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Multiple *Wolbachia* determinants control the evolution of cytoplasmic incompatibilities in *Culex pipiens* mosquito populations

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Abstract

***Wolbachia* are maternally inherited endosymbionts that can invade arthropod populations through manipulation of their reproduction. In mosquitoes, *Wolbachia* induce embryonic death, known as cytoplasmic incompatibility (CI), whenever infected males mate with females either uninfected or infected with an incompatible strain. Although genetic determinants of CI are unknown, a functional model involving the so-called *mod* and *resc* factors has been proposed. Natural populations of *Culex pipiens* mosquito display a complex CI relationship pattern associated with the highest *Wolbachia* (*w*Pip) genetic polymorphism reported so far. We show here that *C. pipiens* populations from La Réunion, a geographically isolated island in the southwest of the Indian Ocean, are infected with genetically closely related *w*Pip strains. Crossing experiments reveal that these *Wolbachia* are all mutually compatible. However, crosses with genetically more distant *w*Pip strains indicate that *Wolbachia* strains from La Réunion belong to at least five distinct incompatibility groups (or crossing types). These incompatibility properties which are strictly independent from the nuclear background, formally establish that in *C. pipiens*, CI is controlled by several *Wolbachia mod/resc* factors.**

Keywords: *Culex pipiens*, cytoplasmic incompatibility, evolution, *mod/resc* determinants, *Wolbachia*

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Introduction

Wolbachia are maternally inherited alpha-proteobacteria commonly found in arthropods and filarial nematodes (Werren *et al.* 2008). They are observed in 15–25% of insect species (Werren *et al.* 1995; West *et al.* 1998; Werren & Windsor 2000; Duron *et al.* 2008), but a recent meta-analysis taking into account rare infections revised this incidence to 66% (Hilgenboecker *et al.* 2008). The successful spread of *Wolbachia* is, at least in part, achieved through their capacity to manipulate host reproduction either by biasing the host's sex ratio towards the production of females (the transmitting sex) or, more commonly, by impeding the reproduction of uninfected females through a sterility phenomenon called cytoplasmic incompatibility (CI) (Werren *et al.* 2008).

Basically, unidirectional CI occurs when infected males mate with uninfected females, while the reciprocal cross is fully fertile. This phenomenon confers a reproductive advantage to infected females, enabling *Wolbachia* to rapidly invade populations (reviewed in Engelstadter & Telschow 2009). A more complex situation occurs when individuals are infected by different *Wolbachia* strains. Crosses between hosts may be (i) compatible, producing viable offspring; (ii) incompatible in both directions (bidirectional CI); or (iii) incompatible

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in one direction only (unidirectional CI) depending on the *Wolbachia* infection. CI disrupts the first mitotic division following fertilization, a phenomenon shown to be associated with defects in paternal chromatin condensation and segregation (review in Serbus *et al.* 2008). These defects occur even though *Wolbachia* are excluded from the mature sperm, indicating that *Wolbachia* induce a long-lasting perturbation of paternal material, which can be suppressed if infected males mate with compatible infected females. Although the proximate mechanism involved in CI has not yet been identified, a functional lock and key model has been proposed (Werren 1997; Poinsot *et al.* 2003). It assumes the existence of two bacterial functions: modification (the lock: *mod*), which modifies sperm during spermatogenesis, and rescue (the key: *resc*), which takes place in eggs and rescues the embryo development by counteracting the modified sperm (Lassy & Karr 1996; Callaini *et al.* 1997; Presgraves 2000; Tram & Sullivan 2002; Ferree & Sullivan 2006). According to this model, the *mod* and *resc* functions differ between *Wolbachia* strains and interact in complex ways as illustrated by crosses between individuals infected by different *Wolbachia* strains (Rousset & Solignac 1995; Duron *et al.* 2006a). Theoretical assumptions suggest that *mod* and *resc* are controlled by different genetic loci and may evolve independently (Charlat *et al.* 2001, 2005).

In the mosquito *Culex pipiens*, several (in)compatibility groups have been identified, although all typed *Wolbachia* (*w*Pip) strains belong to a very small clade within the *Wolbachia* B supergroup (Guillemaud *et al.* 1997), making this host species an excellent model to study the early evolution of *mod/resc* properties. To date, no *Wolbachia* multiple infection has been reported in *C. pipiens* individuals (Rasgon & Scott 2003; Duron *et al.* 2005, 2007a), in contrast to other host species such as the mosquito *Aedes albopictus* (Kittayapong *et al.* 2002), *Drosophila simulans* (Mercot *et al.* 1995), the cherry fruit fly *Rhagoletis cerasi* (Riegler & Stauffer 2002) or the wasp *Nasonia vitripennis* (Bordenstein & Werren 2007) where double infections occur. *w*Pip strains display very complex CI patterns with high frequency of uni- and bidirectional CI reported between mosquitoes from various geographical origins, as illustrated by Laven (1967) who described 17 different crossing types and by Duron *et al.* (2006a) who described 13 crossing types. In *C. pipiens*, no age effect was observed (Rasgon & Scott 2003; Duron *et al.* 2007b), but a host nuclear genome effect on CI was found once (Sinkins *et al.* 2005), suggesting a possible involvement of host genome on CI expression. Furthermore, when artificially introduced into *Ae. albopictus* mosquitoes, a *w*Pip strain induces high CI level either with uninfected or with naturally *w*AlbA and *w*AlbB bi-infected strains, showing its capability to express CI even within different nuclear backgrounds (Calvitti *et al.* 2010).

*w*Pip strains are so closely related that the multilocus sequence typing MLST genes normally used to construct *Wolbachia* phylogeny are monomorphic and thus not informative (Guillemaud *et al.* 1997; Baldo *et al.* 2006). Recent studies have allowed discriminating *w*Pip strains on a finer scale with new polymorphic markers, such as ankyrin genes (Duron *et al.* 2007a; Walker *et al.* 2007) and mobile genetic elements (MGE), including transposable elements (Duron *et al.* 2005; Sanogo *et al.* 2007) and prophages (Sanogo & Dobson 2004; Duron *et al.* 2006b). These markers revealed a spectacular *w*Pip genetic diversity, in particular in southern Europe with about 50 distinct characterized strains, in contrast to a low variability in north Africa where only few closely related strains were identified (Sanogo & Dobson 2004; Duron *et al.* 2006b). However, this variability did not correlate with CI patterns.

We are interested in the evolution of CI properties in *C. pipiens*. For this purpose, we study the *Wolbachia* infecting *C. pipiens* natural populations from La Réunion (an island of 2511 km^2 located in the southwest of the Indian Ocean). We assumed that the geographical isolation should lead to a reduced *Wolbachia* genetic diversity. We first acquired an exhaustive picture of the *Wolbachia* genetic diversity in mosquitoes from La Réunion and further investigated CI relationships among strains from this area. We measured *w*Pip strains' genetic variability using a set of MGE markers and polymorphic sequences. We constructed several local isofemale lines and analysed their CI properties by performing mutual crosses and crosses with genetically more distant strains. We show that *w*Pip strains from La Réunion are genetically closely related and fully compatible. However, they display variable CI properties towards genetically more distant strains, indicating that CI in *C. pipiens* is controlled by multiple *Wolbachia* mechanisms. In addition, we show that this striking pattern is independent of the nuclear background.

Materials and methods

Mosquito collection and laboratory strains

Culex pipiens specimens were collected as larvae or pupae from 15 natural breeding sites in La Réunion Island (Fig. 1). Larvae were reared to adulthood for species identification and some adults stored at −70 °C.

Three samples from la Réunion (Sainte Suzanne, Saint Leu and Saint Pierre) were kept alive to establish laboratory isofemale lines. Larvae collected in these three breeding sites were reared to adulthood in the laboratory, and females were blood-fed and allowed to lay

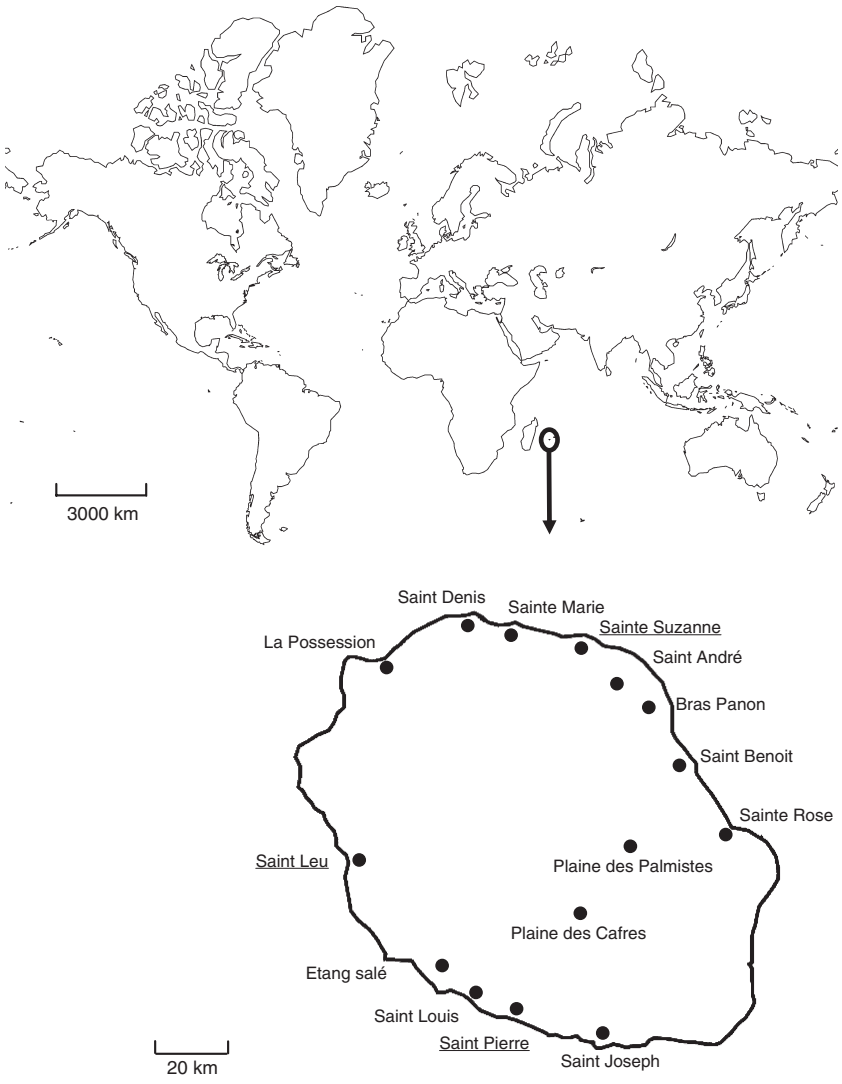

Fig. 1 Locations of the *Culex pipiens* populations sampled in La Réunion. Locations of the populations from which isofemale lines were derived to perform crossing experiments and *Wolbachia* strain sequencing are underlined.

eggs. Each egg-raft (normally 100–300 eggs stuck together) was individually isolated for hatching, and the molecular identification of its *Wolbachia* strain was achieved by analysing a mixture of 10 first-instar larvae (L1). Overall, 250 egg-rafts were screened, and depending on the *Wolbachia* strain identified, offspring were used to establish isofemale lines. For each identified *w*Pip strain, two isofemale lines were maintained (from two different locations when possible). A total of 10 isofemale lines were isolated from La Réunion samples.

Four laboratory *C. pipiens* lines of various geographical origins and infected by different *Wolbachia* strains were used as reference. The Slab and MaClo lines were collected in California in 1950 and 1984, respectively (Georghiou *et al.* 1966; Duron *et al.* 2006b); the line LaVar was collected in France in 2003 (Duron *et al.* 2005) and the Istanbul line in Turkey in 2003 (Duron *et al.* 2005). Mosquitoes were reared in 65-dm^3 screened cages kept in a single room at 22–25 °C, under a 12-h light/12-h dark cycle. Larvae were fed with a mixture of shrimp powder and rabbit pellets while adults were fed with honey solution.

Molecular identification of Wolbachia *strains*

Mosquito DNA was extracted using a CetylTrimethylAmmonium Bromide protocol (Rogers & Bendich 1988). *Wolbachia* genotyping was performed by analysing the molecular pattern of presence or absence of PCR products from 11 MGE (listed in Table S1) as previously described (Duron *et al.* 2005, 2006b). Note that this method does not detect variations in the integration

sites. *Wolbachia* strains were characterized by the combination of the 11 markers and named arbitrarily following Duron *et al.* (2006b). PCR was performed with 0.5 ng of genomic DNA solution in a 40-μL final volume reaction for 33 cycles (94 °C 5 min, 94 °C 30 s, 52 °C 30 s, 72 °C 1 min). Amplified fragments were run on 1.5% agarose gel electrophoresis. In all PCR sets, two previously characterized *w*Pip strains were included as positive controls. The quality of the DNA template was checked by the amplification of the *C. pipiens ace-1* gene (Table S1) for the individuals in which *Wolbachia* DNA could not be amplified.

Sequencing of Wolbachia *polymorphic markers*

Divergence between *w*Pip strains infecting the mosquitoes of isofemale lines was estimated from sequences of five polymorphic genes (Table S1), namely the three ankyrin domain genes *ank2* (WPa_0652 in the *w*Pip (Pel) genome), *pk1* (three identical copies WPa_0256, WPa_0315 and WPa_1308) and *pk2* (two identical copies WPa_0299 and WPa_0413) (Sinkins *et al.* 2005; Duron *et al.* 2007a) and the phage-related proteins *GP12* (four identical copies WPa_0258, WPa_0317, WPa_0429 and WPa_1310) and *GP15* (WPa_1322). The published sequence of the *w*Pip (Pel) strain was used as reference (Klasson *et al.* 2008).

PCR products were obtained as described earlier and further purified with the QIAquick gel extraction kit (QIAGEN, Valencia, CA) before direct sequencing on an ABI Prism 3130 sequencer using the BigDye Terminator Kit (Applied Biosystems). *GP12* and *GP15* sequences were submitted to Genbank under accession numbers GU827985–GU827991.

Mosquito mitochondrial DNA variability

Mitochondrial variability was analysed by DNA sequencing the cytochrome b gene (*cytb*) of La Réunion isofemale lines (primers listed in Table S1). PCR and sequencing were performed as previously described. Sequences were deposited in the Genbank database under accession numbers HM852087-HM852088.

Statistical and network analysis

Distribution of *w*Pip variations in host populations was examined using Wright *F*-statistics (F_{st}) and Fisher's exact tests with GENEPOP 3.4 (Raymond & Rousset 1995). *F*st estimates population differentiation, based on the distribution of genetic polymorphism between populations. A sequential Bonferoni adjustment correction for multiple testing was applied, based on the number of comparisons.

Each *Wolbachia* locus sequence was tested for recombination using the phylogeny-based method RDP (Martin & Rybicki 2000) and the substitution-based method Geneconv (Padidam *et al.* 1999). We used RDP3 3.41 (Martin *et al.* 2005) with default settings and the highest acceptable *P* value (0.001).

Sequences of the five *Wolbachia* markers were aligned using ClustalW (Thompson *et al.* 1994) implemented within the MEGA Software version 4.0 (Tamura *et al.* 2007). Ambiguously aligned sites of each individual gene were removed using the GBLOCKS 0.91b program (Castresana 2000) freely available from the phylogeny.fr web server (Dereeper *et al.* 2008).

The GBLOCKS method selects sequence blocks that fulfil requirements with respect to the number of contiguous conserved positions, lack of gaps and high conservation of flanking positions. We used stringent settings, which do not allow many contiguous nonconserved positions. Eight deletions in the original alignment (from single base to 100 bp because of the lack of one ankyrin domain) were considered as single events. The resulting individual sequence alignments were then concatenated. Network analysis was performed using a final sequence alignment made of 4621 aligned positions after Gblocks removal of 279 sites.

The neighbour-net method was applied to uncorrected *p*-distances calculated from the final concatenation using SPLITSTREE 4.10 (Huson & Bryant 2006). This distance-based method constructs a graphical representation of weighted splits (a split is a partition of all strains into two blocks) in the form of a phylogenetic network. Each edge in the network corresponds to the average shared splits with a length corresponding to their weight. Boxes represent splits containing conflicting data that might result from recombination.

Crossing experiments

Isofemale lines were reared for at least four generations before crossing to allow acclimation to laboratory conditions and to optimize mating and blood feeding. Reciprocal mass crosses were performed with 25–50 females and an equivalent number of males. All individuals were 2–5 days old and virgin. Females were allowed to blood-feed 5 days after mating. Egg-rafts were collected and stored individually until hatching. The CI status of each cross was determined from the egg-hatching rate (HR), quantified by counting larvae and eggs under a binocular microscope. Egg-rafts were checked for fertilization through observation of embryo development: unfertilized egg-rafts show an absence of embryo development, whereas incompatible egg-rafts show developed embryos (Duron & Weill 2006).

Wolbachia strains from La Réunion isofemale lines were introduced into Slab nuclear background through eight backcrosses (100–200 virgin females crossed with 50–100 Slab males), expected to restore 96% of Slab nuclear genes. Reciprocal mass crosses between these new lines were performed as elsewhere.

Results

Low level of Wolbachia *MGE polymorphism in La Réunion field populations*

The variability of *Wolbachia* infecting *C. pipiens* from 15 locations in La Réunion was analysed (Fig. 1). All 360 field mosquitoes (19–32 individuals per location) were found to be infected by *Wolbachia*, supporting further the notion that infection is nearly fixed worldwide in *C. pipiens* field populations (Rasgon & Scott 2003; Duron *et al*. 2005, 2006b). Genetic analysis based on presence/absence of eleven MGE markers revealed low levels of *Wolbachia* genome variability (Table 1): eleven *w*Pip strains were identified from the 360 field insects and a 12th strain (*w*133) was found during the establishment of isofemale lines. This diversity is much lower than the one observed in European populations where 49 very divergent *w*Pip strains were found by genotyping only 103 mosquitoes (Duron *et al*. 2006b). These *w*Pip strains differed from one another by one to four genetic markers, and *w*11 seems to be the strain from which all the others were derived (Fig. S1). Two strains (*w*11 and *w*118 with different *GP3d* alleles) accounted for over 91% of the infections and were found in 296 (82.2%) and 31 (8.6%) individuals, respectively (Table 2). Among the ten remaining *w*Pip strains, six were detected in single locations (*w*65, *w*133, *w*134, *w*135, *w*137, *w*138) and another one in two locations (*w*58). Genetic differentiation among La Réunion populations was assessed through F_{st} analysis, which detected no significant differentiation between 14 of the 15 populations once the *P*-values were corrected using sequential Bonferroni's correction (Hochberg 1988). The only population to stand out was Sainte Rose, showing significant F_{st} with five other populations (see Table S2). Overall, this low differentiation indicates that there is no clear geographical structure in the distribution of the *w*Pip strains over the Réunion Island.

Wolbachia *strains from La Réunion are genetically closely related and are bidirectionally compatible*

Ten isofemale lines infected by six distinct *w*Pip strains (Pie-11, Pie-58, Leu-58, Su-132, Leu-132, Su-118, Leu-118, Su-122, Leu-122 and Su-133) were established. They were used to measure *Wolbachia* sequence polymorphism and to investigate their CI relationships.

Five known variable markers were sequenced in the ten isofemale lines from La Réunion and four laboratory strains. Sequences of the *w*Pip(Pel) strain were used as references. Four *ank2* alleles, four *pk1* alleles, two *pk2* alleles, three *GP12* alleles and four *GP15* alleles were identified (Table 3). Sequence electrophoregrams showed no ambiguous position (all copies of a given gene were identical within individuals, as it is the case in the *w*Pip(Pel) genome). This suggests the absence of intra-individual variability previously detected with the MGE *Tr1* marker displaying codominant alleles (Duron *et al*. 2005). However, if multiple *Wolbachia* infection is present in *C. pipiens* lines, then the least frequent strain

Table 1 Genotyping of *w*Pip strains from La Réunion Island. (+) and (−) indicate positive and negative PCR reactions for *Tr1* and WO prophage markers. Strains were named arbitrarily and shaded boxes indicate deviations from *w*11, the most frequent strain used as a reference. *Tr1* alleles are described following Duron *et al*. 2005

	Markers used for *Wolbachia* genotyping										
*w*Pip strain	*Tr1*	*GP1b*	*GP2a*	*GP2b*	*GP2e*	*GP3a*	*GP3b*	*GP3c*	*GP3d*	*GP15a*	*GP15b*
*w*11	3	−	+	+	+	+	+	−	+	+	−
*w*58	3	−	+	+	+	−	+	−	+	+	−
*w*65	3	+	+	+	+	+	+	−	+	+	−
*w*118	3	−	+	+	+	+	+	−	−	+	−
*w*122	3	−	+	+	−	+	+	−	+	+	−
*w*132	−	−	+	+	+	+	+	−	+	+	−
*w*133	3	−	+	−	+	+	+	−	+	+	−
*w*134	−	−	+	+	+	+	+	−	−	+	−
*w*135	−	−	+	−	−	+	+	−	+	+	−
*w*136	3	−	+	+	+	+	+	+	+	+	−
*w*137	3	−	+	+	+	+	−	−	+	+	−
*w*138	3	−	+	+	−	+	+	−	−	+	−

Table 2 Frequency of the *w*Pip strains identified in *C. pipiens quinquefasciatus* samples from La Reunion. N, sample size

			Frequency of *w*Pip strains										
Population	Year	N	*w*11	*w*58	*w*65	*w*118	*w*122	*w*132	*w*134	*w*135	*w*136	*w*137	*w*138
Saint Denis	2007	24	0.63 (15)	0	0	0	0	0.13 (3)	0	0	0.2 (5)	0.04 (1)	0
Sainte Marie	2009	24	1 (24)	0	0	0	0	0	0	0	0	0	0
Sainte Suzanne	2007	24	0.88 (21)	0.04 (1)	0	0.04 (1)	0.04 (1)	0	0	0	0	0	0
Saint André	2007	24	1 (24)	0	0	0	0	0	0	0	0	0	0
Bras Panon	2007	32	0.81 (26)	0.03 (1)	0	0.13 (4)	0	0	0	0.03 (1)	0	0	0
Saint Benoît	2007	24	0.92 (22)	0	0	0.04 (1)	0	0	0	0	0.04 (1)	0	0
Sainte Rose	2007	24	0.38 (9)	0	0	0.5 (12)	0.04 (1)	0	0.08 (2)	0	0	0	0
Plaine des Palmistes	2007	22	1 (22)	0	0	0	0	0	0	0	0	0	0
Plaine des Cafres	2009	24	0.84 (20)	0	0	0	0	0.08 (2)	0	0	0	0	0.08 (2)
Saint Joseph	2007	19	0.74 (14)	0	0	0.11 (2)	0	0	0	0	0.15 (3)	0	0
Saint Pierre	2009	24	0.92 (22)	0	0	0.04 (1)	0.04 (1)	0	0	0	0	0	0
Saint Louis	2009	23	0.91 (21)	0	0	0.09 (2)	0	0	0	0	0	0	0
Etang Salé	2009	24	0.62 (15)	0	0	0.21 (5)	0	0.17 (4)	0	0	0	0	0
Saint Leu	2007	24	0.71 (17)	0	0.04 (1)	0.13 (3)	0.08 (2)	0.04 (1)	0	0	0	0	0
La Possession	2007	24	1 (24)	0	0	0	0	0	0	0	0	0	0
Total		360	0.82 (296)	0.006 (2)	0.003 (1)	0.086 (31)	0.014 (5)	0.028 (10)	0.006 (2)	0.003 (1)	0.025 (9)	0.003 (1)	0.006 (2)

might not exceed a frequency of 10%, the threshold for the detection of polymorphism by direct sequencing method (Wolford *et al.* 2000).

Analysis of the genetic divergence confirmed that *w*Pip strains from La Réunion are genetically very similar. Indeed, the five markers revealed low levels of polymorphism in nine of the ten strains, the only exception being *w*Pip(Su-133), from which complete *GP15* failed to amplify. As expected from the MGE clustering, alleles from La Réunion were very similar, and sequence analysis disclosed their extreme divergence from the four reference laboratory strains Istanbul, Slab, LaVar and MaClo (Table 3). Intragenic recombination events in *pk1*, *GP12* and *GP15* markers were observed with both RDP and Geneconv methods (all $P < 0.0001$). A *w*Pip genetic tree was constructed from interstrain uncorrected *p*-distances using the network neighbour-net method, which revealed four main groups (Fig. 2). The central box represents conflicting signals generated by recombination. This makes clear that the *w*Pip strains from La Réunion formed a robust monophyletic group, including *w*Pip(Pel), clearly distinct from Slab/MaClo, LaVar and Istanbul *w*Pip strains. To further strengthen this conclusion, we examined mitochondrial DNA variability in the ten isofemale La Réunion lines by sequencing 853 bp of the polymorphic *cytb* gene. We found only two mitochondrial haplotypes differing by a single mutation. The mosquito lines Pie-11, Leu-58, Su-118, Su-132 and Su-133 shared one haplotype, while Pie-58, Su-122, Leu-118, Leu-122 and Leu-132 shared the second one.

CI relationships were investigated among La Réunion *w*Pip strains by carrying out all bidirectional mass crosses (i.e. 90 crosses). No incompatible cross (HR all above 90%, Table 4a) was observed, indicating that all La Réunion strains are mutually fully compatible.

Various crossing types in La Réunion detected by crosses with genetically more distant strains

Next, CI relationships between strains from La Réunion and genetically more distant strains sampled worldwide were assessed. As *C. pipiens* populations display complex CI patterns, crossing type characterization for a given strain depends upon the number and types of strains used in the crosses. Here, we arbitrarily chose the four laboratory reference strains (Istanbul, Slab, LaVar and MaClo). Eighty reciprocal crosses were performed between the four reference strains and the ten isofemale lines from La Réunion, which identified 47 incompatible crosses (HR = 0%), 33 compatible ones (HR > 90%) and no intermediate hatching rates (Table 4). Crosses were split into male and female types to get a clearer picture of the *mod* and *resc* abilities.

Table 3 Polymorphism of five *Wolbachia* genes in La Réunion and laboratory strains. Variants are indicated by small case letters. Accession numbers of each variant are bracketed. The dash in Su-133 indicates an absence of the PCR product with oligonucleotides used for sequencing. Sequences of *w*Pip(Pel) strain were used as references (AM999887). Sequences acquired in this study are underlined

Mosquito line	*w*Pip strain	*ank2*	*pk1*	*pk2*	*GP12*	*GP15*
Pel		a	a	a	a	a
Pie-11	*w*11	a (AM397068)	a (AM397075)	a (DQ000472)	a (GU827985)	a (GU827988)
Su-132	*w*132	a	a	a	a	a
Leu-132	*w*132	a	a	a	a	a
Leu-118	*w*118	a	a	a	a	a
Su-118	*w*118	a	a	a	a	a
Leu-58	*w*58	a	a	a	a	a
Pie-58	*w*58	a	a	a	a	a
Su-122	*w*122	a	a	a	a	a
Leu-122	*w*122	a	a	a	a	a
Su-133	*w*133	a	a	a	a	-
LaVar	*w*5	e (AM397072)	c (AM397077)	a	b (GU827986)	b (GU827989)
MaClo	*w*10	b (AM397069)	b (AM397076)	b (DQ000471)	b	c (GU827990)
Slab	*w*1	b	b	b	b	c
Istanbul	*w*31	c (AM397070)	d (AM397078)	a	c (GU827987)	d (GU827991)

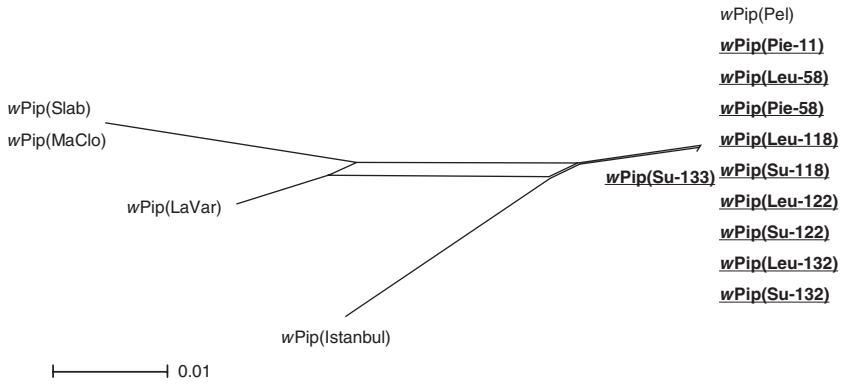

Fig. 2 Neighbour-net analysis of 4621 *Wolbachia* sites from 15 *Culex pipiens* isofemale lines (generated from five concatenated genes listed in Table 3). The network represents the genetic polymorphism and relationships between *Wolbachia* strains. Strains from La Réunion are underlined. Parallel edges represent the same split.

Three distinct male groups (i, ii and iii, *mod* abilities) and two female groups (1 and 2, *resc* abilities) were detected among La Réunion strains, while the more distant four reference strains contributed to three additional *mod* (iv, v & vi) and *resc* groups (3, 4 & 5) (Table 4a). Considering together the *mod* and *resc* abilities of each strain, we identified five distinct crossing types among La Réunion strains, of which crossing type II (Su-132, Leu-132, Pie-58, Leu-122 and Su-133) was the most abundant (Table 4b). We found no correlation between crossing types and the genotypes delineated by the eleven MGE markers (Fisher's exact test $P = 0.76$). For example, Pie-58 and Leu-58 strains, both infected by *w*58, belong to distinct *mod* and *resc* groups (*mod* ii vs. iii, *resc* 1 vs. 2, Table 4). We found no correlation between mitochondrial variability and crossing types. For example, Pie-11, Leu-58, Su-118, Su-132 and Su-133 strains share the same mitochondrial haplotype and belong to four different crossing types. Therefore, although closely related genetically and fully compatible, La Réunion strains display at least five crossing types, revealed through their crossing behaviour towards the four distantly related reference strains.

Nuclear backgrounds do not influence CI

The presence of different crossing types among genetically related and mutually compatible strains can in principle result from two nonexclusive processes: crossing types might be affected by nuclear genes, polymor-

Table 4 Crossing relationships between La Réunion and reference laboratory *Wolbachia* strains. (a) Males and females from La Réunion and reference laboratory strains were crossed with each other. Crosses were classified either compatible (C, hatching rate (HR) > 90%) or incompatible (IC, HR = 0%, shaded). Bidirectionally incompatible crosses are underlined. The number of egg-rafts collected in each cross is bracketed. Crosses between reference strains correspond to data from Duron *et al.* 2006a. (b) Summary of the crossing types, *mod* and *resc* groups identified from strains analysed in (a). La Réunion and reference strains delineate six *mod* (i–vi) and five *resc* groups (1–5). La Réunion strains delineate five crossing types, i.e. specific *mod* and *resc* associations (I–V), distinct from those delineated by the reference strains

(a)

	Males	Pie-11	Su-132	Leu-132	Leu-118	Su-118	Leu-58	Pie-58	Su-122	Leu-122	Su-133	Istanbul	Slab	LaVar	MaClo
	mod	i	ii	ii	iii	ii	iii	ii	iii	ii	ii	iv	iii	v	vi
Females	*resc*														
Pie-11	1		C (6)	C (9)	C (8)	C (8)	C (7)	C (7)	C (8)	C (7)	C (9)	IC (13)	C (13)	IC (4)	IC (5)
Su-132	1	C (7)		C (9)	C (10)	C (6)	C (9)	C (9)	C (12)	C (9)	C (11)	IC (11)	C (7)	IC (9)	IC (10)
Leu-132	1	C (7)	C (8)		C (12)	C (6)	C (8)	C (11)	C (12)	C (8)	C (7)	IC (10)	C (12)	IC (12)	IC (7)
Leu-118	1	C (8)	C (7)	C (4)		C (5)	C (8)	C (7)	C (8)	C (6)	C (5)	IC (7)	C (5)	IC (9)	IC (12)
Su-118	2	C (8)	C (7)	C (7)	C (5)		C (6)	C (9)	C (6)	C (3)	C (4)	IC (9)	C (7)	C (3)	C (9)
Leu-58	2	C (7)	C (11)	C (5)	C (9)	C (10)		C (4)	C (7)	C (5)	C (10)	IC (12)	C (10)	C (32)	C (30)
Pie-58	1	C (9)	C (10)	C (7)	C (10)	C (8)	C (12)		C (7)	C (13)	C (7)	IC (9)	C (9)	IC (6)	IC (7)
Su-122	1	C (6)	C (8)	C (5)	C (6)	C (6)	C (7)	C (8)		C (7)	C (8)	IC (5)	C (6)	IC (8)	IC (9)
Leu-122	1	C (10)	C (7)	C (8)	C (13)	C (13)	C (6)	C (12)	C (8)		C (9)	IC (6)	C (5)	IC (6)	IC (6)
Su-133	1	C (6)	C (4)	C (4)	C (4)	C (4)	C (8)	C (5)	C (5)	C (5)		IC (6)	C (7)	IC (6)	IC (5)
Istanbul	3	C (45)	IC (19)	IC (16)	IC (12)	IC (16)	IC (8)	IC (12)	IC (14)	IC (15)	IC (17)		IC (34)	IC (40)	C (31)
Slab	4	C (18)	IC (22)	IC (22)	C (17)	IC (28)	C (12)	IC (21)	C (12)	IC (29)	IC (24)	C (33)		IC (30)	IC (99)
LaVar	5	C (11)	IC (12)	IC (12)	C (7)	IC (12)	C (9)	IC (12)	C (12)	IC (18)	IC (9)	IC (26)	C (8)		C (10)
MaClo	2	C (15)	C (13)	C (10)	C (10)	C (11)	C (14)	C (15)	C (21)	C (15)	C (13)	IC (53)	C (43)	C (36)	

(b)

Crossing types	*mod*	*resc*	Strains
I	i	1	Pie-11
II	ii	1	Su-132, Leu-132, Pie-58, Leu-122, Su-133
III	iii	1	Leu-118, Su-122
IV	ii	2	Su-118
V	iii	2	Leu-58
VI	iv	3	Istanbul
VII	iii	4	Slab
VIII	v	5	LaVar
IX	vi	2	MaClo

phic within La Réunion island, and/or CI might be encoded by different combinations of *mod* and *resc Wolbachia* factors, genetically unlinked with the molecular markers used in the present study.

To examine the influence of the mosquito nuclear genome, we introduced through eight repeated backcrosses the cytoplasms of Pie-11, Leu-58, Leu-118, Su-118 and Su-132 lines, representing five distinct crossing types (I–V, Table 4) into the Slab nuclear background. The original *Wolbachia* genotypes were confirmed by further checking the MGE markers. The five backcrossed lines were then crossed to each other (20 crosses) and to the reference strains (39 crosses). In all cases, HR and compatibility types were identical to those observed with the natural nonbackcrossed strains (Table S3). Crossing types found in La Réunion strains are thus independent from nuclear backgrounds and likely result from *mod* and *resc* interactions entirely attributable to the infecting *Wolbachia*. Moreover, the crossing types as characterized here are stable over time.

Multiple mod *and* resc *abilities can explain the presence of cryptic crossing types*

The distinction between the male and female perspectives on CI (the *mod/resc* properties) has led to several functional models, among which the lock and key model emerged from several studies (reviewed in Poinsot *et al.* 2003). In this model, embryonic development depends on the physical compatibility of a lock, encoded by a *mod* locus altering the paternal material, and a key, encoded by a *resc* locus and expressed in the oocyte. Given the absence of transitivity of the observed CI relationships in *C. pipiens* strains, we formally interpreted our results by proposing that each key can rescue one or several locks. For example, a key '*resc* A' only rescues lock '*mod* A', whereas '*resc* AB' can rescue '*mod* A', '*mod* B' or '*mod* AB' but not '*mod* C'. Specificities were then sequentially assigned to the *mod* and *resc* types of each strain until a consistent pattern emerged, in which four specific states (A–D) are sufficient to describe the nine crossing types (Table 5). Although minimal but probably not unique, this pattern establishes that CI in *C. pipiens* can fit a combinatorial model.

Discussion

The use of the recently developed sensitive MGE markers highlights the variability within the *w*Pip strains infecting *C. pipiens* and offers the opportunity to study evolutionary dynamics of CI in natural populations. Three major observations were made and are discussed in the following sections.

Table 5 Complexity of *mod* and *resc* groups in *C. pipiens Wolbachia* strains. Crossing data from Table 4 are summarized. Incompatible crosses (IC) are dark-shaded. The nine crossing types identified are numbered (I–IX), with the La Réunion crossing types light-shaded. Features of modifications (*mod*) and rescues (*resc*) are indicated by upper case letters. Crosses can be compatible (C) only when all *mod* features from the male are included in the *resc* features of the female. Although not deduced from the crossing data, *mod*+ was assigned to all strains because *C. pipiens* males always induce cytoplasmic incompatibility when mated with uninfected tetracycline-treated females

	i	ii	iii	iv	v	vi
Males	*mod*+	*mod*+^{A}	*mod*+^{B}	*mod*+^{C}	*mod*+^{BD}	*mod*+^{D}
Females						
1 *resc*+^{AB}	C (I)	C (II)	C (III)	IC	IC	IC
2 *resc*+^{ABD}	C	C (IV)	C (V)	IC	C	C (IX)
3 *resc*+^{CD}	C	IC	IC	C (VI)	IC	C
4 *resc*+^{BC}	C	IC	C (VII)	C	IC	IC
5 *resc*+^{BD}	C	IC	C	IC	C (VIII)	C

Only genetically related w*Pip strains are found in La Réunion Island*

The *w*Pip strains identified in La Réunion are genetically very similar and closely related to the *w*11 strain (or *w*11-like), a strain also observed in Tunisia and Crete (Duron *et al.* 2006b). Observing twelve closely related *w*Pip strains among 360 specimens in La Réunion represents a very low diversity compared to the situation described in southern Europe, where 49 *w*Pip strains differing from each other by several markers had been identified out of 103 samples (Duron *et al.* 2006b; Wilcoxon test, $W = 0$; $P < 10^{4}$). Two nonexclusive hypotheses may explain this situation. First, the drift hypothesis: a recent colonization of La Réunion Island by *Culex pipiens quinquefasciatus* (XVIth century in Mascareignes Islands; Brygoo & Brunhes 1971), a reduced population size and a low immigration from other regions might have simply favoured genetic drift up to fixation of a single *w*Pip strain. Furthermore, *w*Pip strain homogeneity might have been hastened by bottlenecks generated by the constant and massive use of insecticides (Tantely *et al.* 2010). Second, the selection hypothesis: the majority *w*11-like distribution in La Réunion could reflect particular invasive properties that have facilitated its spread and the selective elimination of other *Wolbachia* strains. The low mitochondrial DNA variability observed is also in agreement with a recent expansion of *C. pipiens* in La Réunion Island. A *Wolbachia* infection sweep was previously reported in uninfected *Drosophila simulans* populations in California (Turelli & Hoffmann 1991) and *Laodelphax striatellus* populations in Japan (Hoshizaki & Shimada 1995),

while a superinfection sweep was reported in mono-infected *Rhagoletis cerasi* populations in Europe (Riegler & Stauffer 2002). However, no clear invasive properties emerged from our crossing data. Besides, the wide *w*11-like distribution might be associated with increased host fitness, such as increased fecundity, as previously reported for *Aedes albopictus* (Dobson *et al.* 2002) or a protection against viral infection (Hedges *et al.* 2008; Teixeira *et al.* 2008; Osborne *et al.* 2009).

w*Pip strains from La Réunion are mutually compatible*

Full compatibility was observed between all La Réunion *w*Pip strains. This situation is unusual as *C. pipiens* lines were previously shown to display high CI frequencies (Laven 1967; Magnin *et al.* 1987; O'Neill & Paterson 1992; Guillemaud *et al.* 1997; Duron *et al.* 2006a) and compatible and incompatible *w*Pip strains have already been found within the same host populations (Barr 1980; Duron *et al.* 2006a). However, theoretical studies suggest that incompatible crossing types present within a same host population enter into competition until one strain or a set of compatible strains invade (Dobson 2004; Engelstadter & Telschow 2009). The presence of a large set of compatible *w*Pip strains in La Réunion populations thus fits with this expectation. According to theoretical models, mutations on *mod* and *resc* functions can generate incompatible *Wolbachia* strains from ancestral ones (Charlat *et al.* 2001; Dobson 2004). Although new CI-inducing *w*Pip strains may thus regularly appear by mutation in La Réunion, their frequency may not exceed the threshold allowing invasion and they are likely to be counterselected by other *w*11-like incompatible strains.

Genetic similarity of w*Pip strains does not fully predict their CI status*

Although fully compatible with each other, *w*Pip strains from La Réunion split into five distinct crossing types when crossed with the four laboratory *w*Pip strains, Istanbul, Slab, LaVar and MaClo. This situation wherein closely genetically related and mutually compatible strains develop distinct CI patterns towards more genetically distant strains was previously described for Tunis and Kol *w*11 strains (Duron *et al.* 2006a). This implies that *w*11-like strains contain a variability that cannot be detected from their mutual crosses or MGE genotyping.

One straightforward possibility to explain this cryptic variability is that strains from La Réunion have their CI properties hindered locally by nuclear restorers. Indeed, because CI generates a high cost on host reproduction, hosts may select for nuclear restorers that prevent – even partially – the expression of CI (Rousset *et al.* 1991). However, when homogenized in the same Slab nuclear background, La Réunion *w*Pip strains were still mutually compatible and displayed crossing types identical to those observed in their natural nuclear backgrounds. This does not support the involvement of nuclear restorers, which have been found only once so far (Sinkins *et al.* 2005). We can conclude that the full compatibility between La Réunion strains results strictly from cytoplasmic factors.

Another possibility is the presence of intra-individual variability (i.e. infection by multiple *Wolbachia*), in which strains at low frequency might participate in CI. Indeed, cases of multiple infections were reported previously in other host species but so far, they only pertained to genetically very divergent *Wolbachia* strains (Mercot *et al.* 1995; Kittayapong *et al.* 2002; Bordenstein & Werren 2007). We do not favour this possibility because no intra-individual variability was found by the genotyping approach. However, although MGE represent the most polymorphic *w*Pip markers (Duron *et al.* 2006b), we cannot formally exclude the presence of variability in other loci.

A more likely possibility is the presence of multiple *mod/resc* functions within each *w*Pip strain, as shown recently for the *Wolbachia* strain *w*Tei naturally infecting *Drosophila teissieri* (Zabalou *et al.* 2008). According to this scheme, all *w*11-like strains should be identical for those *mod/resc* features that allow their mutual compatibility and might be variable for other *mod/resc* features that induce incompatibility with distant strains because they are not submitted to the same selection pressure.

With these assumptions, we propose a pattern consistent with the crossing experiments presented here, in which *mod* and *resc* are each described by four characteristics (A–D, Table 5). Although not deduced from the crossing data, *mod*+ was assigned to all strains because *C. pipiens* males always induce CI when mated with uninfected tetracycline-treated females (Duron & Weill 2006; Duron *et al.* 2006a). These data represent the minimum number of characteristics present in La Réunion *w*Pip strains as the 10 lines were crossed with only four distant reference strains. More characteristics would have been probably detected when crossing with additional strains. This pattern raises several issues: First, the *mod* and *resc* generally display an equal number of groups (6 vs. 5), in agreement with the current vision of the 'lock and key' model (Charlat *et al.* 2005). However, the *mod* function appears more specialist (up to two characteristics, Table 5), whereas the *resc* function appears more generalist (two to three characteristics). This fits the 'all or nothing' behaviour of *w*Pip strains, i.e. no CI (>90% hatching) or full CI induction (0% hatching), which might rely on presence or absence of specific *mod/resc* pairs. Second, why do La Réunion Su-118 and Leu-58

*w*Pip strains contain the *resc* D ability whereas no corresponding *mod* was detected? One possibility is that *mod* D is present in the island but was not sampled in the isofemale lines selected. Alternatively, assuming that *mod* D was lost recently, the *resc* D ability might still be present, reflecting a remnant adaptation to past infections.

Finally, the most important issue is how can we reconcile the low apparent genetic variability of La Réunion strains and the presence of at least five crossing types? A simple explanation might be a limitation of the MGE genotyping, as this method only scores the presence of mobile elements and ignores any change in their integration sites. *w*11-like strains might thus well contain identical mobile elements but distributed at different loci, and because these elements are present in multiple copies in *Wolbachia* genomes (Wu *et al.* 2004; Klasson *et al.* 2008, 2009), this might represent a substantial source of variability and affect expression of many genes, in particular those involved in CI. More sensitive genotyping methods are needed to address the variability of MGE integration sites and their relevance to the establishment of CI.

Conclusion

The *Wolbachia* infecting *C. pipiens* mosquito populations in La Réunion are genetically similar, suggesting a common origin and show much less variability than in southern European populations. This low genetic variability suggests either genetic drift that has favoured fixation or invasive properties of *w*11-like *w*Pip strains. Although expressing CI towards genetically distant *w*Pip strains, La Réunion strains are all mutually compatible and no nuclear restorer was observed. Our findings support the conjecture that several *mod* and *resc* factors underlie CI properties, whose combinations might explain the complex network and the rapid evolution of CI relationships within *C. pipiens* populations.

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This work forms part of CMA's PhD thesis, supervised by MW CMA investigates the evolutionary dynamics of *Wolbachia* and cytoplasmic incompatibility (CI) in the mosquito *C. pipiens*. OD works on the evolutionary dynamics of host-parasite relationships, especially of reproductive parasites. PT investigates the role of mosquitoes in transmission of arboviruses in the Indian Ocean. NP has general interest in population genetics of *C. pipiens* mosquitoes. PF works on evolutionary aspects of cell signaling in differentiation. Most of MW's current research focuses on molecular evolution of *C. pipiens-Wolbachia* symbiosis and evolution of resistance to insecticides in mosquitoes.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of the primers used for *Wolbachia* strain identification

Table S2 Genetic differentiation among *C. pipiens* populations from La Réunion

Table S3 No effect of the host nuclear genome on CI expression

Fig. S1 Schematic representation of the MGE genetic differences among La Réunion *w*Pip strains.

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