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Wolbachia prevalence, diversity, and ability to induce cytoplasmic incompatibility in mosquitoes

Mathieu Sicard, Manon Bonneau and Mylène Weill

To protect humans and domestic animals from mosquito borne diseases, alternative methods to chemical insecticides have to be found. Pilot studies using the vertically transmitted bacterial endosymbiont *Wolbachia* were already launched in different parts of the world. *Wolbachia* can be used either in Incompatible Insect Technique (IIT), to decrease mosquito population, or to decrease the ability of mosquitoes to transmit pathogens. Not all mosquito species are naturally infected with *Wolbachia*: while in *Culex pipiens* and *Aedes albopictus* almost all individuals harbor *Wolbachia*, putative infections have to be further investigated in *Anopheles* species and in *Aedes aegypti*. All *Wolbachia*-based control methods rely on the ability of *Wolbachia* to induce cytoplasmic incompatibility (CI) resulting in embryonic death in incompatible crossings. Knowledge on CI diversity in mosquito is required to find the better *Wolbachia*-mosquito associations to optimize the success of both 'sterile insect' and 'pathogen blocking' *Wolbachia*-based methods.

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Introduction

Mosquitoes are vectors for major pathogens such as arboviruses, nematodes and protozoans. To protect humans and domestic animals from these pathogens, strategies targeting the vectors aim at decreasing vector population density and/or at diminishing their ability to transmit pathogens [1•]. Presently, the most common vector control actions are intended to decrease the longevity and the density in vector populations, mainly by using chemical insecticides, which have reached their limits because of genetic resistance and negative

consequence on non-targeted invertebrate species [2]. In this context, new vector control strategies, hopefully more environmental friendly, have to be proposed. Bio-control strategies of vectors based on the knowledge of their microbiota are promising [3], and particularly those based on α -proteobacteria of the genus *Wolbachia* which manipulate many aspects of their mosquito host biology [4–6]. Because these symbionts can influence both mosquito reproduction and their pathogen loads, *Wolbachia*-based control methods can be deployed to reduce vector populations and/or to diminish their capacity to transmit pathogens.

The *Wolbachia*, which are maternally transmitted endosymbionts, have long been studied because of their ability to manipulate their host reproduction to increase their prevalence within host populations [4]. Cytoplasmic incompatibility (CI) is the most frequent manipulation used by *Wolbachia* to spread within insect populations [4]. During the invasion process, CI occurs when males infected with a given *Wolbachia* breed with uninfected females which, then, produce non-viable embryos (Figure 1). As crossings between individuals infected with compatible *Wolbachia* give normal viable embryos, the consequence of CI is that the prevalence of *Wolbachia* increases within the host population so that prevalence can reach 100%.

The ability of *Wolbachia* to induce CI is the cornerstone of the two major *Wolbachia*-based control methods developed to date (Box 1). The first method, called 'Incompatible Insect Techniques' (IIT), is related to the classical sterile insect techniques (SIT) [1•], and aims at decreasing mosquito population size by releasing *Wolbachia* infected males that are incompatible with local females. In this strategy, the local females produce non-viable embryos resulting locally and temporally in the vector population crash-down [7,8,9•,10]. The second method uses CI induction by *Wolbachia*, not to reduce the density of a focal vector population, but to sustainably replace its uninfected individuals by *Wolbachia* infected ones. Indeed it has been shown that *Wolbachia* can interfere negatively with the transmission of disease pathogens including the major arboviruses Chikungunya, Dengue, Rift Valley, West Nile, Zika, and soon [11•,12•,13•,14–18,19•]. In this strategy, CI allows the progressive invasion of local vector population with individuals harboring *Wolbachia* which mediate blocking of arboviruses transmission [20–22].

Wolbachia being a promising weapon against mosquitoes, we synthesize in this review the current knowledge

Figure 1

The different types of CI in mosquitoes.

(a) The simplest type of CI occurring between *Wolbachia* infected males and uninfected females allows *Wolbachia* to invade uninfected populations. In *Culex pipiens* and *Aedes albopictus*, *Wolbachia* has reached fixation in nature; this type of CI is, thus, only observed in laboratory conditions when females are artificially cured of their *Wolbachia* with an antibiotic treatment. This type of CI was also observed when *Ae. aegypti* and *An. stephensi* males were transinfected with a *Wolbachia* strain from other species and crossed with naturally uninfected females. (b–c) Other types of CI can occur between males and females both infected with different *Wolbachia* strains. In such crosses CI can be unidirectional (i.e. only one of the reciprocal crosses is compatible, the other is incompatible) (b) or bidirectional (both reciprocal crosses are incompatible) (c). Unidirectional CI can be observed in *Ae. albopictus* when bi-infected males (infected with both *wAlbA* and *wAlbB*) are crossed with laboratory females infected only with *wAlbA*. In *Culex pipiens*, really complex CI crossing types are observed, including unidirectional and bidirectional CI depending on the *wPip* strains present in crossed individuals.

accumulated on *Wolbachia* prevalence, diversity, and ability to induce CI in the major vector mosquito species of *Aedes*, *Anopheles*, and *Culex* genera.

Prevalence and diversity of *Wolbachia* in mosquitoes

The diversity hidden behind the term ‘*Wolbachia pipientis*’ in arthropod and nematode hosts is presently organized in 17 phylogenetic clades called supergroups (A to Q) [23–27]. Within each supergroup, the unit of diversity is called a ‘strain’. Most of the *Wolbachia* strains were named according to their host species (e.g. *wPip* in *Culex pipiens* and *wAlb* in *Aedes albopictus*). If genetic differences are identified within an already defined ‘strain’, new information including sampling location or phylogenetic position can be added to name the new strains. The increase in genomic analyses to

investigate more and more accurately *Wolbachia* diversity, between and within host species, may lead to important changes in the definition of new ‘strains’ in the near future [28].

Anopheles mosquitoes, the major vectors of *Plasmodium*, were considered to be exempt of *Wolbachia* because classic PCR diagnostic tests were always found negative [29–31]. However, very deep sequencing of *Wolbachia*-specific 16S rRNA recently suggested putative natural infections of *Anopheles coluzzii* and *Anopheles gambiae* in Burkina Faso [32*,33]. The *Wolbachia* 16S sequences obtained were attributed to a new strain named ‘*wAnga*’. Positive mothers did not produce only positive offspring ruling out both an insertion in host genome and a perfect vertical transmission of *Wolbachia* [32*]. Such a genetic detection of *Wolbachia* has now been extended to

Box 1 *Wolbachia* anti-vectorial methods: either decrease the density or modify the physiology of mosquitoes

- a In the Incompatible Insect Technique (IIT) large numbers of *Wolbachia*-infected males are released. The *Wolbachia* harbored by these males has to be carefully chosen to ensure that these males will be able to kill, due to CI, embryos of females from the focal population. This requires that females either do not harbor the same *Wolbachia* strain (for instance in *Ae. albopictus* and *C. pipiens* cases) or that are putatively not infected with *Wolbachia* (for instance in *Ae. aegypti* and *An. gambiae*). After repeated releases of incompatible males, the vector population will decrease. To be successful, the *Wolbachia* strain in the released males should be involved in bidirectional CI with the *Wolbachia* strain from the targeted population (Figures 1 and 2). This way, the released *Wolbachia* has almost no chance to settle in the introduced environment because even if females are concomitantly released with infected males, they would not be able to produce offspring with the local males. The release of the same line of *Wolbachia* infected males can stay efficient through years. For even more efficiency, IIT may be combined with sterile insect technique (SIT) by irradiating *Wolbachia*-infected mosquitoes.
- b *Wolbachia* can also be used to modify the physiology of mosquito. In this method, both *Wolbachia* infected male and female mosquitoes are released. CI allows the progressive invasion of local vector population with individuals harboring *Wolbachia* which mediates blocking of arboviruses transmission. To be successful, the *Wolbachia* of the released individuals have (i) to block viruses and (ii) to exhibit a unidirectional CI relationship with the targeted populations that allows the spread and sustainability of protective *Wolbachia* (Figure 2).

Anopheles funestus from Senegal [34], *Anopheles arabiensis* in Tanzania [35], 16 *Anopheles* species among a total of 25 in Gabon [36], and in 5 species among 17 from Ghana, Democratic Republic of the Congo (DRC), Guinea, Uganda and Madagascar [37]. *Wolbachia* 16S sequences exhibited much larger diversity than usually expected within one strain suggesting multiple infections. Indeed, these sequences can be clustered with those from *Wolbachia* strains belonging to the supergroups A, B and even, more surprisingly, to supergroup C [36,38**]. The reported proportions of positive individuals vary among species and localities but remain low for most of the *Anopheles* species. Besides the interesting case of *Anopheles moucheti* in Gabon and DRC, for which the prevalence of *Wolbachia* seems very high, calls for further studies [36,37]. The low detection of *Wolbachia* in most *Anopheles* species could be due to a low prevalence of the symbiont that required more individuals and screened populations to be detected. However, if *Wolbachia* are really present in these *Anopheles* species their density must be very low as several screening techniques revealed discordant results, even requiring nested PCR or quantitative PCR with a very high number of amplification cycles for detection [34]. The main problem is that the putative presence of *Wolbachia* in all these *Anopheles* species is mostly based on its genetic detection which is not an actual proof of real infection and could result from contaminations, at least

for certain species [38**]. To our knowledge, no electronic microscopy observations that would provide a direct proof of infections, have been yet conducted. Only one study reported fluorescent *in situ* hybridization (FISH) labelling to monitor the presence of *Wolbachia*, found at low density in the ovaries of some *An. coluzzii* [33].

Aedes aegypti and *Ae. albopictus*, the major arboviruses vectors, although belonging to the same genus exhibit strongly different patterns in terms of *Wolbachia* infection. As for *Anopheles*, classic PCR tests were always negative on *Ae. aegypti* placing this species among uninfected ones. However, deep sequencing of *Wolbachia*-specific 16S rRNA from both larvae and adults in USA and Thailand were recently found positive, indicating the putative presence of *Wolbachia* in some individuals [39,40]. Nevertheless, as it is the case with the *Anopheles*, if *Wolbachia* cells are present in this vector it must be at low prevalence and at a 'cryptic' load [41]. Further investigations including symbiont visualization must be conducted in the future to confirm the presence of *Wolbachia* at low prevalence and titer in *Ae. aegypti*.

In contrast, *Ae. albopictus* is found infected with *Wolbachia* everywhere in the world [42,43]. All individuals are usually infected with two *Wolbachia* strains namely wAlbA and wAlbB belonging to the supergroups A and B, respectively [44]. However, a polymorphism of the infection status exists: (i) wAlbB mono-infected males (but not females) have been reported in La Réunion Island and Madagascar field populations [45], and (ii) wAlbA mono-infected laboratory lines were obtained from individuals initially sampled in Thailand and Mauritius [46,47]. The genetic variation within both wAlbA and wAlbB strains is yet considered to be low as no variation was detected within each strain based on 16S rRNA, wsp and ftsZ gene sequences [42,46–48] suggesting that *Wolbachia* could have recently, invaded and spread throughout populations of this mosquito species to finally reach fixation [42].

In *C. pipiens* (s.l.), all individuals are infected with wPip *Wolbachia* that were also found non-polymorphic using MLST genes [23,24]. However, MLSTs including a larger number of highly polymorphic genes (MutL, ank2, pk1, pk2, GP12, GP15, and RepA) allowed to uncover a previously hidden diversity [49]. All wPip strains are monophyletic and closely related, and they form five groups from wPip-I to wPip-V. As the thousands *C. pipiens* individuals tested around the world [50–53] harbored a wPip strain belonging to one of the five groups, the infection is considered to have reached fixation in this species. However, few individuals in South Africa, France, Scotland and Tunisia were found negative to *Wolbachia* genetic tests [54,55]. A phylogenetic analysis based on mitochondrial markers demonstrated that all these uninfected mosquitoes form a new species named ‘

Culex juppi nov. sp.’ independent from all the infected *C. pipiens* [55].

CI induction in natural *Wolbachia*-mosquito associations

In *Anopheles* no cytoplasmic incompatibility has been shown in laboratory crosses between males putatively infected with *Wolbachia* and uninfected females [33]. Such laboratory observations are in accordance with the low detection of the symbionts in *Anopheles* natural populations. However, an acceleration of egg laying in *Wolbachia* positive females has been reported [33]. In *Ae. albopictus*, both *wAlbA* and *wAlbB* were reported to increase host fecundity [8]. CI does not occur between individuals from lines originating from distant parts of the world since most individuals are bi-infected with *wAlbA* and *wAlbB* showing no or low polymorphism [43] (Figure 1). Consequently, there is only one dominant crossing type in *Ae. albopictus* natural populations all over the world, resulting in compatibility between all lines. Nevertheless, females mono-infected with only *wAlbA* strain produce unviable embryos when crossed with normally bi-infected males resulting in unidirectional CI [46,47] (Figure 1). This clearly demonstrated that *wAlbB* strain is able to induce CI but that this CI phenotype rarely occurs in nature because of the high frequency of bi-infections with *wAlbA* and *wAlbB*.

In contrast to the absence of CI recorded in *Anopheles* and the poor crossing type diversity observed in *Ae. albopictus*, the hundreds of crosses performed between *C. pipiens* lines sampled worldwide have revealed an unrivaled diversity of crossing types [56,57,58] (Figure 1). Genetic diversity within the *wPip* clade is responsible for this unique CI polymorphism since (i) no other manipulative endosymbiont was detected in this host species, (ii) the host genetic background did not influence the crossing types, and more importantly (iii) *C. pipiens* lines harboring *wPip* belonging to the same phylogenetic group (*wPip*-I–V) are generally compatible, whereas ‘inter-group crosses’ are more likely to be incompatible [57]. Infected males harboring a *wPip* strain (from any *wPip* group) induce total CI (i.e. no embryo will develop) when crossed with uninfected females while the reciprocal crossing is fertile [59–61,62]. Such unidirectional CI pattern between uninfected and infected individuals has certainly prevailed during the spread of the *wPip* infection in *C. pipiens* populations, but is no longer observed in the wild since infection reached fixation. To date, crosses can only occur between (i) individuals infected with the same *wPip* group (usually resulting in normal reproduction) or (ii) individuals harboring *wPip* from two different groups. Such ‘inter-group crossings’ can have three outcomes (Figure 1): (i) production of living offspring; (ii) unidirectional CI (one cross direction is compatible while the

reciprocal one is incompatible) or (iii) bidirectional CI (both cross directions are incompatible).

CI induction in artificial *Wolbachia*-mosquito associations

When *Wolbachia* have been experimentally introduced by transinfection in two ‘non-infected mosquito species’ namely *Ae. aegypti* and *Anopheles stephensi*, CI has been observed showing that *Wolbachia* molecular targets responsible for CI are present in these species. Indeed, *Ae. aegypti* has been successfully transinfected independently with eight *Wolbachia* strains (*wMel*, *wMelPop-CLA*, *wMelCS*, *wRi*, *wAu*, *wAlbA*, *wAlbB*, and *wPip* [14,19,63–65]) (Figure 2); and all induced unidirectional CI with natural uninfected *Ae. aegypti* except *wAu* which is a *Wolbachia* strain from *D. simulans* that also does not induce CI in its natural host [19]. *An. stephensi* has also been successfully transinfected with *wAlbB* from *Ae. albopictus* which induced CI enabling *Wolbachia* to invade uninfected laboratory populations [66]. Transinfections have also been conducted in *Ae. albopictus*, which is naturally infected, in order to create new crossing types. Both *wPip* and *wMel* strains have been introduced in *Wolbachia*-cured lines resulting in bidirectional incompatibility between transinfected lines and naturally infected ones [67–69]. Moreover, a triple-infected (*wAlbA*, *wAlbB*, and *wPip*) *Ae. albopictus* line has been established; it expresses unidirectional CI when crossed with naturally double-infected mosquitoes.

C. pipiens has not yet been transinfected with other *Wolbachia* since the natural crossing type diversity demonstrated in this species can provide with unidirectional and bidirectional crossing types required in *Wolbachia*-based control methods (e.g. [53]).

Cellular mechanism of CI in mosquitoes

The cellular mechanism of CI has only been yet studied in details in *C. pipiens* [62]. To do so, the early embryogenesis was monitored using fluorescence confocal microscopy in (i) fertile intra-group crosses, (ii) incompatible crosses between infected males and infected females (i.e. inter-group crosses), and (iii) incompatible crosses between infected males and uninfected females. Despite the diversity of the crosses involving various *wPip* strains, common embryonic defects resulting in the death of the embryos were detected. These defects consisted in paternal chromatin condensation and segregation impairments during the first embryonic division as for *Drosophila* and *Nasonia* [62,70–74] (Figure 3).

***Wolbachia* genes involved in CI in mosquitoes**
Cytological observations in *C. pipiens* suggest that a toxin, deposited in maturing sperm, would prevent the development of embryos by impairing paternal

Figure 2

A synthetic view of *Wolbachia* knowledge in mosquitoes.

Arrows represent *Wolbachia* transinfection from one mosquito donor species to a recipient mosquito species or from *Drosophila* to a mosquito recipient species. The *Anopheles* transinfected species was *An. stephensi* [66]. Solid arrows represent strains that were able to induce CI when transferred to a recipient host while the dashed arrow represent the strain wAu that does not induce CI.

chromatin normal segregation unless they are rescued by an antidote [75]. A combination of approaches on different insects demonstrated that the *Wolbachia* *cidA* and *cidB* genes, first identified by the presence of CidA protein in *C. pipiens* sperm [76], were the determinant in the induction and rescue of CI in insects [77[•], 78[•]]. Biochemical analyses revealed that CidB protein could act as toxin since it encodes a putatively toxic deubiquitylase (DUB). Convincingly, when *cidA* and *cidB* were transgenically expressed in uninfected *Drosophila* males, these males were incompatible with uninfected females: embryos were unviable, and the first embryonic mitosis displayed the same characteristics as in CI

[77[•], 78[•]]. CidA is most probably the antidote against the toxic activity of CidB since its expression during early oogenesis restored the viability of uninfected eggs fertilized by *Drosophila* infected males [79]. Both *cidA* and *cidB* genes are monomorphic in wAlbB. No genomic data are yet available on wAlbA and on the putative strain wAnga from *Anopheles*. However, in *C. pipiens*, these genes are amplified and diversified within each wPip genome constituting the fuel for the diversity of crossing types described in this species [80[•]]. This *cidA/cidB* gene amplifications and diversifications in wPip may also account for the impressive CI penetrance observed in *C. pipiens* [62[•]].

Figure 3

CI cellular phenotype as observed after the first embryonic division in *Culex pipiens*.

Paternal chromatin and maternal chromatin were labelled using propidium iodide and were observed under confocal microscopy. In the picture, we can see that paternal chromatin (♂) failed to segregate during the first mitotic embryonic division while maternal chromatin (♀) did segregate. Because of this defect in the first division, the embryos will not be able to develop normally into larvae. Scale bar is 10 mm.

Conclusion

Prevalence and diversity of *Wolbachia* are quite contrasted between mosquito species. *Ae. albopictus* and *C. pipiens* individuals all harbor diverse *Wolbachia* that can induce CI and influence their life history traits at each generation. In contrast, the major arboviruses vector *Ae. aegypti* and the major malaria vectors, *Anopheles* spp., are only suspected to be infected. Further studies are required to investigate infection status of these last species. Recent studies on *C. pipiens* along with those conducted on *Drosophila* brought new elements on CI mechanisms, both at cellular and molecular levels that constitute the cornerstone for an efficient use of *Wolbachia* genetic resources in vector control.

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