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Transposable element polymorphism of *Wolbachia* in the mosquito *Culex pipiens*: evidence of genetic diversity, superinfection and recombination

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Abstract

***Wolbachia* is a group of maternally inherited endosymbiotic bacteria that infect and induce cytoplasmic incompatibility (CI) in a wide range of arthropods. In contrast to other species, the mosquito *Culex pipiens* displays an extremely high number of CI types suggesting differential infection by multiple *Wolbachia* strains. Attempts so far failed to detect *Wolbachia* polymorphism that might explain this high level of CI diversity found in *C. pipiens* populations. Here, we establish that *Wolbachia* infection is near to or at fixation in worldwide populations of the *C. pipiens* complex. *Wolbachia* polymorphism was addressed by sequence analysis of the *Tr1* gene, a unique transposable element of the IS5 family, which allowed the identification of five *C. pipiens Wolbachia* strains, differing either by nucleotide substitution, presence or absence pattern, or insertion site. Sequence analysis also showed that recombination, transposition and superinfection occurred at very low frequencies. Analysis of the geographical distributions of each *Wolbachia* strain among *C. pipiens* populations indicated a strong worldwide differentiation independent from mosquito subspecies type, except in the UK. The availability of this polymorphic marker now opens the way to investigate evolution of *Wolbachia* populations and CI dynamics, in particular in regions where multiple crossing types coexist among *C. pipiens* populations.**

Keywords: *Culex pipiens*, cytoplasmic incompatibility, transposable element, *Wolbachia*

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Introduction

Wolbachia is an α-Proteobacteria widespread among arthropods and filarial parasitic nematodes, which act as key manipulators of host reproduction. These maternally inherited bacteria are associated with different host reproductive phenotypes, including feminization of chromosomal males, thelytokous parthenogenesis, male-killing, and cytoplasmic incompatibility (CI) (for reviews see, e.g. Werren 1997a; Stouthamer *et al*. 1999; Stevens *et al*. 2001; Bourtzis & Miller 2003). *Wolbachia*-induced CI leads to embryonic mortality (up to 100%) that occurs when infected males mate with either uninfected females or with females infected by other *Wolbachia* strains (Yen & Barr 1973). In a mixed-population with infected and uninfected hosts, females carrying a *Wolbachia*-free cytoplasm have a disadvantage when they mate with infected males. The above phenomenon facilitates the spreading up to fixation of those *Wolbachia* that induce CI (Rousset & Raymond 1991; Turelli & Hoffmann 1999).

In *Drosophila melanogaster*, infections occur at varying rates throughout the world (Solignac *et al*. 1994; Clancy & Hoffmann 1996), and polymorphism is at equilibrium because of partial CI levels and incomplete transmission of the bacteria to eggs (Hoffmann *et al*. 1994). The prevalence

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of *Wolbachia* in the mosquito, *Culex pipiens* (*w*Pip, Rousset & de Stordeur 1994) has never been investigated worldwide, although Californian populations were shown to be fully infected (Rasgon & Scott 2003). Crosses between mosquitoes from various origins revealed a high frequency of uni- or bidirectional incompatibilities (Laven 1951, 1967; Barr 1966; Subbarao 1982; Magnin *et al*. 1987; Guillemaud *et al*. 1997). Incompatibility was found higher between mosquito strains from distant regions than between strains from the same country or continent (Service 1956; Laven 1967; Magayuka & White 1971; Thomas 1971; Espinola & Consoli 1972; Guillemaud *et al*. 1997). However, high CI has also been observed between mosquitoes from restricted areas, especially in Europe where CI exhibits an extreme pattern (Laven 1967; Magnin *et al*. 1987). Heterogeneity of cytotypes has been described among the offspring of individual females (Barr 1980), suggesting multiple infections as a possible explanation for some of the observed CI patterns.

The major hypotheses for the highly complex CI pattern in *C. pipiens* are the presence of different *Wolbachia* strains or the occurrence of uninfected insects in natural populations. However, no polymorphism was observed in *w*Pip using the *ftsZ* (Guillemaud *et al*. 1997) and *16S rRNA* (Stouthamer *et al*. 1993) genes.

The purpose of this study was thus to evaluate the extent of *w*Pip infection in *C. pipiens* populations worldwide and to identify polymorphic genetic markers. To this aim, the complete *D. melanogaster Wolbachia* genome (*w*Mel) (Wu *et al*. 2004) was screened for genes that could be prone to polymorphism in *w*Pip. In particular, *w*Mel displays very high number of transposable elements (TEs) that might prove useful for strain discrimination (Wu *et al*. 2004). TEs are mobile and discrete segments of DNA that replicate and spread into the genome through either DNA-mediated or RNA-mediated transposition (Kidwell & Lisch 1997; Kidwell & Lisch 2001). They constitute a large fraction of the genome of many organisms and have the ability to promote mutations, affect gene regulation and alter genome size. Using a PCR approach, we identified *Tr1*, a member of the TEs IS5 family, which turned out to display a level of polymorphism suitable for population studies.

Materials and methods

Mosquitoes

Mosquitoes were collected in breeding sites and raised to adult stage. They were either stored in liquid nitrogen for further analyses (field samples), or bred in the laboratory (strains). For each sample, the putative subspecies, the geographical origin, the year of collection and the reference are indicated (see Appendix). Three subspecies are currently formally recognized in the complex: *Culex pipiens quinquefasciatus*, *Culex pipiens pipiens* and *Culex pipiens molestus*. *C. p. quinquefasciatus* and *C. p. pipiens* are the southern and northern house mosquitoes that are ubiquitous in tropical and temperate regions, respectively. *C. p. pipiens* and *C. p. molestus* are found in the same geographical area but differ by physiological and behavioural traits, consequently to *C. p. molestus* adaptation to underground environments associated with human activity (Fonseca *et al*. 2004). The subspecies were characterized using different methods. Some samples were determined with genetic markers as acetylcholinesterase *ace-2* gene (Bourguet *et al*. 1998) and microsatellites (Fonseca *et al*. 2004). Ecological criteria (epigeous or hypogeous habitat) and geographical origin were used for identified remaining samples (Appendix). In order to generate strains free of *Wolbachia*, a modification of the technique described by Portaro & Barr (1975) was used: larvae were reared for three generations in a solution containing the antibiotic tetracycline hydrochloride at 10^{-4}, 2.10^{-4} and 4.10^{-4} M for the first, second and third generation, respectively. Loss of *Wolbachia* was assayed by polymerase chain reaction (PCR) using the *wsp* amplimer (see succeeding discussion). These *Wolbachia*-free strains are referred as Tc-treated.

Cytoplasmic transmission of *Tr1* was investigated by using reciprocal crosses between two infected mosquito strains, harbouring each a different *w*Pip strain. Randomly sampled F_1 larvae from each cross were screened by PCR for the presence of the *Tr1* gene.

PCR and sequencing

Mosquito DNA was extracted using a cetyltrimethylammonium bromide (CTAB) protocol (Rogers & Bendich 1988). Assays for *Wolbachia* infection were performed by PCR amplification of a 151-bp fragment of the *wsp* gene using the specific primers *wolpipdir* and *wolpiprev* described by Berticat *et al*. (2002). PCR was run for 30 cycles (94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s). PCR products were separated on 0.8% agarose gel. To confirm the specificity of amplification, sequences were performed directly on PCR products on an ABI Prism 310 sequencer using the BigDye Terminator Kit (Applied Biosystems). Control DNA corresponding to uninfected individuals (from Tc-treated strains) was included in each group of PCR. All mosquitoes negative for *Wolbachia* infection were controlled for the quality of their DNA using the acetylcholinesterase *ace-2* gene amplification (Weill *et al*. 2000).

For screening of *Tr1* gene polymorphism, a set of six internal primers and four external primers were designed (internal and external refer to their position relative to *Tr1* flanking regions). PCR was ran for 30 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s to 1 min and 40 s depending on the fragment size. Internal oligonucleotides were: *Tr1i-F1* (5′-ATGAGAAAAAAGTATCCAACAGAT-3′), *Tr1i-F2* (5′-GATAGAGAGTGGGTTTTGATAG-3′), *Tr1i-F3* (5′-AAAGGAGGAAGGCCRCCAAA-3′), *Tr1i-R1*

(5′-CCATCATARCCTTTGATCCC-3′), *Tr1i-R2* (5′-CCCAAAAYCTRCATGGAGGCCTT-3′), *Tr1i-R3* (5′-GGATCCCGTTGTGGCAATAG-3′). External oligonucleotides were: *Tr1e-F1* (5′-ACTTTAGAGGGGTGCTTTCT-3′), *Tr1e-F2* (5′-TTCAGTAACGCAGCAATAGG-3′), *Tr1e-R1* (5′-TTCATGGAGCTGAAGGTAT-3′), *Tr1e-R2* (5′-ACAAACAACGGCACAGATT-3′). In case of ambiguous readings indicating multiple infections, PCR products were TA-cloned in pCR4-TOPO (Invitrogen) and sequenced.

Tr1 *diagnostic assay*

To unambiguously identify the different *Wolbachia* strains, we developed a specific PCR/RFLP (restriction fragment length polymorphism) assay. *Sty*I digestion of the 1321-bp PCR fragment amplified with *Tr1e-F1* and *Tr1e-R1* primers allowed discrimination of *w*Pip2-A from *w*Pip1 and *w*Pip4: *Sty*I cuts twice *w*Pip1 and *w*Pip4 *Tr1* (positions +628 and +986, see Fig. 1) and only once *w*Pip2-A (+628). *Dra*I digestion of the same PCR fragment allowed discrimination of *w*Pip4 from *w*Pip1 and *w*Pip2-A: *Dra*I cuts twice *w*Pip1 and *w*Pip2-A (+468 and +998) and three times *w*Pip4 (+468, +998 and +1119). In combination with PCR using internal primers, which discriminates *w*Pip2-B strain, this assay identifies unambiguously each of the five *w*Pip strains.

Southern blotting

DNA for Southern blotting was extracted from a pool of 100 adults (Raymond *et al*. 1989), RNase-treated and digested with *Kpn*I restriction enzyme in a total volume of 20 µL. Digested DNA was fractionated onto 0.8% agarose gel and transferred onto nylon membrane. The membrane was hybridized at 65 °C with a ^{32}P-labelled probe derived from a 722 bp *Tr1* PCR product (using *Tr1i-F1* and *Tr1i-R3* primers), and washed at high stringency (0.1X SSC) at 65 °C before autoradiography.

Accession numbers

The *w*Pip nucleotide sequences encoding *Tr1* and its flanking regions have been submitted to GenBank with accession mo5 AJ646884 (*w*Pip1), AJ646885 (*w*Pip2-A) and AJ646886 (*w*Pip4). Corresponding flanking regions without *Tr1* have been submitted with accession mo5 AJ646887 (*w*Pip3). Unique sequence of *DTr* has been submitted with accession mo5. AJ646888.

Results

Extent of Wolbachia *infection in the* C. pipiens *complex*

Polymerase chain reaction assay using *wsp* primers detected the presence of *Wolbachia* in all mosquitoes tested (i.e. 531 mosquitoes). When more than one mosquitoes were tested for each laboratory strain it was further considered as a single field individual (Table 1). Exact number of mosquitoes tested was reported in Appendix. Analysis of the *wsp* PCR products of 30 individuals from 15 different populations confirmed the absence of sequence polymorphism. These results show that *Wolbachia* infection seems close to, if not at fixation, in all forms of the *Culex pipiens* complex from Europe, Africa, the Americas, Asia and Australia.

Identification of Tr1 *sequence*

Using primers designed to amplify TEs identified in *w*Mel, we amplified a 918-bp-long fragment from *w*Pip DNA that exhibited hallmarks of genuine TEs (Fig. 1): presence of 17-bp-long terminal inverted-repeat sequences (IRs) and of a transposase (Tpase) made of two overlapping open reading frames, probably translated as a single protein through programmed translational frameshifting, a mechanism critical for controlling the transposase activity (reviewed in Mahillon & Chandler 1998). The encoded Tpase called *Tr1* displays the N2, N3 and C1 domains, in which stretches of conserved amino acids critical for the catalytic activity are found, including the 'DDE' consensus (Fig. 2). Comparison of the DDE regions with those of known Tpases identified *Tr1* as a member of the IS1031 subfamily (IS5 family of TEs). In *w*Mel, 13 strictly identical copies homologous to *Tr1* exist (accession mo5 WD0045, WD0044, WD0137, WD0138, WD0216, WD0215, WD0328, WD0327, WD0456, WD0457, WD0517, WD0516, WD0546, WD0547, WD0588, WD0587, WD0646, WD0647, WD0910, WD0909, WD0920, WD0919, WD0933, WD0934, WD1225, WD1226, Wu *et al*. 2004). *Tr1* ORFs from *w*Pip and *w*Mel share 81% DNA identity (not shown) and encode proteins 93% similar (84% identical) (Fig. 2).

A second 199-bp fragment was coamplified during the *Tr1* PCR (using *Tr1i-F1* and *Tr1i-R2* oligonucleotides). This fragment was similar to *Tr1* except that a 400-bp segment of the central region (not shown) was deleted. This locus, termed *DTr* for Degenerated Tr1, shares more similarity with *w*Mel *Tr1* than with *w*Pip *Tr1* (78% and 83% vs. 69% and 75%, for the 5′- and 3′ regions, respectively). This suggests that *DTr* resulted either from a horizontal transfer of *Wolbachia* from *Drosophila* to *Culex* or that *Wolbachia* was transferred to both insects from another host. *DTr* was identical in mosquitoes (n = 20) from 10 populations of different geographical origins and was not further studied.

Variability of the Tr1 *copy number in field populations*

The presence of *Tr1* was investigated in 531 individuals coming from 67 populations. Using the internal primers *Tr1i-Fx* and *Tr1i-Rx* (Fig. 1), *Tr1* was detected in 143 mosquitoes from 25 populations but appeared absent in 388 mosquitoes (73%) from 42 populations (Table 1). The

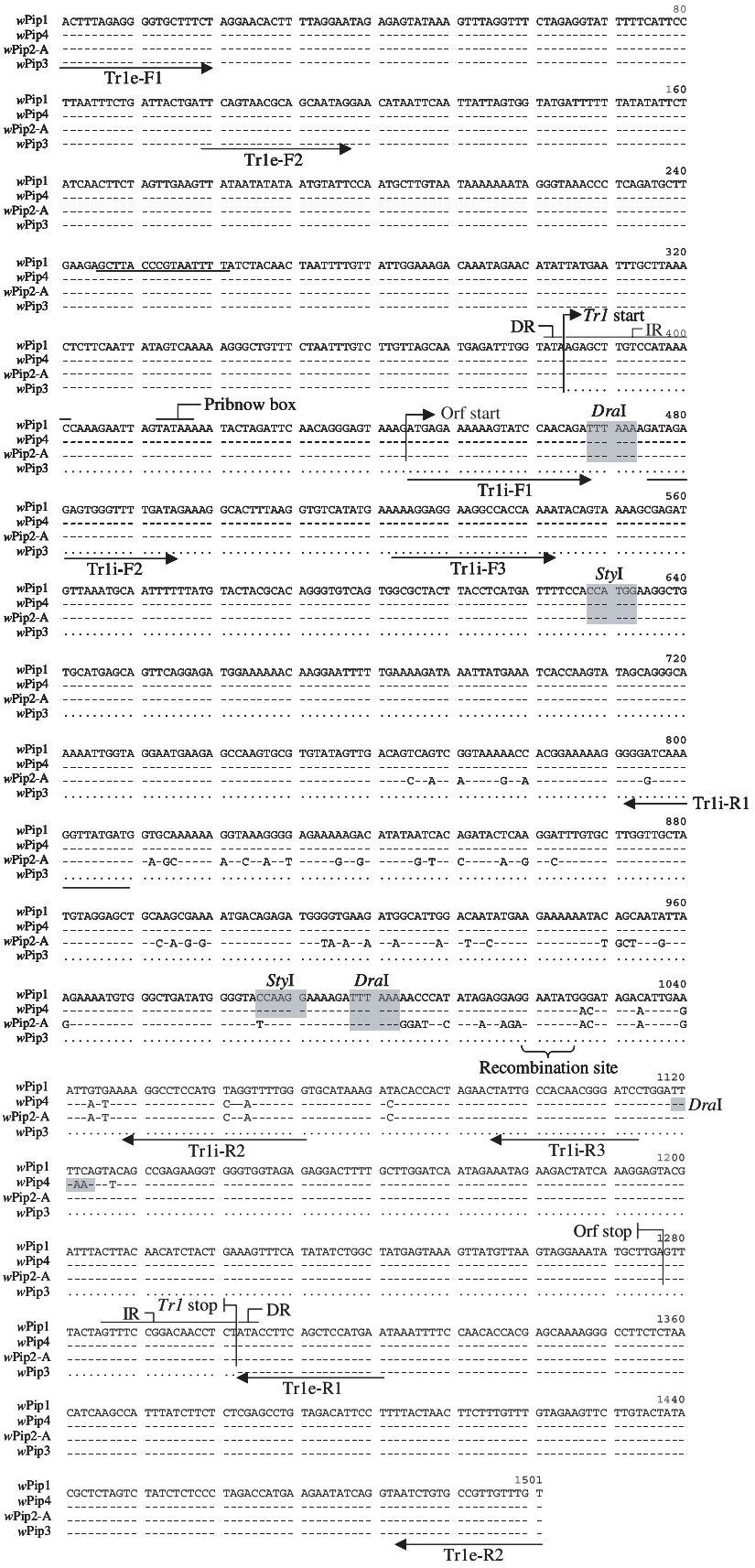

Fig. 1 Alignment of *Tr1* and flanking regions of *w*Pip1, *w*Pip2-A, *w*Pip3 and *w*Pip4. *Tr1* organization is summarized, including short inverted-repeats (IRs), directly repeats (DRs), Pribnow box and the transposase *orf*. The positions of the recombination site, of the *Sty*I and *Dra*I restriction enzyme sites used in the identification assay, and of each primer are indicated.

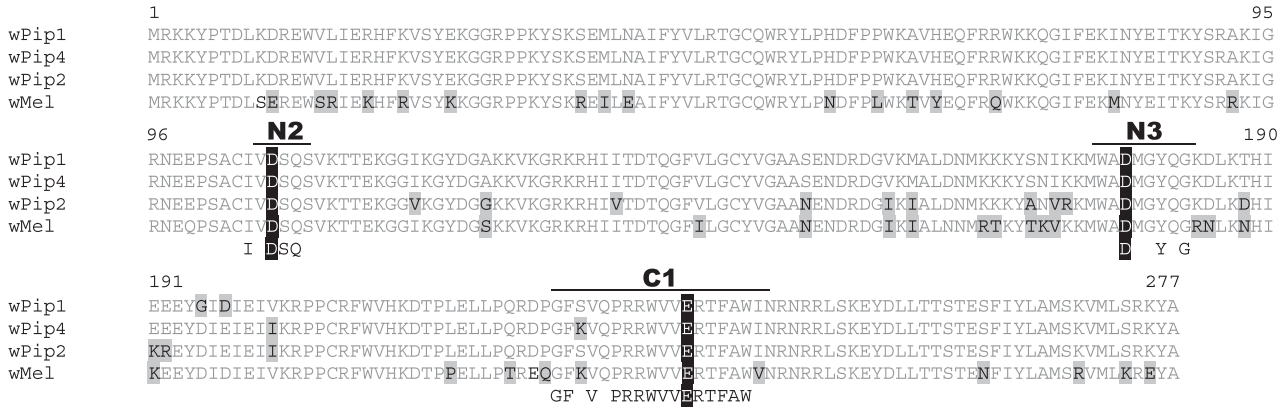

Fig. 2 Alignment of the deduced amino acids sequences of *Tr1* from *w*Pip1, *w*Pip2, *w*Pip4 and *w*Mel. Conserved domains N2, N3, and C1 including the DDE motif (white in black boxes) are indicated. The DDE signature of the IS 1031 family is shown below the *w*Mel sequence. Identical residues are in grey, conserved residues are in black in grey background and nonconserved residues are in black.

Table 1 *Tr1*-genotyping of *Wolbachia* infecting *Culex pipiens* strains used in the study. *w*Pip1, *w*Pip2-A and *w*Pip4 correspond to different *Tr1* alleles inserted in the same location. *w*Pip2-B is the same allele as *w*Pip2-A inserted elsewhere in *Wolbachia* genome, whereas *w*Pip3 corresponds to a lack of *TR1*. Landmasses, countries and areas of origin as well as numbers of populations and samples analysed are indicated. Laboratory strains are considered as single field individuals but at least more mosquitoes were analysed for each strain. See Appendix for further details on the mosquito origins

				Wolbachia strain				
Landmass	Country or area	Populations sampled	Individuals sampled	*w*Pip1	*w*Pip2-A	*w*Pip2-B	*w*Pip3	*w*Pip4
North America	California	6	6		—	—	—	X
	Minnesota	1	1	X	—	—	—	X
	Florida	1	1	X	—	—	—	—
Central and South America	Martinique	1	5	—	—	—	X	—
	Brazil	2	28	—	—	—	X	—
Europe	Portugal	4	32	X	—	—	X	—
	Spain	3	28	X	—	—	X	—
	France	12	143	X	—	—	X	—
	Italy	1	12	—	—	—	X	—
	Switzerland	1	8	—	—	—	X	—
	Belgium	1	14	—	—	—	X	—
	UK	4	21	—	X	—	X	—
	Holland	1	13	—	—	—	X	—
	Greece	1	16	—	—	—	X	—
	Turkey	1	16	—	—	—	X	—
	Cyprus	4	31	—	—	X	—	—
Africa	Tunisia	4	20	—	—	—	X	—
	Zimbabwe	1	13	—	—	—	X	—
	Côte d'Ivoire	2	12	—	—	—	X	—
	South Africa	1	9	—	—	—	X	—
Asia	Pakistan	1	5	—	—	—	X	—
	Vietnam	1	5	—	—	—	X	—
	China	7	50	—	—	—	X	—
	Philippines	2	12	—	—	—	X	—
Oceania	Australia	2	13	—	—	—	X	—
	French Polynesia	2	17	—	—	—	X	—

lack of *Tr1* sequences in these samples was supported by Southern blot analysis (Fig. 3). While this work was in progress, raw *w*Pip DNA sequences were made available (*Wolbachia pipientis* genome project, Beowulf Genomics, Sanger Institute). BLAST analysis showed the presence of a unique *w*Pip *Tr1* sequence strictly identical to the one we identified. The contig containing *Tr1* was then used to delineate flanking primers (*Tr1e-F1* and *Tr1e-R2*) that led in

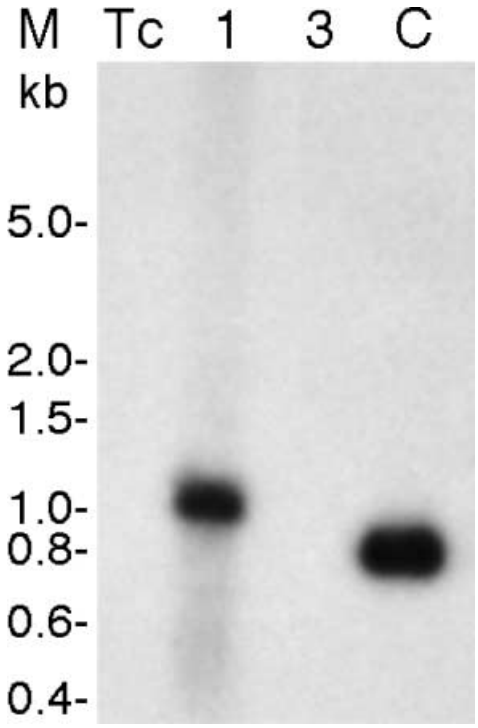

Fig. 3 Southern blotting of *Kpn*I-digested DNA from the SLAB *w*Pip1 *Tr1* positive strain (1) or from the Barriol *w*Pip3 negative for *Tr1* (3). DNA from Tc-treated strain (Tc) and from the 722 bp *Tr1* PCR product (C) are included as negative and positive controls, respectively.

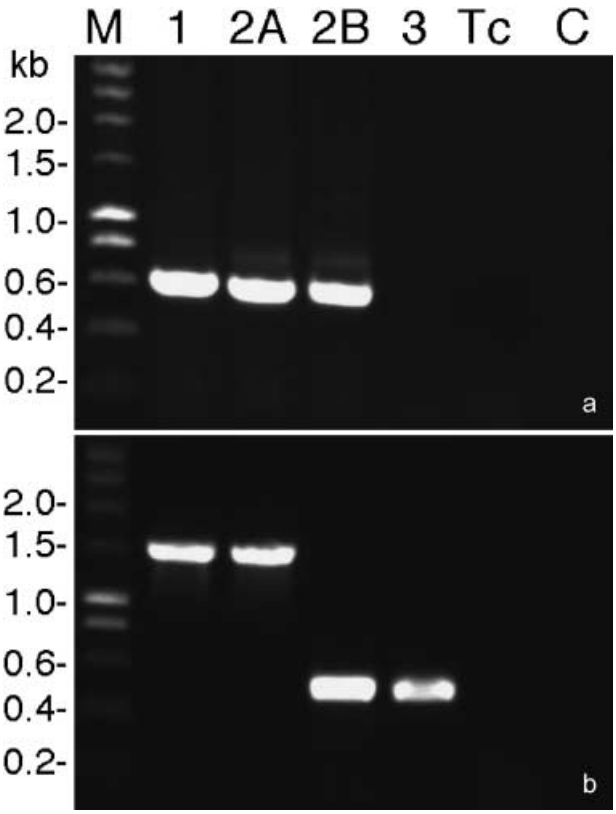

Fig. 4 PCR detection of *Tr1* using the internal *Tr1i-F1* and *Tr1i-R3* (panel a) or the external *Tr1e-F1* and *Tr1e-R2* primers (panel b). Internal primers amplify *Tr1* in *w*Pip1 (1), *w*Pip2-A (2A) and *w*Pip2-B (2B), but not in *w*Pip3 (3), whereas external primers amplified *Tr1* and its flanking regions only in *w*Pip1 and *w*Pip2-A. DNA from Tc-treated strain (Tc) and DNA-free sample (C) were used as negative controls.

all *Tr1* positive samples to the amplification of a 1501-bp fragment comprising the entire TE. An 'ATA' trinucleotide was found both 5′ upstream and 3′ downstream of *Tr1*, which probably corresponds to the short direct repeated sequences (DR) generated upon insertion, a general feature of TEs (review in Mahillon & Chandler 1998). For all *Tr1* negative samples, the PCR produced a 583-bp fragment, corresponding to the *Tr1* flanking sequences only (Fig. 4). The 'ATA' DR motif was also present in all samples, suggesting that the absence of *Tr1* resulted from a secondary loss event. These data thus establish that *Tr1* is unique in the *w*Pip genome and displays a presence/absence polymorphism in field populations.

Single nucleotide and insertion site polymorphisms of Tr1

DNA sequence analysis of two to eight individuals from the 25 *Tr1*-positive populations revealed the presence of three distinct *Tr1* alleles that showed 2–7% variation, thus specifying three types of *Wolbachia*, called *w*Pip1, *w*Pip2 and *w*Pip4 (Fig. 1). Sequence comparison indicated that the first 582 bp and last 251 bp of *w*Pip4 share 100% and 94% identity, respectively, with those of *w*Pip1, and 92% and 99% identity with those of *w*Pip2 (Fig. 1). Thus, *w*Pip4 appears as a *w*Pip1-*w*Pip2 hybrid, suggesting the occurrence of a recombination event.

For Cyprus mosquitoes, whereas PCR using internal primers produced a 918 bp *Tr1w*Pip2 sequence, the short 583-bp fragment indicating the absence of *Tr1* was obtained with the external primers. This indicates that in Cyprus samples, *Tr1* is inserted in a locus distinct from that of *w*Pip1, *w*Pip4 and *w*Pip2 from UK (Fig. 4). In this case, the 583 bp fragment also contained the 'ATA' DR motif suggesting a transposase loss event at this position. To validate the use of *Tr1* as a *Wolbachia* marker, maternal transmission was checked by reciprocal crosses between *w*Pip2-A or *w*Pip3 strains. F_1 larvae from each cross were PCR-screened for the presence of the *Tr1* gene with external primers. All larvae (randomly sampled, $n = 10$) produced by females infected by *w*Pip2-A and males infected by *w*Pip3 displayed the 1501-bp fragment indicating the presence of *Tr1*, whereas those (randomly sampled, $n = 10$) produced by females infected by *w*Pip3 and males infected by *w*Pip2-A only displayed the 583 bp characteristics of *w*Pip3 (not shown).

These data demonstrate that *Tr1* is a *Wolbachia* marker that can discriminate up to five strains in *C. pipiens*: *w*Pip1,

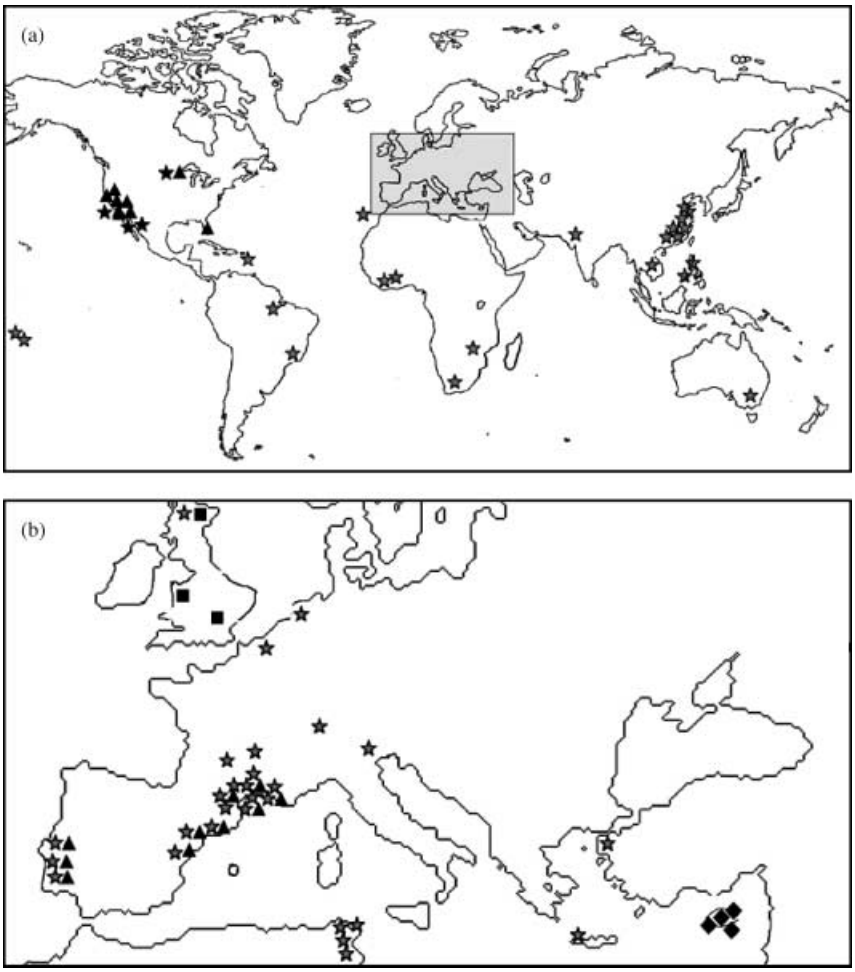

Fig. 5 Distribution of *Wolbachia* strains in *Culex pipiens* populations. (a) World distribution except Europe and a part of North Africa. (b) Detail of the distribution in Europe and North Africa. Each sampled population is figured by a single symbol except for: (i) Portugal, Spain and south of France where two different *Wolbachia* strain occur in sympatry; (ii) North America where super infection occurs. Triangle: *w*Pip1; square: *w*Pip2-A; lozenge: *w*Pip2-B; shaded star: *w*Pip3; black star: *w*Pip4.

*w*Pip2-A and *w*Pip4, which contain distinct *Tr1* alleles, *w*Pip2-B, which contains the same *Tr1* sequence as *w*Pip2-A but at a distinct locus, and *w*Pip3, which lacks *Tr1*.

w*Pip strain geographical distribution based on* Tr1 *occurrence*

We next examined the geographical distribution of the five *w*Pip strains (Fig. 5 and Table 1). The most widespread strain was *w*Pip3, occurring in all geographical areas but was not found North America. *w*Pip1 was widely distributed in North America and overlapped with *w*Pip3 in 10 different populations of Spain, Portugal and southern France. The remaining *w*Pip strains were found in restricted areas: *w*Pip2 was detected only in UK (*w*Pip2-A) and in Cyprus Island (*w*Pip2-B) while *w*Pip4 was found in North America exclusively. These data show that the distribution of the five *Wolbachia* strains is strongly structured within worldwide populations of *C. pipiens*. No evidence of infection by different *Wolbachia* strains according to *C. pipiens* subspecies was found, except in UK (see Appendix).

Occurrence of superinfection in North American populations

We analysed individual mosquitoes from populations of North America in which we had detected the presence of *w*Pip1 and *w*Pip4 using the *Tr1* diagnostic assay (Fig. 6). Among the eight *C. pipiens* populations examined, three were infected by *w*Pip1 and *w*Pip4 (SLAB, TRANS-P and MINNESOTA). Both *w*Pip1 and *w*Pip4 strains were detected simultaneously in all individuals ($n = 27$) of these populations. The presence of both *w*Pip1 and *w*Pip4 *Tr1* alleles in a single *Wolbachia* genome seems improbable as this duplication involves the flanking regions of *Tr1*, which are not supposed to be mobile. Superinfection by two *Wolbachia* types remains the simplest mechanism to explain this result.

Discussion

Availability of genetic markers and knowledge of the status of infection, probable superinfection, represent pivotal information for understanding *Wolbachia* evolution

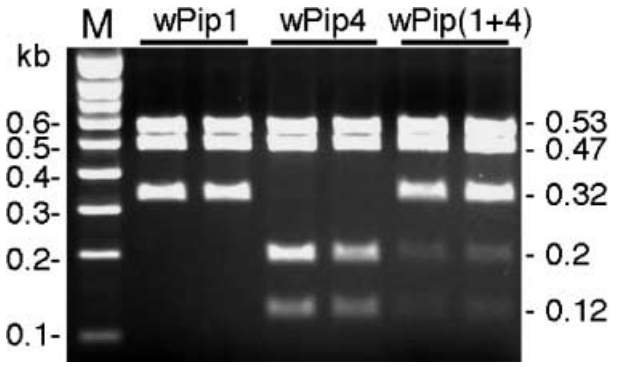

Fig. 6 Identification of *w*Pip1 and *w*Pip4 superinfection in individual mosquitoes from North America. DNA from single samples was submitted to the *Dra*I diagnostic test that discriminates *w*Pip4 (see text). Restriction of *w*Pip1 DNA produced three fragments (0.32, 0.47 and 0.53 kb) while that of *w*Pip4 produced four fragments (0.12, 0.2, 0.47 and 0.53 kb). Digestion of DNA from super infected individuals produced a mixture of five fragments [*w*Pip(1 + 4)].

and the highly complex CI pattern that affects *Culex pipiens* populations throughout the world. The purpose of this study was (i) to investigate the frequency and world distribution of *Wolbachia* infection in *C. pipiens;* (ii) to find *Wolbachia* genetic polymorphism and to detail its geographical distribution; and (iii) to evaluate the correlation between genotypic markers and pattern of CI previously described.

World distribution of Wolbachia *infection*

Wolbachia was found in all mosquitoes tested ($n = 531$), indicating that infection seems fixed in the populations sampled in this study. This is consistent with studies of California populations, which reported vertical transmission above 99%, complete CI levels and no observable effect of infection on female fecundity, predicting a stable equilibrium point of 100% (Rasgon & Scott 2003). Prevalence of the infection we observed in *C. pipiens* populations thus fits the model according to which *Wolbachia* use CI to increase their frequencies. In apparent conflict with our data, studies reported the presence of *Wolbachia*-free *C. pipiens* populations in two specific areas. In South Africa, *Wolbachia* infects the subspecies *Culex pipiens quinquefasciatus* but not *Culex pipiens pipiens* (Cornel *et al*. 2003). This situation was already reported by Irving-Bell 20 years ago (cited in Miles & Paterson 1979) suggesting a stable situation. *Wolbachia* also infect *Culex pipiens molestus* near to fixation in Russia, whereas *C. p. pipiens* is uninfected (Vinogradova *et al*. 2003). Both situations may reflect local particularities of the *C. pipiens* complex. Indeed, *C. p. quinquefasciatus* and *C. p. pipiens* populations do not cross in South Africa, whereas these two forms usually display large hybrid zones wherever they met, especially in North America (Cornel *et al*. 2003). Similar reproductive isolation has been reported between *C. p. pipiens* and *C. p. molestus* (Fonseca *et al*. 2004), the latter being generally considered as an ecotype of uncertain status. Our analysis performed at a worldwide scale clearly indicates that *Wolbachia* infection is largely prevalent in the *C. pipiens* complex.

Wolbachia *polymorphism*

Although crosses between different *C. pipiens* populations exhibit considerable variations in their hatching rates (Laven 1967; Magnin *et al*. 1987; Guillemaud *et al*. 1997), the genomes of the infecting *Wolbachia* (*w*Pip) show low levels of polymorphism, contrasting with those of *Wolbachia* infecting other insects (Rousset & Solignac 1995; Perrot-Minnot *et al*. 1996). The level of mitochondrial DNA polymorphism was also reported to be low in *C. pipiens* compared to other insects, indicating either a recent divergence of *C. pipiens* forms or the existence of a selection affecting mitochondria (Guillemaud *et al*. 1997).

We identify here five *w*Pip strains by analysing the polymorphism of *Tr1*, a transposable element of the IS5 family. Crossing experiments established the cytoplasmic inheritance of *Tr1* thereby confirming its *Wolbachia* origin. Although *Tr1* exhibits all characteristics of a functional and thus mobile TE, our data combined with the available sequences from the *w*Pip genome project indicate that *Tr1* probably never occurs more than once per genome. This contrasts with the 13 copies of the *Tr1* homologue present in the *w*Mel genome. All *w*Mel *Tr1* copies are strictly identical, suggesting a very recent expansion by transposition. Although rare, transposition nevertheless occurred in *w*Pip, as illustrated by *w*Pip2A and *w*Pip2B, which contain the same *Tr1* allele at distinct loci. Besides, an abortive transposition event is probably responsible for the loss of *Tr1* in *w*Pip3. The uniqueness of *Tr1* in *w*Pip combined with a low transposition rate and with the presence of polymorphism makes it a valuable genetic marker to trace *w*Pip populations.

Tr1 *geographical distribution*

Genetic diversity of *w*Pip *Tr1* appears geographically structured, strains occurring mostly alone either in large areas (*w*Pip3) or in very limited areas (*w*Pip2-A and *w*Pip2-B groups). In North America and west–south of Europe, *w*Pip1 was found. *w*Pip2 was found in UK (*w*Pip2-A) and in Cyprus (*w*Pip2-B). The most widespread strain is *w*Pip3, which is present in all major geographical areas, except in North America. Pip4 found only in North American mosquitoes, where superinfection with *w*Pip1 seems to occur, was *w*Pip4 found. Surprisingly, although *w*Pip4 *Tr1* likely results from a recombination event between the genomes of *w*Pip1 and *w*Pip2, these two strains were not found in sympatry. The ecological and possibly historical context and the mechanisms responsible for this recombination thus remain to be identified. However, survey does not permit

to exclude the existence of new strains in limited geographical areas (like those observed in UK and Cyprus).

The distribution of *Wolbachia* strains correlates with geographical criteria but not with *C. pipiens* subspecies. It is interesting to note that *C. p. molestus* (Heteren, Killcare) have the *w*Pip3 strain; *C. p. pipiens* is infected by either *w*Pip1 (St Bauz, Ganges), *w*Pip2 (Keo, Rothamsted), *w*Pip3 (La Var, Perrin) or double infected by *w*Pip1 and *w*Pip4 (Minnesota); *C. p. quinquefasciatus* is infected by *w*Pip1 (Miami), *w*Pip3 (Harare, Manille) or double infected by *w*Pip1 and *w*Pip4 (Slab, Trans-P), but not by *w*Pip2. However, in UK, both *w*Pip2-A and *w*Pip3 are present but in distinct types of *Culex* populations. All mosquitoes infected by *w*Pip2-A indeed belong to the *C. p. pipiens* form, while *w*Pip3 was found in the *C. p. molestus* form. This supports the notion that *C. p. pipiens* and *C. p. molestus* are genetically separated in UK (Fonseca *et al*. 2004). For the time being, we have no indication as to whether or not the reproductive isolation of both subspecies is a consequence of a *Wolbachia*-induced CI (see Werren 1997b).

Four *Wolbachia* strains (*w*Pip1, *w*Pip2-A, *w*Pip2-B and *w*Pip3) identified in this study are found in Europe, at what seems to be a unique situation in the world. If the maximum genetic diversity can be used to infer the geographical origin of a group, it could then be proposed that the *Wolbachia* that infects *C. pipiens* originated from Europe. However, this situation may result from biased sampling as Europe has been more surveyed than other continents. Intrapopulational polymorphism is frequent in Portugal, Spain and southern France, where mosquitoes infected either by *w*Pip1 or by *w*Pip3 occur in sympatry (Fig. 5B). It is predicted however, that the coexistence of multiple bacterial variants is not stable within a population if these variants generate CI (Rousset *et al*. 1991). This would imply that either *w*Pip1 and *w*Pip3 do not generate CI in these areas, or that the coexistence of *w*Pip1 and *w*Pip3 is transient or restricted geographically, for instance to the borderline between the two groups. Previous studies have indeed revealed that cytotypes generating CI coexist in southern France (Raymond *et al*. 1986; Magnin *et al*. 1987).

Congruence between CI patterning and Tr1 *polymorphism*

The five *w*Pip strains identified by *Tr1* are not sufficient to explain the high cytotypes number deduced from crosses between European, North American, Asian and African strains (Laven 1967). This number might be overestimated because most studies describing high CI never verified the fecundation status of females which is crucial with natural populations (Rasgon & Scott 2003). Additional factors like host genotype or bacterial density may also contribute to the expression of CI phenotype (Bourtzis *et al*. 1996; McGraw *et al*. 2001; Veneti *et al*. 2004).

The presence of CI in North America was never demonstrated (Farid 1949; Sundararaman 1949; Rozeboom 1958; Laven 1967; Cornel *et al*. 2003), except by Barr (1980) who reported cytotype heterogeneity among the offspring of individual Californian females, suggesting the occurrence of multiple infections. We show here that several mosquitoes coming from California and Minnesota seem indeed coinfected by *w*Pip1 and *w*Pip4. Superinfection has also been reported in other species, like in the mosquito *Aedes albopictus* in which naturally double infected males turned out incompatible with single infected females of either type (Rousset & Solignac 1995; Sinkins *et al*. 1995; Perrot-Minnot *et al*. 1996). Double infected females are predicted to present a reproductive advantage relative to single or uninfected females because they are compatible with all types of males (Frank 1998). Although this immediate advantage may facilitate the spreading and the fixation of *Wolbachia* multi-infection, double infection seems not fixed in North America, suggesting a counterbalance by natural selection or by partial maternal transmission. The *w*Pip strains that are identified in this study may contain several distinct *Wolbachia*, not revealed by *Tr1* and by other markers.

While this manuscript was in preparation, variability in the number of *orf7* copies of the WO prophage was reported in the *C. pipiens* complex (Sanogo & Dobson 2004). Some of the strains used in the present study showed different *orf7* patterns: strains infected by *w*Pip3 (Kunu, Tunis and Espro) contain the three identified *orf7a, b, c* sequences whereas strains superinfected by *w*Pip1 and *w*Pip4 (Slab and Crisse) lack the *orf7b* sequence. Unfortunately, using the presence or absence of *orf7* as a supplementary marker does not delineate more than the five *w*Pip strains already defined by *Tr1*. Furthermore, phylogenies drawn from WO prophage and other *Wolbachia* genes have been reported to be discrepant (Masui *et al*. 2000), suggesting that the WO phage is prone to horizontal transfer between *Wolbachia* strains.

The high number of transposable element copies in the *w*Mel genome suggests that these might be useful markers for strain discrimination (Wu *et al*. 2004). The polymorphism revealed here using the transposon *Tr1* indeed represents a step toward the identification of the *w*Pip strains associated with the complex CI pattern that affects *C. pipiens* mosquitoes. Furthermore, the mutagenic potential of TEs (frequently inserted in other genes and consequently disrupting their activities), might also play a key role in shaping the evolution of *Wolbachia*. We are currently addressing the functional contribution of TEs to the establishment of *w*Pip-induced cytoplasmic incompatibility.

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Appendix

Name, countries or areas of origin, year of sampling, references, status (P: natural population, S: reared strain), numbers of analysed individuals and *Tr1 Wolbachia* types of the *Culex pipiens* used for experiments. *Culex* subspecies was determined using either genetic markers as acethylcholinesterase *ace-2* gene (*; Bourguet *et al*. 1998) and microsatellites (**; Fonseca *et al*. 2004), ecological criteria as epi/hypogeous habitat (***), or geographical origin (****).

						Wolbachia strain					
Name	Country or area	Year	Reference	P/S	*n*	*w*Pip1	*w*Pip2-B	*w*Pip3	*w*Pip2-A	*w*Pip4	*C. pipiens* subspecies
SLAB	California	1950	(Georghiou *et al*. 1966)	S	20	X	—	—	—	X	*quinquefasciatus**
TEM-R	California	1978	(Georghiou & Pasteur 1978)	S	6	X	—	—	—	—	*quinquefasciatus***
EDIT	California	1988	(Guillemaud *et al*. 1999)	S	6	—	—	—	—	X	*quinquefasciatus/pipiens****
SELAX-B	California	1984	Unpublished data	S	20	—	—	—	—	X	*quinquefasciatus****
TRANS-P	California	1975	(Priester & Georghiou 1978)	S	6	X	—	—	—	X	*quinquefasciatus****
PRO-R	California	1963	(Georghiou *et al*. 1966)	S	6	X	—	—	—	—	*quinquefasciatus****
MINNESOTA	Minnesota	1987	G. Georghiou, personal communication	S	1	X	—	—	—	X	*pipiens****
MIAMI	Florida	1991	Unpublished data	P	1	X	—	—	—	—	*quinquefasciatus****
DUCOS	Martinique	2003	Unpublished data	P	12	—	—	—	X	—	*quinquefasciatus****
BRESIL	Brazil	1993	(Guillemaud *et al*. 1997)	P	15	—	—	—	X	—	*quinquefasciatus****
RECIFE	Brazil	1995	A.B. Failloux, personal communication	P	13	—	—	—	X	—	*quinquefasciatus**
BODES	Desert Islands	1994	Unpublished data	P	3	—	—	—	X	—	*pipiens****
FERREIRA	Portugal	1993	Unpublished data	P	9	X	—	—	X	—	*pipiens****
MITRA	Portugal	1993	Unpublished data	P	10	X	—	—	X	—	*pipiens****
PRAIAS	Portugal	1993	(Bourguet *et al*. 1996)	P	10	X	—	—	X	—	*pipiens****
PALMIER	Spain	1996	(Erija & Chevillon 1999)	P	8	X	—	—	X	—	*pipiens****
MENTHE	Spain	1996	(Erija & Chevillon 1999)	P	11	X	—	—	X	—	*pipiens****
LOTO	Spain	1996	(Erija & Chevillon 1999)	P	9	X	—	—	X	—	*pipiens****
NAZ	France	2002	Unpublished data	P	9	X	—	—	X	—	*pipiens****
MAURIN	France	2003	Unpublished data	P	30	X	—	—	X	—	*pipiens****
St BAUZ	France	2003	Unpublished data	P	30	X	—	—	X	—	*pipiens****
CUCULES	France	2003	Unpublished data	P	20	X	—	—	X	—	*pipiens****
GANGES	France	2002	Unpublished data	P	30	X	—	—	X	—	*pipiens****
NADA	France	2002	Unpublished data	S	5	—	—	—	X	—	*pipiens****
MARSEL	France	2002	Unpublished data	S	5	X	—	—	—	—	*pipiens****
BIFA	France	2002	Unpublished data	S	14	X	—	—	—	—	*pipiens****
BARRIOL	France	1990	(Guillemaud *et al*. 1997)	S	6	—	—	—	X	—	*molestus****
SPHAE	France	1994	(Guillemaud *et al*. 1997)	S	4	—	—	—	X	—	*molestus****
AFF	France	2002	Unpublished data	P	5	—	—	—	X	—	*pipiens****
LA VAR	France	2003	Unpublished data	P	14	—	—	—	X	—	*pipiens****
PADOVA	Italy	1994	(Bourguet *et al*. 1997)	P	12	—	—	—	X	—	*pipiens****
PERRIN	Switzerland	2003	Unpublished data	P	8	—	—	—	X	—	*pipiens****
BRUGES-B	Belgium	1991	(Raymond *et al*. 1996)	P	14	—	—	—	X	—	*pipiens**
ROTHAMSTED	UK	1991	Unpublished data	P	7	—	X	—	—	—	*pipiens**
WILLOW	UK	2001	C. Malcolm, personal communication	P	6	—	X	—	—	—	*pipiens***
QUEST	UK	2002	(Fonseca *et al*. 2004)	P	6	—	X	—	—	—	*pipiens***

Appendix *Continued*

Name	Country or area	Year	Reference	P/S	*n*	*Wolbachia* strain					*C. pipiens* subspecies
						*w*Pip1	*w*Pip2-B	*w*Pip3	*w*Pip2-A	*w*Pip4	
MENSTRIE	UK	2001	(Fonseca *et al*. 2004)	P	3	—	—	—	X	—	*molestus***
HETEREN	Holland	1992	(Weill *et al*. 2003)	P	13	—	—	—	X	—	*molestus*/***
KUNU	Creete (Greece)	2002	Unpublished data	P	16	—	—	—	X	—	*pipiens****
ISTANBUL	Turkey	2003	F. Schaffner, personal communication	P	16	—	—	—	X	—	*molestus****
ACER	Cyprus	1993	(Bourguet *et al*. 1997)	S	8	—	—	X	—	—	*pipiens****
KEO	Cyprus	2003	Unpublished data	P	18	—	—	X	—	—	*pipiens****
NENE	Cyprus	2003	Unpublished data	P	6	—	—	X	—	—	*pipiens****
MIRAGE	Cyprus	2003	Unpublished data	P	6	—	—	X	—	—	*pipiens****
ESPRO	Tunisia	1993	(Ben Cheikh & Pasteur 1993)	S	3	—	—	—	X	—	*molestus****
BISMUTH	Tunisia	2003	Unpublished data	P	15	—	—	—	X	—	*pipiens****
BEJA	Tunisia	2004	Unpublished data	P	3	—	—	—	X	—	*pipiens****
TUNIS	Tunisia	1995	(Ben Cheikh *et al*. 1998)	S	18	—	—	—	X	—	*molestus****
HARARE	Zimbabwe	2001	(Weill *et al*. 2003)	P	13	—	—	—	X	—	*quinquefasciatus*****
SUPERCAR	Côte d'Ivoire	1994	(Bourguet *et al*. 1997)	P	6	—	—	—	X	—	*quinquefasciatus**
BOUAKE	Côte d'Ivoire	1986	(Magnin *et al*. 1988)	P	6	—	—	—	X	—	*quinquefasciatus*****
BSQ	South Africa	1993	(Weill *et al*. 2003)	P	9	—	—	—	X	—	*quinquefasciatus**
LAHORE	Pakistan	1988	(Beyssat-Arnaouty *et al*. 1989)	P	5	—	—	—	X	—	*quinquefasciatus**
NHA TRANG	Viet-Nam	1995	(Pasteur *et al*. 2001)	P	5	—	—	—	X	—	*quinquefasciatus*****
LING	China	2001	(Weill *et al*. 2001)	S	2	—	—	—	X	—	*quinquefasciatus*****
CHANG	China	1996	(Martinez-Torres *et al*. 1999)	S	2	—	—	—	X	—	*quinquefasciatus*****
BEIJING	China	1992	(Qiao & Raymond 1995)	P	14	—	—	—	X	—	*quinquefasciatus**
BJBJT	China	2003	Unpublished data	P	10	—	—	—	X	—	*quinquefasciatus*****
BJHY	China	2003	Unpublished data	P	3	—	—	—	X	—	*quinquefasciatus*****
KARAOKE	China	2003	Unpublished data	P	12	—	—	—	X	—	*quinquefasciatus*****
JIN2	China	2003	Unpublished data	P	3	—	—	—	X	—	*quinquefasciatus*****
MANILLE	Philippines	2003	Unpublished data	P	6	—	—	—	X	—	*quinquefasciatus*****
PALAWAN	Philippines	2003	Unpublished data	P	6	—	—	—	X	—	*quinquefasciatus*****
KILLCARE	Australia	1993	(Guillemaud *et al*. 1997)	P	3	—	—	—	X	—	*molestus*/***
AUSTRALIE	Australia	2004	F. Schaffner, personal communication	P	10	—	—	—	X	—	*molestus****
MOOREA	French Polynesia	1992	(Pasteur *et al*. 1995)	P	12	—	—	—	X	—	*quinquefasciatus*****
TABU	French Polynesia	1992	(Pasteur *et al*. 1995)	P	5	—	—	—	X	—	*quinquefasciatus*****

