression in Tunis and Slab stains. Tu(*w*Sl) corresponds to Slab cytoplasm introgressed into the Tunis nuclear genome. For each cross, mean hatching rate (HR) ± standard deviation and number of egg rafts collected (*n*) are reported. The only statistically significant effect ($P < 10^{-2}$) of nuclear genome on CI is indicated in bold

	Male strain					
	Tunis		Slab		Tu(*w*Sl)	
Female strain	HR	*n*	HR	*n*	HR	*n*
LaVar	0.101 ± 0.088	*11*	0.902 ± 0.054	*6*	0.874 ± 0.074	*8*
Slab	0.043 ± 0.040	*17*	0.879 ± 0.098	*14*	0.945 ± 0.071	*5*
Tu(wSl)	**0.278 ± 0.110**	**11**	0.851 ± 0.086	18	0.895 ± 0.021	17

to find a conspicuous correlation, because the Kol and Tunis strains had different CI patterns whereas their *Wolbachia* strains were indistinguishable. However, features of the *Gp15* variants suggested a link with CI (Table 5): (i) crosses between *Gp15*a1 females and males of any other strain were fully compatible ($n = 52$), except for Istanbul ($n = 4$); (ii) crosses between *Gp15*a2 females and males of any other strain were also fully compatible ($n = 26$), except for Keo-B ($n = 2$) and Istanbul ($n = 2$); (iii) all crosses between *Gp15*a strains were fully compatible, whatever the allelic variant considered; (iv) *Gp15*a females displayed lower levels of incompatibility than *Gp15*b females (2.4% and 25.5%, respectively; binomial two-sided test, $P < 10^{-7}$). However, the correlation failed in many crosses, especially when *Gp15*b variants were involved (Table 5).

Discussion

The reasons for the complexity of CI in *C. pipiens* have long remained unresolved. We identified 13 cytotypes in 14 strains, consistent with previous findings that CI occurs at a high frequency in *C. pipiens* (Laven 1967; Magnin *et al*. 1987; O'Neill & Paterson 1992; Guillemaud *et al*. 1997). However, even though at least one incompatible cross was observed for all strains, most crosses were fully compatible (77% of the 196 cross combinations). All incompatibilities in *C. pipiens* depended on *Wolbachia*, with compatibility restored in all cases when the bacteria were eliminated by TC treatment. Bidirectional CI was infrequent (found only with the Istanbul strain), consistent with predictions established in a panmictic unit that rare variants inducing bidirectional CI are unlikely to invade or even to persist in the absence of multiple infections (Turelli 1994).

Cytotype diversity and mono-infection in C. pipiens

More than 60 *Wolbachia* variants have been genetically identified in *Culex pipiens*, though it seems an underestimation (Duron *et al*. 2006a). We have identified 13 cytotypes with only 14 genetic variants, suggesting that a very high number of cytotypes occurs in *C. pipiens*. The 13 cytotypes identified probably resulted from infection with single *Wolbachia* variants rather than from multiple infections, as all WO markers were monomorphic in individual hosts (this study; Duron *et al*. 2006a). This finding is consistent with the model predicting that, if multiple infection occurs and there are T phenotypic incompatibility types, then $N = \log_2(T)$ genetically different *Wolbachia* are sufficient to account for the observation, whereas if only mono-infection occurs, $N = T - 1$ genetically different strains are required (Frank 1998). In this study, 13 variants and 13 cytotypes were identified, consistent with the mono-infection hypothesis. However, this finding challenges the prediction that multi-infected females have a reproductive advantage over mono-infected or uninfected females (Frank 1998) — a situation reported for other insects (Rousset &

Table 5 *Gp15* variability and CI pattern. Strains were pooled according to *Gp15* status and their crossing relationships were reported. The first number corresponds to occurrence of CI (including crosses with intermediate hatching rate, i.e. 70% > HR ≥ 30%), and the second, to the total number of crosses. Occurrence of both CI and compatibility in a same category of cross (e.g. males *Gp15*b3 and females *Gp15*a2) is indicated in bold. *, including one cross with intermediate hatching rate

	Male strain					
Female strain	*Gp15*a1	*Gp15*a2	*Gp15*b1	*Gp15*b2	*Gp15*b3	*Gp15*c
*Gp15*a1 (Bifa-A, Kol, Tunis, Manille-B)	0/16	0/8	0/16	0/4	0/8	4/4
*Gp15*a2 (Kara-C, Manille-A)	0/8	0/4	0/8	0/2	**2/4**	2/2
*Gp15*b1 (Bifa-B, Aus, Slab, MaClo)	**9*/16**	0/8	**4/16**	**1/4**	**1/8**	**3/4**
*Gp15*b2 (LaVar)	**2/4**	0/2	**1/4**	0/1	0/2	1/1
*Gp15*b3 (Keo-A, Keo-B)	**1*/8**	0/4	0/8	0/2	0/4	2/2
*Gp15*c (Istanbul)	4/4	2/2	**2/4**	1/1	**1/2**	0/1

Solignac 1995; Sinkins *et al*. 1995; Werren 1995; Perrot-Minnot *et al*. 1996; Vavre *et al*. 1999; Jeyaprakash & Hoy 2000; Van Borm *et al*. 2001; Jamnongluk *et al*. 2002; Kondo *et al*. 2002). However, in Frank's model all *Wolbachia* variants are mutually incompatible, which was clearly not the case in our study.

All w*Pip variants induce complete CI with* Wolbachia*-free cytoplasm*

All *w*Pip variants induced unidirectional and complete CI with each of the TC-treated, *Wolbachia*-free females tested, consistent with infection being fixed in *C. pipiens* populations worldwide (Duron *et al*. 2005). However, after fixation, *Wolbachia* variants not inducing CI (i.e. infected variants compatible with *Wolbachia*-free cytoplasm) may emerge and spread (Turelli 1994). This situation occurs naturally in *Drosophila simulans* (Merçot & Charlat 2003), although it is unclear whether the non-CI-inducing variants emerged before or after the fixation of infection. The situation is clearly different in *C. pipiens*, suggesting that *Wolbachia* expansion in *C. pipiens* may have occurred too recently for the emergence of non-CI-inducing *Wolbachia*. Consistent with this hypothesis, it has been suggested that the mitochondrial variants of *C. pipiens* diverged 100 000 years ago (Guillemaud *et al*. 1997). However, the large number of cytotypes found in this complex highlights the strong competition between *w*Pip variants, which may result in the maintenance of *mod* capacity.

Wolbachia *does not induce reproductive isolation between geographically distant populations and subspecies*

Laven (1967) and Werren (1998) suggested that *Wolbachia* might induce reproductive isolation between geographically distant populations and subspecies. It was supposed that proximate populations that exchange a lot of migrants displaying: (i) unidirectional CI could be infected sooner by a same cytotype that have replaced another one; (ii) bidirectional CI could enter into a source-sink spiral and the demographically weaker one will go to extinct. So, both uni- and bidirectional CI should be more frequently found at larger distances. However, our data did not support this hypothesis. Indeed, some strains from different continents were found to be compatible, whereas others from the same country were incompatible. This situation could be explained by a recent expansion in association with human activity. A long-distance gene flow is well established in this species complex (Raymond *et al*. 1991) and could prevent a clear correlation. In addition, the frequency of incompatible crosses did also not differ significantly within and between the subspecies (i.e. *pipiens* and *quinquefasicatus*). These findings are consistent with previous reports of a lack of correlation between cytotypes and subspecies in Australia (Irving-Bell 1983). This may be due to the low frequency of bidirectional incompatibility (observed only with Istanbul), such incompatibility being required for complete reproductive isolation. The original isolation that led to the genetic differences between pure populations of *pipiens* and *quinquefasciatus* may have been influenced by *Wolbachia* and some of the isolation may still be affected by *Wolbachia* but extensive hybridization may have now blurred the picture. The *C. pipiens* complex seems not to display postzygotic barriers because all crosses with uninfected males were fully compatible and hybrids were viable and fertile, as exemplified by the Tu(*w*Sl) backcross (*molestus* and *quinquefasciatus*). Prezygotic barriers as mating behaviour (e.g. stenogamy vs. eurygamy) may then prevent hybrid formation more efficiently.

Intrapopulation cytotype heterogeneity

Several of our laboratory strains had been found to produce a mixture of compatible and incompatible egg rafts. Genotyping with WO markers demonstrated that these strains were heterogeneous, being infected with two

or more *w*Pip variants. The single-*w*Pip lines derived from these strains produced homogeneous egg rafts, ruling out the multi-infection hypothesis. Four of 11 strains had heterogeneous cytotypes and *w*Pip variants, but this proportion may be higher in field populations, because some *w*Pip variants were probably lost during the period of acclimatization (at least 10 generations). For example, local *w*Pip polymorphism is particularly frequent in Southern Europe, with up to 10 genetic variants identified in a single population (Duron *et al*. 2006a). The high frequency of heterogeneous populations highlights the need to check for polymorphism and to use only homogenous substrains, to prevent errors in interpretation.

CI was never observed between substrains isolated from the same population, with the exception of crosses between Bifa-A and Bifa-B, which displayed unidirectional and complete CI. It has been predicted that *Wolbachia* variants generating CI are unlikely to coexist in a stable manner within a population (Rousset *et al*. 1991). However, this situation may arise in several ways. First, a high rate of migration between populations infected with incompatible variants may maintain the polymorphism locally. This hypothesis is supported by long-distance gene flow in *C. pipiens* (Raymond *et al*. 1991) and the coexistence of incompatible cytotypes in proximal populations (Magnin *et al*. 1987). Second, the observed polymorphism may result from a recent invasion, in which case it is likely to disappear eventually. Finally, it may result from strict cytotype-specific homogamy. Although this has never been reported in *C. pipiens* (see Rousset *et al*. 1991), our Bifa strain was maintained for about 15 generations before the A and B substrains were isolated, so homogamy may have occurred in each of the substrains.

Influence of nuclear genome on CI

Although it is generally accepted that selection for nuclear genes restoring compatibility, even only partially, is likely (Rousset *et al*. 1991), our backcross experiments provided no evidence that efficient nuclear restorers were present. In three introgressed lines, only a slight effect of the Tunis nuclear genome was observed in the Tu(*w*Sl) backcross, in which *resc* function was partially modified. It demonstrates a weak effect of maternal nuclear restorer and suggests that CI determinism could be exclusively cytoplasmic in some cases. It corroborates the rarity of maternal nuclear restorer observed in pioneering studies that demonstrated the exclusive maternally inheritance of incompatibility in *C. pipiens* (Ghelelovitch 1952; Laven 1953, 1957, 1967; Barr 1966; Irving-Bell 1983). In a unique case, a complete maternal restorer and a partial paternal one were described in two bidirectionally incompatible Asian strains (Sinkins *et al*. 2005). This heterogeneity of results confirms that the restorer genes are polymorphic in mosquito populations and that their effect depends on strain combinations. Because restorers could reduce selective pressures between cytotypes, and decrease the impact of CI in natural populations, their frequency needs to be investigated and estimated. Rousset *et al*. (1991) suggested that restorers may be lost by genetic drift in strains with a long laboratory history. This implies that CI may be less common between strains recently established from natural populations than between strains established some time ago and maintained in the laboratory. However, our strains established in 2003 (Keo, Istanbul or Manille) gave the same proportion of incompatible crosses as Slab and MaClo, established in 1950 and 1984, respectively.

Variability of mobile genetic elements does generally not correlate with CI

Tr1 and WO genes differ in nucleotide sequence, presence/absence pattern, or insertion site, demonstrating considerable dynamism within the *w*Pip genome. The mobility of these elements masks linkage with CI expression, no correlation being observed for *Tr1* and 14 of the 15 WO genes tested. We have obtained no relation between the variability of Gp3 (orf7) and CI pattern as previously described by Sanogo *et al*. (2005).

However, we found a partial correlation between the *Gp15* gene and CI expression, consistent with previous data acquired for a small number of crosses (Duron *et al*. 2006a) although correlation failed in some crosses. The *Gp15* gene, which has a sequence similar to that of a virulence-related protein gene (see Fujii *et al*. 2004), may be a genuine CI gene but *Gp15* could be just in linkage disequilibrium with a neighbouring CI factor. In addition, as the sex-specific expression of a phage gene has been reported in some strains (Sinkins *et al*. 2005; Sanogo & Dobson 2006), differential production of the CI protein may occur in *w*Pip variants and should be taken into account in future studies.

The absence of linkage observed in *C. pipiens* suggests that WO phage sequences may be or may have been mobile elements. If this proves to be the case, it will be difficult to identify the factors responsible for CI directly, even if they are WO phage sequences, as these mobile elements are the only polymorphic markers currently available. However, even in species in which *Wolbachia* variants (determined using *fts*Z, *wsp* or 16S DNA sequences) clearly correlate with cytotypes, such as *Nasonia vitripennis* (Perrot-Minnot *et al*. 1996), *Drosophila simulans* (Rousset *et al*. 1992) and *Aedes albopictus* (Xi *et al*. 2005), the factors responsible for CI have not yet been identified. Variability in *Wolbachia* density seems not clearly involved in cytotype variability observed in *C. pipiens*. Variable densities of the same *Wolbachia* in *C. pipiens* males do not modify CI outcomes (Duron *et al*. 2006b). It drives the conclusion that quantitative variables (i.e. *Wolbachia* density) represent weak

confounding factors relatively to qualitative variables (i.e. *Wolbachia* variant) for CI expression in *C. pipiens*. Given the high level of diversity of *C. pipiens* cytotypes, CI may depend on a combination of several *w*Pip factors, generating a distorted overall picture that may be difficult to unravel. Functional rather than genetic studies will be necessary to implicate candidate genes directly in CI. In addition, the potential of phages to transfer genetic material among parasitic *Wolbachia* variants may help to increase plasticity and to generate new variant. Such processes could have deep impacts on bacterial evolution and their importance remain to be determined in *Wolbachia*.

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Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2996/MEC2996sm.htm

Table S1 Name, countries or areas of origin, year of collection and bibliographic references for C*ulex pipien*s strains.

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