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Stable coexistence of incompatible *Wolbachia* along a narrow contact zone in mosquito field populations

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Abstract

In Arthropods, the intracellular bacteria *Wolbachia* often induce cytoplasmic incompatibility (CI) between sperm and egg, which causes conditional embryonic death and promotes the spatial spread of *Wolbachia* infections into host populations. The ability of *Wolbachia* to spread in natural populations through CI has attracted attention for using these bacteria in vector-borne disease control. The dynamics of incompatible *Wolbachia* infections have been deeply investigated theoretically, whereas in natural populations there are only few examples described, especially amongst incompatible infected hosts.

Here we have surveyed the distribution of two molecular *Wolbachia* strains (*w*Pip11 and *w*Pip31) infecting the mosquito *Culex pipiens* in Tunisia. We delineated a clear spatial structure of both infections, with a sharp contact zone separating their distribution areas. Crossing experiments with isofemale lines from different localities showed three crossing types: *w*Pip11-infected males always sterilize *w*Pip31-infected females; however, while most *w*Pip31-infected males were compatible with *w*Pip11-infected females, a few completely sterilize them. The *w*Pip11 strain was thus expected to spread but temporal dynamics over

seven years of monitoring shows the stability of the contact zone. We examined which factors may contribute to the observed stability, both theoretically and empirically. Population cage experiments, field samples and modeling did not support significant impacts of local adaptation or assortative mating on the stability of *w*Pip infection structure. By contrast, low dispersal probability and metapopulation dynamics in the host *Cx. pipiens* likely play major roles.

This study highlights the need of understanding CI dynamics in natural populations to design effective and sustainable *Wolbachia*-based control strategies.

Introduction

Wolbachia are maternally inherited intracellular α-proteobacteria that infect many arthropod species (Werren *et al*. 2008). *Wolbachia* are the most frequent bacterial endosymbiont described so far in insects, infecting 20-70% of species (Werren *et al*. 1995; Jeyaprakash & Hoy 2000; Hilgenboecker *et al*. 2008; Zug & Hammerstein 2012). This evolutionary success is mainly attributed to the ability of *Wolbachia* to manipulate the host reproductive system to its own advantage (Werren *et al*. 2008). The most common manipulation is cytoplasmic incompatibility (CI), which triggers embryonic mortality when infected males mate with uninfected females, whereas the reciprocal cross remains compatible. Thus infected females have a reproductive advantage in polymorphic populations which allows the spread of infections. Cases of such spreads were reported in field populations of *Drosophila simulans* in California (Turelli & Hoffmann 1991), planthopper *Laodelphax striatellus* (Hoshizaki & Shimada 1995) and butterfly *Eurema hecabe* in Japan (Hiroki *et al*. 2005).

Wolbachia has attracted much attention as a promising tool to control diseases transmitted by mosquitoes, after the observation that some infections of *Aedes* or *Anopheles* mosquitoes by *Wolbachia* reduce the development time of their host and reduce the transmission of human pathogens such as the dengue and chikungunya viruses (Moreira *et al*. 2009; Walker *et al*. 2011; Blagrove *et al*. 2012) or *Plasmodium* (Kambris *et al*. 2010; Bian *et al*. 2013). Furthermore, *Wolbachia*-infected *Aedes aegypti* released in Australia have readily spread in field populations through CI, as predicted by infection dynamic models (Hoffmann *et al*. 2011). Better knowledge of CI dynamics is thus required to explore the full potential of *Wolbachia* in vector control strategies.

Wolbachia dynamics is made more complex by the presence of several incompatible strains. When males and females are infected with different and incompatible strains, CI occurs and can follow either unidirectional or bidirectional patterns (Riegler & Stauffer 2002; Mercot & Charlat 2004; Duron *et al*. 2006a; Atyame *et al*. 2014). *Wolbachia* dynamics has been largely explored theoretically and three key models parameters describe how CI could influence the spread of *Wolbachia* in a panmictic host population (see Engelstadter & Telschow 2009 for review): (i) the transmission rate- i.e. the proportion of infected offspring produced by an infected female; (ii) the CI mortality (or CI level)- i.e. the proportion of offspring that die in incompatible crosses; and (iii) the effect of *Wolbachia* infection on female fecundity- i.e. on its host fitness (Fine 1978; Hoffmann *et al*. 1990; Turelli & Hoffmann 1995).

Incompatible *Wolbachia* strains cannot stably coexist in an unstructured and panmictic host population, since only compatible strains can resist the CI-driven competition (Rousset *et al*. 1991; Engelstadter & Telschow 2009). The pattern of CI will determine the

competition outcome: in the case of bidirectional CI, the most frequent *Wolbachia* strain always outcompetes the rarest, while with unidirectional CI, the CI-inducing strain is expected to invade the population, once above an initial frequency threshold (Caspari & Watson 1959, Engelstadter & Telschow 2009). Only two studies report on *Wolbachia* strains competing in naturally infected host populations: (i) the cherry fruit fly *Rhagoletis cerasi*, in which flies co-infected with two *Wolbachia* strains invaded populations carrying only one *Wolbachia* infection in southern Europe (Riegler & Stauffer 2002), and (ii) *D. simulans,* in which the *Wolbachia* strain *w*Ri invaded populations infected with the strain *w*Au on the east coast of Australia (Kriesner *et al*. 2013). In this latter case, data were best explained by postulating that both *w*Au and *w*Ri increased females fecundity, highlighting a potential role for selection in the global distribution of *Wolbachia*.

However, invasion thresholds deduced for isolated panmictic populations cannot be directly applied to structured host populations connected by dispersal, as system dynamics and equilibrium states are modified (Barton & Turelli 2011; Hancock & Godfray 2012). Incompatible *Wolbachia* strains can indeed stably coexist in structured parapatric host populations if dispersal is below a critical threshold (Telschow *et al*. 2005; Flor *et al*. 2007).

Culex pipiens complex mosquitoes- whose main subspecies are *Cx. p. quinquefasciatus* and *Cx. p. pipiens*, ubiquitous in tropical and temperate regions, respectively - present an extreme situation. In temperate regions, two *Cx. p. pipiens* forms coexist (the *pipiens* and *molestus* forms), showing behavioral, physiological and genetic specificities (Fonseca *et al*. 2004). *Culex pipiens quinquefasciatus* and *Cx. p. pipiens* mosquitoes are naturally infected by *Wolbachia* strains (*w*Pip) that belong to a unique clade of the B supergroup (Rasgon & Scott 2003; Duron *et al*. 2005; Duron *et al*. 2006b). However,

*w*Pip strains display a high genetic polymorphism at a small evolutionary scale (Duron *et al*. 2006b; Atyame *et al*. 2011a; Dumas *et al*. 2013) and complex CI patterns (Laven 1967a; Duron *et al*. 2006a; Atyame *et al*. 2011b; Atyame *et al*. 2014). A recent survey showed that considerable genetic diversity is stably maintained within geographically close *Cx. pipiens* populations infected by compatible *w*Pip strains (Duron *et al*. 2011).

We report here on a field situation in North Africa where *Cx. pipiens* populations were found infected by only two molecular strains, *w*Pip31 in Algeria and *w*Pip11 in Tunisia (Duron *et al*. 2006b). These two strains were defined using a set of polymorphic molecular markers and laboratory crosses showed CI between them (Duron *et al*. 2006a). This situation was thus a very attractive opportunity to study their evolutionary dynamics in natural populations. We have now mapped the distribution areas of these *w*Pip strains in Algeria and Tunisia, characterized their CI patterns from isofemale lines sampled in different localities, and analyzed CI expression from direct observations in the field. We have still detected only the same two molecular *w*Pip strains. However, we have found three strains in term of CI properties. *w*Pip11-infected males always sterilize *w*Pip31-infected females. While most *w*Pip31-infected males were compatible with *w*Pip11-infected females, a few completely sterilized them. In these conditions, the *w*Pip11 strain is expected to outcompete the *w*Pip31 strains over time. We have thus performed a seven-year follow-up of the *w*Pip infections in field populations, and have found that the contact zone between *w*Pip11 and *w*Pip31 strains has remained stable. By developing a spatially explicit model that better describes the field situation, we have identified critical parameters that might explain the long-term coexistence of these incompatible *w*Pip infections.

Materials and Methods

Mosquito collection and lines maintenance

Culex pipiens mosquitoes were collected as larvae or pupae in ten localities of Algeria (in 1997, June 2006 and 2008) and in 63 localities of Tunisia (in June 1996, 1997, 2003, 2005, 2008, 2009, 2010, October 2010 and June 2011). All localities were not sampled each year (Table S1). Each sample was reared to adulthood in the laboratory and approximately 100 specimens were stored at -20°C or in liquid nitrogen until *Wolbachia* genotyping. Adults from 19 localities were allowed to mate to establish isofemale lines (Table S1). Each egg raft (containing 100-300 eggs) was individually isolated for hatching, and the genotyping of its *Wolbachia* infection was performed by analyzing two first-instar larvae (L1). All isofemale lines (n = 245) were reared in 65 dm^3 screened 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 while adults were fed with honey solution.

Molecular typing

Wolbachia genotyping was performed using two *w*Pip polymorphic markers (among several previously described to differentiate the *w*Pip strains): the ankyrin domains gene *ank*2 (Duron *et al*. 2007) and the DNA mismatch repair protein gene *MutL* (Atyame *et al*. 2011a). These two genes differentiate the *w*Pip strains investigated here on the basis of the size of the PCR amplified fragments: *ank2* displays 313 bp and 511 bp fragment sizes, and *MutL* displays 374 bp and 437 bp fragment sizes for the strains *w*Pip11 and *w*Pip31 respectively (Figure S1).

We also analyzed mitochondrial variability by sequencing 852 bp of the cytochrome b gene (*cytb*) in 12 isofemale lines from Algeria and Tunisia: five *w*Pip11 lines from four localities (Sousse, #25; Zerga, #30; Sokra, #37 and Aïn Tounga, #51) and seven *w*Pip31 lines from seven localities (Harash, #3; Guelma, #6; Douas, #8; Kala, #9; Tabarka, #31; Kef1, #34; Boussalem1, #42).

Finally, we checked for a relationship between *Cx. pipiens* forms (form *pipiens* and form *molestus*) and *w*Pip infection. The *CQ11* microsatellite locus (Bahnck & Fonseca 2006) was used to distinguish the two forms in samples from two *w*Pip11 localities (Ayed, #24 and Riadh, #27) and two *w*Pip31 localities (Tabarka, #31 and Boussalem2, #71). The *CQ11* microsatellite loci was previously found effective for molecular identification of *pipiens* and *molestus* forms, as well as hybrids, in *Cx. pipiens* mosquitoes in Morocco (Amraoui *et al*. 2012).

DNA was extracted from adult mosquitoes and larvae using a CetylTrimethylAmmonium Bromide (CTAB) protocol (Rogers & Bendich 1988). All PCR were performed with 50 ng of genomic DNA solution in a 40 µl final under the following conditions: 94°C for 30s, 52°C for 30s, and 72°C for 1 to 1.5 min for a total of 33 cycles (primers are listed in Table S2). Amplified DNA fragments were separated by agarose gel (1.5%) electrophoresis. For sequencing, PCR products were purified with the QIAquick gel extraction kit (QIAGEN, Valencia, CA) and then directly sequenced with an ABI Prism 3130 sequencer using the BigDye Terminator Kit (Applied Biosystems).

Cytoplasmic incompatibility

In the laboratory

Crosses were carried out with 25-50 virgin females and an equivalent number of males derived from isofemale lines. A first set of crosses was performed between *w*Pip11 and *w*Pip31 lines isolated from localities where only one *Wolbachia* molecular strain was present: five *w*Pip11 lines (three localities: Tunis, #16, Sousse, #25 and Sokra, #37) and 16 *w*Pip31 lines (eight localities: Tabarka, #31; Kef1, #34; Boussalem1, #42 from Tunisia and Guelma, #6; Lac, #7; Douas, #8; Kala, #9; Souk Ahras, #10 from Algeria). Next, crosses were performed between *w*Pip11 and *w*Pip31 lines from the same locality, i.e. between two *w*Pip11 and two *w*Pip31 isofemale lines isolated from the Tunisian samples Zerga (#30) and Ain Tounga (#51). To assess CI phenotypes among *w*Pip11-infected males, isofemale-derived *w*Pip11 males from three localities (El Battan, #43; El Manar, #46 and Aïn Tounga, #51) were crossed with *w*Pip31 females derived from the Har isofemale line (Harash, #3). All individuals were 2-5 days old. Females were allowed to blood feed five days after caging, and their egg rafts were collected after five days and stored individually until hatching. The cytoplasmic incompatibility (CI) status of each cross was determined by examining egg hatching rate (HR) under a binocular microscope. All unhatched egg rafts were checked for fertilization through observation of embryonic development as described by Duron and Weill (2006).

Mating preferences of *w*Pip11 and *w*Pip31 mosquitoes were measured in laboratory cages (65 dm^3), where 100 males and 100 females from a *w*Pip11 isofemale line were placed with an equivalent number of males and females from a *w*Pip31 isofemale line (later identified as a *w*Pip31_U line). Two types of confrontations were set up: Sok × Kef1-1 and Sok × Kef1-2, both showing unidirectional CI (Sok males sterilizing kef1-1 and kef1-2

females). Three replicates were performed for each type of confrontation, so a total of six cages were set up. All individuals were 1-day-old and virgin, and all the mosquitoes were introduced into the cage at the same time. Females were blood-fed 5 days after caging and allowed to oviposit on a water cup. Egg rafts were individually stored and were analyzed as described above.

In field populations

The expression of CI in *Cx. pipiens* natural populations was assessed by sampling egg rafts in Tunisian localities in 2009 and 2010. The egg rafts were carefully removed with a paintbrush from the surface of stagnant water, placed separately in 24-well plates, and brought to the laboratory to observe hatching. As larvae hatch within 36-48 hours after ovoposition at 25°C, HR was evaluated >72 hours after collection under a binocular microscope and egg rafts that had already hatched in the field were easy to qualify.

Population cages

The invasion dynamics of the *w*Pip11 strain was examined in population cages (65 dm^3) in laboratory through confrontations between the *w*Pip11 isofemale line Tn and the *w*Pip31 isofemale line Har (a *w*Pip31_U line), showing unidirectional CI (Tn males sterilizing Har females). To avoid side effects of nuclear genomes, the cytoplasms (including *w*Pip strains) of the Tn and Har lines were introduced into the same nuclear background (from the laboratory line Slab) through eight backcrosses (100-200 virgin females of the Tn line or the Har line crossed with 50-100 Slab males), expected to restore ~ 96% of Slab nuclear genes. Population cages were then initiated using the Tn and Har backcrossed lines, with an initial frequency of 50% (i.e. 100 males and 100 females Tn were mixed with 100

males and 100 females Har). Three replicate were performed and all individuals were 1-day-old and virgin. Mosquitoes were introduced into the cage at the same time. All population cages employed discrete generations by establishing new cages at each generation using newly emerged adults resulting from the previous generation. *Wolbachia* infections frequencies in population cages were monitored by PCR assays as described above.

Data analyses

Field data on the geographic distribution of *w*Pip strains in Tunisia were analyzed with GENEPOP (Raymond & Rousset 1995; Rousset 2008). Wright's F-statistics (F_{st}) was examined to estimate population differentiation, based on the distribution of genetic polymorphism between populations.

All other statistical analyses and modeling were performed using the R software (R Core Team, 2013).

Results

A very narrow contact zone between *w*Pip11 and *w*Pip31 *Wolbachia* strains in Tunisia

We developed a sensitive molecular assay to easily genotype the five *w*Pip groups (Atyame *et al*. 2011a) and screened *Cx. pipiens* collected as larvae or pupae in Algeria and Tunisia in several sampling campaigns from 1996 to 2009 (Table S1). Mosquitoes from all examined samples were only infected by one of the two molecular *w*Pip strains, referred to as *w*Pip11 and *w*Pip31, and belonging to the *w*Pip groups I and IV, respectively (Duron *et al*.

2006b; Atyame *et al*. 2011a). In Tunisia, *w*Pip11 is distributed in the east and south and *w*Pip31 in the north and west, the latter also spread over in Algeria (Figure 1). Structure analysis of the *w*Pip strains frequency was carried out in two independent groups of sampled localities (Clusters I and II, Figure 1, separated by a dry area in which no mosquito breeding site could be found) and evidenced, in both groups, spatial autocorrelations in cytotype frequencies (Mantel test on Spearman rank correlation: b = 0.01, $P = 0.001$ for Cluster-I, and b = 0.01, $P = 0.004$ for Cluster-II). The contact zone was in the center of northern Tunisia, delineated by the Medjerda River along which most samples were infected by one of either strain (Figure 1). The transition between *w*Pip11 and *w*Pip31 distribution was sharp, spanning only a few kilometers, e.g. 3.60 km between Othman (#28, 100% *w*Pip11) and Briss (#38, 100% *w*Pip31). Within the contact zone, 12 localities exhibited a mixture of mosquitoes infected by *w*Pip11 or by *w*Pip31, with a frequency of *w*Pip11 infections ranging from a few percent (e.g. Zerga, #30), 20-30% (e.g. Fontaine, #50; Aïn Tounga, #51) to 91-97% (e.g. Slouguia, #40; Œufs, #44).

Most *w*Pip31 display unidirectional CI with *w*Pip11; a few show bidirectional CI

We first investigated the CI patterns between *w*Pip11 and *w*Pip31 infected mosquitoes from different areas. We used five *w*Pip11 isofemale lines from three localities and 16 *w*Pip31 lines from eight localities (Table S3). Two isofemale lines isolated from a same locality were used as replicates whenever possible. In all crosses, compatibility or incompatibility was associated with full embryonic viability (HR >90%) or mortality (HR = 0%), respectively. We never observed CI in crosses between mosquitoes infected with the same *w*Pip strain (data not shown).

We analyzed the CI patterns between the *w*Pip11 and *w*Pip31 strains (Tables S3 and S4, about 20 egg rafts per cross). *w*Pip11 males sterilized *w*Pip31 females in all crosses (80 crosses); this was further confirmed by crossing isofemale-derived *w*Pip11 males from three other localities (El Battan, #43; El Manar, #46 and Aïn Tounga, #51) with *w*Pip31 females derived from the Har isofemale line (Harash, #3) (Table S5). Most *w*Pip31 males were compatible with *w*Pip11 females (75 of 87 crosses from 20 *w*Pip31 lines; Tables S3B and S4). However, *w*Pip31 males from the Algerian lines Souk1 and Souk2 were incompatible with all *w*Pip11 females assayed (five *w*Pip11 lines tested, Table S3B).

Thus, two strains of *w*Pip31, molecularly indistinguishable for the markers considered, are present in the studied area. The strains where males are respectively compatible or incompatible with the *w*Pip11 females (uni-directional or bi-directional CI) will be thereafter named *w*Pip31_U or *w*Pip31_B (Table 1). Such uni- and bi-directional CIs were also observed between lines from localities where *w*Pip11 and *w*Pip31 strains were sympatric (Zerga, #30; Aïn Tounga, #51; Table S4). Note that all crossed *w*Pip11 lines were compatible among each other, as were the *w*Pip31_U and *w*Pip31_B lines. Since no polymorphic markers can discriminate the two *w*Pip31 cytotypes, we deduced their respective frequencies through extensive crossing experiments. We crossed females from the *w*Pip11 isofemale Sok line (Sokra, #37) with *w*Pip31 isofemale-derived males from geographically distant pure *w*Pip31 (Hamra, #29; Ras Rajel, #57; Khetmine, #62) and mixed *w*Pip31/*w*Pip11 (El Manar, #46; Aïn Tounga, #51) populations. We detected the two *w*Pip31 cytotypes in the five locations, whether near or far from the contact zone. The frequency of the *w*Pip31_B cytotype varied from 3% (1/39; Khetmine, #62) to 20% (7/35; Aïn Tounga, #51) with a mean frequency of 12% (Table 2 and Table S6). Thus, the two *w*Pip31 strains are widespread over Tunisia, but *w*Pip31_U is the most frequent.

In the field, *w*Pip11 does not outcompete *w*Pip31 as predicted

The situation in which *w*Pip11 males induce incompatibility with *w*Pip31 females, whereas *w*Pip31 males are mostly compatible with *w*Pip11 females, is predicted to evolve towards *w*Pip11 invasion if the host population is panmictic and unstructured. We therefore performed additional samplings in 2010 and 2011 along the contact zone delineated in 2009 and compiled the complete data set over the seven-year follow-up (2005-2011). All localities where the initial frequency of *w*Pip11 or *w*Pip31 was 100% remained stable (Table S1). In all localities where *w*Pip11 was majority, *w*Pip31 frequency was very low and did not vary over time. Remarkably, *w*Pip11 frequency did not change in four localities where *w*Pip31 was majority (Zerga, #30; Dougga, #41; Gaafour, #53; Utique, #61) and even decreased in three others (El Manar, #46; Aïn Tounga, #51; Beja Gare, #54). *w*Pip11 frequency increased only in two localities, transiently in Hamra (#29) and more stably in Fontaine (#50) (Table S1). Our data thus indicate an overall stability of the contact zone between the molecular strains *w*Pip11 and *w*Pip31 over the seven-year study.

*w*Pip11 outcompetes *w*Pip31 in population cages

This observed stability of the contact zone prompted us to examine which life history traits may interfere with *w*Pip11 invasion. We first tested the invasive capacity of *w*Pip11 in population cages by confronting *w*Pip11 and *w*Pip31_U infected lines carrying the same nuclear background. Three population cages were initiated using 50/50 as initial frequencies for *w*Pip11 and *w*Pip31_U. Assuming random mating, complete CI, 100% maternal transmission and no differential fitness cost, *w*Pip11 frequency was expected to reach near-fixation (98%) within four generations (Hoffmann *et al*. 1990). *w*Pip11 frequency increased rapidly and consistently with these expectations (Table 3). The observed *w*Pip11 frequencies

were homogeneous between the three cages (Fisher exact test, P = 0.245) and showed no significant deviation from the expected frequencies (Exact binomial tests on pooled replicates, P = 0.28 and 1 for generations 3 and 4, respectively). This result shows that, in the laboratory, *w*Pip11 invades as expected, suggesting that life history traits other than CI penetrance, *Wolbachia* transmission and fitness cost hamper the spreading of *w*Pip11 in the field.

No specific association between *w*Pip strains and *Cx. pipiens* forms

A straightforward explanation might be that *w*Pip11 and *w*Pip31 differentially infect the *pipiens* and *molestus* forms of the *Cx. pipiens* complex. These two forms show behavioral and physiological differences: the *molestus* form mates in confined spaces, remains active during winter and can oviposit without a blood meal, whereas the *pipiens* form mates in open spaces, undergoes winter diapause and requires a blood meal for oviposition (Fonseca *et al*. 2004; Farajollahi *et al*. 2011; Harbach 2012). We genotyped the *CQ11* microsatellite loci of 20 mosquitoes infected by *w*Pip11 (Ayed, #24 and Riadh, #27) and 20 mosquitoes infected with *w*Pip31 (Tabarka, #31; Boussalem2, #71) (Table S7). We identified the two *pipiens* and *molestus* forms and their hybrids (three-level response variable SPEC) in either *w*Pip11- or *w*Pip31-infected mosquitoes (two-level variable INF), in the four populations (four-level variable POP). No evidence for any preferential association was found (multinomial logit model (Venables & Ripley 2002): SPEC=INF+POP+INF.POP; Likelihood Ratio Test (LRT) for INF.POP: χ^2= 0.54, P = 0.76). Therefore the stability of the contact zone cannot be explained by differential infection rates of the *Cx. pipiens* forms.

Assortative mating is insufficient to explain *w*Pip dynamics

Although the kinetics of *w*Pip11 spreading in population cages are compatible with random mating, we further examined the occurrence of assortative mating, known to allow for the stable coexistence of incompatible *w*Pip strains in field populations (Rousset *et al.* 1991). We first addressed this possibility by confronting one *w*Pip11 isofemale line (Sok from Sokra, #37) with two *w*Pip31_U isofemale lines (Kef1-1 and Kef1-2 from Kef1, #34). Assuming random mating, we expected 25% of infertile egg rafts among the offspring (i.e. no hatching (HR = 0%) and 20-80% abortive embryonic development, hallmarks of incompatible crosses). The random mating hypothesis could not be rejected (Table 4), except in one of the six cages, which produced significantly less infertile egg rafts than expected (Fisher exact test, $P = 0.02$). However, this was not significant after a sequential Bonferroni's correction. We next wished to address the occurrence of assortative mating in the field, by measuring the incidence of sterile egg rafts collected in locations where *w*Pip11 and *w*Pip31 are sympatric (Table 5). Overall, we examined 137 to 590 egg rafts per locality, i.e. a total of 1938 egg rafts. In control localities with only *w*Pip11 (Jedaida, #59) or *w*Pip31 (Hamra, #29 and Dougga, #41) infected mosquitoes (allopatric), almost all egg rafts were fertile (<1% infertile, Table 5). In localities where both *w*Pip11 and *w*Pip31 were present (Zerga, #30; Oued Melah, #39; El Manar, #46 and Font Mjez, #69, sympatric), the frequency of infertile egg rafts (INF) was between 3% (7 out of 252) and 7% (18 out of 245). The incidence of infertile egg rafts in sympatric and allopatric localities (two-level variable SYM) was significantly different (generalized linear model with binomial error INF=SYM; LRT: $P < 10^{-6}$), demonstrating that incompatibility is expressed in natural populations.

We then estimated the incidence of assortative mating by maximum likelihood: in the absence of assortative mating, the expected frequency of infertile crosses would be $P_I=p_{11}(1-$

p_{11})(1+p_{31B}), where p_{11} is *w*Pip11 frequency and p_{31B} is *w*Pip31_B frequency among *w*Pip31-infected mosquitoes (i.e. crosses between males *w*Pip11 and females *w*Pip31_U, and crosses between *w*Pip11 and *w*Pip31_B in both directions). Assortative mating entails a reduction of this frequency, described as $P_I(1-F)$, where F is the correlation of cytotypes between mates. F and p_{11} can be jointly estimated using binomial models for the observed frequencies of *w*Pip11 and infertile egg rafts. However, this gives little information on F when strain frequencies are close to 0 or 1, which is the case for the sympatric populations here, even when a large number of egg rafts are examined. We nevertheless jointly estimated by maximum likelihood (i) the *w*Pip11 frequencies in each population, and (ii) a single correlation F, with its confidence interval, for all populations, using $p_{31B} = 0.12$ (i.e. the observed mean frequency of *w*Pip31_B, Table 2). As expected from strain frequencies near to zero, the confidence interval was very wide [-0.08, 0.63], with an estimated value of F of 0.39.

These results show that *w*Pip11 and *w*Pip31 strains clearly mate at random in cage trials and that CI is expressed in the field where those strains are present. The incidence of infertile egg rafts in the field remains compatible either with random or with only moderate assortative mating. Therefore additional factors must be invoked to explain why *w*Pip11 does not supersede *w*Pip31 in Tunisia.

Low dispersal rate and *w*Pip31_B are critical for the stability of the contact zone

The frequency dynamics of the three types can be analyzed as that of two types

Only females transmit *Wolbachia* and *w*Pip31 females display a similar CI pattern toward *w*Pip11 males when infected by *w*Pip31_B and *w*Pip31_U (Table 1, the rows

for *w*Pip31_B and *w*Pip31_U females are identical). This implies that *w*Pip31_U and *w*Pip31_B have the same expected reproductive success and, therefore, that their relative frequency does not change. Thus the evolution of the molecular polymorphism *w*Pip31/*w*Pip11 can be investigated by models previously developed for two cytoplasmic types (e.g. Caspari & Watson 1959; Hoffmann *et al*. 1990), considering complete CI in one direction and low CI in the other direction, in proportion of the frequency of *w*Pip31_B within *w*Pip31.

w*Pip31_B initial frequency is a key factor for* w*Pip11 invasion*

Analysis of *w*Pip molecular types was done in assuming no effect of *w*Pip infections on female fecundity (i.e. no cost of infection) and full maternal transmission of the three cytotypes, as generally observed in *Cx. pipiens* (Rasgon & Scott 2003). These conditions are well known to lead to an unstable equilibrium, at a frequency depending on *w*Pip31_B relative frequency. If the initial frequency of *w*Pip11 is higher than that of *w*Pip31_B, *w*Pip11 is expected to spread to fixation over a few generations, eliminating both *w*Pip31_U and *w*Pip31_B. If the initial frequency of *w*Pip11 is lower than that of *w*Pip31_B, *w*Pip11 should be eliminated, and *w*Pip31_U and *w*Pip31_B would stably coexist. Hence, in this model, no stable coexistence of the three *w*Pip cytotypes is made possible and *w*Pip11 could colonize localities where *w*Pip31 is present only if its frequency exceeds the frequency of *w*Pip31_B. This suggests that dispersal might be a critical factor for the absence of invasion.

Low dispersal rate may prevent w*Pip11 invasion*

We thus investigated the role of dispersal on the stability of the contact zone by modeling evolution of the spatial frequency cline of the three cytotypes (*w*Pip11, *w*Pip31_U and *w*Pip31_B) along a transect orthogonal to the contact zone. We therefore developed a spatially explicit version of earlier two-patch models (Telschow *et al*. 2005; Flor *et al*. 2007). This model (Annex 1) considers the influence of a fecundity (or survival) cost (c) for the *w*Pip11 strain outside its initial range. It assumes that there is no selective difference between *w*Pip31_U and *w*Pip31_B, and that their relative frequency remains constant through time. Migration occurs with probability m between adjacent populations across the contact zone. Adjacent populations in the model represent adjacent localities, about 5 km apart, in the habitat. Numerical iterations were performed during 50 generations (i.e. about 5 years). The model allows estimating the threshold value of the dispersal probability m per generation below which *w*Pip11 cannot invade (i.e. it reaches 50% in a population). This dispersal probability was investigated taking into account different values of b (the frequency of *w*Pip31_B) and c (the cost associated with *w*Pip11 infection when outside its initial range). Schematically, m will increase when b or c increases. Even under the least favorable conditions, i.e. a relatively high fecundity cost (c = 0.2) and a relatively high *w*Pip31_B frequency (b = 0.16), *w*Pip11 invasion can be halted only if dispersal is extremely low (e.g. a dispersal probability m < 0.08). Given the distance between adjacent populations, this represents a mean square axial dispersal distance σ^2 lower than 2 km² per generation.

Discussion

Our analysis of *w*Pip polymorphism in *Cx. pipiens* field populations from Algeria and Tunisia revealed the presence of two molecular strains, *w*Pip11 and *w*Pip31. These strains are

observed in Tunisia with a clear spatial structure: *w*Pip31 over the north and west, and *w*Pip11, over the east and south. Whereas most localities are infected either by *w*Pip31 or by *w*Pip11, they are sympatric along a very narrow contact zone in the center of the northern part of Tunisia.

We found CI in all crosses between *w*Pip11 and *w*Pip31 infected isofemale lines. In all instances *w*Pip11 males sterilized *w*Pip31 females. However, while most of the *w*Pip31 males were compatible with *w*Pip11 females, a fraction of *w*Pip31 males, molecularly indistinguishable induced CI (Table 1). Thus, two *w*Pip31 strains, *w*Pip31_U and *w*Pip31_B, coexist in all localities studied. The differences between the two *w*Pip31 strains may be interpreted in terms of different *mod* factors, as *w*Pip31_U males are incompatible with other *w*Pip strains (Atyame *et al*. 2011b, 2014).

The main result of this study is the stability of the contact zone observed for over seven years. Theoretical models predict that, in an unstructured panmictic population, the CI inducing strain (in the present case *w*Pip11) should invade. We thus explored several mechanisms that could explain why the *w*Pip11 strain does not progress: (i) assortative mating, (ii) local adaptation, (iii) limited dispersal of the *w*Pip11 strain (structuration) and (iv) quality of the breeding sites.

Assortative mating cannot explain the stability of the contact zone

Due to negative effects of CI on host fitness, selection should favor mechanisms that limit or suppress the expression of CI (Rousset *et al*. 1991; Turelli 1994). Among them, assortative mating between individuals infected with the same *Wolbachia* may lead to the

stable coexistence of incompatible strains of *Wolbachia* in field populations. Such mating discrimination has been reported between uninfected *Drosophila subquinaria* females and infected *D. recens* males when they occur in sympatry (Jaenike *et al*. 2006). However, we did not detect any preferential association between *w*Pip11 or *w*Pip31 and either *Cx. pipiens* forms (*pipiens* or *molestus*). In addition, we did not detect assortative mating in laboratory trials (Table 4). These results are in line with previous studies showing that *Cx. pipiens* females cannot discriminate between compatible and incompatible partners (Laven 1967b; Curtis & Adak 1974; Curtis *et al*. 1982; Duron *et al*. 2011). Cage population experiments showed that *w*Pip11 rapidly supersedes *w*Pip31_U, as expected from models assuming random mating, complete CI, 100% *Wolbachia* transmission and no fitness cost. Although the occurrence of moderate assortative mating in the field could not be formally excluded, it would have a limited impact on *w*Pip11 dynamics, even at the maximum value of the confidence interval (Figure S2). Assortative mating may have a significant impact only if at its highest values and combined with a high infection cost, which is not the case here (see below). Thus, if assortative mating occurs in the field, its incidence is too low to explain the stability of the contact zone.

Differential fitness cost cannot explain the stability of the contact zone

The stability of the contact zone can also be explained by local adaptation. Association between *Wolbachia* strains and nuclear or mitochondrial genes may indeed confer an increased fitness of mosquitoes infected by a *w*Pip strain at the expense of immigrant mosquitoes infected by another *w*Pip strain. For instance, local adaptation to climate due to mitochondrial genes has already been described (Blier *et al*. 2001; Ehinger *et al*. 2002; Fontanillas *et al*. 2005; Wallace 2007). Since mitochondria and *Wolbachia* are

maternally co-transmitted, different *Wolbachia* strains could thus be strictly associated with different mitochondrial haplotypes that confer differential local adaptation. In our system, we found two mitochondrial *cytb* haplotypes, each being strictly associated with either *w*Pip11 or *w*Pip31 infections (data not shown), which could potentially participate to local adaptation. However, modeling the cytotype clinal patterns, we showed that *w*Pip11 is expected to invade *w*Pip31 area, even when associated with a very high fitness cost ($c = 0.2$), providing a dispersal probability over 0.08 (Figure 2). Such extreme cost is very unlikely, since *w*Pip11 reached near-fixation in population cages as rapidly as predicted by models assuming no differential cost. Moreover, we also compared the observed *w*Pip11 dynamics in the cages with those expected assuming random mating, complete CI, 100% maternal transmission and a differential fitness cost of 0.2: the dynamics was significantly faster (Exact binomial tests on pooled replicates, $P = 3.10^{-6}$ and 1.10^{-4}, respectively for generations 3 and 4). Thus, it is unlikely that fitness differences associated with *w*Pip11 and *w*Pip31 could explain the observed stability of contact zone.

Low dispersal likely prevents *w*Pip11 invasion

In a panmictic unstructured population the coexistence of the three cytotypes (*w*Pip11, *w*Pip31_U and *w*Pip31_B) is not possible. Because *w*Pip11 is favored by unidirectional CI, *w*Pip31 should obviously be eliminated from localities where *w*Pip11 is the most abundant (i.e. *w*Pip11 > *w*Pip31). *w*Pip11 should also increase up to fixation in localities where *w*Pip31 predominates (i.e. *w*Pip31 > *w*Pip11), when its frequency is higher than *w*Pip31_B. *w*Pip31_B frequency therefore represents the threshold controlling *w*Pip11 invasion. This frequency, estimated in five localities through crossing experiments, ranges from 3% to 20% (mean frequency 12%). Modeling the spatial dynamics of the three cytotypes, assuming a

frequency of *w*Pip31_B $b = 0.16$ and a large cost $c = 0.2$ of *w*Pip11 (which is unlikely high), we deduced that the mean axial dispersal distance σ^2 should be lower than 2 km² per generation to prevent *w*Pip11 progression from one locality to the next within the five-year period (about fifty generations). This is much lower than published dispersal estimates in *Cx. pipiens*, e.g. $\sigma^2 = 43$ km² per generation in southern France (Lenormand *et al*. 1999). This is within the range of low mean dispersal distances (i.e. 1 to 2 km) deduced from recent mark-recapture studies in Hawaï (Lapointe 2008), or New York State (Ciota *et al*. 2012). However, in these studies, traps for recapture were set within 3 and 2 km from the release site respectively, so that more distant events could not be observed. According to the results of our model, low dispersal currently is the parameter that better explains the contact zone stability. Moreover, this is consistent with previous theoretical studies (Telschow *et al*. 2005; Flor *et al*. 2007).

Influence of the quality and/or quantity of breeding sites

Local spatial heterogeneity such as differential host density could also slow down or even block *Wolbachia* progression (Barton & Turelli 2011). However, *w*Pip11 progression in Tunisia could be stalled only if populations infected with *w*Pip31 are far denser than the nearby populations infected by *w*Pip11. This does not correspond to our field observations, based on the survey of breeding larval sites: most sites were sparsely populated, except for a few along the coast in Bizerte (#15) and Tunis (#16).

These *Culex* populations may actually be better described as metapopulations with fluctuating local densities, in which case *Wolbachia* spread could be much slower, as shown by Hancock and Godfray (2012). Mosquito larval and pupal stages require watered breeding

sites to develop and the availability of suitable sites may constitute a limiting parameter. Indeed, *Cx. pipiens* breeding sites in the contact zone are temporary, dry during the summer due to temperatures above 30°C and low rainfall, and filled again from autumn to spring. Extinction-recolonization events thus probably reset every year *w*Pip frequencies to those of the breeding adults present when sites are being rewatered. This may act as a brake on *w*Pip11 progression, despite its CI advantage, and may explain why *w*Pip11 did not invade in most of the sympatric sites where its frequency was above 10% (El Manar, #46; Hamra, #29; Aïn Tounga, #51 and Béja Gare, #54) and even in Fontaine (#50) where the *w*Pip11 frequency appears to level-off at 60-70%.

Conclusion

We highlighted the presence of a very narrow contact zone between the distribution areas of the *w*Pip11 and *w*Pip31 molecular strains in Tunisia. The situation is complex due to the segregation of three *Wolbachia* strains: while the most frequent *w*Pip31 strain is uni-directionally incompatible with *w*Pip11, a *w*Pip31 strain bidirectionally incompatible with *w*Pip11 (*w*Pip31_B) is present at low frequency (~12%). The narrow contact zone between the *w*Pip11 and *w*Pip31 strains appeared stable over a seven-year survey. The situation might have been stable for a longer period of time, since *w*Pip31 infected mosquitoes have been detected in 1996 in Mateur (#14) and Bizerte (#15) and *w*Pip11 in 1997 in Tunis (#16), located 50 to 60 km apart. The observed stability cannot be explained by local adaptation, and likely results from a low dispersal probability strengthened by metapopulation dynamics in *Cx. pipiens*. Although this result has already been predicted theoretically (Telschow *et al.* 2005; Flor *et al.* 2007), we present here the first empirical evidence of such a stable coexistence in the field. This study points out that a thorough knowledge of the host

dynamics and the environmental conditions prevailing in the studied region is required to understand how *Wolbachia* distribution evolves in natural populations and how this might impact *Wolbachia*-based control strategies.

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Data accessibility

Data sets used in this study are available as Supporting Information in the online version of this article.

Supporting information

Table S1. Distribution of the molecular *Wolbachia* strains *w*Pip11 and *w*Pip31 infecting *Culex pipiens* field samples from Algeria and Tunisia. Localities are numbered as in Figure 1. Localities in bold are those where *w*Pip11 and *w*Pip31 infected mosquitoes were sympatric (i.e. observed in a same breeding site); those underlined, i.e. Hamra (#29), Fontaine (#50) and Béja Gare (#54) displayed significant changes of *w*Pip infections with time. Samples G5 (#1), Bled (#11), Gourbi (#12), Menzel (#13), Tunis (#16), Bismuth (#17), Douz (#18) are from Duron *et al*. (2006b). N, sample size. Confidence intervals (c.i.) were calculated from binomial distribution; when frequencies of the *w*Pip11 strain equal 0 or 1, the upper or the lower values are given respectively.

Table S2. List of primers and characteristics of genes used to examine the *Wolbachia* and *Culex pipiens* polymorphisms.

Table S3. Reciprocal crosses between *w*Pip11 and *w*Pip31 infected isofemale lines from allopatric localities. A, *w*Pip11 males and *w*Pip31 females. B, *w*Pip31 males and *w*Pip11 females. The cross is compatible (C), for a HR > 90%; and incompatible (IC) for 0% HR. Incompatible crosses are shaded and bidirectional CI are underlined. The number of egg rafts collected in each cross is in parentheses. The name of mosquito line followed by number, for instance Sou1, Sou2 means that isofemale lines are from the same locality (see Table S1). a, Isofemale lines from Tunisia; b, Isofemale lines from Algeria. Dashes indicate that the cross was not performed.

Table S4. Reciprocal crosses between sympatric *w*Pip11 and *w*Pip31 infected mosquitoes. A, from Zerga (#30). B, from Aïn Tounga (#51). The cross is compatible (C),

for a HR > 90%; and incompatible (IC) for HR = 0%. Incompatible crosses are shaded and bidirectional CI are underlined. The number of egg rafts collected in each cross is in parentheses.

Table S5. Crossing relationships of *w*Pip11 males. This experiment was performed by crossing *w*Pip11 isofemale-derived males from three localities (El Battan, #43; El Manar, #46 and Aïn Tounga, #51) and *w*Pip31 females from the same isofemale line Har from Harash (#3, *w*Pip31_U). All crosses were incompatible (IC) with HR = 0%. The number of egg rafts collected in each cross is in parentheses.

Table S6. **Estimation of the frequencies of *w*Pip31_U and *w*Pip31_B cytotypes among *w*Pip31 infected mosquitoes**. This experiment was performed through unidirectional crosses between *w*Pip31 isofemale-derived males from fives localities (Hamra, #29; El Manar, #46; Aïn Tounga, #51; Ras Rajel, #57 and Khetmine, #62) and *w*Pip11 females from the same line Sok from Sokra (#37). The cross is compatible (C), for a HR > 90%; and incompatible (IC) for 0% HR. Incompatible crosses are shaded. The number of egg rafts collected in each cross is in parentheses.

Table S7. **Occurrence of *pipiens* and *molestus* forms of *Culex pipiens* taxa among *w*Pip11 and *w*Pip31 infected mosquitoes in Tunisia, identified by the *CQ11* microsatellite locus**.

Figure S1. Patterns of *ank2* and *MutL* PCR markers in *w*Pip11 and *w*Pip31 molecular strains.

Figure S2. Effect of dispersal probability on the progression of the *w*Pip11 cytotype into increasingly distant demes, with or without assortative mating. The maximal dispersal probability (m) below which the *w*Pip11 cytotype is prevented from invading a given deme (i.e. from reaching a frequency of 50% of the population) after 50 generations is indicated for different fitness costs associated to the *w*Pip11 cytotype (c) and for different levels of correlation of cytotypes between mates (F). The frequency of the *w*Pip31_B cytotype among all *w*Pip31 individuals was fixed as $b = 0.12$. For example, with $F = 0.39$ and $c = 0.2$, *w*Pip11 will not be able to invade the closest deme (deme 1) as long as $m < 0.09$, the next one (deme 2) as long as $m < 0.13$, the next one (deme 3) as long as $m < 0.2$, etc... Note that for $c = 0.2$ and $F = 0.63$, no value of m allowed *w*Pip11 to invade even the closest deme after 50 generations.

Author Contributions

Conceived and designed the experiments: CMA, OD, NP, PF, MW. Performed the experiments: CMA, MB, PM, OD, ED, PF, MW. Analyzed the data: CMA, PL, FR, OD, NP, PF, MW. Wrote the paper: CMA, PL, FR, NP, AB, PF, MW.

Annex 1: Modeling strains dynamics near the contact zone

The evolution of the spatial frequency cline of the cytotypes along a transect orthogonal to the contact zone was modeled. The transect is represented as a linear array of populations. In agreement with data, we assumed panmixia or partial assortative mating of magnitude F, complete transmission and complete CI. Within each site the change in cytotype i frequency due to incompatibility x'_i can be represented as $x'_i = (x\mathbf{A}x)_i / x\mathbf{A}x$ where x is the vector of cytotype frequencies, and $\mathbf{A}$ is the matrix describing incompatibility, which elements are 1 for compatible crosses, and 0 for incompatible ones (see Table 1). We then

assumed density regulation, followed by dispersal with a probability m to disperse to the two adjacent populations. In addition, a fecundity (or survival) cost c for the *w*Pip11 cytotype was considered.

There is no selective difference between *w*Pip31_U and *w*Pip31_B (Table 1), so that only their total frequency q is modified by incompatibility with *w*Pip11, but the frequency b of *w*Pip31_B among all *w*Pip31 remains constant through time. Thus it is equivalent to model the *w*Pip31_U and *w*Pip31_B cytotypes collectively as a single cytotype, incompatible with *w*Pip11 males, and only weakly incompatible (in proportion to the relative frequency of *w*Pip31_B among *w*Pip31) with *w*Pip11 females. Previously developed results for unidirectional or bidirectional incompatibility between two cytotypes can therefore be adapted to the present model. In particular, the within-population dynamics of *w*Pip11 frequency $p = 1\text{-}q$ can be approximated by a cubic function matching the stable points of the exact dynamics:

$$\Delta p \approx (1+b)p(1-p)\left(p - \frac{b}{1+b}\right).$$

By analogy with eq. (14b) in Barton and Turelli (2011), the cline should then form a travelling wave with speed

$$\approx \sigma\sqrt{(1+b)}\left(\frac{1}{2} - \frac{b}{1+b}\right),$$

where σ is the square-root of the mean square dispersal distance along the transect (axial dispersal). These computations can be readily adapted to take a fecundity cost into account and can be used to argue that limited dispersal is required to explain the apparent stability of

the contact zone, and that a moderate reduction in fecundity of *w*Pip11 has little impact on this conclusion.

Numerical iterations of the recursions were performed to check these different conclusions and to further check that local adaptation of cytotypes, in the form of a larger reduction in fecundity of cytotypes outside their initial range (c), did not affect the conclusions. For simplicity, in these computations, edge effects were avoided by assuming a large circular array, with two symmetric clines evolving simultaneously. The initial state of the contact zone was a step function, all *w*Pip11 on one side and all *w*Pip31 on the other. The initial frequency of *w*Pip31_B within all *w*Pip31 was varied around the observed one (i.e. 12%). Dispersal occurred among adjacent populations on the array.

The results of our model are presented in Figure 2. It displays the maximum amount of dispersal allowed to prevent *w*Pip11 from reaching 50% in a population (i.e. from invading the population) at a given distance from the initial contact zone after 50 generations. It confirms that low dispersal is required to prevent the advance of *w*Pip11 ($m < 0.08$), even if the infection is associated with a relatively high fecundity cost (e.g. $c = 0.2$). Although the qualitative conclusions of the analytical approximation are supported, the approximation can substantially overestimate the speed for small dispersal rate among discrete populations (for example, the approximation overestimate the speed by a factor 2 for $m = 0.02$ in the absence of any fitness cost). Equivalently, estimates of m deduced from an observed advance using the approximation would be too low. To some extent, this is expected since for low enough dispersal rate - even for weakly bidirectional incompatibility - selection against the rare cytotype will be stronger than immigration, so that there will be no wave of advance (Barton & Turelli 2011; Telschow *et al*. 2005).

Breeding sites in the contact zone are distant from each other by about 5 km. According to the analytical approximation, to prevent a progress from one site to the next in 5 years of observations (about fifty generations, i.e. a speed of 1 distance unit for 50 time units) it is required that

$$\sigma^2 < \frac{1}{50^2(1+b)\left(\frac{1}{2}-\frac{b}{1+b}\right)^2}$$

in squared units of intersite distance per generation. That is 5 times more in km^2 per generation. In this equation, $b = 0.12$ implies $\sigma^2 < 0.06$ km² per generation (mean square axial dispersal distance), i.e. $m < 0.0023$. On the other hand, in similar conditions (i.e. no cost), the numerical analysis implies $m < 0.015$ (i.e. $\sigma^2 < 0.375$ km² per generation).

Figure Legends

Figure 1. Distribution of the molecular strains *w*Pip11 and *w*Pip31 in *Culex pipiens* populations from Algeria and Tunisia. In the localities in boxes (Hamra, #29; Fontaine, #50 and Béja Gare, #54), a significant change of *w*Pip frequencies was found over time. Numbers correspond to those in Table S1. Samples were grouped into cluster-I (samples 14-16, 19, 26-29, 36-38, 43-46) and cluster-II (samples 30-42, 47-56) for analyzing the structure of the contact zone. When samples from the same locality were analyzed for several years, we only represented data from the first year (see Table S1). Samples (#1, #11, #12, #13, #16, #17 and #18) are from a previous study (Duron *et al*. 2006b).

Figure 2. Effect of dispersal probability on the progression of the *w*Pip11 cytotype into increasingly distant demes. The maximal dispersal probability (*m*) below which the *w*Pip11 cytotype is prevented from invading a given deme (i.e. from reaching a frequency of 50% of the population) after 50 generations is indicated for different frequencies of the *w*Pip31_B cytotype (*b*) among all *w*Pip31 individuals and for different fitness costs associated to the *w*Pip11 cytotype (*c*). For example, with $b = 0.16$ and $c = 0.05$, *w*Pip11 will not be able to invade the closest deme (deme 1) as long as $m < 0.03$, the next one (deme 2) as long as $m < 0.04$, the next one (deme 3) as long as $m < 0.05$, etc... Note that, for concision and clarity of the figure, only the cases where $m < 0.1$ are presented.

Table 1. Summary of CI patterns occurring between the *w*Pip11 and *w*Pip31 strains.

		Males		
		*w*Pip11	*w*Pip31_U	*w*Pip31_B
Females	*w*Pip11	1	1	**0**
	*w*Pip31_U	**0**	1	1
	*w*Pip31_B	**0**	1	1

1 = compatible cross (all hatching rate (HR) > 90%); 0 = incompatible cross (HR = 0%). Incompatible crosses are bolded. *w*Pip11 males are always incompatible with *w*Pip31_U or *w*Pip31_B females, whilst *w*Pip31_U males were compatible and *w*Pip31_B males incompatible with *w*Pip11 females. So, two crossing types exist between the molecular strains *w*Pip11 and *w*Pip31: unidirectional CI between *w*Pip11 and *w*Pip31_U and bidirectional CI between *w*Pip11 and *w*Pip31_B. Note however, that the females from *w*Pip31_U and *w*Pip31_B display a similar CI pattern (boxed).

Table 2. Frequencies of the *w*Pip31_B cytotype in Tunisia.

Localities	Frequency of *w*Pip31 (n)	N	*w*Pip31_U	*w*Pip31_B	Frequency of *w*Pip31_B	95% *c.i.*
29. Hamra	1 (56)	56	50	6	0.11	(0.04, 0.22)
46. El Manar	0.96 (84)	38	34	4	0.11	(0.03, 0.25)
51. Aïn Tounga	0.90 (40)	35	28	7	0.20	(0.08, 0.37)
57. Ras Rajel	0.98 (66)	23	19	4	0.17	(0.05, 0.39)
62. Khetmine	1 (40)	39	38	1	0.03	(0, 0.13)
Total		191	169	22	0.12	(0.07, 0.17)

Estimations were made through analyzing the crossing relationships between *w*Pip31 males from isofemale lines isolated from samples collected in five localities and *w*Pip11 females of the isofemale line Sok (from Sokra, #37; see details on Table S6). Numbers preceding the localities are as in Table S1 and in Figure 1. The five localities are located in *w*Pip31 area (i.e. localities with *w*Pip31 frequency > *w*Pip11 frequency). Samples used in crossing experiments were collected in June (#57) and October 2010 (#29, #46, #51 and #62) (Table S1). n, total number of mosquitoes examined to estimate the frequency of *w*Pip31; N, the number of *w*Pip31 isofemale lines used in crosses. Confidence intervals (*c.i.*) were calculated from binomial distribution.

Table 3. Population cages showing the invasive capacity of the *w*Pip11 strain.

Generations	Expected frequencies		Observed frequencies (n)					
			Cage 1		Cage 2		Cage 3	
	*w*Pip11	*w*Pip31_U	*w*Pip11	*w*Pip31_U	*w*Pip11	*w*Pip31_U	*w*Pip11	*w*Pip31_U
G0	0.50	0.50	-	-	-	-	-	-
G1	0.67	0.33	-	-	-	-	-	-
G2	0.86	0.14	0.82 (75)	0.16 (16)	0.90 (80)	0.10 (8)	0.93 (75)	0.07 (6)
G3	0.98	0.02	0.97 (70)	0.03 (2)	1 (76)	0 (0)	0.97 (74)	0.03 (2)

Confrontations were performed between the *w*Pip11 strain (from the Tn line) and the *w*Pip31_U strain (from the Har line) introduced into the same nuclear background. Each cage was set up using males (n = 100) and females (n = 100) from the *w*Pip11 line with an equivalent number of males and females from the *w*Pip31_U line. The expected frequencies of *w*Pip11 and *w*Pip31_U strains were estimated assuming no cost and random mating. The number of individuals analyzed by PCR assays to measure the frequency of *Wolbachia* infections frequencies in cages is indicated in brackets (n).

Table 4. **Mating preferences between *w*Pip11 and *w*Pip31 infected mosquitoes in population cages.**

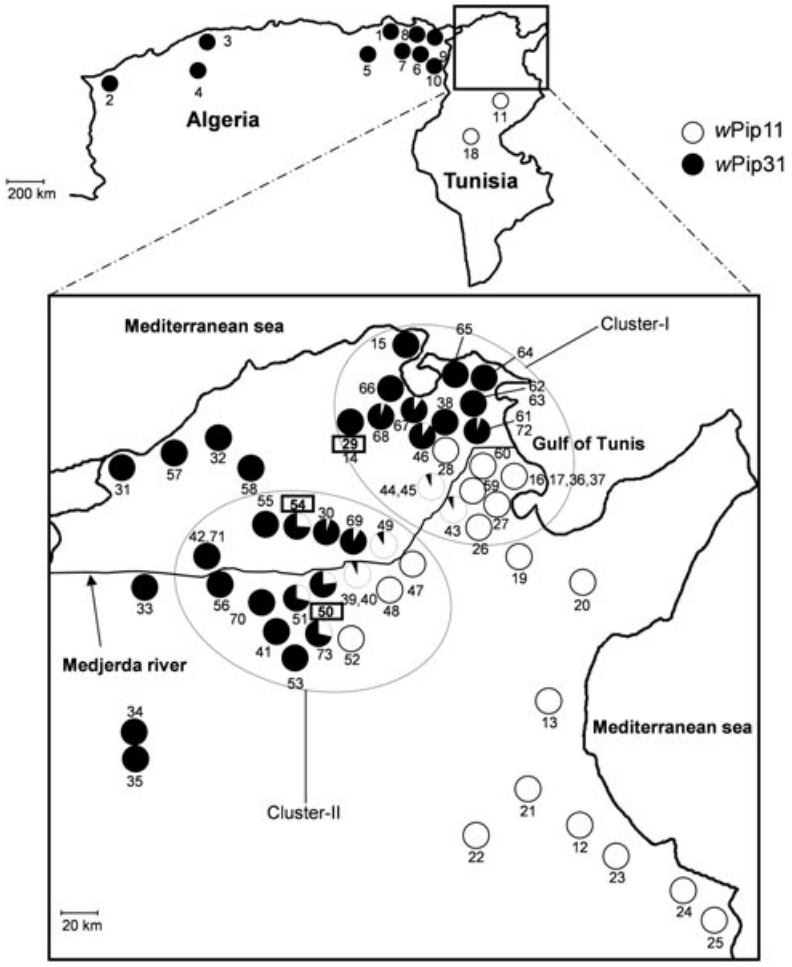
Population	Cage	Number of collected egg rafts	Observed frequency of CI egg rafts (n)	*P*-value[a]
Sok × *Kef1-1*	1	112	0.24 (27)	0.99
	2	79	0.10 (8)	0.02*
	3	110	0.34 (38)	0.18
Sok × *Kef1-2*	1	142	0.23 (32)	0.78
	2	102	0.22 (22)	0.74
	3	74	0.22 (16)	0.85

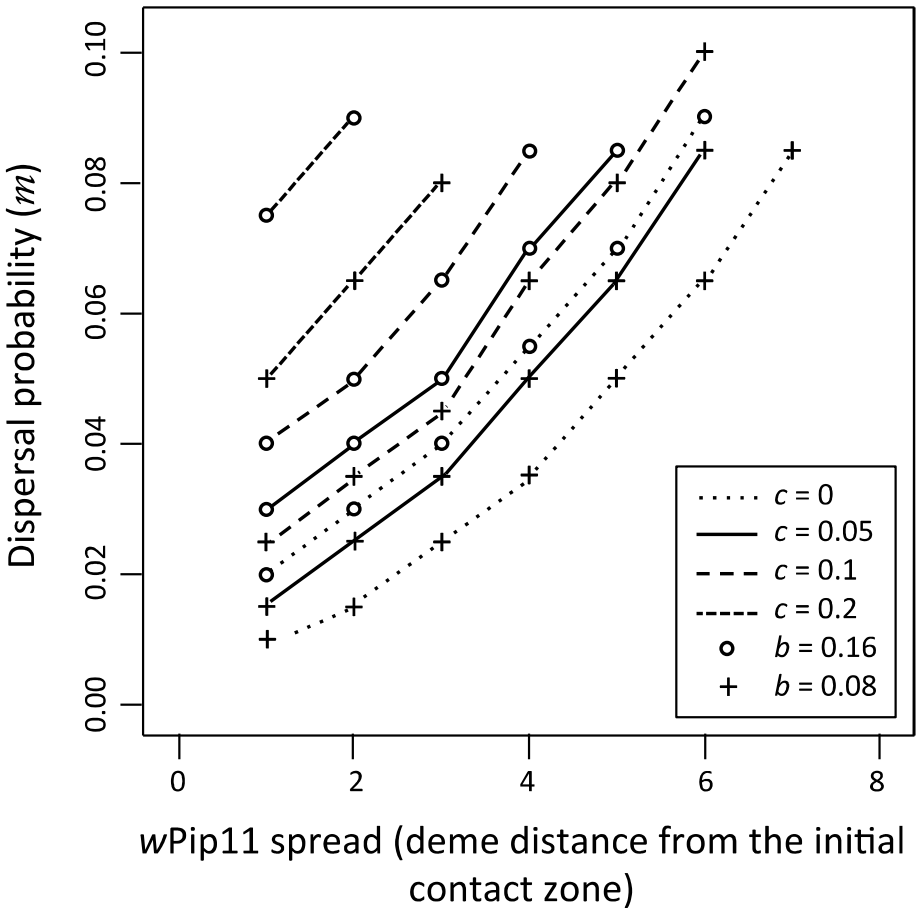
Each cage was set up using males (n = 100) and females (n = 100) from the Sok line (*w*Pip11) with an equivalent number of males and females from the Kef1-1 or Kef1-2 lines (*w*Pip31_U). The expected frequency of infertile egg rafts was 0.25, assuming unidirectional CI and random mating. * indicates significant difference between observed and expected frequencies of infertile egg rafts. [a] Fisher exact test.

Table 5. Status of egg rafts collected in *Culex pipiens* field populations in Tunisia.

Infection status of localities	Localities	Year of sampling	N	Frequency of *w*Pip11	Number of egg rafts examined	Fertile	Infertile
*w*Pip11 > *w*Pip31	39. Oued Melah	2009	42	0.93	245	227	18
	30. Zerga	2009	47	0.02	252	245	7
*w*Pip31 > *w*Pip11	46. El Manar	2010	59	0.1	272	257	15
	69. Font Mjez	2010	33	0.09	590	573	17
*w*Pip11	59. Jedaida	2010	22	1	157	157	0
*w*Pip31	41. Dougga	2009	23	0	137	136	1
*w*Pip31	29. Hamra	2010	41	0	285	284	1

The infection status, the year of sampling, the frequency of *w*Pip11 estimated by PCR on N mosquitoes, the number of egg rafts collected, and whether the egg rafts hatched (fertile) or not (infertile) is indicted for each locality.





Supplementary Information

Stable coexistence of incompatible *Wolbachia* along a narrow contact zone in mosquito field populations

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Table S1

Country	Localities	Coordinates	Year	N	*w*Pip infections				Isofemale lines	
					*w*Pip11	*w*Pip31	Frequency of *w*Pip11	95% *c.i.*	Abbreviation	Number
Algeria	1. G5	-	1997	12	0	12	0	(0.26)		
	2. Tafna	-	2006	5	0	5	0	(0.52)		
	3. Harash	-	2006	24	0	24	0	(0.14)	Har	1
	4. Smar	-	2006	5	0	5	0	(0.52)		
	5. Constantine	-	2006	5	0	5	0	(0.52)		
	6. Guelma	-	2008	30	0	30	0	(0.12)	Guel	2
	7. Lac	-	2008	24	0	24	0	(0.14)	Lac	2
	8. Douas	-	2008	27	0	27	0	(0.13)	Dou	2
	9. Kala	-	2008	28	0	28	0	(0.12)	Kal	2
	10. Souk Ahras	-	2008	30	0	30	0	(0.12)	Souk	2
Tunisia	11. Bled	-	1996	6	6	0	1	(0.54)		
	12. Gourbi	-	1996	16	16	0	1	(0.79)		
	13. Menzel	-	1996	12	12	0	1	(0.74)		
	14. Mateur	N37 02.814 E9 38.738	1996	12	0	12	0	(0.26)		
			2008	21	0	21	0	(0.16)		
			2010	23	0	23	0	(0.15)		
	15. Bizerte	N37 16.756 E9 50.528	1996	12	0	12	0	(0.26)		
			2010	24	0	24	0	(0.14)		
	16. Tunis	-	1997	12	12	0	1	(0.74)	Tn	2
	17. Bismuth	-	2003	17	17	0	1	(0.80)		
	18. Douz	-	2003	2	2	0	1	(0.16)		
	19. Mornag	N36 42.524 E10 16.101	2005	23	23	0	1	(0.85)		
	20. Grombalia	N36 35.454 E10 29.436	2005	23	23	0	1	(0.85)		
	21. Kondar	N35 56.131 E10 18.052	2005	23	23	0	1	(0.85)		

Table S1 continued

Country	Localities	Coordinates	Year	N	*w*Pip infections				Isofemale lines	
					*w*Pip11	*w*Pip31	Frequency of *w*Pip11	95% *c.i.*	Abbreviation	Number
Tunisia	22. Makroud	N35 46.132 E10 07.593	2005	24	24	0	1	(0.86)		
	23. Msaken	N35 43.575 E10 35.946	2005	18	18	0	1	(0.81)		
	24. Ayed	N35 37.586 E10 54.776	2005	24	24	0	1	(0.86)		
	25. Sousse	N35 50.503 E10 37.155	2005	10	10	0	1	(0.69)	Sou	2
	26. Mornaguia	N36 45.805 E10 00.568	2005	23	23	0	1	(0.85)		
	27. Riadh	**N36 50.008 E9 58.910**	2005	24	24	0	1	(0.86)		
			2009	**24**	**23**	**1**	**0.96**	**(0.79, 0.99)**		
			2010	47	47	0	1	(0.92)		
	28. Othman	N37 01.186 E9 51.692	2005	17	17	0	1	(0.80)		
	29. Hamra	**N37 02.668 E9 39.721**	2005	37	0	37	0	(0.09)		
			2009	**33**	**7**	**26**	**0.21**	**(0.09, 0.39)**		
			2010	47	0	47	0	(0.08)		
			2010 (oct)	56	0	56	0	(0.06)	Ham	56
			2011	23	0	23	0	(0.15)		
	30. Zerga	**N36 40.143 E9 26.056**	**2005**	**47**	**3**	**44**	**0.06**	**(0.01, 0.18)**		
			2009	**47**	**1**	**46**	**0.02**	**(0, 0.11)**	**Zer**	**4**
			2011	**24**	**1**	**23**	**0.04**	**(0, 0.21)**		
	31. Tabarka	N36 56.472 E8 45.459	2008	24	0	24	0	(0.14)	Tab	2
			2010	16	0	16	0	(0.21)		
	32. Nefza	N36 59.207 E9 04.733	2008	8	0	8	0	(0.37)		
	33. Souala	N36 31.604 E8 44.839	2008	5	0	5	0	(0.52)		
	34. Kef1	N36 04.054 E8 45.171	2008	17	0	17	0	(0.19)	Kef	2
	35. Kef2	N36 09.573 E8 43.118	2008	30	0	30	0	(0.12)		

Table S1 continued

Country	Localities	Coordinates	Year	N	*w*Pip infections				Isofemale lines	
					*w*Pip11	*w*Pip31	Frequency of *w*Pip11	95% *c.i.*	Abbreviation	Number
Tunisia	36. Ariana	N36 54.111 E10 13.100	2008	29	29	0	1	(0.88)		
			2009	24	24	0	1	(0.86)		
			2011	24	24	0	1	(0.86)		
	37. Sokra	N36 54.123 E10 13.099	2008	14	14	0	1	(0.77)	Sok	1
	38. Briss	N37 02.230 E9 53.887	2008	47	0	47	0	(0.08)		
	39. Oued Melah	**N36 35.032 E9 30.712**	**2008**	**45**	**43**	**2**	**0.96**	**(0.85, 0.99)**		
			2009	**42**	**39**	**3**	**0.93**	**(0.81, 0.99)**		
			2010	46	46	0	1	(0.92)		
			2011	22	22	0	1	(0.85)		
	40. Slouguia	**N36 32.419 E9 24.167**	2008	24	24	0	1	(0.86)		
			2009	**46**	**43**	**3**	**0.93**	**(0.82, 0.99)**		
	41. Dougga	**N36 23.769 E9 14.307**	2008	36	0	36	0	(0.09)		
			2009	23	0	23	0	(0.15)		
			2010	**38**	**1**	**37**	**0.03**	**(0, 0.14)**		
			2011	17	0	17	0	(0.19)		
	42. Boussalem1	N36 34.841 E8 59.096	2008	22	0	22	0	(0.15)	Bou	2
	43. El Battan	**N36 48.450 E9 53.828**	**2009**	**24**	**23**	**1**	**0.96**	**(0.79, 0.99)**		
			2010	47	47	0	1	(0.92)		
			2010 (oct)	20	20	0	1	(0.83)	ElB	19
			2011	24	24	0	1	(0.86)		
	44. Œufs	**N36 52.738 E9 51.876**	**2009**	**30**	**29**	**1**	**0.97**	**(0.83, 0.99)**		
	45. Tebourba	N36 52.738 E9 51.877	2009	24	24	0	1	(0.86)		
			2010	24	24	0	1	(0.86)		
			2011	21	21	0	1	(0.84)		

Table S1 continued

Country	Localities	Coordinates	Year	N	*w*Pip infections				Isofemale lines	
					*w*Pip11	*w*Pip31	Frequency of *w*Pip11	95% *c.i.*	Abbreviation	Number
Tunisia	**46. El Manar**	**N37 01.770 E9 52.207**	**2009**	**45**	**5**	**40**	**0.11**	**(0.04, 0.24)**		
			2010	**61**	**6**	**55**	**0.10**	**(0.04, 0.20)**		
			2010 (oct)	**84**	**3**	**81**	**0.04**	**(0, 0.10)**	**ElM**	**39**
			2011	**24**	**1**	**23**	**0.04**	**(0, 0.21)**		
	47. Goubellat	N36 31.847 E9 40.127	2009	24	24	0	1	(0.86)		
			2011	24	24	0	1	(0.86)		
	48. Chardon	N36 36.098 E9 38.441	2009	24	24	0	1	(0.86)		
	49. Mjez el Bab	**N36 39.484 E9 36.333**	**2009**	**23**	**21**	**2**	**0.91**	**(0.72, 0.99)**		
			2010 (oct)	**27**	**26**	**1**	**0.96**	**(0.81, 0.99)**		
			2011	24	24	0	1	(0.86)		
	50. Fontaine	**N36 32.417 E9 24.161**	**2009**	**31**	**7**	**24**	**0.23**	**(0.09, 0.41)**		
			2010	**55**	**35**	**20**	**0.64**	**(0.49, 0.76)**		
			2010 (oct)	**103**	**71**	**32**	**0.69**	**(0.59, 0.78)**		
			2011	**90**	**52**	**38**	**0.58**	**(0.47, 0.68)**		
	51. Aïn Tounga	**N36 31.361 E9 21.338**	**2009**	**31**	**9**	**22**	**0.29**	**(0.14, 0.48)**		
			2010 (oct)	**40**	**4**	**36**	**0.10**	**(0.03, 0.24)**	**AïnT**	**43**
	52. Buses	N36 22.333 E9 24.924	2009	15	15	0	1	(0.78)		
	53. Gaafour	**N36 20.151 E9 20.343**	2009	8	0	8	0	(0.37)		
			2010	**19**	**1**	**18**	**0.05**	**(0, 0.26)**		
	54. Béja Gare	**N36 43.441 E9 11.437**	**2009**	**19**	**5**	**14**	**0.26**	**(0.09, 0.51)**		
			2010	17	0	17	0	(0.19)		
	55. Beja Oued	N36 43.881 E9 12.315	2009	35	0	35	0	(0.10)		
			2010	48	0	48	0	(0.07)		
			2011	17	0	17	0	(0.19)		
	56. Elevage	N36 33.112 E9 00.491	2009	6	0	6	0	(0.46)		

Table S1 continued

Country	Localities	Coordinates	Year	N	*w*Pip infections				Isofemale lines	
					*w*Pip11	*w*Pip31	Frequency of *w*Pip11	95% *c.i.*	Abbreviation	Number
Tunisia	**57. Ras Rajel**	**N36 57.060 E8 52.784**	**2010**	**66**	**1**	**65**	**0.02**	**(0, 0.08)**	**RasR**	**23**
	58. Ftet	N36 56.658 E9 06.023	2010	5	0	5	0	(0.52)		
	59. Jedaida	N36 48.229 E9 56.544	2010	24	24	0	1	(0.86)		
			2011	22	22	0	1	(0.85)		
	60. Si Thabet	N36 57.189 E10 02.589	2010	23	23	0	1	(0.85)		
	61. Utique	**N37 04.176 E10 00.424**	**2010**	**47**	**2**	**45**	**0.04**	**(0, 0.15)**		
			2010 (oct)	30	0	30	0	(0.12)		
			2011	18	0	18	0	(0.19)		
	62. Khetmine	N37 08.910 E9 59.842	2010	19	0	19	0	(0.18)		
			2010 (oct)	40	0	40	0	(0.09)	Khet	39
	63. El Alia	N37 09.921 E10 02.389	2010	18	0	18	0	(0.19)		
	64. Ras Jebel	N37 13.194 E10 07.017	2010	23	0	23	0	(0.15)		
			2011	21	0	21	0	(0.16)		
	65. Azib	N37 13.358 E9 56.065	2010	13	0	13	0	(0.25)		
			2011	21	0	21	0	(0.16)		
	66. Ichkeul	N37 07.225 E9 44.921	2010	23	0	23	0	(0.15)		
			2011	24	0	24	0	(0.14)		
	67. Jmar	**N37 08.198 E9 53.414**	**2010**	**23**	**2**	**21**	**0.09**	**(0.01, 0.28)**		
	68. Pompe	**N37 04.323 E9 41.457**	**2010**	**43**	**2**	**41**	**0.05**	**(0, 0.16)**		
	69. Font Mjez	**N36 39.612 E9 28.977**	**2010**	**33**	**3**	**30**	**0.09**	**(0.02, 0.24)**		
	70. TBSK font	N36 27.706 E9 14.906	2010	5	0	5	0	(0.52)		
	71. Boussalem2	N36 36.729 E8 58.594	2010	22	0	22	0	(0.15)		
			2011	24	0	24	0	(0.14)		
	72. Utique pont	**N37 02.201 E10 02.437**	**2011**	**23**	**1**	**22**	**0.04**	**(0, 0.22)**		
	73. Nofrancaoui	N36 25.457 E9 19.562	**2011**	**14**	**4**	**10**	**0.29**	**(0.08, 0.58)**		

Table S1. Distribution of the molecular *Wolbachia* strains *w*Pip11 and *w*Pip31 infecting *Culex pipiens* field samples from Algeria and Tunisia. Localities are numbered as in Figure 1. Localities in bold are those where *w*Pip11 and *w*Pip31 infected mosquitoes were sympatric (i.e. observed in a same breeding site); those underlined, i.e. Hamra (#29), Fontaine (#50) and Béja Gare (#54) displayed significant changes of *w*Pip infections with time. Samples G5 (#1), Bled (#11), Gourbi (#12), Menzel (#13), Tunis (#16), Bismuth (#17), Douz (#18) are from Duron *et al*. (2006b). N, sample size. Confidence intervals (c.i.) were calculated from binomial distribution; when frequencies of the *w*Pip11 strain equal 0 or 1, the upper or the lower values are given respectively.

Organism	Gene	Putative product	Primer (5'-3')	Size (bp)	Reference
Wolbachia	*MutL*	DNA mismatch repair protein	F2- GCATAYCCTAGAGGATGATCCGC R2- GTGCATCCAAATAAATCGGAAG	374-437	Atyame *et al*. (2011a)
	ank2	Ankyrin domain protein	F-CTTCTTCTGTGAGTGTACGT R2-TCCATATCGATCTACTGCGT	313-511	Duron *et al*. (2007)
Culex pipiens					
mitochondrial	*cytb*	Cytochrome b	F-CTTTATTAGTAACTGTAAAAATTAC R-ACTAAAGGATTAGCAGGAATGA	852	Atyame *et al*. (2011b)
nuclear	*CQ11*	microsatellite locus	CQ11F2-GATCCTAGCAAGCGAGAAC molCQ11R 5'-CCCTCCAGTAAGGTATCAAC pipCQ11R 5'-CATGTTGAGCTTCGGTGAA	CQ11F2-molCQ11R : 250 CQ11F2-pipCQ11R : 200	Bahnck *et al*. (2006)

Table S2. List of primers and characteristics of genes used to examine the *Wolbachia* and *Culex pipiens* polymorphisms.

References

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A

*w*Pip31 females	*w*Pip11 males				
	Sou1[a]	Sou2[a]	Tn1[a]	Tn2[a]	Sok[a]
Bou1[a]	IC (21)	IC (12)	IC (12)	IC (17)	IC (14)
Bou2[a]	IC (9)	IC (17)	IC (8)	IC (8)	IC (14)
Kef1-1[a]	IC (15)	IC (11)	IC (16)	IC (14)	IC (18)
Kef1-2[a]	IC (15)	IC (17)	IC (17)	IC (12)	IC (11)
Tab1[a]	IC (19)	IC (11)	IC (16)	IC (15)	IC (15)
Tab2[a]	IC (30)	IC (8)	IC (18)	IC (12)	IC (11)
Dou1[b]	IC (12)	IC (7)	IC (22)	IC (12)	IC (23)
Dou2[b]	IC (15)	IC (10)	IC (10)	IC (12)	IC (21)
Guel1[b]	IC (13)	IC (10)	IC (12)	IC (15)	IC (12)
Guel2[b]	IC (21)	IC (20)	IC (19)	IC (21)	IC (24)
Kal1[b]	IC (12)	IC (12)	IC (13)	IC (9)	IC (15)
Kal2[b]	IC (14)	IC (13)	IC (12)	IC (19)	IC (17)
Lac1[b]	IC (13)	IC (12)	IC (16)	IC (12)	IC (15)
Lac2[b]	IC (13)	IC (20)	IC (9)	IC (12)	IC (13)
Souk1[b]	IC (16)	IC (14)	IC (13)	IC (23)	IC (14)
Souk2[b]	IC (11)	IC (15)	IC (12)	IC (22)	IC (10)

B

*w*Pip11 females	*w*Pip31 males															
	Bou1[a]	Bou2[a]	Kef1-1[a]	Kef1-2[a]	Tab1[a]	Tab2[a]	Dou1[b]	Dou2[b]	Guel1[b]	Guel2[b]	Kal1[b]	Kal2[b]	Lac1[b]	Lac2[b]	Souk1[b]	Souk2[b]
Sou1[a]	C (30)	C (16)	C (9)	C (13)	C (13)	C (18)	C (9)	C (17)	C (23)	C (18)	C (14)	C (11)	C (21)	C (15)	IC (24)	IC (21)
Sou2[a]	C (18)	C (20)	C (18)	C (8)	C (22)	C (18)	C (16)	C (17)	C (14)	C (22)	C (21)	C (29)	C (17)	C (19)	IC (20)	IC (16)
Tn1[a]	C (22)	C (16)	C (19)	C (25)	C (35)	C (24)	C (23)	C (19)	C (22)	C (18)	C (14)	C (20)	C (22)	C (12)	IC (19)	IC (24)
Tn2[a]	C (21)	C (21)	C (18)	C (23)	C (22)	C (18)	C (18)	C (15)	C (18)	C (18)	C (15)	C (26)	C (20)	C (15)	IC (23)	IC (16)
Sok[a]	C (16)	C (18)	C (14)	C (12)	-	C (12)	C (16)	C 12)	C (15)	C (12)	C (12)	C (14)	C (23)	C (17)	IC (14)	IC (20)

Table S3. **Reciprocal crosses between *w*Pip11 and *w*Pip31 infected isofemale lines from allopatric localities**. A, *w*Pip11 males and *w*Pip31 females. B, *w*Pip31 males and *w*Pip11 females. The cross is compatible (C), for a HR > 90%; and incompatible (IC) for 0% HR. Incompatible crosses are shaded and bidirectional CI are underlined. The number of egg rafts collected in each cross is in parentheses. The name of mosquito line followed by number, for instance Sou1, Sou2 means that isofemale lines are from the same locality (see Table S1). a, Isofemale lines from Tunisia; b, Isofemale lines from Algeria. Dashes indicate that the cross was not performed.

A

		Males			
		*w*Pip11		*w*Pip31	
Females		Zer1	Zer2	Zer3	Zer4
*w*Pip11	Zer1	-	-	C (21)	C (12)
	Zer2	-	-	C (18)	C (26)
*w*Pip31	Zer3	IC (9)	IC (11)	-	-
	Zer4	IC (15)	IC (16)	-	-

B

		Males			
		*w*Pip11		*w*Pip31	
Females		AïnT1	AïnT2	AïnT3	AïnT4
*w*Pip11	AïnT1	-	C (12)	C (20)	IC (26)
	AïnT2	C (25)	-	C (20)	IC (21)
*w*Pip31	AïnT3	IC (16)	IC (20)	-	C (18)
	AïnT4	IC (9)	IC (16)	C (17)	-

Table S4. **Reciprocal crosses between sympatric *w*Pip11 and *w*Pip31 infected mosquitoes**. A, from Zerga (#30). B, from Aïn Tounga (#51). The cross is compatible (C), for a HR > 90%; and incompatible (IC) for HR = 0%. Incompatible crosses are shaded and bidirectional CI are underlined. The number of egg rafts collected in each cross is in parentheses.

*w*Pip11 males from El Battan × *w*Pip31 females from Har		*w*Pip11 males from El Manar × *w*Pip31 females from Har		*w*Pip11 males from Aïn Tounga × *w*Pip31 females from Har	
ElB1	IC (13)	ElM39	IC (12)	AïnT40	IC (16)
ElB2	IC (12)			AïnT41	IC (15)
ElB3	IC (15)			AïnT42	IC (18)
ElB4	IC (14)			AïnT43	IC (14)
ElB5	IC (16)				
ElB6	IC (15)				
ElB7	IC (21)				
ElB8	IC (15)				
ElB9	IC (14)				
ElB10	IC (12)				
ElB11	IC (14)				
ElB12	IC (17)				
ElB13	IC (14)				
ElB14	IC (12)				
ElB15	IC (16)				
ElB16	IC (13)				
ElB17	IC (12)				
ElB18	IC (14)				

Table S5. **Crossing relationships of *w*Pip11 males**. This experiment was performed by crossing *w*Pip11 isofemale-derived males from three localities (El Battan, #43; El Manar, #46 and Aïn Tounga, #51) and *w*Pip31 females from the same isofemale line Har from Harash (#3). All crosses were incompatible (IC) with HR = 0%. The number of egg rafts collected in each cross is in parentheses.

*w*Pip31 males from Hamra × *w*Pip11 females from Sok		*w*Pip31 males from El Manar × *w*Pip11 females from Sok		*w*Pip31 males from Aïn Tounga × *w*Pip11 females from Sok		*w*Pip31 males from Ras Rajel × *w*Pip11 females from Sok		*w*Pip31 males from Khetmine × *w*Pip11 females from Sok	
Ham1	C (20)	ElM1	C (17)	AïnT5	IC (14)	RasR1	IC (18)	Khet1	C (17)
Ham2	C (19)	ElM2	C (22)	AïnT6	C (17)	RasR2	C (12)	Khet2	C (16)
Ham3	C (19)	ElM3	IC (15)	AïnT7	IC (17)	RasR3	C (15)	Khet3	C (14)
Ham4	C (15)	ElM4	C (20)	AïnT8	C (18)	RasR4	C (22)	Khet4	IC (16)
Ham5	C (18)	ElM5	IC (18)	AïnT9	IC (12)	RasR5	C (5)	Khet5	C (18)
Ham6	C (14)	ElM6	C (23)	AïnT10	C (20)	RasR6	C (14)	Khet6	C (13)
Ham7	C (18)	ElM7	C (19)	AïnT11	C (18)	RasR7	C (20)	Khet7	C (16)
Ham8	C (15)	ElM8	C (18)	AïnT12	IC (17)	RasR8	C (19)	Khet8	C (13)
Ham9	C (17)	ElM9	C (18)	AïnT13	C (15)	RasR9	C (14)	Khet9	C (13)
Ham10	C (18)	ElM10	C (18)	AïnT14	C (24)	RasR10	C (11)	Khet10	C (13)
Ham11	C (11)	ElM11	C (20)	AïnT15	C (21)	RasR11	C (20)	Khet11	C (15)
Ham12	C (16)	ElM12	C (22)	AïnT16	C (22)	RasR12	IC (21)	Khet12	C (21)
Ham13	C (24)	ElM13	C (22)	AïnT17	C (13)	RasR13	C (20)	Khet13	C (24)
Ham14	IC (18)	ElM14	C (24)	AïnT18	C (22)	RasR14	C (20)	Khet14	C (16)
Ham15	C (19)	ElM15	C (18)	AïnT19	C (18)	RasR15	C (15)	Khet15	C (21)
Ham16	C (21)	ElM16	C (19)	AïnT20	C (16)	RasR16	IC (15)	Khet16	C (18)
Ham17	C (19)	ElM17	C (20)	AïnT21	C (20)	RasR17	C (20)	Khet17	C (18)
Ham18	C (13)	ElM18	C (15)	AïnT22	C (19)	RasR18	C (21)	Khet18	C (19)
Ham19	C (19)	ElM19	C (16)	AïnT23	C (14)	RasR19	C (14)	Khet19	C (16)
Ham20	C (21)	ElM20	C (16)	AïnT24	C (17)	RasR20	IC (20)	Khet20	C (12)
Ham21	C (14)	ElM21	C (19)	AïnT25	C (23)	RasR21	C (18)	Khet21	C (17)
Ham22	C (18)	ElM22	IC (19)	AïnT26	C (19)	RasR22	C (7)	Khet22	C (13)
Ham23	C (15)	ElM23	C (16)	AïnT27	C (23)	RasR23	C (19)	Khet23	C (13)
Ham24	C (18)	ElM24	IC (21)	AïnT28	C (22)			Khet24	C (19)
Ham25	C (17)	ElM25	C (20)	AïnT29	C (19)			Khet25	C (19)
Ham26	C (21)	ElM26	C (17)	AïnT30	C (18)			Khet26	C (18)
Ham27	C (18)	ElM27	C (23)	AïnT31	C (22)			Khet27	C (16)
Ham28	C (18)	ElM28	C (12)	AïnT32	C (17)			Khet28	C (20)
Ham29	C (14)	ElM29	C (14)	AïnT33	IC (22)			Khet29	C (15)
Ham30	C (11)	ElM30	C (10)	AïnT34	C (20)			Khet30	C (13)
Ham31	C (18)	ElM31	C (19)	AïnT35	IC (18)			Khet31	C (13)
Ham32	IC (16)	ElM32	C (17)	AïnT36	IC (15)			Khet32	C (23)
Ham33	C (19)	ElM33	C (24)	AïnT37	C (17)			Khet33	C (13)
Ham34	C (16)	ElM34	C (12)	AïnT38	C (18)			Khet34	C (17)
Ham35	C (17)	ElM35	C (12)	AïnT39	C (15)			Khet35	C (21)
Ham36	IC (20)	ElM36	C (19)					Khet36	C (20)
Ham37	C (13)	ElM37	C (23)					Khet37	C (16)
Ham38	C (15)	ElM38	C (12)					Khet38	C (22)
Ham39	IC (18)							Khet39	C (20)
Ham40	C (16)								
Ham41	C (13)								
Ham42	C (17)								
Ham43	C (19)								
Ham44	C (13)								
Ham45	IC (15)								
Ham46	IC (18)								
Ham47	C (15)								
Ham48	C (21)								
Ham49	C (17)								
Ham50	C (16)								
Ham51	C (16)								
Ham52	C (16)								
Ham53	C (13)								
Ham54	C (14)								
Ham55	C (16)								
Ham56	C (19)								

Table S6. **Estimation of the frequencies of *w*Pip31_U and *w*Pip31_B cytotypes among *w*Pip31 infected mosquitoes**. This experiment was performed through unidirectional crosses between *w*Pip31 isofemale-derived males from fives localities (Hamra, #29; El Manar, #46; Aïn Tounga, #51; Ras Rajel, #57 and Khetmine, #62) and *w*Pip11 females from the same line Sok from Sokra (#37). The cross is compatible (C), for a HR > 90%; and incompatible (IC) for 0% HR. Incompatible crosses are shaded. The number of egg rafts collected in each cross is in parentheses.

Infection status	Localities	Year of sampling	N	*pipiens* form	*molestus* form	Hybrids
*w*Pip11	Ayed (#24)	2005	10	3	5	2
	Riadh (#27)	2010	10	3	4	3
*w*Pip31	Tabarka (#31)	2010	10	5	5	0
	Boussalem2 (#71)	2010	10	3	2	5

Table S7. **Occurrence of *pipiens* and *molestus* forms of *Culex pipiens* taxa among *w*Pip11 and *w*Pip31 infected mosquitoes in Tunisia, identified by the *CQ11* microsatellite locus**.

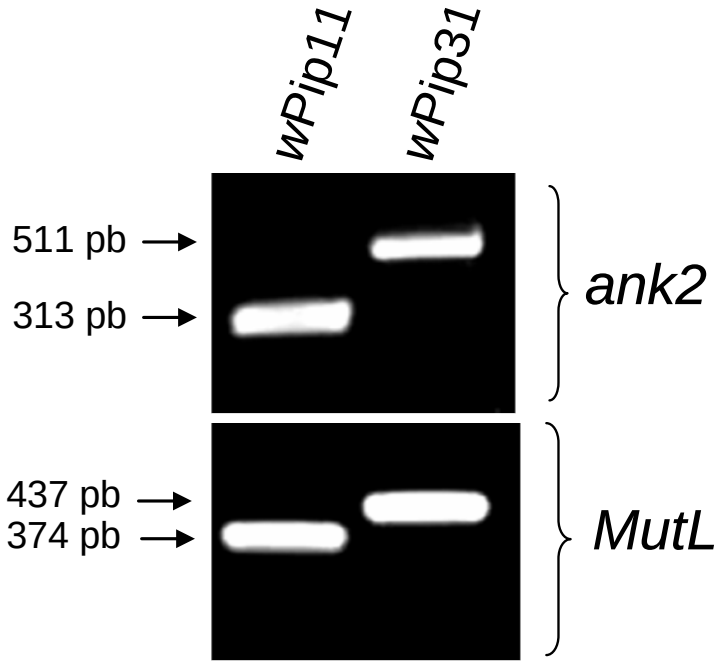

Figure S1. **Patterns of *ank2* and *MutL* PCR markers in *w*Pip11 and *w*Pip31 molecular strains**.

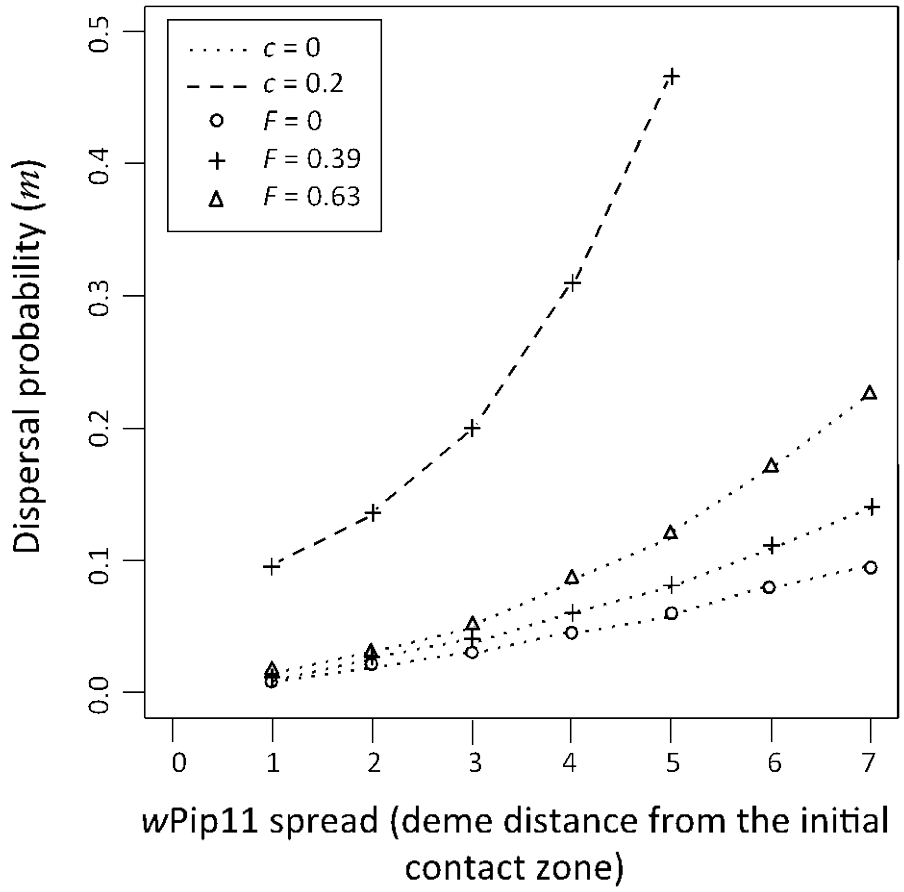

Figure S2. **Effect of dispersal probability on the progression of the *w*Pip11 cytotype into increasingly distant demes, with or without assortative mating**. The maximal dispersal probability (*m*) below which the *w*Pip11 cytotype is prevented from invading a given deme (i.e. from reaching a frequency of 50% of the population) after 50 generations is indicated for different fitness costs associated to the *w*Pip11 cytotype (*c*) and for different levels of correlation of cytotypes between mates (*F*). The frequency of the *w*Pip31_B cytotype among all *w*Pip31 individuals was fixed as $b = 0.12$. For example, with $F = 0.39$ and $c = 0.2$, *w*Pip11 will not be able to invade the closest deme (deme 1) as long as $m < 0.09$, the next one (deme 2) as long as $m < 0.13$, the next one (deme 3) as long as $m < 0.2$, etc... Note that for $c = 0.2$ and $F = 0.63$, no value of m allowed *w*Pip11 to invade even the closest deme after 50 generations.