Contrasting levels of variability between cytoplasmic genomes and incompatibility types in the mosquito *Culex pipiens*

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SUMMARY

Reproductive incompatibilities called cytoplasmic incompatibilities are known to affect a large number of arthropod species and are mediated by *Wolbachia*, a maternally transmitted microorganism. The crossing relationships between strains of potential hosts define their incompatibility types and it is generally assumed that differences between strains of *Wolbachia* induce different crossing types. Among all the described host species, the mosquito, *Culex pipiens*, displays the greatest variability of cytoplasmic incompatibility crossing types. We analysed mitochondrial and bacterial DNA variability in *Culex pipiens* in order to investigate some possible causes of incompatibility crossing type variability. We sequenced fragments of the *ftsZ* gene, and the A+T-rich control region of the mtDNA. We also sequenced the second subunit of the mitochondrial cytochrome oxidase (*COII*) gene, in *Culex pipiens* and a closely related species, *C. torrentium*, in order to verify the usefulness of the A+T-rich region for the present purposes. No variability was found in the *Wolbachia ftsZ* gene fragment, and very limited variation of the mitochondrial marker whatever the compatibility type or the origin of the host. A low variability was found in the A+T-rich region and comparison of divergence of the A+T-rich region and *COII* gene between *C. pipiens* and *C. torrentium* did not reveal any special constraints affecting this region. In contrast to observations in other host species, variability of incompatibility crossing types is not due to multiple infections by distantly related *Wolbachia* strains.

1. INTRODUCTION

In insects, cytoplasmic incompatibilities were first observed in *Culex pipiens* when strains of different geographic origins were crossed, resulting in the failure of egg hatching (Ghelelovitch 1952; Laven 1967*b*). The cytoplasmic factor responsible for these incompatibilities was later shown to be a Rickettsia-like intracellular microorganism (Yen & Barr 1971, 1973). It is now known that this microorganism belongs to the *Wolbachia pipientis* assemblage, and is commonly found in many species of arthropods (O'Neill *et al.* 1992; Juchault & Mocquard 1993; Werren *et al.* 1995). It is transmitted through the eggs and also induces parthenogenesis (Stouthamer 1990) and feminization (Rigaud *et al.* 1991) in some arthropods.

In laboratory strains of *Culex pipiens*, elimination of *Wolbachia* by tetracycline treatment led to uni-directionally incompatible crosses, i.e. infected males and uninfected females were incompatible whereas the reverse crosses were compatible (Yen & Barr 1973). Similar results have now been obtained in several other species (Hoffmann & Turelli 1988; O'Neill 1989; Breeuwer & Werren 1993). However, cytoplasmic incompatibilities may also occur between infected individuals in *Drosophila* (O'Neill & Karr 1990;

Montchamp-Moreau *et al.* 1991; Rousset & Solignac 1995), *Nasonia* (Breeuwer & Werren 1990), and *Aedes* (Kambhampati *et al.* 1993; Sinkins *et al.* 1995*b*). In these cases, they are supposed to be due to differences between strains of *Wolbachia* harboured by the hosts. This hypothesis is supported by the maternal inheritance of incompatibility relationships and the change of incompatibility types after experimental infection by *Wolbachia* (Boyle *et al.* 1993; Braig *et al.* 1994; Rousset & De Stordeur 1994). In *Drosophila* (Rousset & Solignac 1995), as well as in *Nasonia vitripennis* (Perrot-Minnot *et al.* 1996) and *Aedes albopictus* (Sinkins *et al.* 1995*a*), incompatibilities are observed either when the hosts harbour different bacterial clones or when there is a double infection by different bacteria in one of the hosts. In all species, the crossing relationships between strains define the incompatibility type (or cytotype) of each strain relative to the others.

In *Culex pipiens*, crosses of mosquitoes from various origins have revealed a high frequency of incompatibilities which can be uni- or bidirectional (Laven 1951; Laven 1967*a,b*; Subbarao 1982) which is in sharp contrast to the relatively lower levels of variability observed in other hosts. For example, 17 cytotypes were found by Laven (1967*b*) when considering

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allopatric mosquitoes from distant geographic origins. A high polymorphism of incompatibility types has also been observed in mosquitoes from very restricted areas. Barr identified three cytotypes in natural populations of southern California (1980), and Magnin *et al.* (1987) found at least six cytotypes among eight strains from southern France. Barr (1982) suggested a rapid divergence of *Wolbachia* to explain this unusual situation, and more recently, Clancy & Hoffmann (1996) suggested multiple infections as a possible explanation for some of the cytoplasmic incompatibility type variations.

Here, we have attempted to better characterize some possible causes of such a variability in analysing the mitochondrial and bacterial variation in *Culex pipiens*. For *Wolbachia*, we sequenced the most rapidly evolving known molecular marker, the *ftsZ* gene. For mitochondria, we sequenced the A+T-rich control region, which is commonly considered as the fastest evolving marker of this organelle. However, comparisons of evolutionary rate between this region and other mitochondrial loci have not been extensively made in insects (Simon *et al.* 1994). We therefore sequenced a gene which would be expected to evolve more slowly, the second subunit of the cytochrome oxidase (*COII*), in *Culex pipiens* and a closely related species, *C. torrentium*, in order to assess the choice of the A+T-rich region as a marker for the present study rather than another mitochondrial gene.

2. MATERIAL AND METHODS

(*a*) *Mosquito strains*

Ten laboratory strains of the *Culex pipiens* complex, differing in their geographical origin and habitat ecotype, were used. Six strains belong to the form *Culex pipiens pipiens*: BARRIOL (Chevillon *et al.* 1995*b*) and SPHAE (Sinègre *et al.* 1994) were collected in hypogeous habitats, i.e. underground man-made habitats (for details see Chevillon *et al.* 1995*a*), in the south of France in 1990 and 1994; ESPRO (Ben Cheikh & Pasteur 1993), and PGI were collected in epigeous habitats, i.e. open air habitats, in Tunisia (in 1993) and Portugal (in 1991). KILLCARE is a strain provided by S. L. O'Neill (Yale University, CT), collected in 1993 in Australia and reared for three generations in the laboratory before being stored in liquid nitrogen (S. L. O'Neill, personal communication). TC is a *Wolbachia*-free strain derived from BARRIOL by rearing larvae and pupae in tap water containing 0.2 mg l⁻¹ of tetracycline for nine consecutive generations. It began to be used for this study after four generations of treatment, when PCR experiments failed to detect *Wolbachia* genome. Four strains belong to the form *Culex pipiens quinquefasciatus*: MART collected in Martinique in 1993 (Bourguet *et al.* 1996); SELAX derived from a 1984 sample from California (Raymond *et al.* 1987); THAÏ, an isofemale line from Bangkok, Thailand, collected in 1991 and reared for a few generations before storage in liquid nitrogen; BRESIL, a laboratory strain, collected in Brazil in 1993 (Silva-Filha *et al.* 1995). Finally, a *Culex torrentium* strain from Sweden, collected in 1994 (Raymond 1995), was also used.

(*b*) *Crossing experiments*

All the strains except THAI, KILLCARE and BRESIL were crossed in order to determine their compatibility types. Forty to 60 reciprocal individual crosses between males and

virgin females were set for each pair of strains. Crosses were made with two-day old males in order to avoid the effect of male ageing (Krishnamurthy *et al.* 1977). Five days later, females were transferred to cages and blood-fed. The egg rafts (between 50 and 350 eggs per raft) were collected daily and isolated individually during the first three days in order to avoid an effect of sperm ageing in spermathecae on incompatibility. The eggs were counted and observed for hatching two days after isolation. When egg rafts did not produce larvae, spermathecae of every female of the cross were dissected under a binocular microscope and checked for the presence of spermatozoa. Crosses including uninseminated females were discarded.

(*c*) *Molecular analyses*

DNA from egg rafts or dissected ovaries was extracted using the protocol described by Breeuwer *et al.* (1992) with slight modifications (see Tsagkarakou *et al.* 1996 for details), or a CTAB protocol (Rogers & Bendich 1988). In the latter procedure, fresh material was homogenized and incubated for 30 min in CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris pH 8) at 60 °C. DNA was extracted with an equal volume of chloroform-isoamyl alcohol (24:1). After centrifugation (6000 *g* for 10 min), the aqueous phase was removed and DNA was precipitated with an equal volume of isopropanol. DNA was resuspended in 20 μl of double distilled water. Five microlitres of each DNA sample were used without further dilution for PCR experiments.

Primers used for PCR and sequencing of the *ftsZ* gene have been defined from the *Wolbachia* sequence of *D. melanogaster* (Holden *et al.* 1993). They are (from 5′ to 3′ with position of the *D. melanogaster* symbiont) AAT GCT GTG AAT AAC ATG AT (82–102), AAT ACC GAT GCT CAA GCG TT (139–159), TCA ATT GAT GAG ATT ATG GAG CA (262–285), GTT CCA GTA CCA CCA CCC AT (344–321), TTT GCC CAT CTC GCT CAT (714–693), GCA TCA ACT TCA AAC AGA GTC AT (878–855) and CCA GTT GCA AGA ACA GAA AC (989–969).

The A+T-rich control region of the insect mitochondria was amplified using primers ATF: ATA ATA GGG TAT CTA ATC CTA GTT T and T1N8: CTA TCA AGG TAA CCC TTT TTA TCA GGC A (Simon *et al.* 1994). Primer ATF was modified from primer SRJ-14612 of Simon *et al.* (1994). Sequences were made with primers GGT ATA ACC GCG ACT GCT GGC in position 14766–14786 on the *Anopheles gambiae* sequence of Beard *et al.* (1993) and primers directly defined from the A+T-rich region sequences obtained in *Culex pipiens*. These primers are from 5′ to 3′: AAA CCC CTA ATT TTT TTT TGT, ATC CCT ATT TAT ATA TAA CTA TCA, and GGT CTT AAG TGT AAC TTA AAA.

The mitochondrial *COII* gene was amplified and partially sequenced using primers described by Ho *et al.* (1995). The amplification reactions were carried out in a final volume of 100 μl with 0.2 nmols of each primer, 20 nmoles of each dNTP, 2.5 units of *Taq* polymerase (Eurobiotaq, Eurobio, Les Ulis, France) with 1 × enzyme buffer supplied by the manufacturer and 75 nmoles of MgCl₂. A negative control with no DNA template was always included.

After an initial denaturation step at 95 °C for 5 min, the cycling reaction consisted of 35 amplifications with 1 min at 94 °C, 1 min at 50 °C, 48 °C and 46 °C, respectively for *ftsZ*, the mitochondrial A+T-rich control region and the *COII* gene, and 1.5 min at 72 °C followed by 10 min at 72 °C. The 100 μl PCR products were purified (GeneClean II Kit, Bio 101 Inc, La Jolla, CA, USA) and suspended in 20 μl of double distilled water. The DNA sequences were obtained by

a direct sequencing method according to Rousset *et al.* (1992). The A+T-rich region sequences of SELAX and *C. torrentium* and *COII* sequence of *C. torrentium* were submitted to Genbank database and their accession numbers are U69572, U69573 and U69574, respectively.

3. RESULTS

(*a*) *Cytoplasmic incompatibility types*

In spite of repeated attempts, the crossing type of the PGI strain could not be determined because very few egg rafts were obtained in all matings, including within strain matings. Moreover, females of the PGI strain tend to lay eggs between two to three weeks after blood feeding so that sperm aging could affect crossing results. In all other experiments, age of males was controlled so that male aging did not influence the measurements of incompatibility. The results included five strains plus the *Wolbachia*-free strain TC. This last strain was used as a control and was only crossed with BARRIOL, the strain from which it derived. As expected, no offspring was obtained between infected males (BARRIOL) and non-infected females (TC) while the reciprocal cross was compatible.

The strains BARRIOL, ESPRO and MART were compatible (table 1), ESPRO and SELAX showed bidirectional incompatibility, and SPHAE and SELAX displayed total incompatibility when SPHAE females were crossed with SELAX males and partial incompatibility (72.4% of hatching) in the reverse cross. If we except this last cross, SPHAE behaved in crosses as if it was an uninfected strain. However, this strain is infected (see below) and its males are incompatible with infected females from several other strains (C. Bernard, personal communication). The two strains BARRIOL and MART displayed the same crossing relationships with the other strains. Among the five strains tested, four incompatibility types were found.

(*b*) *FtsZ gene analysis*

The symbionts were found to be present in all strains except TC by PCR assays. Sequences were obtained for each strain for positions 103–797 based on the sequence of Holden *et al.* (1993). They were all strictly identical when compared (from position 150 onwards) to the sequence of *ftsZ* from *Culex pipiens* obtained by Werren *et al.* (1995). No ambiguously readable nucleic acid sites which could have indicated multiple infections of a strain by different bacteria were observed.

(*b*) *Mitochondrial DNA variability*

The entire A+T-rich control region of mitochondrial DNA (742 bp) was obtained for each strain except SPHAE that does not present specific interest in terms of geographical origin. Four variable sites were detected by direct sequencing between the eight strains. Three sites were located in three regions of repeated motifs and their variabilities corresponded to length variation that consisted of a deletion of one motif: a deletion of one of the six TA repeats between position 294 and 305 in MART, a deletion of one of the nineteen T between position 365 and 383 in KILLCARE and PGI, and an insertion of one of the 10 T between position 512 and 521 in BRESIL. Finally, the C in position 125 is substituted by a A in BRESIL and PGI.

We investigated the usefulness of the A+T region as a fast evolving marker in *Culex* species by sequencing it in *Culex torrentium*, a closely related species (Dahl 1988; Miller *et al.* 1996) and comparing the level of divergence for this gene to that of another mitochondrial molecular marker, the *COII* gene, in the two species. This would allow us to detect atypical behaviour of the mitochondrial marker used in this study.

Sequencing of the A+T-rich control region of *C. torrentium* revealed a substantial divergence between the two closely related species. Three insertion-deletion events and 43 nucleotide substitutions were observed and this variability was almost completely localized to the 5′ end of this region since 38 mutations were present in the first 317 bp. Sequencing of 657 bp of the *COII* gene in *Culex pipiens* and *C. torrentium* revealed 14 variable nucleotide sites, i.e. three times less nucleotide divergence than that of the A+T-rich control region.

Table 1. *Incompatibility relationships between different* Culex pipiens *strains*

(Mean proportion of hatched eggs±standard errors among egg rafts laid by the *n* females that laid eggs for each cross.)

female strain	male strain					
	TC	BARRIOL	SELAX	ESPRO	SPHAE	MART
TC	0.954±0.020	0±0				
n	9	11				
BARRIOL	0.937±0.018	0.910±0.025	0.887±0.020	0.920±0.008	0.865±0.029	0.910±0.029
n	15	16	9	54	28	9
SELAX		0.008±0.008	0.960±0.003	0.170±0.038	0.724±0.051	0.003±0.002
n		22	13	12	26	14
ESPRO		0.922±0.009	0.006±0.005	0.985±0.002	0.944±0.013	0.969±0.008
n		18	9	18	13	2
SPHAE		0.001±0.001	0.003±0.003	0.002±0.001	0.931±0.013	0±0
n		27	9	38	23	45
MART		0.882±0.013	0.938±0.020	0.967±0.010	0.891±0.021	0.881±0.052
n		37	14	14	9	19

Because the *COII* protein is slowly evolving (Simon *et al.* 1994), a more relevant comparison uses third codon positions. In contrast to the *Anopheles gambiae* complex (Caccone *et al.* 1996) where it was found that the A + T-rich region evolved more slowly than third codon positions of the *COII* gene, the A + T region in *Culex* is evolving at about the same rate.

4. DISCUSSION

(a) *mtDNA variation in* Culex pipiens

The data obtained from the comparison of the A + T-rich control region sequences from *C. pipiens* and *C. torrentium* showed a high degree of differentiation between the two species. In contrast, the sequences of the *COII* gene presented very few divergent sites between these species. The A + T-rich region evolves at about the same rate as third codon positions of the *COII* protein-coding gene in the mitochondria. The pattern of variation of this marker was similar to that previously shown in *Drosophila* species (Monnerot *et al.* 1990; Monforte *et al.* 1993), i.e. the variability was concentrated in the 5′ end of this region, near the 12S RNA. The comparison with the sequence from *Anopheles gambiae* revealed little if any similarity between the two genera.

The A + T-rich region often shows substantial size variation in insects, as observed in *Drosophila melanogaster* (Lewis *et al.* 1994), *Pissodes* (Boyce *et al.* 1989), *Gryllus* (Rand & Harrisson 1989), and *Cicada* species (Martin & Simon 1990), and also reveals sequence variation in *Anopheles gambiae* (Caccone *et al.* 1996) and *Jalmenus* species (Taylor *et al.* 1993). Here, the variability observed over eight sequences is lower than that observed within the species of the *Jalmenus* Lepidopterans (Taylor *et al.* 1993) that have the shortest known A + T-rich region (350 bp). For example, in *J. daemeli*, sequences of six individuals were all different by insertion/deletion events within a 34 bp region and by substitutions at five other variable sites (Taylor *et al.* 1993). Our results are in agreement with a study of *Culex pipiens* mtDNA restriction fragment length polymorphism using eleven restriction enzymes which revealed very little variability (C. Chevillon, personal communication).

Thus, mitochondrial divergence observed within *Culex pipiens* is low compared to other insects and this may be due to the existence of a recent common ancestor of the mitochondria. As usual, it is difficult to estimate rates of evolution and put reliable dates on events. Estimates of nucleotide sequence divergence in insect mtDNA all give rates slightly above 1 % per million years (2 % for pairwise divergence) (Venanzetti *et al.* 1993; Brower 1994), which are mostly due to silent sites. The highest reported rate is 5.7 % for four-fold degenerate sites in *Drosophila* (Tamura 1992). Taking 1 % as a minimum rate, the observed 2.1 % pairwise sequence divergence (*COII* gene) corresponds to a divergence time of roughly a million years, or less, between the mtDNA of the two species, *C. pipiens* and *C. torrentium*. The five mutation events among *Culex pipiens* sequences (A + T-rich region) can be compared

to the 44 between the two species, and this suggests a total divergence time of about 100 000 years for these mitochondria (and probably less if we take homoplasy and heterogeneity of substitution rates between sites into account).

(b) *ftsZ variation and cytoplasmic incompatibility types*

The extent of cytoplasmic incompatibility type variability is known to be very high in *Culex pipiens*, not only between mosquitoes from distant origins (Laven 1967 *b*) but also from very close localities (Barr 1980; Magnin *et al.* 1987). In this study, we have found four incompatibility types among the five strains tested, including compatibility, bidirectional and unidirectional incompatibilities. No sequence variation of the *ftsZ* gene was found between these strains. These results contrast with the variability observed between *Wolbachia* associated with different incompatibility types in *Nasonia* (Perrot-Minnot *et al.* 1996) or *Drosophila* (using the more slowly evolving 16S ribosomal DNA (Rousset & Solignac 1995)). Therefore, there is no evidence for infection by distantly related clones of *Wolbachia* in *Culex pipiens*.

It is important to note that, considering the rate of evolution of the *ftsZ* gene, the monomorphism encountered only indicates that the divergence of existing *Wolbachia* of *C. pipiens* is not older than two million years (Werren *et al.* 1995). This lapse of time may have been long enough to allow the differentiation of faster evolving genes, including those which confer incompatibility properties. We can suggest different alternative hypotheses for the extremely high cytotype variability observed in *C. pipiens*. Host effects, e.g. nuclear and mitochondrial genome effects, could be involved in the differentiation of some cytoplasmic incompatibility crossing types. This hypothesis remains to be tested carefully since previous studies only focused on nuclear effects and involved very few mosquito strains in crossing experiments (e.g. Laven 1967 *b*; review in Rousset *et al.* 1991). Environmental effects could affect the cytoplasmic incompatibility properties (Sinkins *et al.* 1995 *b*); however, the different mosquito strains used in our crossing experiments were reared in standard conditions for more than ten generations in the same laboratory, thereby excluding most environmental variability. Variability in bacterial density in the hosts could be a possible explanation for some of the variability observed (Sinkins *et al.* 1995 *b*). It cannot account for the whole variation (e.g. bidirectional incompatibility) but may be an additional source of cytotype variability. Finally, the possibility that extrachromosomal genetic elements relative to *Wolbachia* chromosome could also explain a part of the cytotype variability cannot be rejected at present.

The low variability of *Culex pipiens* cytoplasmic genes, i.e. mitochondrial and bacterial genes, is unusual and may be explained by three different causes.

(1) A recent expansion in the geographic range of *C. pipiens*, as suggested by Van Dine (1904) and Ross (1964), will result in a reduction of variability in both

cytoplasmic and nuclear genomes. Such a hypothesis has been proposed to explain the lack of mitochondrial variation in another mosquito species, *Aedes albopictus* (Kambhampati & Rai 1991). However, nuclear genes display considerable polymorphism in *Culex pipiens*. For example, Guillemaud *et al*. (1996) and Raymond *et al*. (1995) have shown that the nucleotide polymorphism of *esterase A* and *esterase B* genes is one of the largest so far described. For *esterase A*, the estimation of nucleotide diversity per site is $\pi = 0.07$, which is about five times larger than values reported in *Drosophila* species for the *Xdh* (Riley *et al*. 1992), *Adh* (Schaeffer & Miller 1992) or *esterase* 5-*B* loci (Veuille & King 1995). Urbanelli *et al*. (1980, 1985) analysed the electrophoretic variability of African, European and American populations of *Culex pipiens* mosquitoes. They reported the following estimations of heterozygosity (H) and percentage (P) of polymorphic loci: $H = 0.18$ and $P = 0.61$ in the first study and $H = 0.14$ and $P = 0.52$ in the second one. These values are larger than those found on average in *Drosophila* species where $H = 0.12$ and $P = 0.48$ (Nevo *et al*. 1984). This excludes a recent bottleneck of *C. pipiens* populations as an explanation for the low variability of mtDNA.

(2) A selection affecting the mitochondrial genome, which is now recognized in *Drosophila* species (Aubert & Solignac 1990; Jenkins *et al*. 1996), could induce the lack of variability observed by hitchhiking.

(3) A sweep involving *Wolbachia* can result in the reduction of the mitochondrial variability by hitchhiking, as shown by Turelli & Hoffmann (1995) in *Drosophila simulans*. This could also explain the low mitochondrial polymorphism found in *Aedes albopictus* (Kambhampati & Rai 1991). The two latter hypotheses, which could account for the situation found in *Culex pipiens*, are not exclusive—selection can act on both cytoplasmic genomes—and these hypotheses were also proposed by Ballard & Kreitman (1994) to explain departures from neutral evolution of the mitochondrial genome of *Drosophila simulans*, and *D. melanogaster*.

This study has focused on the analysis of *Culex pipiens* which is a species known to display the greatest variability of cytoplasmic incompatibility crossing types. Molecular analysis of cytoplasmic genomes and crossing experiments between strains of *Culex pipiens* have revealed an unexpected discrepancy between the extremely high cytotype variability and the lack of variability of the bacterial genome. Therefore, in contrast to other host species, variability of incompatibility crossing types is not due to multiple infections by distantly related *Wolbachia* strains, but might involve other mechanisms which remain to be determined. Moreover, the low variability of cytoplasmic genomes suggests the existence of a selection affecting mitochondria or a selective sweep by *Wolbachia*.

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