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Hypervariable prophage WO sequences describe an unexpected high number of *Wolbachia* variants in the mosquito *Culex pipiens*

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Wolbachia are maternally inherited endosymbiotic bacteria that infect many arthropod species and may induce cytoplasmic incompatibility (CI) resulting in abortive embryonic development. Among all the described host species, mosquitoes of the *Culex pipiens* complex display the highest variability of CI crossing types. Paradoxically, searches for polymorphism in *Wolbachia* infecting strains and field populations hitherto failed or produced very few markers. Here, we show that an abundant source of the long-sought polymorphism lies in WO prophage sequences present in multiple copies dispersed in the genome of *Wolbachia* infecting *C. pipiens* (*w*Pip). We identified up to 66 different *Wolbachia* variants in *C. pipiens* strains and field populations and no occurrence of superinfection was observed. At least 49 different *Wolbachia* occurred in Southern Europe *C. pipiens* populations, and up to 10 different *Wolbachia* were even detected in a single population. This is in sharp contrast with North African and Cretan samples, which exhibited only six variants. The WO polymorphism appeared stable over time, and was exclusively transferred maternally. Interestingly, we found that the CI pattern previously described correlates with the variability of Gp15, a prophage protein similar to a bacterial virulence protein. WO prophage sequences thus represent variable markers that now open routes for approaching the molecular basis of CI, the host effects, the structure and dynamics of *Wolbachia* populations.

Keywords: *Wolbachia*; *Culex pipiens*; cytoplasmic incompatibility; prophage; WO

1. INTRODUCTION

Wolbachia are maternally inherited endocellular bacteria widespread among arthropods and filarial parasitic nematodes. In several species like the mosquito *Culex pipiens*, *Wolbachia* induce cytoplasmic incompatibility (CI) leading to embryonic mortality that occurs when infected males mate either with uninfected females or with females infected by incompatible *Wolbachia* strain(s) (Yen & Barr 1973). Thus, in a mixed population, *Wolbachia* that induce CI get a selective advantage and are predicted to spread up to fixation (Rousset & Raymond 1991; Turelli & Hoffmann 1999). In support of this hypothesis, the prevalence of *Wolbachia* infecting *C. pipiens* (called *w*Pip) was investigated worldwide, and appeared fixed in 67 populations studied (Duron *et al.* 2005). Crosses between mosquitoes from various origins revealed a high level of incompatibilities (Laven 1951, 1967; Barr 1966; Subbarao 1982; Magnin *et al.* 1987; Guillemaud *et al.* 1997) contrasting with other insect situations (Werren 1998). However, the higher CI level was observed between mosquitoes from Europe, where it exhibits an extreme pattern (Laven 1967; Magnin *et al.* 1987; Guillemaud *et al.* 1997).

A straightforward hypothesis to explain the complex CI pattern in *C. pipiens* is the presence of different *Wolbachia* strains. However, no polymorphism was observed in strains displaying incompatibilities using the *ftsZ* gene (Guillemaud *et al.* 1997), driving the authors to conclude that factors other than *Wolbachia* variants were responsible for incompatibility in this species. Similarly, no polymorphism could be detected either in the 16S *r*RNA sequences of *Wolbachia* infecting different *C. pipiens* subspecies (Rousset *et al.* 1992) or in the fastest *Wolbachia* evolving gene *wsp* of mosquitoes sampled worldwide (Duron *et al.* 2005). Recent searches for polymorphism in *w*Pip have produced a few markers, all affecting mobile genetic elements. Sanogo & Dobson (2004) delineated three *w*Pip variants among 11 laboratory strains by analysis of the variability of *orf7* copy number in the WO prophage. Sinkins *et al.* (2005) described two *w*Pip variants based on polymorphism of ankyrin-repeat encoding genes (*pk* genes) associated with a prophage region. We identified previously five distinct *w*Pip strains from 531 mosquitoes by analysing the polymorphism of *Tr1*, an apparently functional transposable element of the IS5 family (Duron *et al.* 2005). *w*Pip *Tr1* genetic diversity appeared geographically structured and independent of the *C. pipiens* subspecies status, and affected mostly European populations (four variants detected). Nevertheless, neither *orf7* nor *pk* or *Tr1* markers were sensitive enough to explain the 17 cytotypes present in Laven's (1967) crosses.

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The purpose of this study was to identify polymorphic markers that could describe the complex CI pattern found in *C. pipiens*. To this end, we examined the variability of prophage sequences, described as major contributors of genomic flux in bacteria. WO prophage sequences have been shown to be widespread in the genomes of 35 *Wolbachia* infecting diverse arthropods and to transfer at high rates between divergent lineages (Bordenstein & Wernegreen 2004; Gavotte *et al.* 2004). This led to the suggestion that bacteriophages could drive significant gene transfer and evolutionary changes in the genomes of *Wolbachia* and that phage proteins might be linked to CI directly. Here, we report an unprecedented level of *Wolbachia* polymorphism from the analysis of 15 WO prophage sequences in 12 laboratory strains and 19 natural populations.

2. MATERIAL AND METHODS

(a) *Mosquito collections*

Mosquitoes were collected in breeding sites and raised to the adult stage. They were either stored in liquid nitrogen for further analyses (field samples), or bred in the laboratory (strains). For each sample, references, subspecies (only for strains), years and countries of origin are indicated in tables S1 and S2 in the electronic supplementary material.

(b) *Prophage transmission*

Cytoplasmic transmission of WO prophage markers was investigated by using reciprocal crosses between two fully compatible mosquito strains (Keo-A and LaVar), infected by distinct *Wolbachia* (50 males crossed with 50 females). F_1 larvae (randomly sampled, $N=10$ for each cross) were screened by PCR for the presence of maternal- or paternal-specific WO prophage markers (Gp1b, Gp3a and Gp7d).

A second inheritance analysis was performed on F_6 larvae from backcrosses between Istanbul females and Keo-B males. In the first generation, 100–200 females were crossed with equal number of males. In the next generation, F_1 females were crossed with Keo-B males. The same procedure was repeated in each generation. F_6 adults ($N=15$) were screened by PCR for the presence of maternal- or paternal-specific WO prophage markers (Gp1b, Gp3b, Gp3d, Gp7d and Gp15b).

Horizontal phage transfer was tested between Keo-A and LaVar strains by feeding Keo-A larvae with crushed LaVar mosquitoes (larvae and adults) as a unique source of food from the first to the adult stages. Fifteen adults were screened by PCR for the presence of maternal- or paternal-specific WO prophage markers (Gp1b, Gp3a and Gp7d).

(c) *Database analysis*

The *w*Pip genome was searched for CauB WO prophage homologues using the Sanger's Institute Web facilities (http://www.sanger.ac.uk/Projects/W_pipientis/). The *w*Sim genome (partial assembly) was searched by Blast analysis on a local computer. Homologues to Gp 1 to Gp 13 (2 copies): AAGC01000-020;-246;-599;-315;025;-248;-172;-391;-122;-350;-372;-035;-368;-596;-469;-142. Homologues to Gp14 to 24 (single copy): AAGC01000-104;-360;-424;-355;-322;-531;-494;-139;-449;-541;-363;-529.

(d) *PCR and sequencing*

Mosquito DNA was extracted using a CTAB protocol (Rogers & Bendich 1988). Assays for WO prophage sequences were performed by PCR amplification using specific primers listed in table S3 of the electronic supplementary material. The main issue was to amplify unique open reading frames (ORFs) from multiple copies. For example, designing new primers was necessary to amplify specifically the recently described Gp3c gene product (termed as *orf7c* in Sanogo & Dobson 2004), since the published primers turned out not to be specific and amplified Gp3c, Gp3d and Gp3e copies concomitantly. The PCR was run for 30 cycles (94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min). At least four mosquitoes were assayed for each laboratory strain. To study sequence polymorphism, sequencing was performed directly on PCR products on an ABI prism 310 sequencer using the Big Dye Terminator kit. Two Control DNAs corresponding to positive and negative strains were included in each group of PCR.

Loss of *Wolbachia* in tetracycline treated strains was assessed by PCR amplification of a fragment of the *wsp* gene using the specific primers wolpipdir and wolpiprev described by Berticat *et al.* (2002). In all mosquitoes negative for *Wolbachia* infection after treatment the quality of their DNA was checked using the acetylcholinesterase *ace-2* gene amplification (Weill *et al.* 2000).

3. RESULTS

(a) *Multiple WO prophage open reading frames copies in the wPip* genome

A recent paper reported the nearly complete DNA sequence of a WO phage isolated from *Wolbachia* infecting the almond moth *Cadra cautella* (*w*CauB; Fujii *et al.* 2004). Twenty-four ORFs were described, including a structural gene module and genes for replication and lysogenic conversion. We took opportunity of the recently available genome of *w*Pip to search for sequences homologous to the 24 gene products (Gp). We identified 22 clusters (labelled A–V) encoding variable numbers of proteins, from one unique partial sequence up to 15 clustered complete ones (table 1). It is noteworthy that the *w*Pip genome assembly is still underway and determination of the final picture of the WO prophage family must await completion of the assembly. For clarity, for each cluster, Gp are numbered as those of the *w*cauB1 WO phage and for each Gp, variants detected by DNA sequencing are identified by a lower case letter. The number of ORF copies varied from 2 to 6 in the *w*Pip genome, whereas only two WO prophage clusters were found in the *w*Mel genome, one complete and the other partial (Gp1 to Gp13; Wu *et al.* 2004). Blast analysis of the partially assembled genome of *Wolbachia* infecting *Drosophila simulans* (*w*Sim, Salzberg *et al.* 2005) detected two copies for Gp1 to Gp13 and a single one for Gp14 to 24, suggesting a WO prophage organization identical to *w*Mel (see table 1).

(b) *Polymorphism of WO prophage ORF copies*

(i) *Presence/absence of polymorphism*

We addressed WO prophage variability by analysis of the presence or absence of specific Gp copies (markers) on individual mosquitoes from 12 *C. pipiens* strains derived from populations sampled worldwide (table 2 and S1). All markers were absent from tetracycline-treated *Wolbachia*-free mosquitoes strains, which confirmed the bacterial origin of the WO phage clusters (not shown). Unexpectedly, all markers were

Table 1. Schematic representation of WO phage ORFs in the *w*Pip, *w*Mel and *w*Sim genomes. (For each cluster, the accession number (http://www.sanger.ac.uk/Projects/W_pipientis/), the identifier used in this study (A–V) and the occurrence of WOcauB1 ORF homologues is indicated. ORF numbering refers to Fujii *et al*. (2004). Variant ORFs copies are identified by a small case letter. Shading indicates clusters and ORFs analysed in the study. Gp3c corresponds to an additional variant referred to as *orf7* (Sanogo & Dobson 2004), absent from the *w*Pip genome (Pel strain; Amin & Pereis 1990). At the bottom are shown *w*CauB WO homologues encoded in the *w*Mel genome (cluster (A, B) and single loci (C, D), Wu *et al*. 2004) and the in the *w*Sim genome (Salzberg *et al*. 2005), without taking into account ORF organization. *w*Pip genome description corresponds to the current situation and will certainly change in term of clusters and copies number when the assembling is achieved.)

	ORF	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	No. loci	5	6	7	6	6	5	5	5	4	5	5	7	4	4	6	2	2	2	2	5	3	2	2	2
	Clusters																								
A	176e09.p1k	a	a	a	a	a	a	a	a	a	a														
B	62f06.p1k	a	b	b	b	b	b	b	b	b	b	b	b	b											
C	171c03.p1k	b	c												b	a	a	a	a	a	a	a	a	a	a
D	8g08.q1ka	c	d	d	c	c	c	c	c	c	c	b	b	b	b	a					a				
E	96g11.p2kA12			e	d	d																			
F	ends-02h07.q1k	a																							
G	158g06.p1k		f	f																					
H	ends-03a06.p1k		e	f																					
I	139c09.q1k			b																					
J	6b07.p2kA12				e	e																			
K	138b03.q1k				e	d	d	d	c	c	c	b	b													
L	116h03.p1k						a	e	a																	
M	ends-02b01.p1k										a	a	a	b											
N	140f11.p1k											c	c												
O	8e03.q1ka												d	c											
P	ends-01f06.q1k												e												
Q	159e01.p1k														b	a									
R	180e07.p1k														c	b	b	b	b	b	b	b			
S	8e06.q1ka															a					a				
T	6b05.q1k															c									
U	101g02.p1k																				a				
V	77h04.q1k																					a	b	b	b
	orf-7c			c																					
	Nb loci	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	2	1	1	1	1
	wMel WO-A/wSim	b	b	b	b	b	b	b	b	b	b	b	b	b												
	wMel WO-B/wSim	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a		a	a	a	a
	wMel WO-C/wSim																				a				
	wMel WO-D/wSim																				a				

polymorphic in the 12 laboratory strains (table 2). Apart from Tunis, Bismuth, Bifa-A and Kol which displayed the same pattern, all strains were different, from two copies (e.g. Slab and LaVar) up to 11 copies (e.g. Tunis and Ducos; table 2). This analysis also showed that 4 of the 12 mosquito strains (Bifa, Keo, Kara and BJBJT) displayed a mixture of *Wolbachia* variants that can differ by up to 12 markers (Bifa-A and Bifa-B). Interestingly, Gp15a and Gp15b fragments were found to be mutually exclusive in all examined strains, whereas they both are present in the *w*Pip genomic sequences (table 1).

(ii) *Single nucleotide polymorphism*

To get a more accurate view of WO phage polymorphism, we determined and compared the sequences of PCR products for the 15 markers. A high level of single nucleotide polymorphism was found in eight markers (table 2). This suggests that the absence of some PCR products is most likely a consequence of mutations in the priming regions. This was demonstrated for the Istanbul Gp15, which was amplified with Gp14 and Gp16 primers and whose DNA sequence showed divergent Gp15 priming sites (not shown). Ninety per cent of the mutations found in ORFs were conservative, indicating that the encoded proteins are subject to selective constraints and are probably functional (data not shown). Pairwise analysis of cluster A and B markers revealed no significant linkage desequilibrium (GENEPOP software; Raymond & Rousset 1995), suggesting a high rate of mutations and/or recombination events. For each individual, products amplified and sequenced were all monomorphic, showing the absence of multiple infections in our samples.

Table 2. Patterns of presence or absence of WO phage PCR markers in *C. pipiens* strains. (For each strain, PCR was performed using the amplimer sets listed in table S3. Shaded boxes indicate positive PCR reactions. Allelic sequences of PCR products are identified by numbers. The Pel strain pattern (deduced from the *w*Pip genome analysis) was chosen as a reference. When several *Wolbachia* variants were identified in the same mosquito strain, distinction was made using a suffix-letter added to the mosquito strain name. References, subspecies, years and countries of collection are reported in table S1.)

cluster	C	A	B	H	A	B	*orf-7c*	D	K	A	B	C-D-Q	R	C	V
	GP1b	GP2a	GP2b	GP2e	GP3a	GP3b	GP3c	GP3d	GP7d	GP9a	GP9b	GP15a	GP15b	GP24a	GP24b
Slab	1	1	_	1	2	_	1	_	1	1	_	_	1	1	2
La Var	1	1	_	1	_	_	2	_	1	1	_	_	2	1	_
Bifa-A	_	1	1	1	2	1	_	1	1	1	1	1	_	1	1
Bifa-B	_	3	_	1	2	_	2	_	_	_	_	_	1	_	_
MaClo	1	1	_	_	3	1	2	_	1	1	_	_	1	1	2
Istanbul	1	_	1	1	4	1	3	3	1	1	1	_	_	1	_
Tunis	_	2	1	1	2	1	_	1	1	1	1	1	_	1	1
Bismuth	_	2	1	1	2	1	_	1	1	1	1	1	_	1	1
Kol	_	2	1	1	2	1	_	1	1	1	1	1	_	1	1
Ducos	1	_	_	1	2	_	1	_	1	1	_	_	1	_	_
Keo-A	_	_	2	1	2	_	3	_	_	1	1	_	3	1	_
Keo-B	_	_	2	1	_	_	3	_	_	1	1	_	3	1	_
Kara-A	1	_	_	_	3	_	_	2	1	1	_	2	_	1	_
Kara-B	_	_	_	_	3	_	_	2	1	1	_	2	_	1	_
Kara-C	1	_	_	_	_	_	_	2	_	1	_	2	_	1	_
BJBJT-A	1	_	_	1	2	1	_	_	_	1	1	1	_	1	_
BJBJT-B	1	_	_	1	2	_	_	2	_	1	1	1	_	1	_
BJBJT-C	1	_	_	_	2	1	_	_	_	1	1	1	_	1	_
Pel	1	1	1	1	1	1	_	1	1	1	1	1	1	1	1

(c) *WO prophage transmission*

Phage inheritance was investigated using reciprocal mass crosses between two fully compatible mosquito strains (Keo-A and LaVar), infected by distinct *Wolbachia* (table 2). All F_1 larvae (randomly sampled, $N=10$ for each cross) displayed a maternally inherited pattern. A second inheritance analysis was performed on F_6 larvae from backcrosses between Istanbul females and Keo-B males. All 13 F_6 adults tested displayed the Istanbul pattern, confirming maternal phage transmission.

We next investigated horizontal phage transfer through feeding Keo-A larvae with crushed LaVar mosquitoes (larvae and adults) as unique source of food from the first to the adult stages. All 15 adults tested showed a Keo-A pattern. Paternal or horizontal transfers, thus seem to occur at extremely low levels if any.

(d) *WO prophage polymorphism in field populations*

Inter-individual variability within strains suggested that WO polymorphism exists in natural populations. To address this issue, we examined field populations from southern Europe and North Africa. For simplification, we restricted the analysis to 10 phage markers that discriminate all our strains (see legend of table S4 for details). Analysis of 183 mosquitoes from 19 locations (figure 1, table S2) revealed extreme *Wolbachia* variability, identifying up to 36 additional variants (table S4). The same analysis using the *Tr1* transposase (Duron *et al.* 2005) generated 7 additional variants.

Altogether with those already found in the laboratory strains, the combination of WO prophages and *Tr1* PCR assays thus discriminated up to 61 variants in 207 insects from strains and field populations. This represents an underestimation considering the high single nucleotide polymorphism found in strains.

In southern Europe, WO prophage variability was detected in all populations despite low sample sizes. Forty-nine different variants were observed in 103 mosquitoes tested. For most locations, 2–10 variants differing by 1 to 6 markers were observed, a value within the range of the observed inter-strain variations (table S4, electronic supplementary material). Moreover, inter-population polymorphism was unexpectedly high between neighbouring populations sampled during the same period, as illustrated by the Portuguese populations 1 and 2, or the Spanish populations 3 and 4 (figure 1). French populations 7 and 8 were sampled at the same place with an interval of 11 years but display distinct variants.

A dramatically contrasting picture emerged from the analysis of Algerian, Cretan and Tunisian *C. pipiens* populations, in which only six variants were identified. All variants were distinct from southern European ones. In Tunisia variant 11 appeared nearly at fixation, identified in 65 of 68 mosquitoes analysed from six locations spreading over 500 km. The three remaining variants were found only once in population 16, sampled in 1996 near Monastir. In contrast to southern European populations, North African polymorphism appeared stable over time: we found a strictly identical pattern for the Tunis populations 14 and 15, sampled in 1992 and 2003, respectively, and for the Tunis strain, maintained in the laboratory since 1992. This strengthens the notion that WO prophage transmission is stable across several generations.

(e) *WO prophage and cytoplasmic incompatibilities*

Previous work by Guillemaud *et al.* (1997) identified four incompatibility types among five *C. pipiens* strains (Barriol, Mart, Espro, Selax and Sphae). Barriol, Mart, and Espro strains were compatible, whereas Espro and

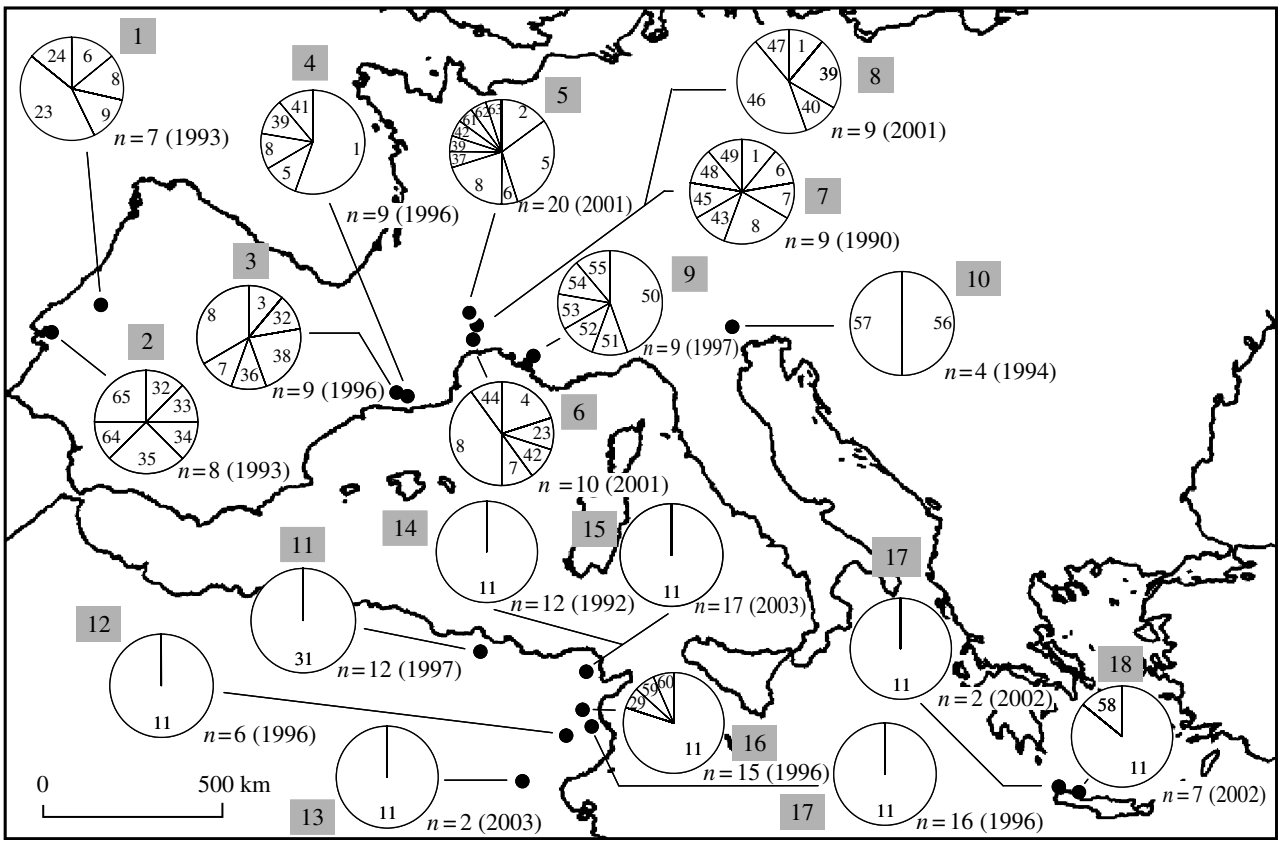

Figure 1. Distribution of *Wolbachia* variants in *C. pipiens* field populations. For each population, identified variants are indicated within circles by numbers referring to table S4. Below circles are indicated the sizes (*n*) and the years of sampling. Populations are numbered (shaded box) as reported in table S2.

Selax showed bi-directional incompatibility. All other possible crosses were unidirectionally incompatible. Since no sequence polymorphism of the *ftsZ* gene had been found by the authors, we examined WO phage polymorphism in the *Wolbachia* infecting the same strains at the time of the crossing experiment (kept in liquid nitrogen). All strains produced distinct presence/absence patterns, generating seven variants (only two were previously found; table S4). Two strains (Selax and Sphae) were infected by a mixture of distinct *Wolbachia*. Barriol and Mart, which displayed identical compatibility towards the other strains, differed from six markers indicating that distinct *Wolbachia* strains thus do not necessarily generate CI. Matching WO polymorphism to the CI pattern reported in Guillemaud *et al.* (1997) failed to show a correlation, except for Gp15. All crosses between Gp15a strains (Barriol, Espro and Mart, table 3) were fully compatible bi-directionnally. Crosses between Gp15a males and Gp15b (Selax) females were all incompatible. Inter-strain crosses with Gp15-negative (Sphae) females were all incompatible but Gp15-negative males did not induce CI in all cases (table 3). However, the correlation failed for crosses between Gp15b males and Gp15a females that were either compatible or incompatible.

4. DISCUSSION

Availability of genetic markers is crucial to understand *Wolbachia* evolution and the highly complex CI pattern that affects *C. pipiens* populations throughout the world. The purpose of this study was to find *Wolbachia* polymorphic DNA regions and to combine them with those already described in order to genotype precisely *Wolbachia* strains, to evaluate their role in CI and to approach *Wolbachia* dynamics in field populations.

Several recent studies revealed that prophage WO is widespread in *Wolbachia* genomes (Bordenstein & Wernegreen 2004; Gavotte *et al.* 2004; Wu *et al.* 2004) and the detection of particles has lent support to the idea that WO prophage genes are activated and mobile (Masui *et al.* 2000; Masui *et al.* 2001). This notion was strengthened by the lack of congruence between *Wolbachia* and phage phylogenies established with the *orf7* sequence (ORF Gp3 in our study), suggesting horizontal phage exchange between bacteria co-infecting the same intracellular environment (Bordenstein & Wernegreen 2004; Gavotte *et al.* 2004; Sanogo & Dobson 2004). Since all polymorphic regions found so far in *w*Pip DNA are mobile genetic elements (Sanogo & Dobson 2004; Duron *et al.* 2005), the 'real' *Wolbachia* genes being monomorphic (Rousset *et al.* 1992; Guillemaud *et al.* 1997; Duron *et al.* 2005), it was proposed that these mobile genetic elements could favour genome fluidity and enhance rapid adaptation, especially in the highly diversified CI system of *C. pipiens*. For all these reasons we decided to study WO prophage organization and variability in the *w*Pip genome.

(a) *WO putative proteins multiple copies or multiple infections?*

For each of the 24 WO ORFs studied, 2–6 copies were found dispersed in the *w*Pip genome (table 1). The situation is clearly different in the *w*Mel and *w*Sim genomes (Wu *et al.* 2004; Salzberg *et al.* 2005), which

Table 3. Correlation between Gp15 gene product and CI pattern. (Incompatibilities between strains from Guillemaud *et al.* (1997) were marked by grey area, all other crosses being compatible. The first letter corresponds to the Gp15 status in the male strain, and the second one to the Gp15 status in the female strain. Ø indicates lack of Gp15 PCR product. All crosses with the same parental combination display the same CI pattern, excepted b : a crosses.)

female strain	male strain				
	Barriol	Mart	Espro	Selax	Sphae
Barriol	a : a	a : a	a : a	b : a	Ø : a
Mart	a : a	a : a	a : a	b : a	Ø : a
Espro	a : a	a : a	a : a	b : a	Ø : a
Selax	a : b	a : b	a : b	b : b	Ø : b
Sphae	a : Ø	a : Ø	a : Ø	b : Ø	Ø : Ø

contain only two WO prophage clusters, one complete and the other partial (table 1). The higher copy number in *w*Pip may be overestimated due to a mixture in the *Wolbachia* used for the *w*Pip genome project. Indeed, the Gp15a and Gp15b WO variants (this study) and the *w*Pip1 and *w*Pip3 *Tr1* transposase variants (Duron *et al.* 2005), each mutually exclusive in all examined strains and field mosquitoes, are both present in the *w*Pip genome sequences (table 1). This strongly suggests that the Pel strain used for the sequenced library was infected by different *Wolbachia* variants or contaminated by other strains (a contamination with the Bei strain is now mentioned on the *w*Pip genome web page). Several short unassembled WO prophage clusters presented in table 1 might thus derive from different genomes.

Among the six different copies of Gp3 protein, four copies (Gp3a, b, c and d) were found concomitantly in individual mosquitoes from the MaClo strain (table 2). These four copies could derive from a single *Wolbachia* genome or from different *Wolbachia* genomes co-infecting the same mosquito. Co-infection has indeed been reported in other insect species, in which doubly infected males turned out to be incompatible with single infected females of either type (Rousset & Solignac 1995; Sinkins *et al.* 1995; Perrot-Minnot *et al.* 1996; Vavre *et al.* 1999). However, two issues do not support the co-infection hypothesis: PCR products amplified from single MaClo individuals were all monomorphic, and a strict mutually exclusive presence of Gp15 and *Tr1* is observed. The presence of multiple WO ORFs copies thus probably results from multi-insertion in a single *Wolbachia* genome. Along this line, we previously proposed that the concomitant presence of the *Tr1* transposase variants *w*Pip1 and *w*Pip4 in Slab and several North American samples resulted either from superinfection or from a duplication event (Duron *et al.* 2005). WO analysis of the same strain and samples showed a unique pattern, which strongly favours the duplication hypothesis.

Taken together, our data call for an extremely low level of super-infection in *C. pipiens*. This does not fit the prediction that doubly or multi-infected females should have a reproductive advantage over single or uninfected females, which would facilitate their spreading and fixation (Frank 1998). This suggests that multi-infection in *C. pipiens* is sharply limited by selective constraints that remain to be identified.

(b) *Contrasted WO prophage polymorphism levels in southern Europe and North Africa*

The combination of 10 WO and *Tr1* markers allowed the detection of an unexpected level of polymorphism, discriminating up to 66 variants in a sample of moderate size ($N=208$) and even not including allelic polymorphism from sequence data. Variability mostly affects southern European populations, where 51 *Wolbachia* strains were identified, up to 10 variants being found in population 5. Availability of these markers now opens access to population dynamics to examine whether WO polymorphism is neutral or maintained by selection. However, since the coexistence of multiple bacterial variants that generate CI is predicted to be unstable within a population (Rousset *et al.* 1991), it is likely that many of the *Wolbachia* variants identified in these regions will not generate CI. The CI patterns observed in southern Europe (Laven 1967; Magnin *et al.* 1987; Guillemaud *et al.* 1997) would thus be restricted to contact areas between particular cytotypes, a possibility that could be evaluated by the frequency of incompatible egg-rafts in natural populations.

In contrast with southern European populations, North African populations showed a very low level of WO prophage polymorphism. This situation appears stable over time, as exemplified by Tunis samples which have kept the same WO pattern over at least the last 10 years. The reasons why a single WO variant is present all over Tunisia remain speculative. First, the *Wolbachia* variant 11 might generate CI that has facilitated its spread. Alternatively, the narrow *C. pipiens* habitat between the Mediterranean sea and the Sahara desert might have reduced migration and favoured genetic drift up to the fixation of a single *Wolbachia* variant. Lastly, a strong population bottleneck generated by the constant and massive use of insecticides may have occurred. All Tunisian populations indeed carry the G119S resistant allele at the *ace-1* resistance locus, except Menzel wherein the additional variants were found (not shown; Weill *et al.* 2003).

(c) *WO prophage and cytoplasmic incompatibility*

The discovery of so many variants in southern Europe might now help explain the complex CI situation found in *C. pipiens*. Although the WO and *Tr1* marker patterns do not correlate globally with the CI patterns already described in Guillemaud *et al.* (1997), we found an interesting but not fully consistent linkage between Gp15 variability and CI. This putative secretory protein was reported to share sequence homology with a virulence-related protein of a pathogenic bacteria and proposed to be responsible for sexual alteration (Fujii *et al.* 2004). Analysis of a much larger number of crosses will be necessary to unambiguously establish the correlation between CI and the Gp15 protein. Besides, the implication of *Wolbachia* variants in the establishment of CI does not preclude the influence of others factors like nuclear genes, in particular restorer genes (Rousset *et al.* 1991; Sinkins *et al.* 2005). Construction of strains harbouring distinct *Wolbachia* variants in a same nuclear genetic background is currently underway to address this latter issue.

In conclusion, our data demonstrating the high variability of WO prophage sequences give new insights into the *C. pipiens* and *Wolbachia* relationship: (i) different *Wolbachia* variants do not necessarily generate CI. (ii) *Wolbachia* genotyping is an obligatory step before addressing the CI status between mosquito strains. Indeed, the mixture of variants found in several strains may induce a wrong CI interpretation and hinder the finding of markers correlated with CI. (iii) The extreme and apparently stable over time level of polymorphism of *Wolbachia* infecting southern European mosquitoes, in contrast with the quasi-monomorphism found in North African *Wolbachia* populations, raises novel issues on the dynamics of *Wolbachia* infection and warrants the use of the WO markers to analyse the genetic structure of *Wolbachia* infecting field populations. This may open routes for the control of vector-borne diseases since *Wolbachia* are considered as a promising driving force for manipulating gene pools of mosquito populations (Sinkins 2004).

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