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Rapid evolution of *Wolbachia* incompatibility types

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In most insects, the endosymbiont *Wolbachia* induces cytoplasmic incompatibility (CI), an embryonic mortality observed when infected males mate either with uninfected females or with females infected by an incompatible *Wolbachia* strain. Although the molecular mechanism of CI remains elusive, it is classically viewed as a modification–rescue model, in which a *Wolbachia mod* function disables the reproductive success of the sperm of infected males, unless eggs are infected and express a compatible *resc* function. The extent to which the modification–rescue model can predict highly complex CI pattern remains a challenging issue. Here, we show the rapid evolution of the *mod–resc* system in the *Culex pipiens* mosquito. We have surveyed four incompatible laboratory isofemale lines over 50 generations and observed in two of them that CI has evolved from complete to partial incompatibility (i.e. the production of a mixture of compatible and incompatible clutches). Emergence of the new CI types depends only on *Wolbachia* determinants and can be simply explained by the gain of new *resc* functions. Evolution of CI types in *Cx. pipiens* thus appears as a gradual process, in which one or several *resc* functions can coexist in the same individual host in addition to the ones involved in the self-compatibility. Our data identified CI as a very dynamic process. We suggest that ancestral and mutant *Wolbachia* expressing distinct *resc* functions can co-infect individual hosts, opening the possibility for the *mod* functions to evolve subsequently. This gives a first clue towards the understanding of how *Wolbachia* reached highly complex CI pattern in host populations.

Keywords: symbiosis; cytoplasmic incompatibility; *Wolbachia*; *Culex pipiens*

1. INTRODUCTION

Cytoplasmically inherited bacteria have profoundly changed our view of insect sexuality within the last decade [1–3]. These bacteria are generally transmitted only by female hosts, through the egg cytoplasm, and have evolved complex interactions with insects. They are found to be widespread [4], and the most common of them, the alpha-proteobacterium *Wolbachia*, is suspected to infect more than 65 per cent of insect species [5]. *Wolbachia* is also well known to have evolved a sperm–egg incompatibility known as cytoplasmic incompatibility (CI) promoting its own spread [3,6]. Among the most far-reaching effects of CI is the facilitation of speciation, making *Wolbachia* an important driver of insect evolutionary ecology [1,3].

The most spectacular effect of CI is that it specifically kills the embryos of uninfected females mated with infected males, so that infected females have a reproductive advantage. Cytological studies have shown that, in incompatible crosses, the paternal chromosomes are damaged and lost during the first mitotic divisions [7]. Although the genetic basis of CI is still unknown, it can be interpreted as a 'modification–rescue' model: sperm is modified by a *mod* function in infected males, and the infected eggs rescue the modified sperm with a *resc* function, enabling the progeny to develop normally [8,9]. Apart from this simple case, CI can also occur between males and females carrying incompatible *Wolbachia* strains, a situation explained by assuming that each strain carries its own *mod–resc* pair, resulting in its particular CI type, as observed in the fruitfly *Drosophila simulans* [8].

Understanding the evolution of new CI types was mainly addressed using a combination of theoretical and empirical approaches. Mathematical investigations have produced a variety of predictions, with the assumption that CI can be explained with a single pair of *mod* and *resc* loci [10–14]. In such models, a mutation affecting either the *mod* or the *resc* locus will generate a new CI type incompatible with the ancestral type. Hence, the evolution of new CI types is classically viewed as the sudden transition of a *Wolbachia* strain from one CI type to another one that will be incompatible with the ancestral type. However, recent studies on *Wolbachia* strains from *Drosophila* spp. [15] and from mosquitoes of the *Culex pipiens* complex [16,17] suggested that a *Wolbachia* strain may use different *mod–resc* mechanisms, and therefore loci, to explain its compatibility pattern with other strains. This feature will condition the potential for evolution of CI types: increasing the number of *mod* and *resc* loci implies that CI types can change gradually rather than suddenly, with new CI types remaining compatible with ancestral ones.

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Here, we report on a rapid evolution of CI types induced by *Wolbachia* strains, known as *w*Pip strains, infecting *Cx. pipiens*. A wealth of CI types has been documented in *Cx. pipiens* populations for many years, with crossing relationships exhibiting frequently either unidirectional CI (one direction of a cross is sterile) or bidirectional CI (both directions of a cross are sterile) [16,18–20]. In this study, we showed that *w*Pip can evolve new CI types in less than 50 host generations. The evolution of these new CI types can be explained by the accumulation of different *resc* functions by individual hosts, without apparent change in their *mod* functions. As a result, the new CI types remain compatible with their respective ancestral types.

2. MATERIAL AND METHODS

(a) *Mosquito lines*

Four isofemale *Cx. pipiens* lines, each descended from a single founder female, were used in this study: Lv, Is, Ko and Tn (see the electronic supplementary material, table S1). They harboured different *Cx. pipiens* genetic background and were naturally infected by different *w*Pip strains, namely *w*Pip(Lv), *w*Pip(Is), *w*Pip(Ko) and *w*Pip(Tn). In addition, one *Wolbachia*-uninfected line (Sl-TC), artificially created by antibiotic treatment as described by Duron *et al.* [21], was used (see the electronic supplementary material, table S1). Mosquito lines were kept in standard laboratory conditions in 65 dm^3 cages at a constant temperature of 25°C, under a 12 L : 12 D cycle. Larvae were fed with a mixture of shrimp powder and rabbit pellets; adults were fed with honey solution.

(b) *Backcrossing*

The cytoplasms of the Lv, Is, Ko and Tn lines, including their respective *w*Pip strains, were separately introduced into the Sl-TC nuclear background through eight generations of backcrosses, a procedure that should result in at least 99 per cent genome replacement of the original lines by the Sl nuclear genome. A first cross was made using 200 virgin females and 250 Sl-TC males. For the following generations, 200 hybrid females were backcrossed with 250 Sl-TC males. Using this protocol, we obtained the Sl^{wLv}, Sl^{wIs}, Sl^{wKo} and Sl^{wTn} lines that carry the Sl-TC nuclear genome and the *w*Pip(Lv), *w*Pip(Is), *w*Pip(Ko) and *w*Pip(Tn) strains, respectively.

(c) *Crossing experiments*

The CI types of *Cx. pipiens* lines were determined through crossing experiments. Mass crosses were carried out using an equal number of 2- to 5-day-old virgin males and females. All crosses were performed at least twice independently, and the results pooled for analysis. Note that CI was previously shown to be expressed at the same intensity throughout the *Cx. pipiens* male lifespan (i.e. without age effect) [22,23]. Females were allowed to blood feed 5 days after caging and their clutches were stored separately until hatching at 25°C ± 2°C. Hatching rates (HRs) were scored 72 h after egg collection to determine the CI phenotype. All unhatched clutches were checked for fertilization through observation of embryonic development following the procedure of Duron & Weill [24].

(d) *Molecular typing*

The *w*Pip infections were characterized through the sequencing of four *Wolbachia* markers: the ankyrin domains encoding genes *pk1* and *ank2* [25], one putative guanylate kinase encoding gene (*WPa_679*; this study) and one phage-related gene (the putative secreted protein gene *VrlC*, also known as *GP15*; electronic supplementary material, table S2) [26]. These markers are not amplified from the *Wolbachia*-uninfected line (Sl-TC) and discriminate the four *w*Pip strains examined here (see the electronic supplementary material, table S3). Independent assays for infection by four other endosymbionts commonly found in insects (i.e. *Cardinium*, *Rickettsia*, *Arsenophonus* and *Spiroplasma*) were performed using specific polymerase chain reaction (PCR) amplifications as described by Duron *et al.* [4] (see the electronic supplementary material, table S2).

The *Cx. pipiens* mitochondrial haplotypes were determined through sequencing of an 852 bp fragment from the cytochrome b (*cytb*) gene (see the electronic supplementary material, table S2). The examination of the *Cx. pipiens* nuclear genome was assessed by PCR/restriction fragment length polymorphism (RFLP) tests based on *ace-2* and $Ester^2$ genes (see the electronic supplementary material, table S2). The *ace-2* gene is located on chromosome I and encodes acetylcholinesterase 2 [27]. The $Ester^2$ gene is located on chromosome II and encodes a carboxylester hydrolase [28]. A PCR/RFLP test on *ace-2* using the restriction enzyme *Sca*I (37°C, 3 h [29]) allows the discrimination between the Lv, Is, Ko, Tn (two fragments: 230 and 470 bp) and the Sl-TC (three fragments: 120, 230 and 350 bp) nuclear genomes. We developed a PCR/RFLP test on $Ester^2$ using the *AvaII* enzyme (37°C, 3 h), which generated different restriction fragments for the Lv (four fragments: 92, 176, 313 and 519 bp), Is (three fragments: 37, 519 and 544 bp), Ko (three fragments: 37, 519 and 544 bp), Tn (three fragments: 37, 519 and 544 bp) and Sl-TC (two fragments: 519 and 581 bp) nuclear genomes.

Mosquito DNA was extracted using a cetyltrimethylammonium bromide protocol [30]. All PCRs were performed with approximately 20 ng of genomic DNA solution in a 40 µl final volume reaction for 35 cycles (94°C, 5 min; 94°C, 30 s; 52°C, 30 s; 72°C, 1 min). Primers are listed in electronic supplementary material, table S2. Direct sequencing of PCR products was performed on an ABI Prism 3130 sequencer using the BigDye Terminator Kit (Applied Biosystems) after purification with the QIAquick gel extraction kit (Qiagen, Valencia, CA). Sequence alignment and analyses were carried out using software MEGA [31]. The sequences produced in this study are deposited in GenBank (accession numbers: JX188403–JX188404).

3. RESULTS

(a) *New cytoplasmic incompatibility types evolve in laboratory* Culex pipiens *lines*

We analysed experimentally the evolution of CI types over five years (2005–2009), which is roughly 50 mosquito generations. To this end, four isofemale lines of *Cx. pipiens*, designated as Lv, Is, Ko and Tn, and infected by the *w*Pip(Lv), *w*Pip(Is), *w*Pip(Ko) and *w*Pip(Tn) strains, respectively (electronic supplementary material, table S1), were crossed with each other and their CI types examined in 2005. The same crosses were repeated in 2009.

Although no self-incompatibility was observed between mosquitoes of the same line, there was clear evidence that some CI types had evolved. In 2005, the 16 reciprocal mass crosses between the four infected lines revealed a variety of crossing relationships with bidirectional CI (e.g. Lv × Is, Ko × Is and Tn × Is), unidirectional

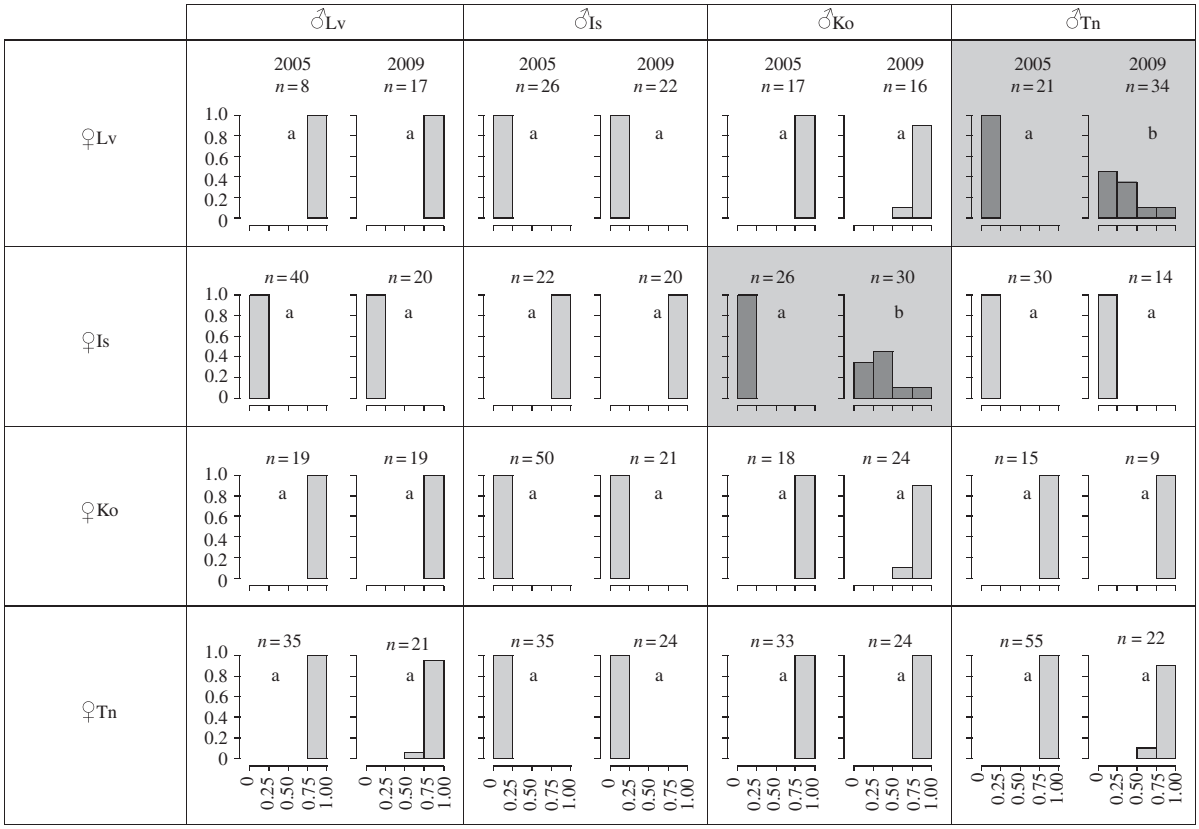

Figure 1. Crossing relationships between the four infected lines of *Culex pipiens* in 2005 and 2009. Histograms give the distribution of hatching rates (*x*-axis, HR; *y*-axis, proportion of clutches). The number of clutches observed (*n*) is indicated for each cross (at least 1000 eggs were examined per cross). Crosses showing variable outcomes between 2005 and 2009 are shaded in grey. Variations of HR between 2005 and 2009 were tested through Fisher exact tests (letters a and b refer to statistical groups).

CI (e.g. Lv × Tn) and bidirectional compatibility (e.g. Lv × Ko and Ko × Tn; figure 1). In 2009, we repeated the same crosses, using the same methodology, but obtained different outcomes in two crosses, ♂Tn × ♀Lv and ♂Ko × ♀Is. Both crosses were incompatible and gave very few progeny in 2005, but they showed partial compatibility in 2009, producing a continuum from incompatible to compatible clutches (figure 1; electronic supplementary material, figure S1). We verified that no inadvertent contamination had occurred between 2005 and 2009 in the four strains by examining an array of genetic markers (electronic supplementary material, table S2). (i) The molecular typing of *Cx. pipiens* mitochondrial and nuclear backgrounds confirmed that the mosquito lines used in 2009 were derived from those initially used in 2005 (electronic supplementary material, table S3). (ii) Multi-locus *Wolbachia* sequencing showed that the *w*Pip strains observed in 2009 descended from those characterized in 2005, and that no other *Wolbachia* strain was present (electronic supplementary material, table S3). In addition, we tested for the presence of a range of inherited bacteria also known to manipulate insect reproduction (i.e. *Cardinium*, *Arsenophonus*, *Rickettsia* and *Spiroplasma*), but did not find any of them.

Thus, the changes of CI types observed in 2009 when compared with 2005 were clearly due to the evolution of *Cx. pipiens* lines and/or to their infecting *w*Pip strains. Two non-exclusive mechanisms could explain the observed variations of CI properties: the presence of a host restorer gene that prevents the expression of CI, and the emergence of new mutated *w*Pip strain types.

(b) *Cytoplasmic incompatibility properties are only due to* Wolbachia

Each *Cx. pipiens* line induced unidirectional CI with an uninfected line, Sl-TC. In 2005 as well as in 2009, infected males always sterilized uninfected females, producing no progeny, whereas the reverse crosses were always fertile, with normal HRs (electronic supplementary material, figure S2). An antibiotic treatment of Lv, Is, Ko and Tn had restored full compatibility with Sl-TC females in 2006, indicating that the sterility was due to *w*Pip infection rather than to nuclear incompatibility [18]. Thus, it can be concluded that each *w*Pip strain expressed a constitutive *mod*.

In 2009, we introduced separately each *w*Pip infection into the Sl-TC nuclear background through eight generations of cytoplasmic introgression using Sl-TC males. The backcrossed lines, designated Sl^{wLv}, Sl^{wIs}, Sl^{wKo} and Sl^{wTn}, shared a homogenized Sl-TC nuclear background but harboured distinct cytoplasm, as confirmed by examining *w*Pip, mitochondrial and nuclear *Cx. pipiens* genetic markers (electronic supplementary material, table S3). Through crossing experiments, we found that the four *w*Pip strains exhibited the same CI types in the Sl-TC nuclear background as in their original nuclear backgrounds (figure 2; electronic supplementary material, figure S2). Thus, the variations of CI types were dependent only on *w*Pip factors and not on *Cx. pipiens* nuclear factors.

(c) *Mutations affecting* resc *loci explain the evolution of cytoplasmic incompatibility types*

Having established that the 2005–2009 CI differences observed in the crosses ♂Tn × ♀Lv and ♂Ko × ♀Is were

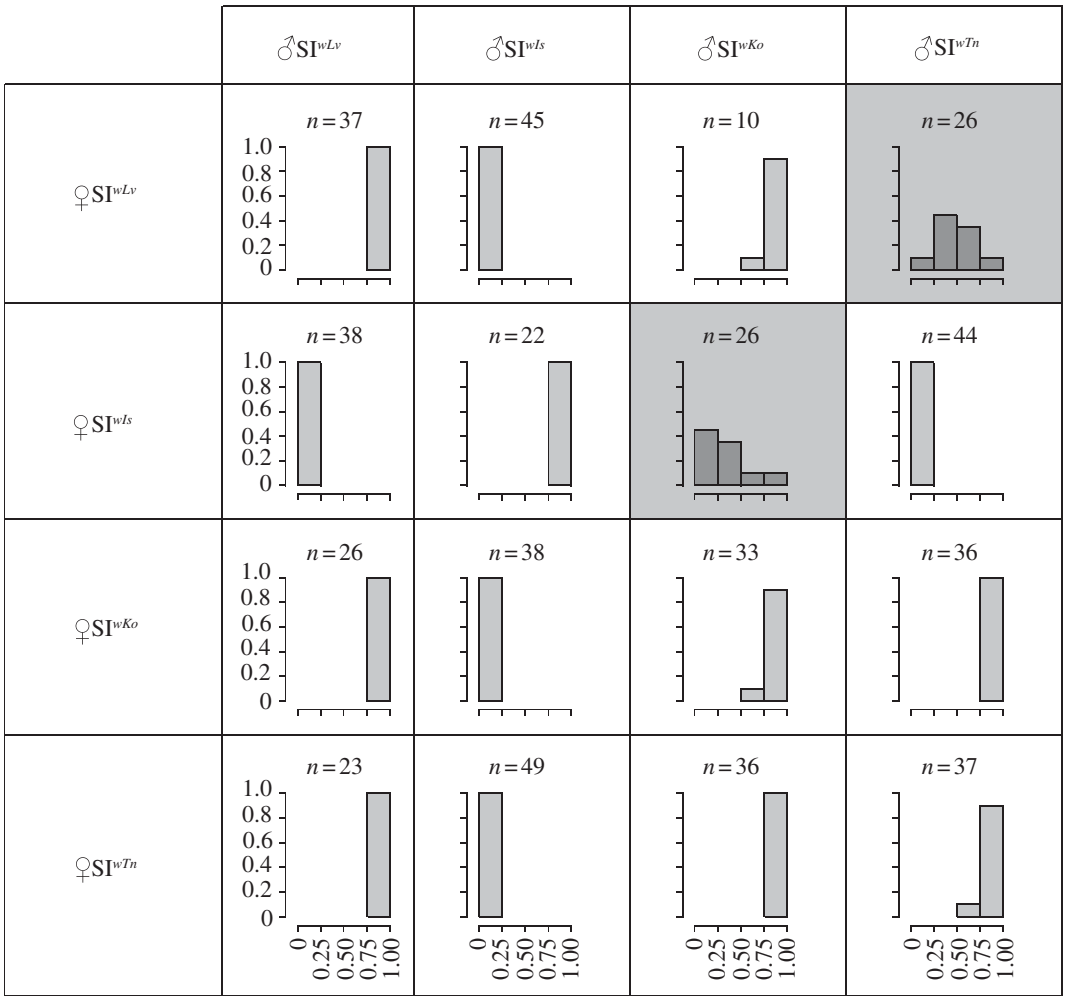

Figure 2. Crossing relationships between the four backcrossed lines (Sl^{wLv}, Sl^{wIs}, Sl^{wKo} and Sl^{wTn}) sharing a homogenized Sl-TC nuclear background. Histograms give the distribution of hatching rates (*x*-axis, HR; *y*-axis, proportion of clutches). The number of clutches observed (*n*) is indicated for each cross (a minimum of 1200 eggs was examined per cross). No significant variation of HR was observed between the crosses made in 2009 using the original nuclear backgrounds and the crosses using the Sl-TC background (Fisher exact tests, all $p > 0.10$). Crosses showing significant variation with the 2005 dataset are shaded in grey (Fisher exact tests, all $p < 0.005$).

due to the evolution of their *Wolbachia*, we next examined which *w*Pip functions had changed—either the *mod* functions of the *w*Pip(Tn) and *w*Pip(Ko) strains or the *resc* functions of *w*Pip(Lv) and *w*Pip(Is), or both. We sub-cloned each of the four *Cx. pipiens* lines in 10–14 new isofemales lines, and determined their respective CI types when crossed with original lines (see figure 3*a–d*). The crosses of females from 14 Lv sub-lines (named Lv-sub1 to Lv-sub14) with Tn males from the original line showed that two distinct CI types were coexisting among the females of the Lv original line (figure 3*a*; electronic supplementary material, figure S3*a*). Females from six Lv sub-lines exhibited the ancestral CI type (complete incompatibility) initially observed in 2005. Females of the eight other Lv sub-lines exhibited the new CI type (partial compatibility) observed in 2009; for instance, Lv-sub10 females mated with Tn males produced clutches with an HR continuum from low to high. Similarly, two CI types were also found among females of the Is line (figure 3*b*; electronic supplementary material, figure S3*b*). Females from five Is sub-lines exhibited the ancestral CI type, whereas females of the six other sub-lines exhibited the new CI type. By contrast, crosses between males from 12 Tn sub-lines and Lv females from the original line produced clutches with a HR continuum, suggesting that only one CI type occurred among males of the Tn line (figure 3*c*); an identical observation was made for the males of the Ko line using males from 10 Ko sub-lines crossed with Is females (figure 3*d*). That there is no variation among Tn and Ko males is also suggested by crosses with Lv and Is females exhibiting the ancestral CI types: all these crosses are incompatible, showing that all Tn and Ko males exhibit only the ancestral CI type (figure 3*a*,*b*).

The crossing patterns exhibited by isofemale sub-lines showed that the differences in CI patterns observed between 2005 and 2009 were due to changes in the *resc* function of the *w*Pip(Lv) and *w*Pip(Is) strains, rather than in the *mod* function of *w*Pip(Tn) and *w*Pip(Ko). These new mutated *resc* functions were found in approximately half of the examined isofemale sub-lines, and they only partially restored the compatibility, exhibiting an HR continuum from low to high. These data showed that the ancestral and the new mutated *resc* functions coexisted within females from whom sub-lines originated.

(d) *Characterization of the new* resc *functions*

We first examined if the new CI types (called N hereafter) remained compatible with their ancestral counterparts

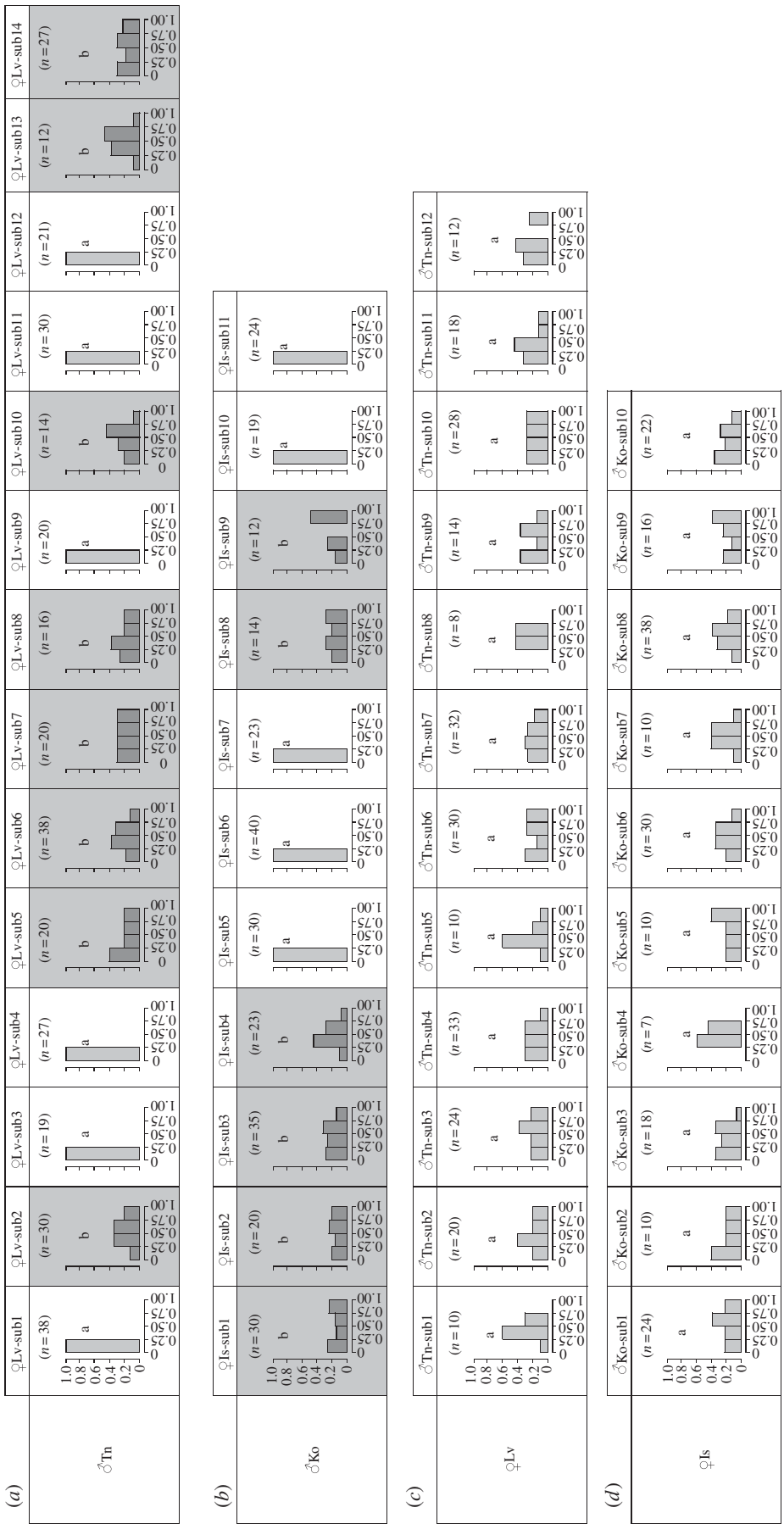

Figure 3. Crossing relationships of (*a*) females from the 14 Lv sub-lines (named Lv-sub1–14) with Tn males; (*b*) females from the 11 Is sub-lines (Is-sub1–11) with Ko males; (*c*) males from the 12 Tn sub-lines (Tn-sub1–12) with Lv females, (*d*) males from the 10 Ko sub-lines (Ko-sub1–10) with Is females. Histograms give the distribution of HR (*x*-axis, HR; *y*-axis, proportion of clutches). The number of clutches observed (*n*) is indicated for each cross (a minimum of 1000 eggs was examined per cross). Variations of HR among sub-lines were tested through Fisher exact tests (letters a and b refer to statistical groups). For (*a*) and (*b*), sub-lines exhibiting the new CI type are shaded in grey.

(called A hereafter) in Lv and Is mosquito lines. Reciprocal crosses between individuals from the Lv-sub12 (A) and Lv-sub10 (N) sub-lines showed full compatibility (electronic supplementary material, figure S4*a*). An identical observation was made in crosses with the Is-sub5 (A) and Is-sub1 (N) sub-lines (electronic supplementary material, figure S4*b*). These results indicated that two *resc* functions were present in the Lv and Is N CI types: (i) N CI types must have retained their ancestral *resc* function, able to restore the ancestral *mod* function (otherwise, the crosses between males with A CI types and females with N CI types would have been incompatible); (ii) in addition, N CI types have acquired new *resc* functions able to restore compatibility of Lv and Is females with Tn and Ko males, respectively. An alternative interpretation is that a single *resc* function can do all this in the N CI types. Under this hypothesis, the ancestral *resc* function has evolved towards a more generalist *resc* function that can restore distinct *mod* functions. In the case of Lv, the new *resc* function can then restore the Lv and Tn *mod* functions while the ancestral can restore only the Lv *mod* function. Similarly, a new and generalist *resc* function could also exist in the Is line. Note that this evolution of *resc* function in Lv and Is *Wolbachia* strains was independent of Lv and Is *mod* functions, which had not varied since 2005 (figure 1), confirming that the A and N CI types share the same *mod* function in each strain.

We then surveyed the new CI types in the four freshly isolated Lv and Is sub-lines. In general, we found that the A and N CI types of Lv and of Is were stably expressed over 10 host generations (electronic supplementary material, figure S5*a–d*). It is thus obvious that the new *resc* functions were permanently gained by the N Lv and by the N Is CI types: females from Lv-sub10 and Is-sub1 sub-lines crossed with Tn and Ko males, respectively, always produced clutches with an HR continuum from incompatible to compatible over 10 generations, while females from Lv-sub12 and Is-sub5 sub-lines remained incompatible (electronic supplementary material, figure S5*a–d*). However, there was also evidence that N CI types were still evolving from the A CI type: the incompatible cross ♂Ko × ♀Is-sub5 produced only incompatible clutches in G1 and G2, but few clutches with intermediate to high HR appeared in the next generations (electronic supplementary material, figure S5*c*).

Finally, new Lv and Is females that produce only fully compatible clutches when crossed with Tn and Ko males, respectively, were selected during two generations. As F_2-selected females mated with incompatible males still produced clutches with low to high HR in the same proportions as at the F_0 generation (Wilcoxon two-tailed tests; $p = 0.26$ for comparisons between F_0 and F_2 Lv females; $p = 0.17$ for comparisons between F_0 and F_2 Is females), the experiment was discontinued.

4. DISCUSSION

The evolution of *Wolbachia* CI types is classically viewed as a sharp transition from one CI type to a new one, incompatible with the former. In this study, we showed that changes in CI properties of *Wolbachia* infecting *Cx. pipiens* can be observed on a laboratory time scale, and support the notion that complexity of the *mod–resc* system allows a more gradual transition [32,33]. Within 5 years, new CI types emerged independently in two *Cx. pipiens* lines with a similar phenotypic scheme. Ancestral (A) and new (N) CI types remain mutually compatible, but they display different CI properties with specific strains, termed discriminating (D) strains. Appearance of the N CI types is independent of the host nuclear background and is driven only by variations of *Wolbachia resc* factors.

Shifts in CI properties had already been reported in pioneering work on laboratory lines [34–37]. In particular, Sasa *et al.* [36] described how unidirectional CI between two *Cx. pipiens* lines maintained in the laboratory was modified after an unspecified number of generations, producing a mixture of compatible and incompatible clutches as in our study. Rapid change of CI types could thus represent a general feature of *Wolbachia*, at least for *w*Pip. Each new CI type observed here resulted from the gain of a new *resc* function with no apparent change in its *mod* function. This supports the notion that the *mod* and *resc* functions vary independently, in agreement with the fact that these functions are encoded by different genes of the *Wolbachia* genome [8,11,12,15,16].

How were the new *resc* functions acquired in the *w*Pip strains? A first possibility could be that their *resc* factors have been modified either qualitatively (punctual mutation) or quantitatively (increased expression). Punctual mutations within *resc* sequences could reduce their specificity and thus allow the rescue of additional *mod* types. Additionally, mutant *Wolbachia* could carry two different *resc* loci inserted into their genome. Such genomic evolution could arise either through duplication of the existing *resc* locus, followed by divergence through point mutation, or through genetic exchange with another *Wolbachia* strain, resulting in the introduction of a new *resc* locus onto the genome. On the other hand, a higher level of *resc* expression might also lower their specificity. We do not favour this last hypothesis, because the new *resc* function in Lv and Is N lines each rescued only a single specific D strain, whereas one would have expected the rescue of more *mod* functions. A second possibility is the activation of a hitherto silent *resc* factor of distinct specificity. Expression of such additional functions was proposed to rely on the transposition activity of mobile genetic elements (MGEs), expected either to modify the expression of genes located close to their insertion sites, or to break or restore the function of genes in which they are inserted [26,38–41]. The potential implication of MGEs as modifiers of *resc* functions may thus also hold true for the present study, and the emergence of N CI types in Lv and Is *Cx. pipiens* lines may result from the activation of a new *resc* factor on the same *Wolbachia* genome. The fact that the two N CI types each rescue only one D strain also suggests that the number of genetic determinants of CI in a single *Wolbachia* genome could actually be larger than originally thought. This situation is reminiscent of our recent study of *Cx. pipiens* populations on Réunion Island, in which closely related *Wolbachia* were found to encode a variety of *resc* functions in addition to the one involved in their self-compatibility [16].

The thorough examination of compatibility pattern suggests the presence of single infection in A CI types and of co-infection in N CI types. Interestingly, A CI types represent around half of isofemale Lv and Is

sub-lines; they were incompatible with the D males and remained so across generations, suggesting that they were mainly infected by the ancestral *Wolbachia*. It is likely that the other half of isofemale sub-lines (N CI types) were co-infected by a mixture of ancestral and mutant *Wolbachia*. Such a co-infection can easily explain that these females produced a continuum from incompatible to compatible clutches, depending on the abundance ratio of ancestral and mutant *Wolbachia* they carried. Thus, the compatibility with the D strain probably requires a threshold frequency of the mutant *Wolbachia* responsible for the N CI type. Following that logic, females harbouring a majority of mutant *Wolbachia* will be compatible with D males, but not female hosts harbouring a majority of ancestral *Wolbachia*. Worth noting is that no female host producing only compatible clutches with D males was observed, suggesting that none was mono-infected by the mutant *Wolbachia*. In other words, double infection seems thus maintained over several host generations, meaning that the efficiency of maternal transmission has the potential to impede segregation from double to single infection. Overall, it appears that the mutant *Wolbachia* have spread as neutral variants in Lv and Is lines where they coexist with the ancestral *Wolbachia* in about half of the females with a proportion sufficiently high to allow compatibility with their respective D strains.

Whether the co-infection of ancestral and mutant *Wolbachia* is a general feature in *Cx. pipiens* populations is still unknown. As long as the molecular mechanism of CI is not known and CI markers are not available, the only way to find out is to maintain strains with different CI types in laboratory conditions and detect variants by crossing with discriminating D strains. However, it is very likely that the presence of several *resc* functions within individual hosts is common, a feature that may facilitate the gradual evolution of the CI types. Indeed, expression of a new *resc* factor is neutral for the host, because the A and N CI types remain fully compatible, as in Lv and Is lines. The situation would be opposite for the *mod* factors, because a mutant *Wolbachia* expressing a new *mod* function is expected to be self-incompatible, and thus to be rapidly counter-selected at the population level until it evolves a new *resc* function. This probably explains why changes only in *resc* functions have been identified from our study. Co-infection should also allow for the emergence of mutant *Wolbachia* that no longer express the ancestral *resc* factor, because the production of the ancestral *resc* factor will be ensured by the co-infecting bacteria. This opens up the possibility for a gradual and neutral transition towards a greater complexity of CI types.

In conclusion, the present study provides experimental support for the rapid evolution of CI types through changes that separately affect the *mod* and *resc* functions of *Wolbachia*. It also demonstrates that multiple *resc* functions can be expressed by individual hosts, probably as a result of co-infection. With the increasing ability to genetically characterize *Wolbachia* [26,38–40], it is likely that infections currently defined as clonal will be shown to be a mixture of closely related *Wolbachia* strains, and deep genomic analysis should be valuable for the understanding of the genetic basis of CI.

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