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Variation in *Wolbachia cidB* gene, but not *cidA*, is associated with cytoplasmic incompatibility *mod* phenotype diversity in *Culex pipiens*

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Abstract

Endosymbiotic *Wolbachia* bacteria are, to date, considered the most widespread symbionts in arthropods and are the cornerstone of major biological control strategies. Such a high prevalence is based on the ability of *Wolbachia* to manipulate their hosts' reproduction. One manipulation called cytoplasmic incompatibility (CI) is based on the death of the embryos generated by crosses between infected males and uninfected females or between individuals infected with incompatible *Wolbachia* strains. CI can be seen as a modification-rescue system (or *mod-resc*) in which paternal *Wolbachia* produce *mod* factors, inducing embryonic defects, unless the maternal *Wolbachia* produce compatible *resc* factors. Transgenic experiments in *Drosophila melanogaster* and *Saccharomyces cerevisiae* converged towards a model where the *cidB Wolbachia* gene is involved in the *mod* function while *cidA* is involved in the *resc* function. However, as *cidA* expression in *Drosophila* males was required to observe CI, it has been proposed that *cidA* could be involved in both *resc* and *mod* functions. A recent correlative study in natural *Culex pipiens* mosquito populations has revealed an association between specific *cidA* and *cidB* variations and changes in *mod* phenotype, also suggesting a role for both these genes in *mod* diversity. Here, by studying *cidA* and *cidB* genomic repertoires of individuals from newly sampled natural *C. pipiens* populations harbouring *w*PipIV strains from North Italy, we reinforce the link between *cidB* variation and *mod* phenotype variation fostering the involvement of *cidB* in the *mod* phenotype diversity. However, no association between any *cidA* variants or combination of *cidA* variants and *mod* phenotype variation was observed. Taken together our results in natural *C. pipiens* populations do not support the involvement of *cidA* in *mod* phenotype variation.

KEYWORDS

cidA, *cidB*, Culex pipiens, cytoplasmic incompatibility, *Wolbachia*

1 | INTRODUCTION

Wolbachia are maternally inherited endosymbiotic bacteria commonly found in arthropods and filarial nematodes (Ferri et al., 2011; Taylor, Bandi, & Hoerauf, 2005; Werren, Baldo, & Clark, 2008). More than 40% of terrestrial arthropod species are thought to be infected (Weinert, Araujo-Jnr, Ahmed, & Welch, 2015; Zug & Hammerstein, 2012). The pervasiveness of this bacterial genus is mostly attributed to its ability to manipulate host reproduction, facilitating its spread within arthropod populations (Rousset & Raymond, 1991; Turelli & Hoffmann, 1991; Werren et al., 2008). The most commonly described *Wolbachia*-induced phenotype in arthropods is cytoplasmic incompatibility (CI; Werren et al., 2008). CI is a form of conditional sterility resulting in embryonic lethality when infected males mate with uninfected females or with females infected with a different, incompatible *Wolbachia* strain (Atyame, Duron, et al., 2011; Bonneau, Landmann, et al., 2018; Bordenstein, O'Hara, & Werren, 2001; Breeuwer & Werren, 1990; Callaini, Riparbelli, Giordano, & Dallai, 1996; Duron et al., 2006; Laven, 1967; O'Neill & Karr, 1990). In *C. pipiens* where all males and females are infected, CI may be unidirectional (crossing is compatible in one direction but incompatible in the other) or bidirectional (crosses in both directions are incompatible; Atyame et al., 2014; Dumas et al., 2013; Laven, 1967; Rasgon & Scott, 2003; Sicard, Bonneau, & Weill, 2019). CI can be seen as a toxin-antidote model or modification-rescue model (*mod-resc*) in which the *Wolbachia* present in the male produce a toxin (*mod* factors) during spermatogenesis which induces CI through embryonic defects after fertilization unless the *Wolbachia* present in the eggs produce compatible antidotes (*resc* factors) (Hurst, 1991; Poinsot, Charlat, & Merçot, 2003; Werren, 1997). Both sterile insect and pathogen blocking *Wolbachia*-based methods to fight against arthropod pests and vectors rely on the ability of *Wolbachia* to induce CI. Knowledge on CI diversity in mosquito is required to find the better *Wolbachia*-mosquito associations to optimize the success of biological control (Flores & O'Neill, 2018; Sicard et al., 2019).

Recent works have implicated the syntenic *Wolbachia* genes *cidA* and *cidB*, from *w*Mel and *w*Pip-Buckeye strains infecting *Drosophila melanogaster* and *Culex pipiens* respectively, in CI (Figure 1; Beckmann, Ronau, & Hochstrasser, 2017; LePage et al., 2017). These genes exhibit many typical features of toxin-antidote models (Beckmann et al., 2019a, 2019b, 2017). In both transgenic yeasts and flies, $cidA^{wPip}$ and $cidB^{wPip}$ genes were proposed to encode interacting proteins acting in a toxin-antidote fashion with the toxicity of $CidB^{wPip}$ being rescued by the expression of $CidA^{wPip}$ (Beckmann et al., 2017). However, both $cidA^{wMel}$ and $cidB^{wMel}$ were required to induce CI in transgenic *D. melanogaster* (LePage et al., 2017; Shropshire & Bordenstein, 2019) and the expression of $cidA^{wMel}$ in transgenic *D. melanogaster* females was necessary and sufficient to resume the *resc* function (Figure 1; Shropshire & Bordenstein, 2019; Shropshire, On, Layton, Zhou, & Bordenstein, 2018). Based on these findings a two-by-one model was proposed, in which $cidA^{wMel}$ acts as a *mod* factor when expressed in males and as a *resc* factor when expressed in females, while $cidB^{wMel}$ acts only as a *mod* factor in *D. melanogaster* (Shropshire & Bordenstein, 2019; Shropshire et al., 2018).

Cytoplasmic incompatibility in *Culex pipiens* mosquitoes is characterized by its unprecedented diversity of compatibility and incompatibility relationships that is based on the diversity of the *Wolbachia* strains infecting this species (Atyame et al., 2014; Duron et al., 2006; Laven, 1967). All the *Wolbachia* infecting *C. pipiens* belong to the monophyletic group *w*Pip inside the supergroup B and are closely related to *w*Bol, the *Wolbachia* strain infecting the butterfly *Hypolimnas bolina* (Atyame, Delsuc, Pasteur, Weill, & Duron, 2011; Bleidorn & Gerth, 2018). The *w*Pip group is divided into five groups *w*PipI-V and mosquitoes infected with *Wolbachia* from two different *w*Pip groups are more likely to be incompatible than mosquitoes infected with *w*Pip strains from the same *w*Pip group (Atyame et al., 2014). An analysis conducted on multiple crosses showed that each *w*Pip genome must contain several *mod* and *resc* factors to account for the diversity of CI phenotypes in *C. pipiens* (Atyame, Duron, et al., 2011; Nor et al., 2013). These multiple *mod* and *resc* factors could theoretically be encoded by different copies (i.e., variants) of the same *mod/resc* genes or different *mod* and *resc* genes within the same *w*Pip genome as already proposed in some CI models (Atyame, Duron, et al., 2011; Nor et al., 2013; Poinsot et al., 2003). To investigate the genetic basis of the unprecedented diversity of CI phenotypes found in *C. pipiens* mosquitoes we studied the *cidA* and *cidB* genes of *w*Pip *Wolbachia* strains belonging to the groups *w*PipI, II, III and IV (Bonneau, Atyame, et al., 2018). All these *w*Pip genomes exhibited several polymorphic copies of $cidA^{wPip}$ and $cidB^{wPip}$ genes (Bonneau, Atyame, et al., 2018; Bonneau, Landmann, et al., 2018). To be responsible for the CI phenotype diversity observed in *C. pipiens*, $cidA^{wPip}$ and $cidB^{wPip}$ genes must have different sequences in *w*Pip strains that induce different CI phenotypes (i.e., showing different incompatibility relationships when crossed with individuals harbouring other strains). We analyzed the $cidA^{wPip}$ and $cidB^{wPip}$ variant repertoires of *w*Pip strains showing different CI phenotypes and showed that they exhibited distinct $cidA^{wPip}$ and $cidB^{wPip}$ variants in their genomes supporting the implication of these two genes in CI phenotype diversity in *C. pipiens* (Bonneau, Atyame, et al., 2018).

The putative roles of the $cidA^{wPip}$ and $cidB^{wPip}$ genes in CI phenotype diversity have been further investigated by analyzing variation of $cidA^{wPip}$ and $cidB^{wPip}$ in isofemale lines infected with *Wolbachia* from the *w*PipIV phylogenetic group and exhibiting well differentiated *mod* phenotypes (Atyame et al., 2015; Bonneau, Atyame, et al., 2018). In *C. pipiens*, difference in *mod* phenotypes refers to differing compatibility between crosses involving focal studied males (i.e., from which we aim to infer the *mod* phenotype) and females infected with reference *w*Pip strains (Bonneau, Atyame, et al., 2018). As such crosses only differ from each other because of the *w*Pip strain harboured by the males, the difference in compatibility (i.e., egg-raft hatching vs. no egg-raft hatching) between these crosses allows us to qualify the *mod* phenotype. This way, we demonstrated that mosquitoes infected with *w*PipIV present two different *mod* phenotypes: the incompatible *mod* phenotype, in which males cannot produce viable progenies with females infected with *w*Pip from group

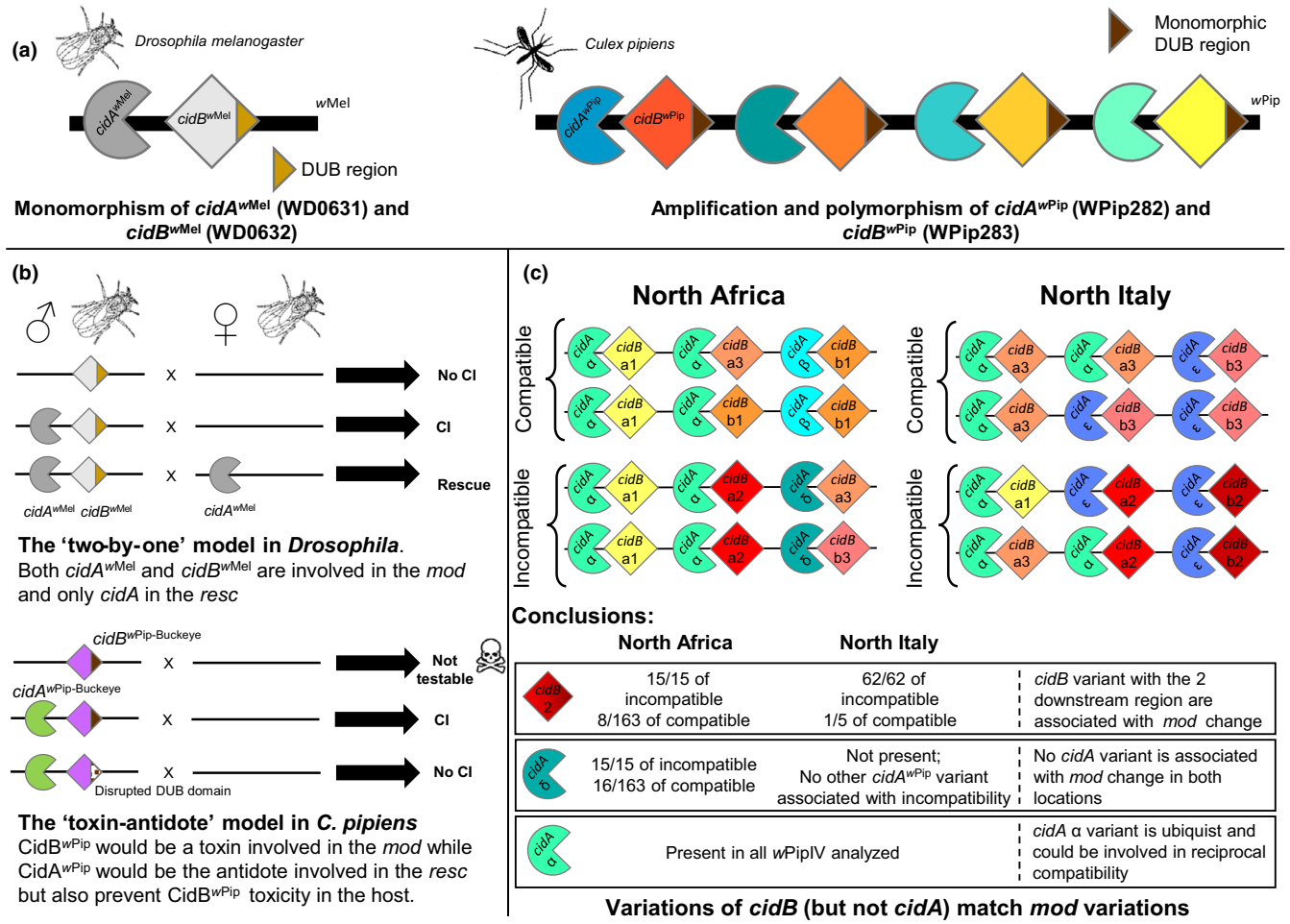

FIGURE 1 Summary of transgenic experiments and natural population studies conducted on *cidA* and *cidB*. (a) Hypothetical representation of the portion of prophage WO containing *cidA* and *cidB* CI genes in *w*Mel and *w*Pip *Wolbachia* strains. The genome of *w*Mel contain only one copy of *cidA* and *cidB* genes (Lindsey et al., 2018). Genomes of all the *w*Pip strains from several *w*Pip groups investigated contain several different copies of *cidA* and *cidB* genes (Bonneau, Atyame, et al., 2018). The deubiquitinase (DUB) region is the catalytic domain of CidB protein (Beckmann et al., 2017). This region is conserved between the $cidB^{wPip}$ variants (Bonneau, Atyame, et al., 2018). (b) transgenic expression of *cidA* and *cidB* from either *w*Mel or *w*Pip-Buckeye genomes in *Drosophila melanogaster* flies. The expression of both $cidA^{wMel}$ and $cidB^{wMel}$ in *D. melanogaster* males is required to induce CI, neither $cidA^{wMel}$ or $cidB^{wMel}$ alone can induce CI (LePage et al., 2017; Shropshire & Bordenstein, 2019). The expression of $cidA^{wMel}$ alone in *D. melanogaster* females is sufficient to rescue CI (Shropshire et al., 2018). A two-by-one model of CI was proposed in *D. melanogaster* in which $cidA^{wMel}$ acts as a *mod* factor when expressed in males and as a *resc* factor when expressed in females (Shropshire & Bordenstein, 2019; Shropshire et al., 2018). The production of viable transgenic male flies expressing only $cidB^{wPip}$ was not possible suggesting a toxic effect of the $CidB^{wPip}$ protein (represented by a skull) while flies expressing both $cidA^{wPip}$ and $cidB^{wPip}$ were viable and capable of CI induction. The male flies expressing $cidA^{wPip}$ and $cidB^{wPip}$ with a disrupted catalytic DUB domain were not capable to induce CI suggesting that the DUB region is functionally involved in CI. The current transgenic data in *Culex pipiens* support a toxin-antidote model where $cidB^{wPip}$ would encode a toxin involved in the *mod* function while $cidA^{wPip}$ would encode the antidote involved in the *resc* but also prevent the producer from the toxicity of $CidB^{wPip}$ protein. (c) $cidA^{wPip}$ and $cidB^{wPip}$ variants repertoires in natural populations of *C. pipiens* infected with *w*PipIV strains. Full variant names are not shown (they all belong to the group *w*PipIV) and only the letter/number of the variant appear. For instance *cidA* α referring to *cidA*_IV(α) and *cidB* a2 to *cidB*_IV(a/2). The number of pairs of genes as well as their disposition in the genome might not reflect the reality as these informations are still under investigation. Males from North African and North Italian natural populations are either compatible or incompatible with females from the Tunis isofemale line depending on the *w*PipIV strain they carry. All the *w*PipIV strains carry several $cidA^{wPip}$ and $cidB^{wPip}$ variants inside their genomes. The variants *cidB*_IV(2) [i.e., *cidB*_IV(a/2) and *cidB*_IV(b/2)] were found associated with the incompatible *mod* phenotype in both geographical areas while the variant *cidA*_IV(δ) was found associated with the incompatible *mod* phenotype only in the North African population. Furthermore, no other *cidA* variant or combination of variants was found associated with *mod* phenotype variation in North Italian populations suggesting that only *cidB* plays a role in the *mod* phenotype variations in *C. pipiens*. Finally, the *cidA*_IV(α) variant was detected in all the *w*PipIV strains regardless of their *mod* phenotype and their geographical origins. The ubiquity of the *cidA*_IV(α) variant could be responsible for the reciprocal compatibility always observed between mosquitoes infected with different *w*PipIV strains and suggests a role of *cidA* in the *resc* function [Colour figure can be viewed at wileyonlinelibrary.com]

I, II or III, and the compatible *mod* phenotype, in which males produce viable progenies with such females (Atyame et al., 2014, 2015; Bonneau, Atyame, et al., 2018). The association of a specific *cidB*^{wPip} variant (variant *cidB*_IV[a/2]) with the incompatible *mod* phenotype in 180 isofemale lines (Figure 1), together with the results of functional studies in *D. melanogaster* (Beckmann et al., 2017; LePage et al., 2017), demonstrated the involvement of *cidB*^{wPip} in *mod* function and in the diversity of the CI *mod* phenotype in *C. pipiens* (Bonneau, Atyame, et al., 2018). However, the implication of *cidA*^{wPip} in CI *mod* and/or *resc* function remained unclear in *C. pipiens*. Indeed, an association was found between a specific *cidA*^{wPip} variant (*cidA*_IV[δ]) and the incompatible *mod* phenotype, suggesting a possible role for *cidA*^{wPip} in *mod* phenotype diversity (Figure 1) (Bonneau, Atyame, et al., 2018). However, the ubiquitous presence of *cidA*_IV(α) in all the *w*PipIV strains might account for reciprocal compatibility (systematic rescue) between them suggesting a putative role for *cidA*^{wPip} in the *resc* function (Figure 1) (Bonneau, Atyame, et al., 2018). Overall, the diversity of CI phenotypes described in *C. pipiens* might result from differential interactions between specific CidA and CidB variants. We have already proposed a zone of interaction between CidA and CidB proteins located in the polymorphic regions shown to be correlated with CI variation (Bonneau, Atyame, et al., 2018). It is thus possible that the association of the *cidA*_IV(δ) variant with the *w*PipIV incompatible *mod* phenotype results from codiversification with the *cidB*_IV(a/2) variant for binding adjustment, as predicted for toxin-antidote systems.

Here, we further investigated the link between *cidA* and CI phenotype diversity, by studying the *cidA*^{wPip}/*cidB*^{wPip} variant repertoires in *w*PipIV strains from newly-sampled natural populations with possibly different evolutionary histories. We screened the *mod* phenotype variation in four North Italian populations presenting a mixture of *w*Pip group IV strains inducing either compatible or incompatible *mod* phenotype. The *cidA*_IV(α), thought to be associated with intra-*w*PipIV compatibility was present in all the lines, regardless of their *mod* phenotype suggesting the putative implication of *cidA*^{wPip} in the *resc* (antidote) function. The presence of the *cidB*_IV(a/2) variant in all incompatible isofemale lines supports the role of *cidB* in *mod* phenotype variation. In the analyzed North African natural populations approximately 5% of isofemale lines were qualified as incongruent as they displayed the compatible *mod* phenotype but carried the *cidA*^{wPip} and *cidB*^{wPip} variants associated (i.e., in the 95% other cases) with incompatible *mod* phenotypes (Bonneau, Atyame, et al., 2018). We could not study these incongruent lines previously as they were not alive anymore when we studied their *cidA* and *cidB* gene repertoires (Bonneau, Atyame, et al., 2018). In this study, we managed to study one incongruent line (i.e., being compatible while harbouring *cidB*_IV[a/2] variant). This line exhibited both a unique *cidB* repertoire and a lower expression of the *cidB* variant associated with incompatible *mod* phenotype that could contribute to explain such incongruence. Most importantly, no specific *cidA*^{wPip} variant or combination of *cidA*^{wPip} variants was associated with either incompatible or compatible *mod* phenotypes, pointing towards the absence of consequence of *cidA*^{wPip} variation on CI *mod* diversity in *C. pipiens*. Overall, our data do not reject the two-by-one model of CI but have nothing to support it in *C. pipiens*. However, the variations of *cidB* that match changes in *mod* and the ubiquity of a *cidA* variant between compatible strains fit the expectation of a classic toxin-antidote model.

2 | MATERIALS AND METHODS

2.1 | Mosquito collection and the construction of isofemale lines

Culex pipiens larvae and pupae were collected from four natural breeding sites in North Italy in 2017 (Roveré della Luna, San Michele all'Adige, Zambana and Mezzocorona sites, Data S1) and reared to adulthood in the laboratory. Females were then fed turkey blood (bcl Wholly Wild World) with a Hemotek membrane feeding system (Discovery Workshops, UK), to enable them to lay eggs, from which isofemale lines were established. Each egg-raft (containing 100–250 eggs) was individually isolated for hatching, and the *Wolbachia* group present was determined by performing the pk1 PCR-RFLP test (Altinli, Gunay, Alten, Weill, & Sicard, 2018) on two first-instar larvae (L1) after extracting DNA using an acetyl trimethylammonium bromide (CTAB) protocol (Rogers & Bendich, 1989). Isofemale lines were created by rearing the offspring resulting from a single egg-raft (thus from a single female). We established 67 isofemale lines for this study (Data S1). Isofemale lines were reared in 65 dm^3 screened cages in a single room maintained at 22–25°C, under a 12 hr light/12 hr dark cycle. Larvae were fed with a mixture of shrimp powder and rabbit pellets, and adults were fed on honey solution.

2.2 | Determination of CI phenotypes

2.2.1 | CI phenotype of the isofemale lines resulting from field collection in North Italy

To be able to associate *cidA* and *cidB* variants with CI phenotype variation, the CI *mod* phenotype of each of the 67 North Italian isofemale lines was determined. CI *mod* phenotypes were characterized by crossing in the same cage males (25–50 virgin males) from each of the studied isofemale lines with females (25–50 virgin females) from the Tunis laboratory isofemale line infected with a *Wolbachia* strain from the *w*PipI group (Table S1; Duron et al., 2005). After five days in cages, the females were fed a blood meal and, five days later, egg-rafts were collected and deposited into 24-well plates. The CI *mod* status of each cross was determined by assessing eggs-raft hatching status. All unhatched eggs-rafts were checked for fertilization by observing embryonic development with a light microscope (Axiophot2, Zeiss), as described by Duron and Weill (2006). Two type of crosses were found: crosses with only fertilized unhatched eggs-rafts which were qualified as incompatible and crosses with only hatched eggs-rafts which were qualified as compatible. No crosses resulting in both fertilized hatched and fertilized unhatched eggs-rafts were found. Thus isofemale lines in which the males were incompatible

with females from the Tunis line were described as incompatible isofemale lines, whereas isofemale lines in which the males were compatible with females from the Tunis line were described as compatible isofemale lines.

2.2.2 | Capacity of Michele26 line to induce CI

Because Michele26 males were not able to induce CI when crossed with Tunis females, the capacity of Michele26 to induce CI at all was tested. The capacity of Michele26 line to induce CI was tested by crossing 25 virgin males with 50 virgin uninfected females from the SlabTC laboratory line. SlabTC line was obtained from the Slab laboratory line treated with tetracycline as described in Duron et al. (2006).

2.2.3 | Reciprocal compatibility of isofemale lines infected with *w*PipIV strains

To have a better support of the hypothesis that the *cidA*_IV(α) variant might be associated with the reciprocal compatibility of the isofemale lines infected with *w*PipIV, crosses between males and females infected with different *w*PipIV strains all harbouring *cidA*_IV(α) variant were performed. The reciprocal compatibility from seven laboratory *w*PipIV infected lines showing different *cidA*-*cidB* repertoires but all exhibiting *cidA*_IV(α) variant (Table S1) was tested by crossing 25 virgin males with 50 virgin females. This way, 20 different crosses were performed between these seven different lines (Table S2).

2.3 | Cloning and Sanger sequencing of *cidA* and *cidB* variants

The *cidA* and *cidB* genes of seven isofemale lines (Luna1, Luna3, Luna8, Luna27, Michele26, Michele1 and Mezzo9) were cloned and Sanger sequenced, as described by Bonneau, Atyame, et al. (2018), starting from the same DNA samples used to determine *Wolbachia* phylogenetic group. For each gene of each isofemale line, 24 clones were sequenced on average (the detail of numbers of clones sequenced per isofemale line and gene are presented in the Data S2). Moreover, we confirmed the presence of the variants detected in the clones by Sanger sequencing the *cidA* and *cidB* fragment amplified from each isofemale line before cloning. This allowed us to verify the polymorphism found in the different *cidA* and *cidB* clones. However, even with this double-checking system, we cannot exclude that some variants might not have been reported. Michele26 was chosen for cloning and sequencing because it was the only incongruent isofemale line found and the six other lines amenable to sustainable rearing under our utilized laboratory conditions. The Muscle alignment tool (Edgar, 2004) implemented in SEAVIEW 6.4.1 software (Gouy, Guindon, & Gascuel, 2010) was used to align variant sequences.

The *cidB*_IV(a/2) variant previously found associated with the incompatible *mod* phenotype in natural populations from North Africa in the study by Bonneau, Atyame, et al. (2018) was undoubtedly identified only by the cloning and Sanger sequencing because the PCR-RFLP test (see below) was designed to only discriminate the downstream polymorphic region of *cidB* variant as it was the one found associated with *mod* phenotype variation in Bonneau, Atyame, et al. (2018). We therefore named a variant as *cidB*_IV(a/2) only in situations in which cloning and Sanger sequencing experiments were performed.

2.4 | Screen of *cidA* and *cidB* variants in natural populations from North Italy

2.4.1 | Detection of *cidA*_IV(δ) and *cidB*_IV(2) variants

We investigated the presence of these variants in the 67 isofemale lines, using the same DNA samples used to determine *Wolbachia* phylogenetic group. We used the PCR-RFLP tests described by Bonneau, Atyame, et al. (2018). The *cidA*_IV and *cidB*_IV variants described by Bonneau, Atyame, et al. (2018) have two polymorphic regions: an upstream and a downstream region. For instance, *cidA*_IV(δ/1) and *cidA*_IV(δ/2) have the same upstream sequence (δ) but two different downstream sequences (1/2), whereas *cidB*_IV(a/2) and *cidB*_IV(b/2) have two different upstream sequences (a/b) but the same downstream sequence (2) (Figures S1 and S2). Only the upstream polymorphic region of *cidA*_IV variants was previously found associated with the CI *mod* phenotype (Bonneau, Atyame, et al., 2018). The *cidA*_IV PCR-RFLP test was, therefore, designed to distinguish between the various upstream polymorphic sequences – *cidA*_IV(α), *cidA*_IV(β) and *cidA*_IV(δ) – regardless of the downstream sequences present. The detection with this test of *cidA*_IV(δ) in an isofemale line accounts for the presence of *cidA*_IV(δ/1) and/or *cidA*_IV(δ/2) (Figure S1). Only the downstream polymorphic region of *cidB*_IV variants was previously found associated with the CI phenotype (Bonneau, Atyame, et al., 2018). A PCR-RFLP test was, therefore, designed to distinguish between the *cidB*_IV(1), *cidB*_IV(2), and *cidB*_IV(3) sequences, regardless of the upstream sequences present. Thus, the detection, in isofemale lines, of *cidB*_IV(2), accounts for the presence of the *cidB*_IV(a/2) and/or *cidB*_IV(b/2) variants (Figure S2).

These tests only allowed us to detect variants previously described with the cloning and Sanger sequencing experiment and any variants that were not uncovered with this method would be missed.

In addition to our PCR-RFLP test, a specific presence/absence PCR test was also designed to detect the presence of *cidB*_IV(2) (which accounts for the *cidB*_IV(a/2) and/or *cidB*_IV(b/2) variants), to confirm the PCR-RFLP test results in isofemale lines. A 107 bp fragment was amplified with the primers CidB_QPCR_spe_2_dir3 (5′-GGG-AAT-AGT-GCT-TTT-GAT-AGA-GAG-TA) and CidB_QPCR_spe_rev1 (5′-GTT-AAA-CAT-CTT-AAA-CCC-TCA-TCA-CC), under the following PCR conditions: 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 30 s, with a final elongation for 5 min at 72°C. To check the specificity, a PCR was performed with CidB_QPCR_spe_2_dir3 and CidB_QPCR_spe_2_rev1 primers on clones carrying either *cidB*_IV(1), *cidB*_IV(2) or *cidB*_IV(3)

with the conditions described above and only the clones carrying *cidB*_IV(2) were amplified demonstrating that our PCR was specific of this variant.

2.5 | Real-time quantitative PCR

2.5.1 | Quantification of *Wolbachia* density in male testes

We quantified the density of *Wolbachia* in the testes of males from the Michele26 and Mezzo9 lines, by quantitative PCR with the LightCycler 480 system (Roche). Specific primer pairs and procedures were described by Berticat, Rousset, Raymond, Berthomieu, and Weill (2002). Each DNA template was obtained from pools of three pairs of testes from six-day-old males, with the DNeasy Blood & Tissue Spin-Column kit (Qiagen; bench protocol: animal tissues). Five independent DNA templates were used for each line (Data S3). We estimated the number of *Wolbachia* bacteria per mosquito testis, by amplifying two different genes for each sample: the *C. pipiens*-specific *ace-2* locus (Weill, Berticat, Raymond, & Chevillon, 2000) and the *Wolbachia*-specific single-copy *wsp* locus (Berticat et al., 2002). Standard curves were generated with dilutions of a pBluescriptKS vector containing single copies of the *ace-2* and *wsp* genes. Each DNA template was analyzed in triplicate for the quantification of both *wsp* and *ace-2*. If a triplicate had an error above 0.5 it was removed from the *wsp/ace-2* estimation. As both genes were present as single copies per haploid genome, the ratio of the *wsp* and *ace-2* signals could be used to estimate the relative number of *Wolbachia* genomes per *Culex* genome, thus correcting for mosquito size and DNA quality.

2.5.2 | Expression of the *cidA* and *cidB* genes

For the Mezzo9 and Michele26 lines, we extracted RNA from 10 six-day-old males with Trizol (Life Technologies). The RNA was then treated with DNase with the TURBO DNA-free Kit (Life Technologies), in accordance with the manufacturer's instructions. The absence of residual DNA was confirmed by performing a PCR specific for *cidA* and *cidB* loci with the primers describe in Bonneau, Atyame, et al. (2018). We subjected 2–5 µg of each total RNA sample to reverse transcription with the SuperScript III Reverse Transcriptase Kit and 30 ng of random oligomer primers ([RP]10; Invitrogen, Life Technologies). Four different quantitative PCRs were performed on the resulting cDNA, according to the procedure described by Berticat et al. (2002). The first was specific for the *wsp* locus, as described by Berticat et al. (2002) and was chosen because (a) it is present in a single copy in the *w*PipPel reference genome and (b) was the reference gene used for *Wolbachia* density estimation (Berticat et al., 2002). The second was specific for a 189 bp fragment of the *cidA* gene conserved in all sequenced *w*Pip strains, and was performed with the primers *w*Pip_0282_QPCR_2_Dir (5′-AGG-TCC-TGT-ATT-TGA-TTT-CTG-GA) and *w*Pip_0282_QPCR_2_Rev (5′-TGA-ACG-CGA-GAA-AGA-GCA-AG). The third was specific for a 135 bp fragment of the *cidB* gene conserved in all sequenced *w*Pip strains and was performed with the primers *w*Pip_0283_QPCR_1_Dir (5′-TGA-GTG-TTT-GGA-GAA-TGA-AGG-A) and *w*Pip_0283_QPCR_1_Rev (5′-TTC-CCA-AAA-GCA-AAA-CCA-GTT). The fourth was specific for the 107 bp fragment of *cidB*_IV(2) described above. As *w*Pip strains carry multiple *cidB* variants we checked that the real-time quantitative PCR was specific of the *cidB*_IV(2) variant by performing real-time quantitative PCR (a) on isofemale lines infected with *w*PipIV strains lacking *cidB*_IV(2) but carrying *cidB*_IV(1) and *cidB*_IV(3) (Ichkeul 13 and Harash lines) which represent our negative controls that tested for nonspecific amplification and (b) on isofemale lines infected with *w*PipIV strains carrying *cidB*_IV(2) (Istanbul and Ichkeul 09) which represented our positive controls. Amplifications were only observed in Istanbul and Ichkeul 09 samples as well as Michele26 and Mezzo9 samples, with melting curve of these samples checked for single product amplification. Each DNA template was analyzed in triplicate for *wsp*, *cidA*, *cidB* and *cidB*_IV(2). Standard curves were generated for the *cidA*, *cidB*, *cidB*_IV(2) and *wsp* genes, by diluting the PCR products for these four genes. Expression levels for the *cidA*, *cidB* and *cidB*_IV(2) genes were estimated relative to that of the *wsp* gene (Data S4).

2.6 | Statistical analysis

All analyses were performed with R version 3.4.4 software (R Core Team, 2018). Comparisons between the real-time quantitative variables of the Michele26 and Mezzo9 isofemale lines were performed with the nonparametric Wilcoxon rank-sum test (Bauer, 1972). The test was chosen because we were comparing two sets of independent data not all normally distributed.

3 | RESULTS

3.1 | Only *cidB*_IV(2) variants are associated with the incompatible *mod* phenotype in North Italy

3.1.1 | Both compatible and incompatible *mod* phenotypes are present in North Italy

A total of 67 isofemale lines were established from larvae collected at four sites in the province of Trento in the North-East of Italy (San Michele all'Adige, Roveré Della Luna, Mezzocorona and Zambana: Data S1), because it was already known that *w*Pip strains from the *w*PipIV group occurred in this area (Dumas et al., 2013). The PCR-RFLP test as described in Altinli et al. (2018) confirmed that all isofemale lines were infected with *w*PipIV strains. Crosses between males from these 67 isofemale lines and females from the *w*PipI Tunis laboratory line (reference line used for the screening) led to sort the lines according to the two *mod* phenotypes previously described (Atyame et al., 2015; Bonneau, Atyame, et al., 2018): the males from 62 isofemale lines from North Italy were found incompatible with Tunis females (qualified as incompatible isofemale lines) and five lines exhibited males compatible with Tunis

TABLE 1 *cidA* and *cidB* variant repertoires for seven *w*PipIV strains from North Italy

		*cidA*_IV									
Line name[(a)]	***mod* phenotype**	**α1**	**α2**	**β1**	**β2**	**γ1**	**γ2**	**δ1**	**δ2**	**ε1†**	**ε2†**
Luna 8	Compatible	P	P	A	A	A	A	A	A	P	P
Luna 27	Compatible	P	P	A	A	A	A	A	A	P	P
Luna 1	Incompatible	P	A	A	A	A	A	A	A	P	P
Luna 3	Incompatible	P	A	A	A	A	A	A	A	P	P
Michele 1	Incompatible	P	P	A	A	A	A	A	A	P	P
Mezzo 9	Incompatible	P	P	A	A	A	A	A	A	P	P
Michele 26	**Compatible**	**P**	**P**	**A**	**A**	**A**	**A**	**A**	**A**	**P**	**P**

		*cidB*_IV							
Line name[(b)]	***mod* phenotype**	**a1**	a2	**a3**	**b1**	b2	**b3**	**c1†**	**c3†**
Luna 8	Compatible	A	A	P	A	A	P	A	A
Luna 27	Compatible	A	A	P	A	A	P	A	A
Luna 1	Incompatible	A	**P**	P	A	**P**	A	A	A
Luna 3	Incompatible	A	**P**	P	A	**P**	P	A	A
Michele 1	Incompatible	P	**P**	P	P	**P**	A	A	A
Mezzo 9	Incompatible	A	**P**	P	A	**P**	P	A	A
Michele 26	**Compatible**	**P**	**P**	**P**	**A**	**A**	**A**	**P**	**P**

Note: All *cidA*_IV (a) and *cidB*_IV (b) variants from Bonneau, Atyame, et al. (2018) and the present study (indicated with †) are compiled. Variants were either present in a *w*Pip strain (P in green) or absent (A in black). Variants matching the *mod* phenotype are highlighted in bold letters and have a larger font. Michele 26 is in a darker color because it is the incongruent line.

females (qualified as compatible isofemale lines: Data S1). In summary, 92.5% of the isofemale lines in North Italy exhibited the incompatible *mod* phenotype.

3.1.2 | *cidA* and *cidB* variant repertoires

For investigation of the diversity of *cidA*_IV and *cidB*_IV genes in North Italian *w*PipIV-infected *C. pipiens* populations and identification of the *cidA*_IV and *cidB*_IV variants putatively associated with compatible or incompatible *mod* phenotypes, we first cloned and Sanger sequenced PCR amplification of *cidA* and *cidB* genes from two compatible (Luna 8 and Luna 27) and four incompatible (Luna 1, Luna 3, Michele 1 and Mezzo 9) isofemale lines (Table 1). For each of the six *w*Pip strains studied, we detected several combinations of *cidA* and *cidB* variants further referred as repertoires of *cidA* and *cidB* variants. To name the different *cidA* and *cidB* variants, we used the following nomenclature: the first number corresponds to the group of *w*Pip. Here, as all strains belonged to *w*PipIV group, all variants were named *cidA*_IV or *cidB*_IV. Furthermore in all *cidA*_IV and *cidB*_IV variants, two polymorphic regions were detected: the upstream region which is identified with a letter (Greek for *cidA* variants and Latin for *cidB* variants) and the downstream region which is identified with a number (Figure S1 [Bonneau, Atyame, et al., 2018]). Consequently, the variants *cidA*_IV(α/1) and *cidA*_IV(α/2) share the same sequence for the upstream region but carry a different sequence for the downstream region. The same reasoning was applied to *cidB* variants.

The presence of *cidA*_IV(α) and the absence of *cidA*_IV(δ) was observed in the six isofemale lines analyzed (Tables 1a and 2). A new variant called *cidA*_IV(ε), different from the *cidA*_IV(β) previously described in North Africa due to the replacement of a valine with an isoleucine residue in position 143, was detected in the six isofemale lines studied (Table 1a; Figure S1). Among all the *cidA* variants detected in the North Italy *w*PipIV strains, no specific *cidA* variant or combination of *cidA* variants were found associated with either compatible or incompatible *mod* phenotypes.

The sequencing of *cidB* gene repertoires of the six *w*Pip strains revealed the presence of *cidB*_IV(a/2) and *cidB*_IV(b/2) in the four incompatible isofemale lines and their absence in the two compatible lines (Table 1b). No other *cidB*_IV variants were found putatively associated with difference in *mod* phenotypes (Tables 1b and 2). *CidB*_IV(a/3) and *cidB*_IV(b/3) were detected in both some compatible and incompatible strains (Table 1b).

For extension of *cidA* and *cidB* repertoire analyses at the population scale, we performed a PCR-RFLP screen of *cidA*_IV and *cidB*_IV variants on the 67 isofemale lines originated from the four sites (Data S1). In North Africa natural populations, the upstream region of *cidA* and the downstream region of *cidB* were found associated with *mod* phenotypes variations. Consequently, the PCR-RFLP tests were designed to differentiate between the different upstream sequences of *cidA* (α, β, γ, δ) and the different downstream sequences of *cidB* (1, 2, 3), respectively. Thus, the detection, with this test, of *cidA*_IV(δ) in an isofemale line accounts for the presence of *cidA*_IV(δ/1) and/or *cidA*_IV(δ/2) variants and the detection of *cidB*_IV(2), accounts for

TABLE 2 Distribution of *cidA*_IV(**α**), *cidA*_IV(**δ**) and *cidB*_IV(2) in the 247 isofemale lines infected with *w*PipIV strains from North Africa, Turkey, China and North Italy

***cidA cidB* variants**	***mod* phenotype**	
	« Incompatible» *n* = 79	**« Compatible» *n* = 168**
*cidA*_IV(α)	China: 1/1 North Africa: 15/15 North Italy: 62/62 Turkey: 1/1 **Total: 100%**	- North Africa: 163/163 North Italy: 5/5 - **Total: 100%**
*cidA*_IV(δ)	China: 1/1 North Africa: 15/15 North Italy: 0/62 Turkey: 1/1 **Total: 21.5%**	- North Africa: 16/163 North Italy: 0/5 - **Total: 9.5%**
*cidB*_IV(2)	China: 1/1 North Africa: 15/15 North Italy: 62/62 Turkey: 1/1 **Total: 100%**	- North Africa: 8/163 North Italy: 1/5 - **Total: 5.4%**

Note: The prevalence of each variant of interest is given for compatible and incompatible isofemale lines in each area, together with the total percentage of compatible or incompatible isofemale lines carrying the variant concerned, regardless of geographic origin. -, indicates the absence of isofemale lines with the compatible *mod* phenotype in a given location. The isofemale lines from North Africa, Turkey and China were previously analyzed in Bonneau, Atyame, et al. (2018).

the presence of the *cidB*_IV(a/2) and/or *cidB*_IV(b/2) variants. The *cidA*_IV(α) variant was detected in all the 67 lines. By contrast, the *cidA*_IV(δ) variant which had been previously reported to be associated with the incompatible *mod* phenotype (Bonneau, Atyame, et al., 2018) was not found in any of the 67 lines, including the 62 incompatible isofemale lines. The global distribution of the *cidA* variants of interest in all the natural populations studied in the present study and in those studied in Bonneau, Atyame, et al. (2018) revealed the presence of *cidA*_IV(α) in all the 247 *w*PipIV-infected isofemale lines studied, regardless of *mod* phenotype and geographic origins (Table 2; Figure 1). Overall, *cidA*_IV(δ) was detected in 21.5% (17/79) of the incompatible isofemale lines (Table 2; Figure 1). All together these data show a lack of correlation between *cidA* variants and *mod* phenotype variation.

All the 62 incompatible Italian isofemale lines carried the *cidB*_IV(2) variants, as confirmed by the two independent PCR-based methods (Data S1). However, this variant was also detected in Michele26, one of the five compatible isofemale lines. In compiling the data from China, Turkey, North Africa and North Italy, *cidB*_IV(2) was detected in 100% (79/79) of the incompatible isofemale lines while only in 5.4% (9/168) of the compatible isofemale lines, regardless of geographic origin (Table 2).

3.2 | All lines that carry *cidA*_IV(α) are reciprocally compatible

Because the *cidA*_IV(α) was detected in all studied *w*PipIV strains and all tested mosquito lines infected with *w*PipIV strains were always found mutually compatible (Atyame et al., 2014), we previously hypothesised that this variant might be involved in this reciprocal compatibility and thus in the *resc* function (Bonneau, Atyame, et al., 2018). To investigate further that hypothesis, crosses between all the different mosquito *w*PipIV infected lines present in the laboratory were performed. We were able to perform a total of 20 new crosses between *w*PipIV-infected isofemale lines originated from Turkey, North Africa and Italy which carry the *cidA*_IV(α) variant. These 20 crosses together with the 38 already performed in Atyame et al. (2014) confirmed the compatibility between *w*PipIV strains (Table S2).

3.3 | How can Michele26 be compatible while it carries the *cidB*_IV(a/2) variant associated with incompatibility?

3.3.1 | The Michele26 isofemale line harbours a unique *cidB* variant repertoire

The *cidA* and *cidB* variant repertoires of the *w*PipIV strain infecting the Michele26 isofemale line were cloned, Sanger sequenced, and compared with the repertoires obtained for the four incompatible and two compatible isofemale lines from North Italy (Table 1a). No specific *cidA*_IV variant repertoire was identified for this isofemale line. Indeed, the *cidA*_IV variant repertoire was identical to that of some compatible and incompatible isofemale lines (Table 1a). In contrast the *cidB*_IV variant repertoire of Michele26 was unique by including *cidB*_IV(a/2), but lacking *cidB*_IV(b/2), which was present in all the incompatible lines. Furthermore, two variants unique to Michele26, so called *cidB*_IV(c/1) and *cidB*_IV(c/3), were identified (Table 1b; Figure S2). In summary, the Michele26 isofemale line had a similar *cidA* variant repertoire but a unique *cidB* variant repertoire different from the other compatible and incompatible *w*PipIV strains sampled in the same area.

TABLE 3 Total *cidA* and *cidB* variant expression levels, specific *cidB*_IV(2) expression levels and *Wolbachia* density in the testes of compatible Michele26 and incompatible Mezzo9 males

Line	Ratio			
	***Wolbachia* testes density (*wsp/ace–2*)**	***cidA* expression (*cidA/wsp*)**	***cidB* expression (*cidB/wsp*)**	***cidB*_IV(2) expression (*cidB*_IV(2)/*wsp*)**
Mezzo9	18.10 ± 4.72 (a)	1.67 ± 0.56 (a)	0.72 ± 0.23 (a)	0.06 ± 0.01 (a)
Michele26	10.25 ± 3.42 (b)	1.33 ± 0.31 (a)	0.58 ± 0.18 (a)	0.04 ± 0.02 (b)

Note: For each variable, letters indicate groups of statistical similarity. Mean values are expressed ± standard deviation. *Wolbachia* density and expression level were estimated using real-time quantitative PCR as ratio between target genes (*wsp* for *Wolbachia* density, *cidA*, *cidB* and *cidB*_IV(2) for expression level) and reference genes (ace-2 for *Wolbachia* density and *wsp* for expression level).

3.3.2 | Michele26 males are able to induce CI

The incapacity of males Michele26 to induce CI when crossed with Tunis females might be due to the incapacity of Michele26 males to induce CI at all. Consequently, we checked the capacity of Michele26 males to induce CI by crossing them with females artificially cured from their *Wolbachia* by tetracycline treatment. A total of 21 eggs-rafts were collected and none of them hatched demonstrating that Michele26 males were able to induce CI.

3.3.3 | *cidB*_IV(2) expression is lower in compatible Michele26 males than in incompatible Mezzo9 males

We then investigated possible differences in the expression of the *cidA* and *cidB* genes between compatible Michele26 males and incompatible Mezzo9 males. Indeed, the compatible phenotype of Michele26 could result from an absence of expression of the *cidB*_IV(2) variant associated with incompatibility. The overall levels of *cidA* and *cidB* expression between the two lines were not significantly different (*cidA* Wilcoxon, $W = 62$, $p = .182$ and *cidB* Wilcoxon, $W = 60$, $p = .243$, Table 3; Data S4). The expression of *cidB*_IV(2) was studied by real-time quantitative PCR of a sequence fragment accounting for both the *cidB*_IV(a/2) and *cidB*_IV(b/2) variants (a/2 present in Mezzo9 and Michele26, and b/2 present only in Mezzo9, see Table 1b). Expression of the *cidB*_IV(2) fragment in males from the Michele26 isofemale line was significantly lower than in males from the Mezzo9 isofemale line (0.04 as opposed to 0.06) (Wilcoxon, $W = 12$, $p = .003$, Table 3; Data S4).

3.3.4 | Less *Wolbachia* in the testes of males from the compatible Michele26 isofemale line

As CidB proteins are predicted to be introduced in the sperm during spermatogenesis, we determined *Wolbachia* density in the gonads of both Michele26 and Mezzo9 males. The testes of males from the Michele26 isofemale line contained significantly less *Wolbachia* than those of males from the Mezzo9 isofemale line: 10.25 ± 3.42 and 18.10 ± 4.72 *Wolbachia* per host cell, respectively (Wilcoxon, $W = 1$, $p = .008$, Table 3; Data S3).

4 | DISCUSSION

In the current state of knowledge on CI and its diversity in *C. pipiens*, new investigations on the putative role(s) of $cidA^{wPip}$ were necessary. Indeed, the fact that the coexpression of both $cidA^{wPip}$ and $cidB^{wPip}$ in *D. melanogaster* males was required to induce CI could support the implication of both $cidA^{wPip}$ and $cidB^{wPip}$ in the *mod* function (Figure 1; LePage et al., 2017; Shropshire & Bordenstein, 2019; Shropshire et al., 2018). However, the same requirement for the production of live transgenic *D. melanogaster* and *S. cerevisiae* could suggest that $CidA^{wPip}$ protein may simply serve as an antidote (i.e., *resc*) to $CidB^{wPip}$ protein without being directly involved in the *mod* function (Figure 1; Beckmann et al., 2019b, 2017). As we could not conduct a functional transgenic study of the role of $cidA^{wPip}$ in *C. pipiens*, due to technical restrictions and the amplifications of this gene in the *Wolbachia* harboured by this species, we investigated here the putative link between CI *mod* phenotype diversity and variation in its $cidA^{wPip}$ repertoire by sampling new natural populations in North Italy infected with *w*PipIV *Wolbachia*, a *w*Pip phylogenetic group for which simple *mod* phenotype variations were already screened in North Africa (Atyame et al., 2015; Bonneau, Atyame, et al., 2018). The screening of the 67 isofemale lines obtained from our sampling in Italy revealed the coexistence of the two *mod* phenotypes in these Italian populations. However, unlike North African populations in which 8.4% of the isofemale lines were found incompatible when males from these lines were crossed with *w*PipI-infected females from the Tunis line (Atyame et al., 2015; Bonneau, Atyame, et al., 2018), 92.5% of the isofemale lines from North Italian populations were found incompatible.

In natural populations from North Africa, both *cidA*_IV(δ) and *cidB*_IV(a/2) variants were associated with the incompatible *mod* phenotype (Table 2; Figure 1), a pattern that suggest that both *cidA* and *cidB*, were putatively involved in the *mod* phenotypic variations (Bonneau, Atyame, et al., 2018). In North Italy, *cidB*_IV(2), which accounts for both the *cidB*_IV(a/2) and *cidB*_IV(b/2) variants, was also found associated with the incompatible *mod* phenotype (Tables 1b and 2; Figure 1; Data S1). All our data demonstrate that only *cidB*_IV(2) variants were systematically found in incompatible repertoires, strengthening the link between *cidB* variations and *mod* phenotype variations in *C. pipiens*. By contrast to the natural populations from North Africa, *cidA*_IV(δ) was not detected in any of the *w*PipIV

strains hosting by the 67 isofemale lines in North Italy, demonstrating that this variant was not essential for the incompatible *mod* phenotype (Tables 1a and 2; Figure 1). We were unable to identify any other *cidA* variant or combination of variants associated with either incompatible or compatible *mod* phenotypes (Table 2; Figure 1). Furthermore, we found exactly the same *cidA* repertoire associated with either incompatible or compatible *mod* phenotypes, suggesting that *cidA* plays no role in *mod* phenotype diversity in *C. pipiens* (Tables 1a and 2). We can thus hypothesize that the association between *cidA*_IV(δ)/*cidB*_IV(2) and the incompatible *mod* phenotype in natural populations from North Africa resulted from codiversification of these two variants. As previously suggested, *cidA* and *cidB* may encode a toxin-antidote (TA) system in which CidA acts as the antidote of CidB (Beckmann et al., 2019b, 2019a, 2017; Shropshire et al., 2019). Such TA system may have driven the association of *cidA*_IV(δ) and *cidB*_IV(a/2) variants in North Africa if these variants interact particularly well together independently of any involvement of *cidA* in the *mod* phenotype diversity. However, in the absence of *cidA*_IV(δ) in North Italian isofemale lines, other *cidA*_IV variants may also interact with *cidB*_IV(2).

In all the 247 *w*PipIV infected *C. pipiens* lines yet investigated (Table 2), including the 67 North Italian ones, the *cidA*_IV(α) variant was detected (Figure 1). A total of 58 intra-*w*PipIV group compatible crosses between Turkish, North African and Italian lines, including the 20 crosses from the present study, show the self-compatibility between *w*PipIV-infected isofemale lines previously established (Atyame et al., 2014). As already suggested in Bonneau, Atyame, et al. (2018), this observation supports a role for *cidA* in the *resc* function in *C. pipiens*, as the presence of a ubiquitous *cidA* variant is expected to explain the compatibility of mosquitoes infected with *w*PipIV strains. This conclusion is further supported by the recent findings of Shropshire et al. (2018), revealing the involvement of $cidA^{wMel}$ in the *resc* function in transgenic *D. melanogaster* females (Table 2).

Our results show that $cidB^{wPip}$ variant repertoire is associated with the diversity of *mod* phenotypes observed in *C. pipiens*. Together with functional transgenic data (Beckmann et al., 2017), they clearly demonstrate the involvement of *cidB* in both *mod* function and *mod* phenotype diversity in *C. pipiens* (Figure 1). By contrast, we show here that $cidA^{wPip}$ is not involved in *mod* phenotype diversity, as lines with different *mod* phenotypes had the same $cidA^{wPip}$ repertoire. In *C. pipiens* we have thus far no proof of a two-by-one system as proposed for *D. melanogaster* (Shropshire & Bordenstein, 2019; Shropshire et al., 2018). The *cidA* and *cidB* genes may not, therefore, behave in the same way in the *Wolbachia* bacteria infecting *C. pipiens* and *D. melanogaster*. Further investigations of this putative divergence in the molecular mechanisms of CI induced by $cidA^{wMel}$ and $cidA^{wPip}$ in these two species are required to shed light on this putative difference.

In North Africa, ~5% of isofemale lines were incongruent, i.e., exhibiting a compatible *mod* phenotype while carrying the *cidB*_IV(a/2) variant associated with incompatible *mod* phenotype. This phenomenon could not be further studied in our previous work in North Africa as the lines were not alive anymore when the variants were screened. In Italy, we successfully sampled and maintained one incongruent isofemale line (Michele26). We investigated this discordant isofemale line further to search for possible causes of this apparent dissociation between genotype and phenotype. First of all, the incapacity of Michele26 males to induce CI when crossed with Tunis females could have resulted from the incapacity of males Michele26 to induce CI at all. However, we confirmed that Michele26 was able to induce CI by crossing males with females artificially cured of *Wolbachia* from the SlabTC laboratory line. More importantly, we showed that the *w*PipIV strain harboured by Michele26 mosquitoes presented specific genetic features distinguishing it from both compatible and incompatible lines found in Italy. We found a specific *cidB* variant repertoire, including two variants (*cidB*_IV[c/1] and *cidB*_IV[c/3]) not detected in any other *w*PipIV strains cloned and Sanger sequenced (Table 1b; Figure S2). Such presence of additional *cidB*_IV variants in Michele26 might result in the deregulation of titration or binding with other *Wolbachia* or host targets, preventing incompatibility with *w*PipI females. The *w*PipIV strain harboured by Michele26 also lacked the *cidB*_IV(b/2) variant reported in all incompatible isofemale lines from North Italy. It was tempting to speculate that both *cidB*_IV(a/2) and *cidB*_IV(b/2) were required to induce incompatible phenotype. However, this hypothesis was ruled out by the lack of detection of *cidB*_IV(b/2) in incompatible isofemale lines from North African populations. Michele26 males do express *cidB*_IV(2), so that compatibility cannot be caused by a lack of expression of this variant. However, the levels of *cidB*_IV(2) expression in compatible Michele26 males were lower compared to those in incompatible Mezzo9 males. This is certainly because Michele26 carries only the *cidB*_IV(a/2) variant, whereas Mezzo9 carries both *cidB*_IV(a/2) and *cidB*_IV(b/2) variants. As CidB proteins are probably released into the sperm in the testes during spermatogenesis, we measured the density of *Wolbachia* in the male gonads. We found that the density of *Wolbachia* in Michele26 was significantly lower than in Mezzo9. The lower *cidB* expression level in addition with a lower density of *Wolbachia* in males Michele26 could result in an insufficient amounts of CidB_IV(2) proteins being produced to induce incompatibility with *w*PipI females. A similar dosage-driven hypothesis had already been proposed in a quantitative model where CI phenotype diversity could rely on different *mod* and *resc* genes as well as the amount of these genes products (Nor et al., 2013).

In conclusion, our findings support that $cidB^{wPip}$ variant repertoire is associated with the diversity of *mod* phenotypes observed in *C. pipiens*. Together with functional transgenic data (Beckmann et al., 2017), they clearly suggest the involvement of *cidB* in both *mod* function and *mod* phenotype diversity in *C. pipiens* but further suggest that variation in *w*Pip density and/or *cidB* expression may matter. By contrast, we have no indication that $cidA^{wPip}$ could be involved in *mod* phenotype diversity, as lines with different *mod* phenotypes exhibited exactly the same $cidA^{wPip}$ repertoire. Overall, a toxin-antidote model where *cidB* is a toxin and *cidA* its antidote fits well our current knowledge of *C. pipiens-Wolbachia* interactions.

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AUTHOR CONTRIBUTIONS

M.B., M.S., and M.W. conceptualized and designed the study; M.B., B.C., D.A., M.S., and M.W. field sampled the mosquitoes; M.B., A.L., M. P-S, R.C., S.U. performed the experiments; M.B., M.S., and M.W. analyzed and interpreted the data; M.B., B.C., R.C., M.S., and M.W. wrote the manuscript.

DATA AVAILABILITY STATEMENT

The nucleotide and amino-acid sequences of the *cidA-cidB* variants were deposited in GenBank and accession numbers are provided in Table S3. The authors declare that all other data supporting the findings of this study are available within the article and Supporting Information.

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REFERENCES

Altinli, M., Gunay, F., Alten, B., Weill, M., & Sicard, M. (2018). *Wolbachia* diversity and cytoplasmic incompatibility patterns in *Culex pipiens* populations in Turkey. *Parasites and Vectors*, *11*(1), 1–9. https://doi.org/10.1186/s13071-018-2777-9

Atyame, C. M., Delsuc, F., Pasteur, N., Weill, M., & Duron, O. (2011). Diversification of *Wolbachia* Endosymbiont in the *Culex pipiens* Mosquito. *Molecular Biology and Evolution*, *28*(10), 2761–2772. https://doi.org/10.1093/molbev/msr083

Atyame, C. M., Duron, O., Tortosa, P., Pasteur, N., Fort, P., & Weill, M. (2011). Multiple *Wolbachia* determinants control the evolution of cytoplasmic incompatibilities in *Culex pipiens* mosquito populations. *Molecular Ecology*, *20*(2), 286–298. https://doi.org/10.1111/j.1365-294X.2010.04937.x

Atyame, C. M., Labbé, P., Dumas, E., Milesi, P., Charlat, S., Fort, P., & Weill, M. (2014). *Wolbachia* Divergence and the Evolution of Cytoplasmic Incompatibility in *Culex pipiens*. *PLoS ONE*, *9*(1), e87336. https://doi.org/10.1371/journal.pone.0087336

Atyame, C. M., Labbé, P., Rousset, F., Beji, M., Makoundou, P., Duron, O., … Weill, M. (2015). Stable coexistence of incompatible *Wolbachia* along a narrow contact zone in mosquito field populations. *Molecular Ecology*, *24*(2), 508–521. https://doi.org/10.1111/mec.13035

Bauer, D. F. (1972). Constructing confidence sets using rank statistics. *Journal of the American Statistical Association*, *67*(339), 687. https://doi.org/10.2307/2284469

Beckmann, J. F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Merçot, H., … Charlat, S. (2019a). Caution Does Not Preclude Predictive and Testable Models of Cytoplasmic Incompatibility: A Reply to Shropshire et al. *Trends in Genetics*, *35*(6), 399–400. https://doi.org/10.1016/j.tig.2019.03.002

Beckmann, J. F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Merçot, H., … Charlat, S. (2019b). The Toxin-Antidote Model of Cytoplasmic Incompatibility: Genetics and Evolutionary Implications. *Trends in Genetics*, *35*(3), 175–185. https://doi.org/10.1016/j.tig.2018.12.004

Beckmann, J. F., Ronau, J. A., & Hochstrasser, M. (2017). A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology*, *2*(5), 17007. https://doi.org/10.1038/nmicrobiol.2017.7

Berticat, C., Rousset, F., Raymond, M., Berthomieu, A., & Weill, M. (2002). High *Wolbachia* density in insecticide-resistant mosquitoes. *Proceedings of the Royal Society B: Biological Sciences*, *269*(1498), 1413–1416. https://doi.org/10.1098/rspb.2002.2022

Bleidorn, C., & Gerth, M. (2018). A critical re-evaluation of multilocus sequence typing (MLST) efforts in *Wolbachia*. *FEMS Microbiology Ecology*, *94*(1), 1–11. https://doi.org/10.1093/femsec/fix163

Bonneau, M., Atyame, C., Beji, M., Justy, F., Cohen-Gonsaud, M., Sicard, M., & Weill, M. (2018). *Culex pipiens* crossing type diversity is governed by an amplified and polymorphic operon of *Wolbachia*. *Nature Communications*, *9*(1), 319. https://doi.org/10.1038/s41467-017-02749-w

Bonneau, M., Landmann, F., Labbé, P., Justy, F., Weill, M., & Sicard, M. (2018). The cellular phenotype of cytoplasmic incompatibility in *Culex pipiens* in the light of *cidB* diversity. *PLoS Path*, *14*(10), e1007364. https://doi.org/10.1371/journal.ppat.1007364

Bordenstein, S. R., O'Hara, F. P., & Werren, J. H. (2001). *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature*, *409*(6821), 707–710. https://doi.org/10.1038/35055543

Breeuwer, J. A. J., & Werren, J. H. (1990). Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature*, *346*(6284), 558–560. https://doi.org/10.1038/346558a0

Callaini, G., Riparbelli, M. G., Giordano, R., & Dallai, R. (1996). Mitotic defects associated with cytoplasmic incompatibility in *Drosophila simulans*. *Journal of Invertebrate Pathology*, *67*(1), 55–64. https://doi.org/10.1006/jipa.1996.0009

Dumas, E., Atyame, C. M., Milesi, P., Fonseca, D. M., Shaikevich, E. V., Unal, S., … Duron, O. (2013). Population structure of *Wolbachia* and cytoplasmic introgression in a complex of mosquito species. *BMC Evolutionary Biology*, *13*(1), 181. https://doi.org/10.1186/1471-2148-13-181

Duron, O., Bernard, C., Unal, S., Berthomieu, A., Berticat, C., & Weill, M. (2006). Tracking factors modulating cytoplasmic incompatibilities in the mosquito *Culex pipiens*. *Molecular Ecology*, *15*(10), 3061–3071. https://doi.org/10.1111/j.1365-294X.2006.02996.x

Duron, O., Lagnel, J., Raymond, M., Bourtzis, K., Fort, P., & Weill, M. (2005). Transposable element polymorphism of *Wolbachia* in the mosquito *Culex pipiens*: Evidence of genetic diversity, superinfection and recombination. *Molecular Ecology*, *14*(5), 1561–1573. https://doi.org/10.1111/j.1365-294X.2005.02495.x

Duron, O., & Weill, M. (2006). *Wolbachia* infection influences the development of *Culex pipiens* embryo in incompatible crosses. *Heredity*, *96*(6), 493–500. https://doi.org/10.1038/sj.hdy.6800831

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Ferri, E., Bain, O., Barbuto, M., Martin, C., Lo, N., Uni, S., … Casiraghi, M. (2011). New insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. *PLoS ONE*, *6*(6), e20843. https://doi.org/10.1371/journal.pone.0020843

Flores, H. A., & O'Neill, S. L. (2018). Controlling vector-borne diseases by releasing modified mosquitoes. *Nature Reviews Microbiology*, *16*(8), 508–518. https://doi.org/10.1038/s41579-018-0025-0

Gouy, M., Guindon, S., & Gascuel, O. (2010). SEAVIEW version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*, *27*(2), 221–224. https://doi.org/10.1093/molbev/msp259

Hurst, L. D. (1991). The evolution of cytoplasmic incompatibility or when spite can be successful. *Journal of Theoretical Biology*, *148*(2), 269–277. https://doi.org/10.1016/S0022-5193(05)80344-3

Laven, H. (1967). Speciation and evolution in *Culex pipiens*. In J. Wright, & R. Pal (Eds.), *Genetics of insect vectors of disease* (pp. 251–275). Amsterdam, The Netherlands: Elsevier.

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., … Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247. https://doi.org/10.1038/nature21391

Lindsey, A. R. I., Rice, D. W., Bordenstein, S. R., Brooks, A. W., Bordenstein, S. R., & Newton, I. L. G. (2018). Evolutionary genetics of cytoplasmic incompatibility genes *cifA* and *cifB* in prophage WO of *Wolbachia*. *Genome Biology and Evolution*, *10*(2), 434–451. https://doi.org/10.1093/gbe/evy012

Nor, I., Engelstädter, J., Duron, O., Reuter, M., Sagot, M.-F., & Charlat, S. (2013). On the genetic architecture of cytoplasmic incompatibility: Inference from phenotypic data. *The American Naturalist*, *182*(1), E15–E24. https://doi.org/10.1086/670612

O'Neill, S. L., & Karr, T. L. (1990). Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature*, *348*(6297), 178–180. https://doi.org/10.1038/348178a0

Poinsot, D., Charlat, S., & Merçot, H. (2003). On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: Confronting the models with the facts. *BioEssays*, *25*(3), 259–265. https://doi.org/10.1002/bies.10234

R Core Team (2018). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rasgon, J. L., & Scott, T. W. (2003). *Wolbachia* and cytoplasmic incompatibility in the California *Culex pipiens* mosquito species complex: Parameter estimates and infection dynamics in natural populations. *Genetics*, *165*(4), 2029–2038.

Rogers, S. O., & Bendich, A. J. (1989). Extraction of DNA from plant tissues. In S. B. Gelvin, R. A. Schilperoort, & D. P. S. Verma (Eds), *Plant molecular biology manual* (pp. 73–83). Dordrecht, The Netherlands: Springer. https://doi.org/10.1007/978-94-009-0951-9_6

Rousset, F., & Raymond, M. (1991). Cytoplasmic incompatibility in insects: Why sterilize females? *Trends in Ecology & Evolution*, *6*(2), 54–57. https://doi.org/10.1016/0169-5347(91)90123-F

Shropshire, J. D., & Bordenstein, S. R. (2019). Two-By-One model of cytoplasmic incompatibility: Synthetic recapitulation by transgenic expression of cifA and cifB in *Drosophila*. *PLOS Genetics*, *15*(6), e1008221. https://doi.org/10.1371/journal.pgen.1008221

Shropshire, J. D., Leigh, B., Bordenstein, S. R., Duplouy, A., Riegler, M., Brownlie, J. C., & Bordenstein, S. R. (2019). Models and nomenclature for cytoplasmic incompatibility: Caution over premature conclusions – a response to beckmann et al. *Trends in Genetics*, *35*(6), 397–399. https://doi.org/10.1016/J.TIG.2019.03.004

Shropshire, J. D., On, J., Layton, E. M., Zhou, H., & Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, *115*(19), 4987–4991. https://doi.org/10.1073/pnas.1800650115

Sicard, M., Bonneau, M., & Weill, M. (2019). *Wolbachia* prevalence, diversity, and ability to induce cytoplasmic incompatibility in mosquitoes. *Current Opinion in Insect Science*, *34*, 12–20. https://doi.org/10.1016/j.cois.2019.02.005

Taylor, M. J., Bandi, C., & Hoerauf, A. (2005). *Wolbachia*. Bacterial endosymbionts of filarial nematodes. *Advances in Parasitology*, *60*, 245–284. https://doi.org/10.1016/S0065-308X(05)60004-8

Turelli, M., & Hoffmann, A. A. (1991). Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature*, *353*(6343), 440–442. https://doi.org/10.1038/353440a0

Weill, M., Berticat, C., Raymond, M., & Chevillon, C. (2000). Quantitative polymerase chain reaction to estimate the number of amplified esterase genes in insecticide-resistant mosquitoes. *Analytical Biochemistry*, *285*(2), 267–270. https://doi.org/10.1006/abio.2000.4781

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150249. https://doi.org/10.1098/rspb.2015.0249

Werren, J. H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology*, *42*(1), 587–609. https://doi.org/10.1146/annurev.ento.42.1.587

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751. https://doi.org/10.1038/nrmicro1969

Zug, R., & Hammerstein, P. (2012). Still a host of hosts for *Wolbachia*: Analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE*, *7*(6), e38544. https://doi.org/10.1371/journal.pone.0038544

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