Wolbachia modulates prevalence and viral load of Culex pipiens densoviruses in natural populations

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Wolbachia modulates prevalence and viral load of Culex pipiens densoviruses in natural populations

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Abstract

The inadequacy of standard mosquito control strategies calls for ecologically safe novel approaches, for example the use of biological agents such as the endosymbiotic α-proteobacteria *Wolbachia* or insect-specific viruses (ISVs). Understanding the ecological interactions between these "biocontrol endosymbionts" is thus a fundamental step. *Wolbachia* are transmitted vertically from mother to offspring and modify their hosts' phenotypes, including reproduction (e.g., cytoplasmic incompatibility) and survival (e.g., viral interference). In nature, *Culex pipiens* (*sensu lato*) mosquitoes are always found infected with genetically diverse *Wolbachia* called *w*Pip that belong to five phylogenetic groups. In recent years, ISVs have also been discovered in these mosquito species, although their interactions with *Wolbachia* in nature are unknown. Here, we studied the interactions between a widely prevalent ISV, the Culex pipiens densovirus (CpDV, *Densovirinae*), and *Wolbachia* in northern Tunisian *C. pipiens* populations. We showed an influence of different *Wolbachia* groups on CpDV prevalence and a general positive correlation between *Wolbachia* and CpDV loads. By investigating the putative relationship between CpDV diversification and *w*Pip groups in the different sites, we detected a signal linked to *w*Pip groups in CpDV phylogeny in sites where all larvae were infected by the same *w*Pip group. However, no such signal was detected where the *w*Pip groups coexisted, suggesting CpDV horizontal transfer between hosts. Overall, our results provide good evidence for an ecological influence of *Wolbachia* on an ISV, CpDV, in natural populations and highlight the importance of integrating *Wolbachia* in our understanding of ISV ecology in nature.

KEYWORDS

Culex pipiens, densovirus, insect-specific virus, microbiota interactions, *Wolbachia*

Mylene Weill and Mathieu Sicard are contributed to this work equally.

1 | INTRODUCTION

In the last decade, many insect-specific viruses (ISVs) have been discovered in mosquitoes, in addition to medically important arthropod-borne viruses (arboviruses) (Agboli, Leggewie, Altinli, & Schnettler, 2019). In contrast to arboviruses, which are maintained in nature mainly via horizontal transmission cycles between vertebrate and invertebrate hosts, ISVs are restricted to insect hosts and do not replicate in vertebrates (Agboli et al., 2019). While ISV–insect host interactions have not yet been studied in detail, ISVs are known to be widespread in natural mosquito populations (Altinli, Lequime, et al., 2019; Baidaliuk et al., 2020; Farfan-Ale et al., 2009; Goenaga et al., 2014; Parry & Asgari, 2018). They are found in every life stage of their hosts (Ajamma et al., 2018; Bolling, Olea-Popelka, Eisen, Moore, & Blair, 2012; Haddow et al., 2013; Kawakami et al., 2016; Saiyasombat, Bolling, Brault, Bartholomay, & Blitvich, 2011; Sang et al., 2003) and can exhibit high rates of vertical transmission (Altinli, Soms, et al., 2019; Barreau, Jousset, & Bergoin, 1997; Bolling et al., 2012). Thus, ISVs can putatively have huge impact on mosquitos' life history traits at different stages of their development, on their fitness and on their population dynamics in nature.

The Culex pipiens densovirus (CpDV) is an insect-specific densovirus (*Parvoviridae*) that has been isolated following high mortality in laboratory colonies of *C. pipiens* (Jousset, Baquerizo, & Bergoin, 2000). However, high mortality has not been shown through experimental infections, and persistently infected *C. pipiens* colonies do not exhibit apparently high mortality, in contrast to what has been observed for other mosquito densoviruses (Altinli, Soms, et al., 2019; Jousset et al., 2000; Li et al., 2019). Recently, CpDV has been found to be widespread in *C. pipiens* (*sensu lato* [*s.l.*]) natural populations, with about 50% prevalence in almost 3,000 individual mosquitoes tested worldwide (Altinli, Lequime, et al., 2019). This wide distribution of CpDV in natural populations of *C. pipiens* (*s.l.*) suggests an interaction with their hosts and possibly with their microbiota, in particular with their major endosymbiont *Wolbachia*. Indeed, all *C. pipiens* (*s.l.*) mosquitoes are naturally infected with *Wolbachia* called *w*Pip (Altinli, Gunay, Alten, Weill, & Sicard, 2018; Dumas et al., 2013; Rasgon & Scott, 2003). These endosymbiotic bacteria are mainly vertically transmitted from mother to offspring, and manipulate their host's reproduction by causing cytoplasmic incompatibility (CI). In *C. pipiens* (*s.l.*), CI occurs as a sperm–egg incompatibility resulting in embryonic death in crosses between individuals infected with incompatible *w*Pip strains (Beckmann et al., 2019; Bonneau et al., 2018). *w*Pip strains are closely related worldwide, all belonging to the same phylogenetic clade, and can be separated into five genetically distinct phylogenetic groups, *w*Pip-I to V (Atyame, Delsuc, Delsuc, Pasteur, Weill, & Duron, 2011; Atyame et al., 2014). CpDV was observed, in the same cells, along with *Wolbachia* in mosquito ovaries in laboratory *C. pipiens* (*s.l.*) mosquitoes (Altinli, Soms, et al., 2019). CpDV was vertically transmitted to an average of 20% of the offspring per infected female along with *w*Pip (Altinli, Soms, et al., 2019). Furthermore, in *w*Pip-free mosquitoes (artificially treated with tetracycline in the laboratory), CpDV load and vertical transmission decreased significantly compared to *w*Pip-infected females, suggesting a facilitation of CpDV infection by *w*Pip (Altinli, Soms, et al., 2019). *w*Pip not only coexists with CpDV in the oocytes and enhances the load and vertical transmission of this virus, but its genetic diversity seemed also to affect CpDV density in the ovaries, suggesting their interactions (Altinli, Soms, et al., 2019). In mosquitoes naturally infected with *w*Pip belonging to two distinct *w*Pip groups (*w*Pip-I and *w*Pip-IV), CpDV load was higher in the ovaries of *C. pipiens* lines carrying *w*Pip-IV compared to those carrying *w*Pip-I. This was also true when *w*Pip-IV and *w*Pip-I were introduced in the same mosquito genetic background through backcrosses (Altinli, Soms, et al., 2019).

Wolbachia–virus interactions have attracted considerable attention in the context of *Wolbachia* arbovirus interference. Hence, studies have mainly focused on RNA viruses in medically important mosquitoes such as *Aedes aegypti*, in which the natural presence of *Wolbachia* is controversial (reviewed by Ross et al., 2020; Sicard, Bonneau, & Weill, 2019), and on cell lines that were stably transinfected with *Wolbachia*, especially *w*Mel and *w*MelPop strains from *Drosophila melanogaster*. Interactions between arboviruses and transinfected mosquitoes were shown to be mostly antagonistic as the *Wolbachia* was found to decrease the replication, dissemination or transmission of many arboviruses (e.g., Dengue: Bian, Xu, Lu, Xie, & Xi, 2010; Frentiu et al., 2014; P. Lu, Bian, Pan, & Xi, 2012; Walker et al., 2011, or Zika: Dutra et al., 2016), making *Wolbachia* a promising arbovirus control tool. In contrast, in natural associations, *Wolbachia* did not seem to have an effect on arboviruses (reviewed by Johnson, 2015) and in one case of transiently transinfected mosquitoes it even enhanced arboviral replication (Dodson et al., 2014). In *C. pipiens* (*s.l.*) mosquitoes naturally infected with *w*Pip, *Wolbachia* has been shown to reduce West Nile virus load but only in *C. quinquefasciatus* laboratory lines with high somatic *w*Pip load, and not in recently settled *C. pipiens* lines. Moreover, *w*Pip did not have any effect on RNA arboviruses in transinfected *Aedes* mosquitoes (Fraser et al., 2020).

Contrary to RNA viruses, *Wolbachia*–DNA virus interactions have been much less studied and appear to be less antagonistic. For example, mortality of *Drosophila* when challenged with Invertebrate iridescent virus-6 (*Iridoviridae*) was higher for flies infected with *w*Mel compared to tetracycline-treated flies (Teixeira, Ferreira, & Ashburner, 2008). *w*Mel-infected flies also tended to exhibit higher virus loads (Teixeira et al., 2008). In the African armyworm moth larvae (*Spodoptera exempta*), natural *Wolbachia* infection increased larval susceptibility to Spodoptera exempta nucleopolyhedrovirus (*Baculoviridae*) and the resultant larval mortality (Graham, Grzywacz, Mushobozi, & Wilson, 2012). Recently, a similar facilitation between *Wolbachia* and a mosquito densovirus has also been recorded (i.e., Aedes albopictus densovirus) in *Aedes* mosquito-derived cell lines transinfected with *w*MelPop (from *D. melanogaster*) or *w*AlbB (from *Aedes albopictus*), compared to the tetracycline-treated or nontransinfected cells (Parry, Bishop, De Hayr, & Asgari, 2019).

Although both ISVs and *Wolbachia* are considered as promising vector population and arbovirus control tools (Johnson & Rasgon, 2018; Patterson, Villinger, Muthoni, Dobel-Ober, & Hughes, 2020; Sicard et al., 2019), *Wolbachia*–ISV interactions in nature are mostly unknown.

To our knowledge, only one previous ecological study on the topic suggested a higher insect-specific flavivirus abundance and load in *Aedes aegypti* infected with *w*Mel locally released to fight against Dengue transmission (Amuzu et al., 2018). However, *Wolbachia*–ISV interactions in a completely natural system, where *Wolbachia* and host have co-evolved, have never been studied.

The coexistence of *Wolbachia* and CpDV in almost half of the *C. pipiens* (*s.l.*) tested and even in the same cells (Altinli, Lequime, et al., 2019; Altinli, Soms, et al., 2019) suggests that these endosymbionts could interact and influence their respective prevalence, load and diversity in natural populations. *C. pipiens* (*s.l.*) thus constitutes an interesting model to study the ecology and evolution of natural multipartite interactions between DNA viruses/*Wolbachia* and mosquitoes. To study the influence of *w*Pip strain genetic variations on *C. pipiens*–CpDV interactions, we have focused on an area in North Africa where a stable coexistence of different *w*Pip strains, belonging to the *w*Pip-I and *w*Pip-IV groups, has been shown over at least 7 years (Atyame et al., 2015). In this zone of coexistence, the presence of phylogenetically close CpDV variants from the same clade, CpDV-I, had recently been revealed with more than 50% prevalence (Altinli, Lequime, et al., 2019). In this specific location, we were able to test the hypothesis that genetic diversity of coexisting *w*Pip, variations in their loads and spatial distribution might interfere with CpDV dynamics, distribution and diversification. To this end, we have studied CpDV prevalence and analysed them together with results for the distribution of *w*Pip groups for the same individuals (Atyame et al., 2015). We additionally quantified both CpDV and *w*Pip loads to investigate their potential influence. Lastly, given the experimentally documented occurrence of vertical cotransmission events (Altinli, Soms, et al., 2019), we examined the CpDV genetic diversification in relation to *w*Pip group diversity in the area, by sequencing a polymorphic region of the CpDV genome.

2 | METHODS

2.1 | Biological material

All the samples were collected in June 2010 and 2011 from natural populations of *Culex pipiens* (*s.l.*) in Tunisia during larval stage (Table S1) using a net (Atyame et al., 2015). DNA was extracted from larval samples as described in Atyame et al. (2015) and stored at −80°C after its use for identification of *w*Pip groups as previously described (Altinli et al., 2018; Atyame et al., 2015).

2.2 | Prevalence of CpDV inferred by diagnostic PCR test on NS2 region

Samples that had been identified for their *w*Pip group (Atyame et al., 2015) were used to infer CpDV prevalence in the area. For this, we used a CpDV-specific polymerase chain reaction (PCR)-based diagnostic test as previously described (Altinli, Lequime, et al., 2019). The amplified 238-bp fragment within the conserved NS2 coding region was visualized on an electrophoresis agarose gel to check for the presence or absence of the specific amplicons.

2.3 | qPCR assay to assess CpDV and *Wolbachia* loads

To test whether the *Wolbachia* load differed between mosquitoes harbouring different *Wolbachia* groups (*w*Pip-I and *w*Pip-IV) and whether this affected the CpDV load, we quantified both symbionts in a given individual using real-time quantitative PCR (qPCR), as previously described (Altinli, Soms, et al., 2019). Briefly, we used CpDV *ns2* (CpDVquantiF, CpDVquantiR), *Wolbachia wsp* (wolpipdir, wolpiprev: Berticat, Rousset, Raymond, Berthomieu, & Weill, 2002) and *Culex* Ace-2 locus-specific primers (acequantidir, acequantirev: Weill, Berticat, Raymond, & Chevillon, 2000) to calculate the normalized *Wolbachia* and CpDV amount per Ace-2 (host) copy.

We quantified 137 samples in total, which were chosen to balance the sampling years and *Wolbachia* types from the sampling sites where *w*Pip-I and *w*Pip-IV coexisted (Table S2). Samples that showed a Ct (cycle threshold) value >33 for any of the qPCRs were excluded because of the high uncertainty of the quantification at such low titres. After exclusion of outliers (see Statistical analyses), a total of 111 samples were analysed: *w*Pip-I ($n = 51$) and *w*Pip-IV ($n = 60$), collected in 2010 ($n = 65$) and in 2011 ($n = 46$).

2.4 | Diversity of CpDV by sequencing the variable NS3 region

To study CpDV diversification in geographically close populations, we sequenced the partial NS3 protein coding region, as this part of the genome is expected to be less conserved compared to NS1 and VP that were previously sequenced to study CpDV worldwide diversification (Altinli, Lequime, et al., 2019). For this, we designed primers CpDV_355_F (5′-GGTCTGAATTGGCTGATG C-3′) and CpDV_1902_R (5′-GTGTCCCAGCAACTTCTCGA-3′) that amplified a 1,566-bp-long amplicon. The resulting PCR products were used as a template (i.e., seminested PCR) to amplify a 1,072-bp-long fragment including the NS3 coding region using the same forward primer (CpDV_355_F) and CpDV_1387_R (5′-CGAAATTAGCATTTGAC TCTCC-3′). We then sequenced these amplicons using only the CpDV_1387_R primer, resulting in 800-bp-long sequences (Figure S1, about 14% of the complete CpDV genome).

For both PCRs, the following protocol was used: 94°C for 5 min, 35 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 90 s, and a final elongation at 72°C for 5 min. PCR products were purified using AMPure (Agencourt) with a Biomek 4000, and Sanger sequencing reactions were conducted (96°C for 5 min, 30 cycles of 96°C for 15 s, 50°C for 10 s and 60°C for 4 min). Sequencing products were purified using CleanSEQ (Agencourt) with a Biomek 4000 and analysed with capillary electrophoresis.

In total, 92 samples were sequenced and the sequences are publicly available on GenBank (accession nos.: MK722526–MK722617, Table S3).

2.5 | Phylogenetic analyses of CpDV diversification

Sequences were aligned using MAFFT version 7.273 (Katoh & Standley, 2013) and lack of recombination was verified using RDP4 software (Martin, Murrell, Golden, Khoosal, & Muhire, 2015). Two samples (Elmanar_10_2011 MK722527 and Fontaine_80_2011 MK722526) were excluded from the analyses because of the presence of a large indel. JMODELTEST was used for model selection (Posada, 2008). Phylogenetic analyses were conducted in BEAST 1.10.5 (Suchard et al., 2018) for three data sets: (a) one containing all CpDV sequence data generated in this study, (b) one with CpDV sequence from sampling sites where both *w*Pip-I and *w*Pip-IV coexisted (coexistence zone; Dougga, El Manar, Fontaine, Font Mjez, Nofrancoui, Pompe, Rasrajel, Utique, Utique_Pont[UTP], Zerga), and (c) one where only one *w*Pip group was found (Tabarka, Ouedmelah, El battan, Ariana).

All sequences were considered isochronous and a strict molecular clock with a rate fixed to 1 was set. The evolutionary process was reconstructed under a constant population size model and an HKY85 + I nucleotide substitution model (Altinli, Lequime, et al., 2019; Hasegawa, Kishino, & Yano, 1985; Yang, 1994). *w*Pip group was considered as a discrete trait analysed using the asymmetric diffusion model (Edwards et al., 2011; Lemey, Rambaut, Drummond, & Suchard, 2009). Each continuous-time Markov chain analysis was set in three independent replicates to ensure proper convergence and mixing (effective sample size >200), as verified using TRACER version 1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018), and combined after removal of the burn-in (10% of the samples). The presence of a phylogenetic signal linked to the *w*Pip group was assessed by estimating the number of changes between the two states (Markov jumps) in the posterior distribution of trees. This distribution was then compared to the estimated number of Markov jumps in a set of 10 independent runs were the states (i.e., *w*Pip group) were randomized ("null" distribution). A clear overlap between the 95% highest posterior density (95% HPD) would sign the absence of a phylogenetic signal (Lemey et al., 2009).

2.6 | Statistical analyses

All data analyses were performed in R version 3.3.1.

To analyse the effects of *sampling site*, *sampling year* (2010–2011) and *Wolbachia groups* (*w*Pip-I, *w*Pip-IV) on CpDV prevalence, we first fitted a generalized linear model from the binomial family. Likelihood ratio tests of the full model against the model without a given effect were used to obtain deviance and *p*-values. A subset of the data was then created to include only those sites where both *Wolbachia* strains coexisted and where at least one sample was CpDV positive. Here in each sampling site tested, the probability of a given sample being infected by CpDV was assumed to be independent from the sampling sites where samples were collected. Hence, a binomial generalized linear model was used to test the effect of *sampling year* (2010 or 2011) and *Wolbachia groups* (*w*Pip-I, *w*Pip-IV) on CpDV prevalence.

To investigate the effects of *Wolbachia groups*, *Wolbachia load* and *sampling site* on CpDV load, a generalized linear model was fitted. Most significant outliers ($n = 4$) were removed using a multivariate approach (outlierTest, CAR package) (Fox & Weisberg, 2011). We additionally analysed a second data set where we excluded all the outliers stepwise ($n = 9$) to verify that they did not have a major effect on our results (Figure S2). The effect of a given variable was calculated as described above. The correlation between *Wolbachia* and CpDV loads was further calculated by linear regression. The difference between the slopes of the two *w*Pip groups was calculated by fitting the same model with *w*Pip × *Wolbachia* load interaction.

The prevalence of CpDV-Ia and CpDV-Ib in *w*Pip-I- and *w*Pip-IV-carrying mosquitoes was compared using a χ^2 test, for the sampling sites where *w*Pip-I and *w*Pip-IV did not coexist (Tabarka, Ouedmelah, El battan, Ariana) versus where they both coexisted (coexistence zone; Dougga, El Manar, Fontaine, Font Mjez, Nofrancoui, Pompe, Rasrajel, Utique, Utique_Pont, Zerga) during 2010 and 2011. Confidence intervals (95% CI) were calculated as twice the standard error of the prevalence.

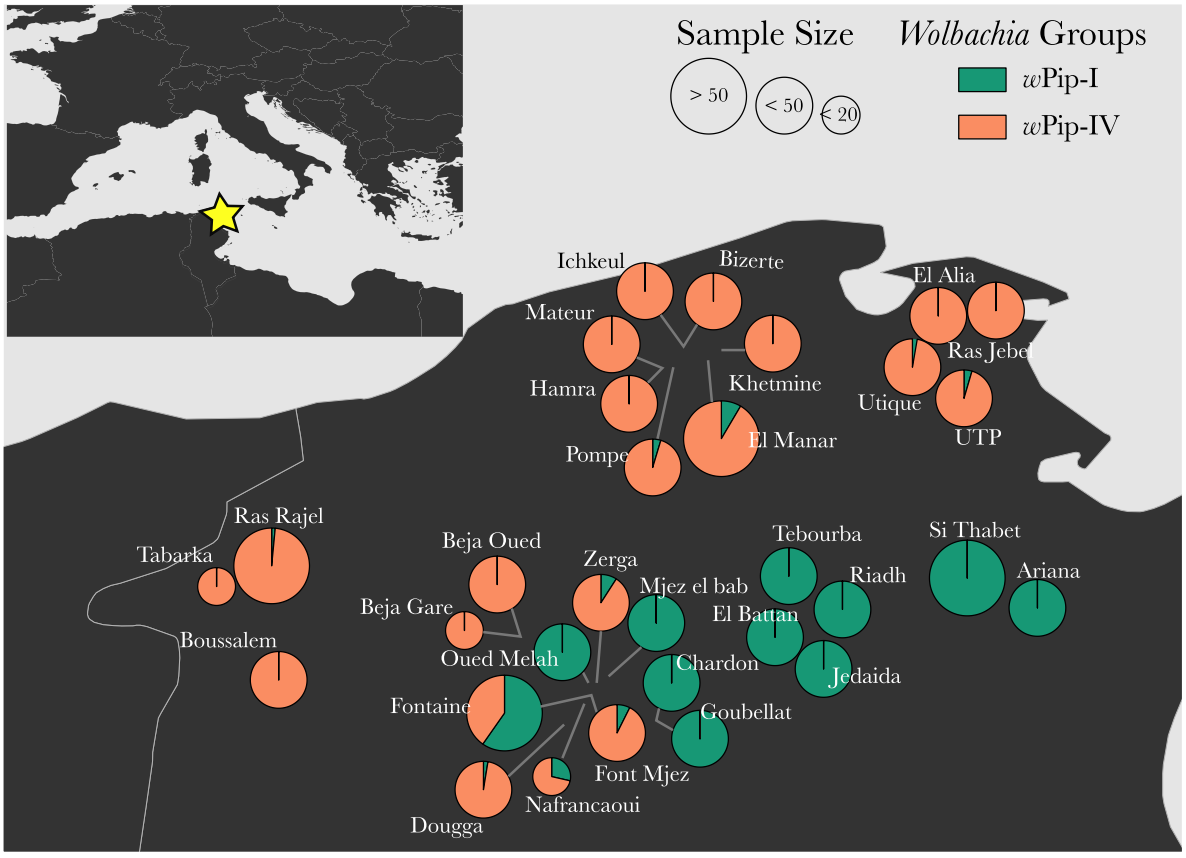
3 | RESULTS

3.1 | CpDV prevalence is dependent on the sampling site

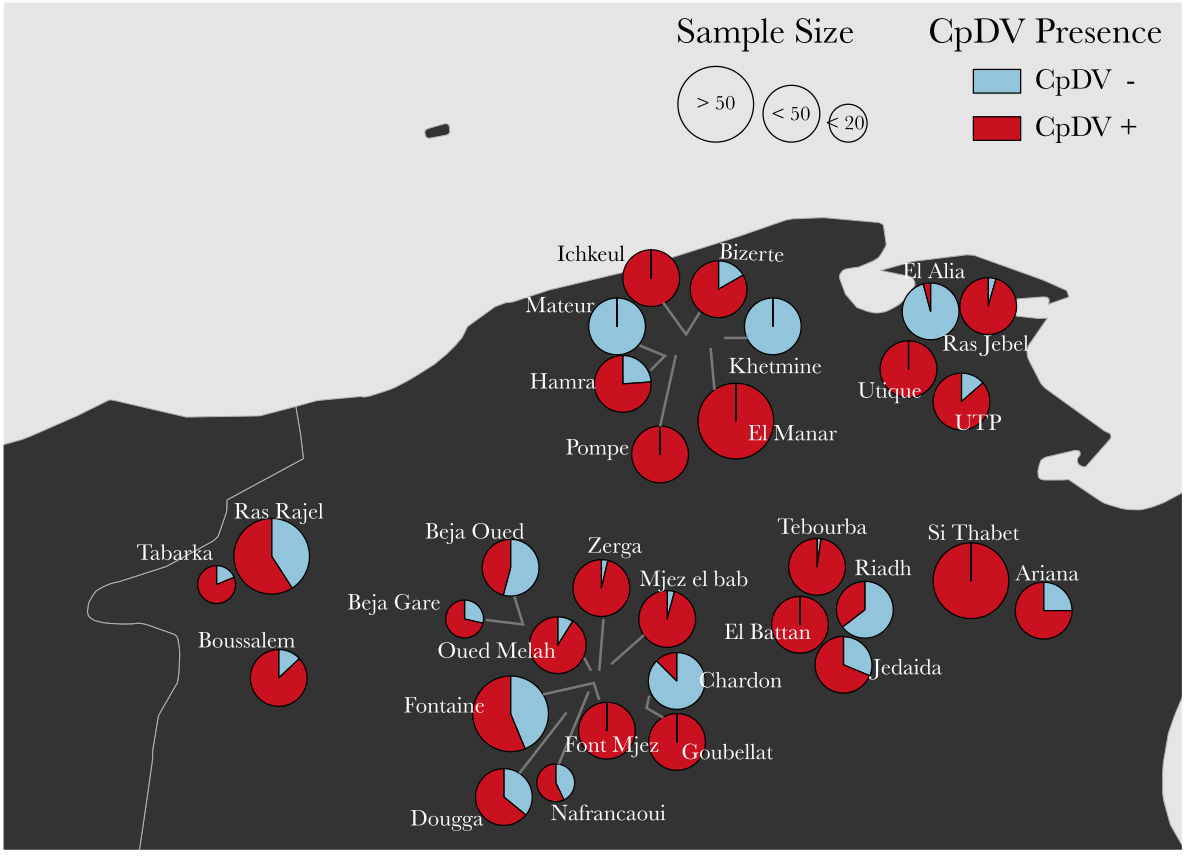
We analysed 1,230 individuals that were previously collected and extracted in 2010 and 2011 from northern Tunisia and qualified for their *w*Pip group infection by Atyame et al. (2015) (Figure 1a). CpDV diagnostic PCR was positive for 74% of these individuals collected in and around the *w*Pip-I/*w*Pip-IV coexistence zone. CpDV prevalence differed significantly between sampling sites (glm, $df = 30$, dev = 592.97, $p < .001$; Figure 2b) and years (glm, $df = 1$, dev = 8.234, $p = .004$). This global-scale analysis including all sampling sites (i.e., not focusing on sites in the coexistence zone where both *w*Pip groups are present in similar ecological conditions),showed no significant

FIGURE 1 *Wolbachia* groups and CpDV prevalence in northern Tunisia. A total of 1,230 individuals were collected from 31 sampling sites in 2010 and 2011. (a) Distribution of *w*Pip groups in the area: *w*Pip-I in southeastern sampling sites and *w*Pip-IV northwestern sampling sites with a zone in between where these two groups coexist. (b) CpDV prevalence and distribution in the area. We detected CpDV in a total of 913 individuals. In the whole area, there was no significant link between CpDV prevalence and *w*Pip group distribution (glm, $df = 1$, dev = 0.108, $p = .741$)

(a)


Sample Size
> 50
< 50
< 20
Wolbachia Groups
wPip-I
wPip-IV
Ichkeul
Bizerte
Mateur
Hamra
Khetmine
Pompe
El Manar
El Alia
Ras Jebel
Utique
UTP
Ras Rajel
Tabarka
Boussalem
Beja Oued
Zerga
Beja Gare
Mjez el bab
Oued Melah
Chardon
Fontaine
Goubellat
Font Mjez
Nafrancaoui
Dougga
Tebourba
Riadh
El Battan
Jedaida
Si Thabet
Ariana

(b)


Sample Size
> 50
< 50
< 20
CpDV Presence
CpDV -
CpDV +
Ichkeul
Bizerte
Mateur
Hamra
Khetmine
Pompe
El Manar
El Alia
Ras Jebel
Utique
UTP
Ras Rajel
Tabarka
Boussalem
Beja Oued
Zerga
Beja Gare
Mjez el bab
Oued Melah
Chardon
Fontaine
Goubellat
Font Mjez
Nafrancaoui
Dougga
Tebourba
Riadh
El Battan
Jedaida
Si Thabet
Ariana

effect of *Wolbachia* group (i.e., *w*Pip-I and *w*Pip-IV) on CpDV prevalence (glm, $df = 1$, dev = 0.108, $p = .741$, Figure 1).

3.2 | In sites where the two *Wolbachia* strains coexist, mosquitoes infected with *w*Pip-IV are more likely to be infected with CpDV

The absence of CpDV in some rare sampling sites (Figure 1) could reflect that these populations had never encountered CpDV or that the environmental conditions were not favourable for the infection. As this could bias global-scale statistical analyses, we performed the analysis on a subset of the data only considering the sites where at least one individual was CpDV-positive (per year/per site) and both *Wolbachia* groups coexisted. In this way, we were able to assess the possible effect of different *w*Pip groups on CpDV prevalence under the same ecological conditions (i.e., the similar probability of getting infected with CpDV for *C. pipiens* larvae harbouring *w*Pip-I or *w*Pip-IV).

The main factor that affected the CpDV prevalence in sampling sites from the coexistence zone was the *Wolbachia* groups (i.e., *w*Pip-I vs. *w*Pip-IV) (glm, $df = 1$, dev = 16.492, $p < .001$, Figure 2). Indeed, mosquitoes harbouring the *w*Pip-IV were 34% more likely to be CpDV-positive (risk ratio [RR] = 1.338; odds ratio [OR] = 2.791, 95% CI: 1.709–4.542). This difference was constant between the two years of sampling that did not significantly differ from each other (glm, $df = 1$, dev = 2.739, $p = .097$, Figure 2).

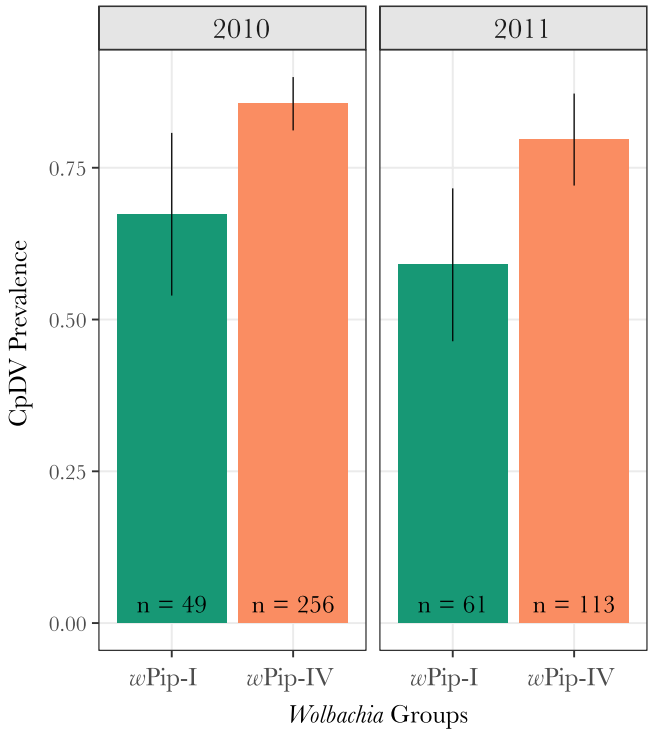


FIGURE 2 *Wolbachia* groups and CpDV prevalence in samples from the coexistence zone. The data set was reduced only to sites where at least one individual was CpDV-positive (per year/per site) and both *Wolbachia* groups coexisted. CpDV distribution was significantly affected by the *Wolbachia* group (glm, $df = 1$, dev = 16.492, $p < .001$). Mosquitoes harbouring *w*Pip-IV were 34% (RR = 1.338; OR = 2.791, 95% CI: 1.709–4.542) more likely to be CpDV-positive. Sampling year did not have a significant effect in the model (glm, $df = 1$, dev = 2.739, $p = .097$). *n* = total sample size per *w*Pip group/year. Lines represent 95% CI for CpDV prevalence

3.3 | CpDV and *Wolbachia* loads are positively correlated

We quantified CpDV and *Wolbachia* loads in some larvae collected from the coexistence zone. Individuals were chosen to balance the sampling years and *w*Pip groups. In one larva collected from El Manar, 10^5 CpDV per host cell were detected (Table S2) and this possibly represented a peak of an acute infection. This sample was removed from further analyses. The rest of the samples (Figure 3a) had lower CpDV loads possibly representing persistent infections. Overall, CpDV load was highly variable between *C. pipiens* larvae, ranging from 10^{-7} to 85 CpDV per host cell. CpDV load differed significantly between the sampling sites (glm, $df = 7$, dev = 182.66, $p < .001$; Figure 3a).

Compared to CpDV, *Wolbachia* load was more constant between larvae, ranging from 0.01 to 3.168 *Wolbachia* per host cell, and only marginally differed between sampling sites ($df = 5$, dev = 2.476, $p = .07$; Figure 3b).

In the general model, there was no significant effect of the different *w*Pip groups on the CpDV load (glm, $df = 1$, dev = 0.268, $p = .414$). Similarly, larvae harbouring different *Wolbachia* groups did not differ significantly in terms of their *Wolbachia* loads (glm, $df = 1$, dev = 0.007, $p = .512$). However, CpDV load differed with respect to *Wolbachia* load (glm, $df = 1$, dev = 4.072, $p = .001$, Figure 3c). We obtained similar results when we excluded all possible outliers from the data (Figure S2): CpDV load was always mainly affected by sampling site (glm, $df = 6$, dev = 147.32, $p < .001$) and *Wolbachia* load (glm, $df = 1$, dev = 1.523, $p = .004$). To visualize the correlation between CpDV and *Wolbachia* loads, we also fitted simple linear regression models. The linear model alone did not explain a large part of the variance in the data, as indicated by the low r^2 values. However, overall CpDV load was positively correlated with *Wolbachia* load (lm, adjusted $r^2 = 0.10$, $df = 109$, $p < .001$, Figure 3c). This significant positive correlation existed for the larvae harbouring both *w*Pip-IV (lm, adjusted $r^2 = 0.12$, $df = 58$, $p = .003$) and *w*Pip-I (lm, adjusted $r^2 = 0.099$, $df = 49$, $p = .014$). While the linear model restricted to *w*Pip-IV harbouring larvae exhibited a stronger r^2, and a visually steeper slope (95% CI, *w*Pip-IV = 0.733–2.66; *w*Pip-I = 0.057–1.68, $p = .194$, $df = 107$) there was no statistically supported difference between *Wolbachia* group influence on CpDV loads.

3.4 | CpDV diversification is not linked to *Wolbachia* groups

CpDV samples were genetically diverse on the sequenced 800-bp-long fragment, that is the partial NS3 protein coding region (Figure S1;

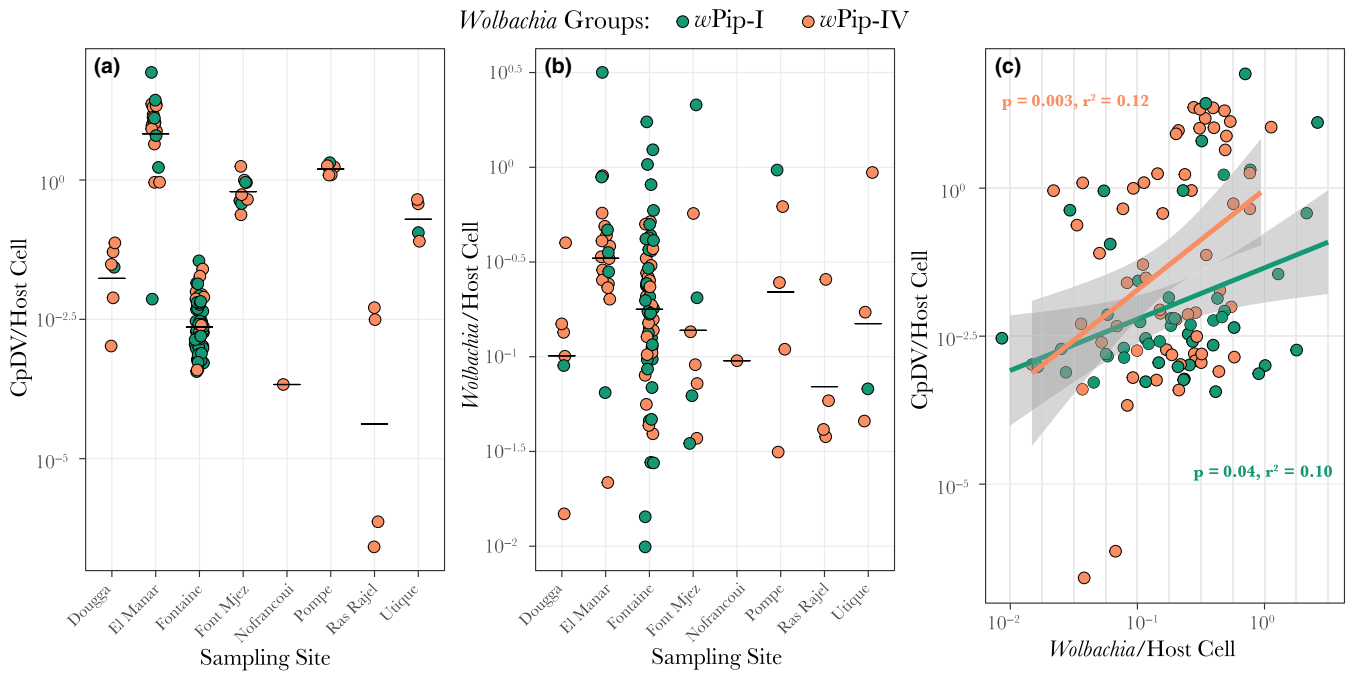


FIGURE 3 *Wolbachia* and CpDV load in samples from the coexistence zone. (a) CpDV load was highly variable ($n = 111$, mean = 3 CpDV per host cell, $SD = 10$). Sampling site (glm, $df = 7$, dev = 182.66, $p < .001$) explained an important part of the variance. (b) *Wolbachia* load. Average *Wolbachia* amount per host cell was 0.319 (±0.426). *Wolbachia* load was marginally influenced by the sampling site ($df = 5$, dev = 2.476, $p = .07$). (c) Correlation between *Wolbachia* and CpDV load. CpDV load was positively correlated with *Wolbachia* load (glm, $df = 1$, dev = 4.072, $p = .001$). This positive correlation existed for both the larvae harbouring *w*Pip-IV and *w*Pip-I with no difference between them

Figure 4a). A Bayesian phylogenetic reconstruction shows that this diversity mainly grouped in two well-supported clades, namely CpDV-Ia and CpDV-Ib, within the previously described CpDV-I clade (Altinli, Lequime, et al., 2019), with the only exception being the Ras Rajel 14 sample (Figure 4a). For some of the sampling sites (i.e., Oued Melah, El Battan, Tabarka), CpDV clustered together in well-supported clades, showing their genomic proximity and recent diversification (Figure 4a).

To investigate the putative relationship between *w*Pip groups and CpDV diversification, using CpDV phylogeny, we compared the state (i.e., carrying *w*Pip-I or *w*Pip-IV) changes through CpDV evolution observed for: (a) the real data set (Figure 4b, shown in red) and (b) data sets where *w*Pip group distributions were randomized (Figure 4b, shown in grey). A total overlap between the estimated numbers of state changes (95% HPD) for both data sets indicated the lack of a phylogenetic signal linked to the *w*Pip groups. This analysis showed that CpDV-Ia and CpDV-Ib strains were found in mosquitoes infected with either one of the *w*Pip groups. Both tree topology together with the lack of a phylogenetic signal linked to the *w*Pip group highlighted the horizontal transmission of CpDV between mosquito-*w*Pip lineages.

In addition to the above phylogenetic approach, we statistically compared the distribution of *w*Pip groups and CpDV-I clades in both the sampling sites outside and inside the coexistence zone. We found that *w*Pip groups and CpDV-I clades exhibited a parallel distribution in the sampling sites outside of the coexistence zone where only *w*Pip-I- (Ariana, Oued Melah, El Battan) or only *w*Pip-IV-carrying mosquitoes (Tabarka) were collected (Figure 5a; $\chi^2 = 9.67$, $df = 1$, $p = .002$). In this subset of data, CpDV-Ia was predominantly found in *w*Pip-I-carrying mosquitoes (CpDV-Ia, $n = 14$, *w*Pip-IV = 1, *w*Pip-I = 13) while CpDV-Ib was found in *w*Pip-IV-carrying mosquitoes (CpDV-Ib, $n = 7$, *w*Pip-IV = 6, *w*Pip-I = 1), suggesting putative local cotransmission of the two symbionts. By contrast, in the coexistence zone, the distribution of CpDV-Ia and CpDV-Ib was random with regard to their *Wolbachia* group infection status, i.e., *w*Pip-I versus *w*Pip-IV (Figure 5b; $\chi^2 = 2e\text{-}31$, $df = 1$, $p = 1$) showing that horizontal transfer occurred. In line with these results, state (i.e., carrying *w*Pip-I or *w*Pip-IV) changes through the CpDV phylogeny revealed a significant link between *w*Pip group and CpDV evolution when analyses were conducted on the data set considering sampling sites where only *w*Pip-I or *w*Pip-IV was found (Ariana, Oued Melah, El Battan or Tabarka) (Figure 5c) but not when performed on the data set from the coexistence zone (Figure 5d).

4 | DISCUSSION

Since its discovery (Hedges, Brownlie, O'Neill, & Johnson, 2008; Teixeira et al., 2008), *Wolbachia's* viral interference has been mainly studied due to its potential in arbovirus control. Most of our knowledge on *Wolbachia*–virus interactions thus comes from these studies generally conducted in transinfected cell and mosquito lines (Flores & O'Neill, 2018). Although this knowledge is crucial for the immediate applied aspects of *Wolbachia*-based arbovirus control methods, studies on natural *Wolbachia*–virus–mosquito interactions are

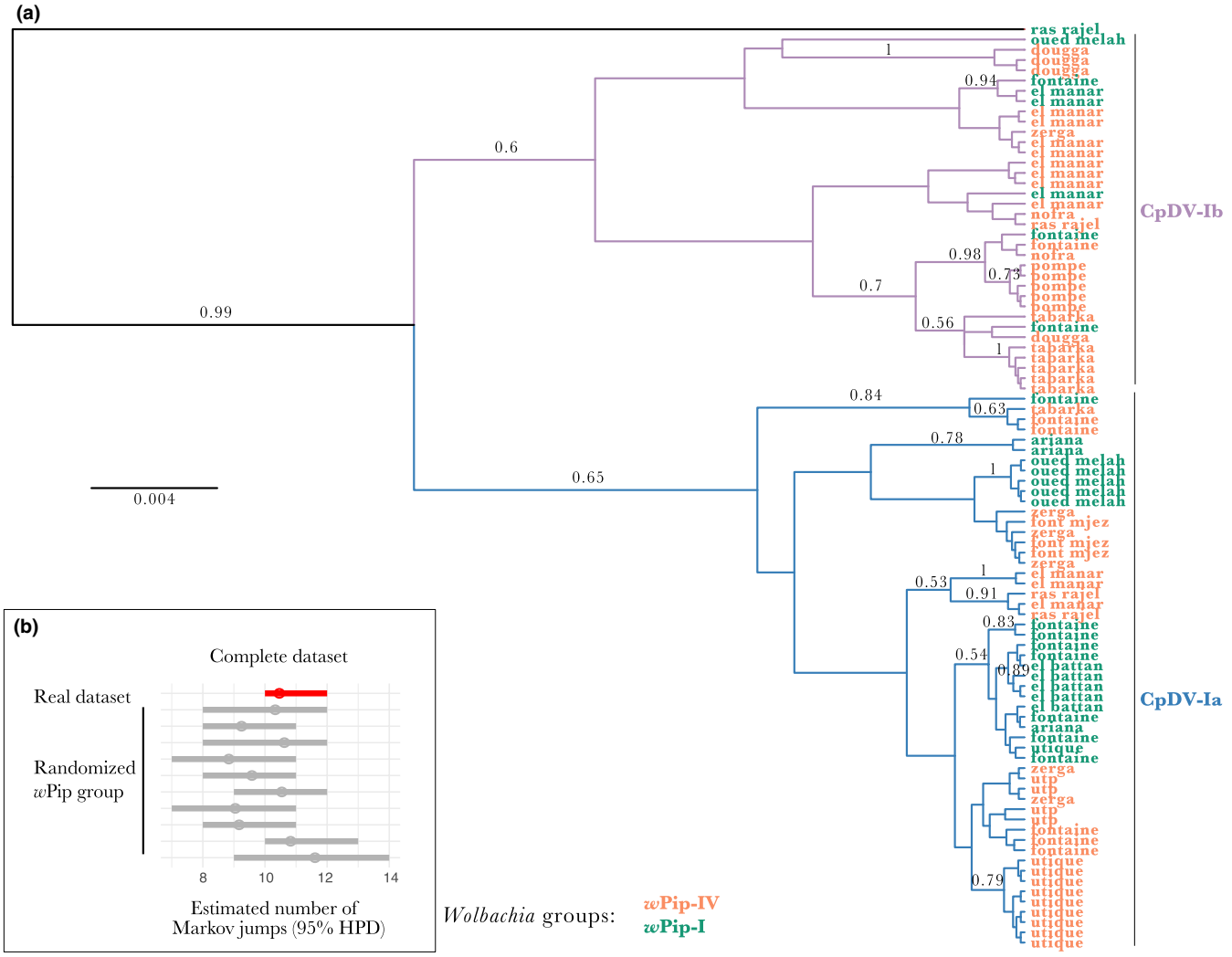


FIGURE 4 CpDV phylogeny in northern Tunisia. (a) Bayesian maximum clade credibility tree based on the CpDV sequences. Node posterior probabilities are only shown when they are higher than 0.5. CpDV samples found in the area grouped into two well-supported clades, CpDV-Ia and CpDV-Ib. CpDV variants from the same sampling sites can be clustered together with high posterior probabilities (e.g., samples from Oued Melah, El Battan and Tabarka). (b) Total overlap between the estimated number of state (i.e., carrying *w*Pip-I or *w*Pip-IV) changes for (i) the real data set (red) and (ii) the data set where *w*Pip groups were randomized (grey) proved the lack of a phylogenetic signal linked to the *w*Pip groups

required to understand their natural ecology and evolution, which would lead to further improvement and sustainability of these methods. To address this knowledge gap, we focused on a widely distributed ISV, CpDV, and its interactions with *Wolbachia* *w*Pip in their natural host *C. pipiens* (*s.l.*) in the field. As it was previously demonstrated that: (a) *C. pipiens* (*s.l.*) are all infected with *w*Pip (Dumas et al., 2013), (b) CpDV is present worldwide in ~50% of samples (Altinli, Lequime, et al., 2019) and (c) both *w*Pip and CpDV can share the same cells and be vertically cotransmitted to some extent (Altinli, Soms, et al., 2019), it could be suspected that these two endosymbionts might intimately interact and influence each other's dynamics. To characterize the putative influence of *Wolbachia* *w*Pip on CpDV prevalence, load and genetic diversification, we focused on a region where two *w*Pip groups stably coexist (Atyame et al., 2015). We hypothesized that mosquitoes harbouring different *w*Pip groups (*w*Pip-I and *w*Pip-IV) or loads might differ in their interactions with CpDV and thus could affect the virus' dynamics and diversification in the region.

We detected a high prevalence of CpDV in the area (i.e., 74%) and a strong heterogeneity between the sampling sites (Figure 1). These sites, water bodies with varying ecological conditions, are subject to extinction–recolonization events due to successions of dry and wet periods that are thought to reset the frequency and the coexistence of the two *w*Pip groups in the breeding spots (Atyame et al., 2015). A similar dynamic is probable for CpDV as well, as during each wet period the CpDV prevalence in larvae will mostly depend on the number of infected adults recolonizing the breeding spots. Such dispersal of a mosquito densovirus to new breeding spots has been shown in laboratory large cage trials for Aedes aegypti densovirus (Wise de Valdez, Suchman, Carlson, & Black, 2010). It is also possible that CpDV remains infective during the dry season in the breeding spots, as some mosquito densoviruses are highly stable in nature and have

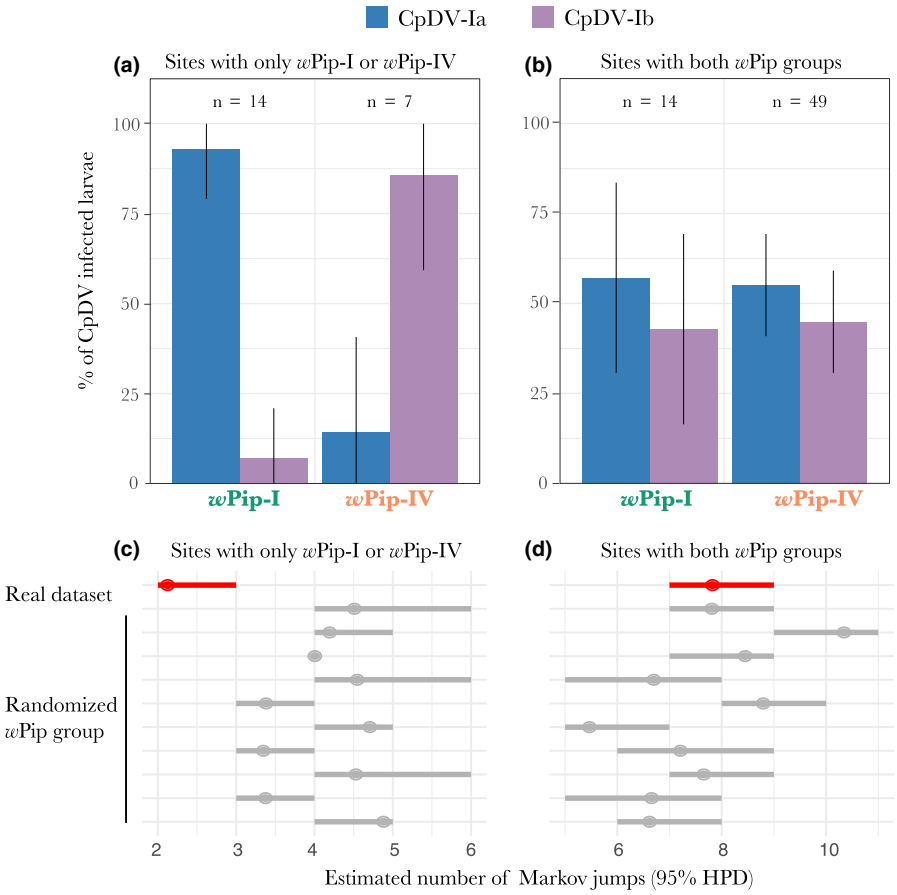


FIGURE 5 (a,b) *w*Pip group – CpDV clade distribution in and outside the coexistence zone. (a) Outside of the coexistence zone *w*Pip and CpDV-I clades exhibited a parallel distribution ($\chi^2 = 9.67$, $df = 1$, $p = .002$). (b) In contrast, in the coexistence zone, the distribution of CpDV-Ia and CpDV-Ib was random with regard to their *Wolbachia* group infection status, i.e., *w*Pip-I versus *w*Pip-IV ($\chi^2 =$ 2e-31, $df = 1$, $p = 1$). Lines represent 95% CI. (c,d) CpDV phylogenetic signal linked to *w*Pip groups in and outside the coexistence zone. The presence of a phylogenetic signal linked to the *w*Pip group was assessed by estimating the number of changes between the *w*Pip-I and *w*Pip-IV states (Markov jumps) in the posterior distribution of trees. A clear overlap of 95% HPD of randomized and real data confirmed the lack of a phylogenetic signal linked to the *w*Pip groups in the coexistence zone (d), while a phylogenetic signal is detected outside this zone (c)

been shown to remain infective for at least a year in water bodies, although their infectivity would decrease with direct sunlight and heat (Buchatsky, 1989). As our samples were collected in water bodies with varying ecological conditions, it was difficult to study the influence of *w*Pip groups on CpDV in the whole area. To circumvent this limitation, and to avoid interpretation bias due to random associations, we focused our analyses on sampling sites where both *w*Pip groups (*w*Pip-I and *w*Pip-IV) and CpDV coexisted, and where the larvae from each water body (i.e., sites) shared similar ecological conditions and probability of becoming infected. In these sites, we observed a strong influence of the *w*Pip groups on CpDV prevalence: larvae infected with *w*Pip-IV were more likely to be infected with CpDV compared to *w*Pip-I-infected larvae (Figure 2). The higher CpDV prevalence in mosquitoes harbouring *w*Pip-IV could be due to facilitation of CpDV transmission specifically by this *Wolbachia* group. In line with this, in a previous paper, we have shown that *w*Pip-IV-infected females exhibited higher CpDV loads in their ovaries compared to *w*Pip-I females, both when these *Wolbachia* groups infected the mosquito lines naturally and when they were introduced into the same mosquito genetic background (Altinli, Soms, et al., 2019). However, whether this increase in viral load would result in a significant increase in vertical transmission is still not proven.

In addition to this higher CpDV prevalence associated with *w*Pip-IV, we observed a general positive correlation between CpDV and *Wolbachia* loads (Figure 3). These results might suggest either an increase in *Wolbachia* amount in response to CpDV infection or a facilitation of CpDV replication by *Wolbachia* presence in *C. pipiens* cells. While our knowledge on virus effects on *Wolbachia* is limited, the latter is in accordance with our earlier results, where CpDV decreased when naturally infected *C. pipiens* laboratory lines were treated with tetracycline to clear their *w*Pip infection (Altinli, Soms, et al., 2019). Such facilitation could potentially arise from direct (e.g., via gene products of *Wolbachia*) or indirect (modulation of the host) interactions between viruses and the rest of the microbiota (Zélé, Magalhães, Kéfi, & Duncan, 2018). The general mechanism of *Wolbachia*–virus interference is not well understood and mostly studied for antagonistic interactions, where *Wolbachia* hampers viral replication. These antagonistic interactions are thought to be linked to the direct competition for resources (Caragata et al., 2013), or through the indirect induction of host immune response by *Wolbachia* (Pan et al., 2012). In many cases *Wolbachia* virus interference has also been linked to *Wolbachia* density (P. Lu et al., 2012; Martinez et al., 2017; Micieli & Glaser, 2014; Osborne, Iturbe-Ormaetxe, Brownlie, O'Neill, & Johnson, 2012). However, there are counter-examples for all the putative mechanisms involved in the *Wolbachia*–virus interference. Indeed, according to a recent study, there was no effect of *Wolbachia* density or *Wolbachia*-mediated immune gene expression on Dengue and West Nile virus interference by different *Wolbachia* strains, including *w*Pip, in the same *A. aegypti* genetic background (Fraser et al., 2020).

These contradictory results highlight the complex nature of the factors that define whether *Wolbachia* facilitates or restricts viral

replication. The outcome of these interactions from the perspective of the virus is so far thought to be related to the fundamental differences between DNA viruses and +ssRNA viruses, as *Wolbachia* seems to decrease the replication of many arboviruses, which are +ssRNA viruses (Bian, Zhou, Lu, & Xi, 2013; Dutra et al., 2016; Frentiu et al., 2014; Lu et al., 2012; Micieli & Glaser, 2014; Walker et al., 2011). However, antagonistic *Wolbachia*–DNA virus (such as densoviruses) interactions have never been shown (Altinli, Soms, et al., 2019; Graham et al., 2012; Parry et al., 2019; Teixeira et al., 2008). A fundamental difference between arboviral RNA viruses and DNA viruses is that while the first replicate in the cytoplasm along with *Wolbachia*, DNA viruses mostly replicate in the nucleus (Novoa et al., 2005; Schmid, Speiseder, Dobner, & Gonzalez, 2014). It is possible that *Wolbachia* interferes with viral replication, entry and encapsidation by inducing stress conditions in the host environment (reviewed by Lindsey, Bhattacharya, Newton, & Hardy, 2018). For instance, *Wolbachia* modulates host cholesterol and actin metabolism, both of which are required for the interaction of viruses with the cytoskeleton and for the proper trafficking of viral and host components to the viral replication centres in the cytoplasm (Barton, Sawicki, & Sawicki, 1991; Caragata et al., 2013; Carro & Damonte, 2013; Geoghegan et al., 2017; Lu, Cassese, & Kielian, 1999; Newton, Savytskyy, & Sheehan, 2015; Sheehan, Martin, Lesser, Isberg, & Newton, 2016). Such an indirect mechanism has previously been proposed to explain densovirus facilitation by *Wolbachia*, observed in mosquito-derived cell lines, through the induction of reactive oxygen species (ROS) by *Wolbachia* presence in the cytoplasm (Parry et al., 2019). Indeed, ROS could stimulate host–DNA repair mechanisms, which in return could increase densovirus replication in the nucleus because they use host–DNA repair mechanisms to replicate (Hristov et al., 2010; Moné, Monnin, & Kremer, 2014; Parry et al., 2019; Wong, Brownlie, & Johnson, 2015).

All the DNA viruses that have been initially studied for their interactions with *Wolbachia* were ISVs. Recent studies have shown that *Wolbachia* not only facilitates DNA ISVs in mosquitoes but also RNA ISVs, showing that facilitation could be a dominant outcome of *Wolbachia*–ISV interactions independent of viral genetic materials. Indeed, in cells, *w*AlbB enhanced the replication of Aedes albopictus Negev-like virus (*Virgaviridae*) (Bishop, Parry, & Asgari, 2020), and neither *w*MelPop nor *w*Mel restricted the replication of Phaesi Chareon-like virus (*Bunyaviridae*) (McLean, Dainty, Flores, & O'Neill, 2019). Facilitation by *Wolbachia* has also been observed for RNA flavivirus infection in *A. aegypti* carrying *w*Mel compared to their *Wolbachia*-free counterparts (Amuzu et al., 2018). In fact, among the studied ISVs, Cell-Fusing Agent virus (*Flaviviridae*) replication was the only mosquito ISV to be restricted by *w*MelPop, *w*Mel and *w*AlbB in transinfected *A. aegypti*-derived cell lines (Bishop et al., 2020; McLean et al., 2019; Schnettler, Sreenu, Mottram, & McFarlane, 2016; Zhang, Etebari, & Asgari, 2016). Therefore, it is possible that ecological differences between ISVs and arboviruses contribute to the different nature of interactions: (a) their presence in all life stages (while arboviruses are mostly restricted to female adults) (Ajamma et al., 2018; Bolling et al., 2012; Haddow et al., 2013; Kawakami et al., 2016; Saiyasombat et al., 2011; Sang et al., 2003); (b) their prevalence (the first studies conducted on ISVs demonstrated that they were highly prevalent (Altinli, Lequime, et al., 2019; Farfan-Ale et al., 2009; Goenaga et al., 2014; Parry & Asgari, 2018); and (c) transmission mode (ISVs being probably more frequently vertically transmitted than arboviruses) (Agboli et al., 2019; Lequime & Lambrechts, 2014; Lequime, Paul, & Lambrechts, 2016).

Using phylogenetic analyses, we investigated the putative relationship between *w*Pip groups and CpDV diversification. For that, we sequenced a polymorphic region of the CpDV genome including the partial NS3 coding region which represents about 14% of the CpDV genome. If CpDV and *Wolbachia* were strictly vertically cotransmitted via oocytes, as is the case for mitochondrion and *Wolbachia* in *C. pipiens* (*s.l.*) (Atyame, Delsuc, et al., 2011; Dumas et al., 2013; Rasgon, Cornel, & Scott, 2006), the different CpDV clades would be expected to match with the two *w*Pip groups (Turelli et al., 2018). Because the two *w*Pip groups coexisted in our study area, it was possible to test whether CpDV diversification and distribution could be, at least partially, explained by *w*Pip group distributions. Samples from some sites formed well-supported clades, indicating that this genetic marker was appropriate to follow CpDV evolution at a local scale (Figure 4). Our results also showed that CpDV variants sampled in the area grouped into two distinct clades (i.e., CpDV-Ia and CpDV-Ib). The distribution of these clades fit with the distribution of *w*Pip groups in the regions where just one of the *w*Pip groups existed (Figure 5). In these regions, *w*Pip-I- and *w*Pip-IV-carrying larvae were more likely to be associated respectively with CpDV-Ia and CpDV-Ib. This could potentially result from the vertical cotransmission of *w*Pip groups with their respective CpDV clades or alternatively from a bias due to limited diversification of the CpDV within sampling sites with only one *w*Pip group. The opportunity to investigate the occurrence of such *w*Pip-CpDV vertical cotransmission in the populations where *w*Pip-I- and *w*Pip-IV-infected larvae coexisted with phylogenetically different CpDV made it possible to rule out a strict CpDV–*Wolbachia* vertical cotransmission. The fact that a phylogenetic signal linked to *w*Pip was absent in these sites pointed that the CpDV–*Wolbachia* vertical cotransmission is not the major force driving the CpDV distribution and suggests (a) the horizontal transmission of CpDV between larvae, (b) transmission of CpDV paternally or venereally from fathers to offspring, in contrast to maternally transmitted *Wolbachia*, and (c) putative rare occurrence of horizontal transfers of *Wolbachia*. Horizontal transmission during the larval stage has been experimentally demonstrated for several mosquito densoviruses and thought to be the main transmission mode for these viruses (Ledermann, Suchman, Black, & Carlson, 2004; Li et al., 2019; Sun et al., 2019), although this has not yet been experimentally shown for CpDV. Venereal transmission from infected males to females has also previously been shown for Aedes albopictus densovirus at very low frequency (Barreau et al., 1997), although whether mosquito densoviruses can also be paternally transmitted remains unknown. Alternatively, this pattern can be caused by horizontal transfers of *Wolbachia*, which are rare events that have been experimentally and genetically investigated in many arthropod species (Baldo

et al., 2008; Le Clec'h et al., 2013; Raychoudhury, Baldo, Oliveira, & Werren, 2009). However, this scenario seems unlikely, as previous studies have shown that *w*Pip strictly codiverged with *C. pipiens* (*s.l.*) mitochondrial genes and not with their host nuclear background, suggesting perfect maternal vertical transmission for *w*Pip (Atyame, Delsuc, et al., 2011; Dumas et al., 2013; Rasgon et al., 2006).

Overall our results shed light on the influence of *Wolbachia* endosymbionts on CpDV infection dynamics in nature. Mosquito densoviruses (Afanasiev, Kozlov, Carlson, & Beaty, 1994; Carlson, Suchman, & Buchatsky, 2006; Johnson & Rasgon, 2018; Ren & Rasgon, 2010) and *Wolbachia* (Atyame, Pasteur, et al., 2011; Frentiu et al., 2014; Hoffmann et al., 2011; Laven, 1967; McGraw & O'Neill, 2013) are considered as promising vector control tools. Although studies have highlighted the effects of *Wolbachia* on arboviral infections, our knowledge on its impact on ISVs is very limited, especially in natural populations. Our results have suggested that introduction of new *Wolbachia* strains into mosquito populations for control reasons is unlikely to affect CpDV transmission due to its efficient horizontal transmission, although different *Wolbachia* strains can facilitate the densovirus infection to varying degrees. Still, further empirical and modelling studies are needed to fully understand the complex multipartite microbiota (including ISVs)–vector interactions. Given the importance of these interactions on the dynamics of vectors and the viruses they transmit, further knowledge on multipartite interactions will also be crucial for field releases of *Wolbachia*-infected or transinfected mosquitoes.

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AUTHOR CONTRIBUTIONS

M.A., M.S. and M.W. conceptualized and designed the study; C.A. and M.W. conducted the sample collection; M.A., C.A. and F.J. performed the experiments; M.A. and S.L. analysed and interpreted the data; M.S. and M.W. supervised the project; M.A. and M.S. wrote the original draft; all authors contributed to the final manuscript and provided critical feedback.

DATA AVAILABILITY STATEMENT

DNA sequences: GenBank accession nos. MK722526–722617. The remaining data used in this study are provided as supporting information (*Wolbachia* groups and CpDV prevalence in Northern Tunisia, Table S1; *Wolbachia* and CpDV loads, Table S2; Sequenced samples and accession codes, Table S3).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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