Wolbachia modulates prevalence and viral load of Culex pipiens densoviruses in natural populations
Mine Altinli, Sebastian Lequime, Célestine Atyame Nten, Fabienne Justy, Mylène Weill, Mathieu Sicard

To cite this version:

HAL Id: hal-02961602
https://hal.umontpellier.fr/hal-02961602
Submitted on 8 Oct 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives| 4.0 International License
Wolbachia modulates prevalence and viral load of Culex pipiens densoviruses in natural populations

Mine Altinli1,2,3 | Sebastian Lequime4 | Celestine Atyame5 | Fabienne Justy1 | Mylene Weill1 | Mathieu Sicard1

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 wPip 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 wPip groups in the different sites, we detected a signal linked to wPip groups in CpDV phylogeny in sites where all larvae were infected by the same wPip group. However, no such signal was detected where the wPip 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

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2020 The Authors. Molecular Ecology published by John Wiley & Sons Ltd
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 Moore, & Blair, 2012; Haddow et al., 2013; Kawakami et al., 2016; Altinli, Soms, 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 mosquitoes’ 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 (Paroviridae) 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 wPip (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 wPip strains (Beckmann et al., 2019; Bonneau et al., 2018). wPip strains are closely related worldwide, all belonging to the same phylogenetic clade, and can be separated into five genetically distinct phylogenetic groups, wPip-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 wPip (Altinli, Soms, et al., 2019). Furthermore, in wPip-free mosquitoes (artificially treated with tetracycline in the laboratory), CpDV load and vertical transmission decreased significantly compared to wPip-infected females, suggesting a facilitation of CpDV infection by wPip (Altinli, Soms, et al., 2019).wPip 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 wPip belonging to two distinct wPip groups (wPip-I and wPip-IV), CpDV load was higher in the ovaries of C. pipiens lines carrying wPip-IV compared to those carrying wPip-I. This was also true when wPip-IV and wPip-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 transfected with Wolbachia, especially wMel and wMelPop strains from Drosophila melanogaster. Interactions between arboviruses and transfected 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 transfected mosquitoes it even enhanced arboviral replication (Dodson et al., 2014). In C. pipiens (s.l) mosquitoes naturally infected with wPip, Wolbachia has been shown to reduce West Nile virus load but only in C. quinquefasciatus laboratory lines with high somatic wPip load, and not in recently settled C. pipiens lines. Moreover, wPip did not have any effect on RNA arboviruses in transfected 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 wMel compared to tetracycline-treated flies (Teixeira, Ferreira, & Ashburner, 2008), wMel-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, Gryzwacz, 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 transfected with wMelPop (from D. melanogaster) or wAlB (from Aedes albopictus), compared to the tetracycline-treated or nontransfected 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 wMel locally released to fight against Dengue transmission (Amuzu et al., 2018). However, Wolbachia-IVS 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 wPip strain genetic variations on C. pipiens–CpDV interactions, we have focused on an area in North Africa where a stable coexistence of different wPip strains, belonging to the wPip-I and wPip-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 wPip, 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 wPip groups for the same individuals (Atyame et al., 2015). We additionally quantified both CpDV and wPip 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 wPip 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 wPip 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 wPip 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 (wPip-I and wPip-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 wPip-I and wPip-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: wPip-I (n = 51) and wPip-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′-GGTCTGAATTGGCTGATGC-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-3′) that amplified 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 wPip-I and wPip-IV coexisted (coexistence zone; Dougga, El Manar, Fontaine, Font Mjez, Nofrancouli, Pompe, Rasrajel, Utique, Utique Pont[UTP], Zerga), and (c) one where only one wPip group was found (Tabarka, Ouedmelah, El batten, 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 + 1 nucleotide substitution model (Altinli, Lequime, et al., 2019; Hasegawa, Kishino, & Yano, 1985; Yang, 1994). wPip 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 wPip 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., wPip 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** (wPip-I, wPip-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** (wPip-I, wPip-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 wPip groups was calculated by fitting the same model with wPip × Wolbachia load interaction.

The prevalence of CpDV-Ia and CpDV-Ib in wPip-I- and wPip-IV-carrying mosquitoes was compared using a χ² test, for the sampling sites where wPip-I and wPip-IV did not coexist (Tabarka, Ouedmelah, El batten, Ariana) versus where they both coexisted (coexistence zone; Dougga, El Manar, Fontaine, Font Mjez, Nofrancouli, Pompe, Rasrajel, Utique, Utique Pont[UTP], 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 wPip group infection by Atyame et al. (2015) (Figure 1a). CpDV diagnostic PCR was positive for 74% of these individuals collected in and around the wPip-I/wPip-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 wPip 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 wPip groups in the area: wPip-I in southeastern sampling sites and wPip-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 wPip group distribution (glm, df = 1, dev = 0.108, p = .741)](image-url)
effect of Wolbachia group (i.e., wPip-I and wPip-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 wPip-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 wPip groups on CpDV prevalence under the same ecological conditions (i.e., the similar probability of getting infected with CpDV for C. pipiens larvae harbouring wPip-I or wPip-IV).

The main factor that affected the CpDV prevalence in sampling sites from the coexistence zone was the Wolbachia groups (i.e., wPip-I vs. wPip-IV) (glm, df = 1, dev = 16.492, p < .001, Figure 2). Indeed, mosquitoes harbouring the wPip-IV were 34% more likely to be CpDV-positive (risk ratio [RR] = 2.791; 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).

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 wPip 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 wPip 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 wPip-IV (lm, adjusted r^2 = 0.12, df = 58, p = .003) and wPip-I (lm, adjusted r^2 = 0.099, df = 49, p = .014). While the linear model restricted to wPip-IV harbouring larvae exhibited a stronger r^2, and a visually steeper slope (95% CI, wPip-IV = 0.733–2.66; wPip-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 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 wPip groups and CpDV diversification, using CpDV phylogeny, we compared the state (i.e., carrying wPip-I or wPip-IV) changes through CpDV evolution observed for: (a) the real data set (Figure 4b, shown in red) and (b) data sets where wPip 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 wPip groups. This analysis showed that CpDV-Ia and CpDV-Ib strains were found in mosquitoes infected with either one of the wPip groups. Both tree topology together with the lack of a phylogenetic signal linked to the wPip group highlighted the horizontal transmission of CpDV between mosquito-wPip lineages.

In addition to the above phylogenetic approach, we statistically compared the distribution of wPip groups and CpDV-I clades in both the sampling sites outside and inside the coexistence zone. We found that wPip groups and CpDV-I clades exhibited a parallel distribution in the sampling sites outside of the coexistence zone where only wPip-I (Ariana, Oued Melah, El Battan) or only wPip-IV-carrying mosquitoes (Tabarka) were collected (Figure 5a; $\chi^2 = 9.67$, df = 1, p = .002). In this subset of data, CpDV-la was predominantly found in wPip-I-carrying mosquitoes (CpDV-la, n = 14, wPip-IV = 1, wPip-I = 13) while CpDV-Ib was found in wPip-IV-carrying mosquitoes (CpDV-Ib, n = 7, wPip-IV = 6, wPip-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., wPip-I versus wPip-IV (Figure 5b; $\chi^2 = 2e-31$, df = 1, p = 1) showing that horizontal transfer occurred. In line with these results, state (i.e., carrying wPip-I or wPip-IV) changes through the CpDV phylogeny revealed a significant link between wPip group and CpDV evolution when analyses were conducted on the data set considering sampling sites where only wPip-I or wPip-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
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 densovirus are highly stable in nature and have

**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.
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 the coexistence zone. Outside the coexistence zone, the distribution of pipeline 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 pipeline-Ia and pipeline-Ib was random with regard to their Wolbachia group infection status, i.e., pipeline-I versus pipeline-IV ($\chi^2 = 2e-31, df = 1, p = 1$). Lines represent 95% CI. (c,d) CpDV phylogenetic signal linked to pipeline groups in and outside the coexistence zone. The presence of a phylogenetic signal linked to the pipeline group was assessed by estimating the number of changes between the pipeline-I and pipeline-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 pipeline groups in the coexistence zone (d), while a phylogenetic signal is detected outside this zone (c).
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 densovirus) 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, Savvytskyy, & 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, wAlbB enhanced the replication of Aedes albopictus Negev-like virus (Virgaviroidae) (Bishop, Parry, & Asgari, 2020), and neither wMelPop nor wMel restricted the replication of Phaesi Charone-like virus (Bunyaviroidae) (McLean, Dainty, Flores, & O’Neill, 2019). Facilitation by Wolbachia has also been observed for RNA flavivirus infection in A. aegypti carrying wMel compared to their Wolbachia–free counterparts (Amuzu et al., 2018). In fact, among the studied ISVs, Cell-Fusing Agent virus (Flavirviroidae) replication was the only mosquito ISV to be restricted by wMelPop, wMel and wAlbB in transfected A. aegypti-derived cell lines (Bishop et al., 2020; McLean et al., 2019; Schnettler, Sreen, 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 wPip 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; Rason, Cornel, & Scott, 2006), the different CpDV clades would be expected to match with the two wPip groups (Turelli et al., 2018). Because the two wPip groups coexisted in our study area, it was possible to test whether CpDV diversification and distribution could be, at least partially, explained by wPip 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 wPip groups in the regions where just one of the wPip groups existed (Figure 5). In these regions, wPip-I and wPip-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 wPip groups with their respective CpDV clades or alternatively from a bias due to limited diversification of the CpDV within sampling sites with only one wPip group. The opportunity to investigate the occurrence of such wPip–CpDV vertical cotransmission in the populations where wPip-I and wPip-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 wPip 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 wPip strictly codiverged with C. pipiens (s.l.) mitochondrial genes and not with their host nuclear background, suggesting perfect maternal vertical transmission for wPip (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 densovirus (Afanasiev, Kozlov, & 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.

ACKNOWLEDGEMENTS

We thank Sandra Unal and Patrick Makoundou for technical support, Dr Philippe Clair for his help in the qPCR experiments which were performed at the “qPCR Haut Debit (qPHD) Montpellier génomIX” platform. Sequencing data were produced at the LabEX “Centre Méditerranéen de l’Environnement et de la Biodiversité” in the GENSEQ platform. We also thank Maxime Courcelle, Dr Emmanuel Douzery, Dr Bram Vrancken, Dr Simon Dellicour and Dr Philippe Lemey for helpful discussions. This work was funded by the French ANR programme (project ANR-16-CE02-0006-01 “CIAWOL,” 2017–2021). Sampling was done with the assistance of the Comité de Coopération Marseille Provence Méditerranée supervised by Dr Robert Bismuth in collaboration with the Tunisian ministry of ecology. M.A. received support from Ministère des affaires étrangères (Campus France) via the French Embassy in Turkey.

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).

REFERENCES


**SUPPORTING INFORMATION**

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