**Wolbachia** in mosquitoes from the Central Valley of California, USA

Ryan Torres¹, Eunis Hernandez¹, Valeria Flores¹, Jose Luis Ramirez² and Andrea L. Joyce¹*

### Abstract

**Background:** Wolbachia bacteria are widely distributed throughout terrestrial arthropod species. These bacteria can manipulate reproduction and influence the vector competence of their hosts. Recently, Wolbachia have been integrated into vector control programmes for mosquito management. A number of supergroups and strains exist for Wolbachia, and they have yet to be characterized for many mosquito species. In this study, we examined Wolbachia prevalence and their phylogenetic relationship to other Wolbachia, using mosquitoes collected in Merced County in the Central Valley of California.

**Methods:** Adult mosquitoes were collected from 85 sites in Merced County, California in 2017 and 2018. Traditional and quantitative PCR were used to investigate the presence or absence and the density of Wolbachia, using Wolbachia-specific 16S rRNA and Wolbachia-surface protein (wsp) genes. The supergroup of Wolbachia was determined, and Multilocus Sequence Typing (MLST) by sequencing five housekeeping genes (coxA, gatB, fisZ, hcpA and fbpA) was also used to determine Wolbachia supergroup as well as strain.

**Results:** Over 7100 mosquitoes of 12 species were collected: *Aedes melanimon*, *Ae. nigromaculis*, *Ae. vexans*, *Ae. aegypti*, *Culex pipiens*, *Cx. stigmatosoma*, *Cx. tarsalis*, *Anopheles franciscanus*, *An. freeborni*, *An. punctipennis*, *Culiseta inornata*. Eight showed evidence of Wolbachia. To our knowledge, this study is the first to report detection of Wolbachia in five of these species (*Ae. melanimon*, *Cx. stigmatosoma*, *Cx. tarsalis*, *Cs. incidens* and *Cs. inornata*). *Culex pipiens* and *Cx. stigmatosoma* had a high frequency and density of Wolbachia infection, which grouped into supergroup B; *Cs. inornata* clustered with supergroup A. MLST comparisons identified *Cx. pipiens* and *Cx. stigmatosoma* as wPip strain type 9 supergroup B. Six species had moderate to low (< 14%) frequencies of Wolbachia. Four species were negative, *Ae. nigromaculis*, *An. franciscanus*, *An. freeborni* and *Ae. aegypti*.

**Conclusions:** New records of Wolbachia detection were found in mosquitoes from Merced County, California. *Culex stigmatosoma* and *Cs. inornata* were new records for Wolbachia supergroup B and A, respectively. Other species with Wolbachia occurred with low frequency and low density. Detection of Wolbachia in mosquitoes can be used to inform potential vector control applications. Future study of Wolbachia within *Cx. stigmatosoma* and *Cs. inornata* in California and through the range of these species could further explore Wolbachia infection in these two species.

**Keywords:** Wolbachia, Strain characterization, Supergroup, 16S rRNA, Multilocus sequence typing (MLST), *Culex pipiens*, *Culex stigmatosoma*, *Culiseta inornata*, *Aedes melanimon*, *Aedes aegypti*, Vector control

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Wolbachia routinely infect their host’s reproductive tissues, and they are capable of surviving in a variety of invertebrates [5–7]. Wolbachia are known to be transmitted vertically through maternal inheritance and have also been shown to transmit horizontally between species, genera, and orders [8–11]. Wolbachia infections can have a diverse range of effects depending on the host species, from commensal, mutualistic, to parasitic interactions [5].

In recent years, Wolbachia have been implemented for population control of vector species [12, 13]. This is largely a result of the reproductive alterations that Wolbachia induce within their hosts in a strain-specific manner [5, 14]. Such reproductive manipulations include termination of male offspring, feminization of genetic males, parthenogenesis, and cytoplasmic incompatibility [5, 15]. Cytoplasmic incompatibility is the only known phenotype to be expressed within mosquito species [16]; when infected males mate with uninfected females, viable offspring are not produced. Furthermore, Wolbachia have been shown to modulate host fitness and vector potential. For instance, studies have shown a protective effect of Wolbachia against infection with pathogenic RNA viruses [12, 14, 17–21]. In addition, Wolbachia-infections have shown other complex host-specific manipulations: they can have increased or decreased rates of reproductive phenotypes; reduced host life-span and egg viability [6, 22]; impact larval survival [23]; decreased female mosquito biting ability [24]; decreased relative abundance of resident bacteria [25]; and in some cases, increased viral susceptibility and host mortality [26, 27]. Artificial infection of this endosymbiont into arthropod vectors has been shown to impact transmission of vector-borne diseases including lymphatic filariasis, West Nile virus, chikungunya, dengue, Zika, and avian malaria [14, 19, 20, 26].

Wolbachia offers a potential effective alternative to traditional chemical pesticide applications for the control of disease vectors, for example through cytoplasmic incompatibility. Cytoplasmic incompatibility was first proposed as a method of biological control for Culex pipiens fatigans in 1967, although initially it was not attributed to Wolbachia [28]. Since then, the use of Wolbachia-mediated incompatible technique strategies have been studied for pest control of a number of insects including Aedes aegypti, Ae. albopictus, Ae. polynesiensis, Ceratitis capitata, Rhagoletis cerasi, Glossina morsitans, Culex pipiens and Cx. quinquefasciatus [28–34]. This method of control aims to reduce vector populations through the introduction of Wolbachia-infected ‘sterile’ males, which compete with uninfected males for mates at the release site. Aedes aegypti do not naturally harbor Wolbachia; when Wolbachia have been detected in Ae. aegypti [35–39], the range of detected strain types suggest they may be due to environmental contamination [40].

Naturally uninfected arthropod species like Ae. aegypti can be amenable to Wolbachia-infection through micro-injection of the endosymbiont from another insect species into developing embryos [41, 42]. Currently, eight novel strains (wAlbA, wAlbB, wAu, wMel, wMelCS, wMelPop-CLA, wPip and wRi) have been transinfected into Ae. aegypti to be evaluated for vector control applications [42–46]. Aedes aegypti is widespread in tropical and subtropical regions globally [47, 48], and since its detection in California in 2011 it has become widespread in southern California and the Central Valley [49, 50]. One example of where Wolbachia-infected Ae. aegypti males have been used to reduce mosquito populations through cytoplasmic incompatibility is through the DeBug Fresno California programme, which released male Ae. aegypti with the wAlbB strain of Wolbachia to reduce local Ae. aegypti populations [34]. A second method of using Wolbachia-infections for mosquito control relies on the introduction of Wolbachia-infected male and female mosquitoes to replace uninfected mosquito populations [12, 51, 52]. Aedes aegypti populations (each with a unique Wolbachia strain, wMel or wAlbB), have been introduced into regions of Australia and Malaysia, respectively [12, 52, 53]. Both strains were shown to reduce the incidence of dengue virus infections [53, 54].

Each Wolbachia strain has particular biological characteristics when moved into another vector, and identification of strains is key. Supergroups are used to differentiate major phylogenetic subdivisions within Wolbachia pipiensis [55]. The 16S rRNA gene and the Wolbachia-surface protein (wsp) have been used to characterize supergroups [55, 56]. Within supergroups, Wolbachia strains are identified and can be characterized by multilocus sequence typing (MLST) which relies on five conserved bacterial housekeeping genes (gatB, coxA, hepA, ftsZ, and fbpA). Strains are commonly characterized based on the host species in which they are first identified [55, 57, 58]. For instance, wPip is the strain of Wolbachia which was identified from the Culex pipiens mosquito species. Mosquitoes can be singly or superinfected with more than one Wolbachia strain, or infected with multiple variants of the same strain [31].

Merced County is located in the Central Valley of California and includes a diverse range of habitats and mosquito species. While previous studies have identified the presence or absence of Wolbachia within some mosquito species throughout California using traditional PCR [58], the current infection status for species in the Central Valley of California and Merced County is unknown. Wolbachia-infected mosquitoes as a method of mosquito
control has great potential globally, and this vector-control method continues to be developed and refined.

The objectives of this study were to determine the presence or absence of *Wolbachia* in twelve mosquito species collected throughout Merced County, and to characterize the *Wolbachia* supergroup and strain for species with detections. Our study expands current knowledge of *Wolbachia* presence in mosquitoes in Merced and in the Central Valley of California, and would aid in the design of future *Wolbachia*-based mosquito control applications.

**Methods**

**Mosquito collections**

Adult mosquitoes were collected weekly from June to September, in both 2017 and 2018, using Encephalitis Vector Survey (EVS) traps (Bioquip, Rancho Dominguez, CA, USA) in Merced County. Traps sites were selected for habitats known to harbor the different species of mosquitoes. The EVS traps contained (1–2 kg) of dry ice (carbon dioxide) per container as an attractant for host-seeking female mosquitoes. Traps were hung on trees or fences in close proximity to a water source. The GPS coordinates of the site were recorded using a Garmin etrex High Sensitivity GPS unit (Garmin Ltd., Olathe, KS, USA). Traps were placed during the early afternoon and retrieved the following morning. Samples were transported on ice to a –20 °C laboratory freezer. Adults were identified on a cold plate using a taxonomic key specific to Californian mosquitoes [59] and stored in 1.5 ml Eppendorf tubes until DNA extraction.

*Aedes aegypti* larvae were collected in addition to adults from several sites in Merced, California. Larvae were collected from water sources, transported to the lab, and reared at laboratory temperature in a BugDorm (MegaView Science, Taichung, Taiwan). Emerged adults were stored at –20 °C and identification was confirmed using a taxonomic key. A map of trapping locations was constructed for 2017 and 2018 using qGIS v3.8.3-Zanzibar [60]. The Census TIGER/Line file for Merced County, California was retrieved (www.census.gov/cgi-bin/geo/shapefiles2010/main), and site location GPS coordinates were overlaid on the county map.

**DNA extraction**

The whole body of the mosquito was used for individual extractions of genomic DNA using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA), following the manufacturer's protocol for tissue extraction with a 2 h incubation at 65 °C [61]. Extracted samples were stored at –20 °C. The DNA quantity was measured using the Qubit® dsDNA HS Assay kit (Life Technologies, Carlsbad, CA, USA). The quantity of DNA in the samples averaged 10–15 ng/µl.

**Screening samples for *Wolbachia* and relative *Wolbachia* density determination**

Presence or absence of *Wolbachia* was determined by amplicon detection of the *Wolbachia*-specific 16S rRNA gene and the general *Wolbachia* surface protein (wsp) via qPCR in individual field-collected mosquitoes. For each mosquito species collected, a subset of individuals was screened for *Wolbachia*, and individuals tested came from multiple sites or collection dates (Additional file 1: Table S1). The primer combinations for the *Wolbachia*-16S rRNA gene and *Wolbachia*-surface-protein (wsp) used in our assays are detailed on Table 1. The qPCR cycling conditions were those recommended for the master mix and consisted of holding at 95 °C for 10 min and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A melt curve stage at the end of the reaction was included. Each sample was analyzed in duplicates (technical replicates), and a non-template control was included. The qPCR assays were run on Applied Biosystems 5700 Fast Real-time PCR (Applied Biosystems, Foster City, CA, USA).

The relative *Wolbachia* density was determined via qPCR for two species, *Culex pipiens* and *Culex stigmatosoma*. Relative density was determined by measuring the signal amplifications of the *Wolbachia* 16S rRNA or wsp gene and the respective reference gene for each mosquito species. The RpS3 gene was used as a reference gene and primers specific for this location (Table 1) were employed to compare *Wolbachia* densities in the collected samples. The RpS3 gene is known to be a single copy gene in mosquitoes [62] and is described to be highly conserved [63]. *Culex pipiens* is known to be naturally infected with *Wolbachia* was used as a control to compare the relative *Wolbachia* density to *Cx. stigmatosoma*. Samples were compared and the data was analyzed post-run using the ΔΔ Ct method [64]. Data were evaluated using the GraphPad Prism 8.4.2 statistical software, comparing the two species using Student’s t-test.

**Determination of *Wolbachia* supergroups**

A subset of samples that screened positive for *Wolbachia* by qPCR were used to characterize the *Wolbachia* supergroup. Samples were run using the *Wolbachia* wsp supergroup A and wsp supergroup B primers [65] (Table 1). Polymerase chain reaction (PCR) was performed using a mixture of 2 µl of DNA, 1 µl of each forward and reverse primer at 10 µM concentration, 1 µl of Taq polymerase, 5 µl of buffer, 1 µl of dNTPs (2.5 µM) (Takara-Clontech Bio, Mountain View, CA, USA) and 40 µl of sterile water to make the reaction volume of 51 µl. The temperature profile for wsp amplification was the following: initial
denaturation for 3 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, a final elongation of 10 min at 72 °C and a final hold at 4 °C, modified from the protocol in Zhou et al. [65]. Amplification was confirmed by visualizing products on an agarose gel. Products were purified using USB Exo-sap-it® (Affymetrix Inc., Santa Clara, CA, USA) PCR cleanup kit. Each forward and reverse sequence reaction was prepared using 1 µM primers, 2 µl purified water, and 10 µl purified PCR product per reaction and sequenced on an Applied Biosystems 3730xl DNA Analyzer at the UC Berkeley DNA Sequencing Facility. Multilocus sequence typing (MLST) was also used to characterize supergroups (described below).

**Strain characterization by multilocus sequence typing (MLST)**

Species with samples which were successfully sequenced for supergroup A or B were also sequenced by multilocus sequence typing (MLST) using the standard primers for five ubiquitous bacterial housekeeping genes: coxA, gatB, ftsZ, hcpA and hcpA [57] (Table 1). The PCR mix for each gene used a mixture of 2 µl of DNA, 1 µl of each forward and reverse primer at 10 µM concentration, 1 µl of Taq polymerase, 5 µl of buffer, 1 µl of dNTPs (2.5 µM) (Takara-Clonetech Bio Inc., Mountain View, CA, USA) and 40 µl of sterile water to make the reaction volume of 51 µl. The PCR temperature profile for four of the genes (coxA, gatB, ftsZ and hcpA) was the following: initial denaturation for 2 min at 94 °C, followed by 37 cycles of 30 s at 94 °C, 45 s at 54 °C, and 1.5 min at 72 °C, a final elongation for 10 min at 72 °C and a final hold at 4 °C [57]; the PCR program for the fbpA gene was identical except the annealing was for 45 s at 59 °C. PCR amplification was visually confirmed on agarose gels, products purified by USB Exo-sap-it®, and sequencing reactions were similar to those previously described.

**Sequence analysis**

*Wolbachia* surface protein (*wsp*) and the MLST genes (coxA, gatB, ftsZ, hcpA and fbpA) sequence files were viewed, edited, and aligned in Geneious Prime 2020.05. Consensus sequences were generated and exported as FASTA files. Consensus sequences were queried using the BLASTn program to find sequences with the highest similarity.

*Wolbachia* supergroup sequences from this study were combined with high similarity sequences from GenBank and others from a study by Carvajal et al. [38] to produce a Neighbor-Joining tree. Included in the tree were consensus sequences of 18 samples from this study [Cx. pipiens (n = 8) Culex stigmatosoma (n = 9) and Culiseta inornata (n = 1)] and an additional 15 wsp sequence

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**Table 1** Primer sequences used for diagnostic testing of *Wolbachia*

| Test                  | Gene target | PCR product (bp) | Primer name | Sequence (5’-3’)                  | References |
|-----------------------|-------------|------------------|-------------|-----------------------------------|------------|
| Wolbachia presence    | 16S rRNA    | 438              | W16S-F      | CATACCTATTCGAAGGGTAG               | [56, 95, 96] |
|                       |             |                  | W16S-R      | GTCGGGAGACTTACCAAAAGCA             |            |
|                       | *wsp* (General) | 185            | *wsp*-F     | GCAATTGGTAYAAATAAGCAG              | [97]       |
|                       |             |                  | *wsp*-R     | GGAGTGATAGGACATCTCTCAA             |            |
| Wolbachia density     | Rps3        | 70               | Rps3-F      | AGCGTGCAGGTCCGATAG                | [98]       |
|                       |             |                  | Rps3-R      | GCTACTTCGGCGGAGCTCTC              |            |
| Supergroup A/B identification | *wsp* (Supergroup A) | 556 | 136F | TGAATTTTTACCTCCTTTT           | [65]       |
|                       |             |                  | 691R        | AATTTTACTCCTTTT                  |            |
|                       | *wsp* (Supergroup B) | 442            | 81F         | TGGTCAATGATGAGAAGAAGAC            | [57]       |
|                       |             |                  | S22R        | ACCAGCTTTTGTGTTGTA               |            |
| Multilocus sequence typing | *gatB* | 396            | *gatB*-F1   | GATTTAACGCCAGGGBGT               | [57]       |
|                       |             |                  | *gatB*-R1   | TGGYAAATCRGGYAAAAGTGA             |            |
|                       | coxA        | 402              | coxA_F1     | TTGGRGCRATYAACTTTTATG            |            |
|                       |             |                  | coxA_R1     | CTAAGAGCTTTKACRCAG               |            |
|                       | hcpA        | 444              | hcpA_F1     | GAAATARCAGTTGTCGAAA              |            |
|                       |             |                  | hcpA_R1     | GAAAGTYRAGCAAGYCTG               |            |
|                       | ftsZ        | 435              | ftsZ_F1     | ATYATGGCARATATAAARGTAG           |            |
|                       |             |                  | ftsZ_R1     | TCRAGYAGATGATRGAT                |            |
|                       | fbpA        | 429              | fbpA_F1     | GCTGCTCCCTTGGYWTGAT              |            |
|                       |             |                  | fbpA_R1     | CRRCCAGARAAAAAYACTATTC          |            |
files from GenBank which represented 11 genera previously confirmed with detections of Wolbachia. The species selected for comparison were Aedes albopictus (AF020058, AF020059), Brugia malayi (AJ252061), Culex pipiens (AF020061), Culex quinquefasciatus (AF020060), Dirofilaria imitis (AJ252062), Drosophila melanogaster (AF020072), Drosophila simulans (AF020070), Glossina austeni (AF020077), Glossina morsitans (AF020079), Muscidifurax urinaptor (AF020082) [38], and three additional sequences (Loxoblemmus sp. MG97910, Myrmecophillus sp. MK995471 and Cerapachys augustae KC137155) of high similarity. These 33 sequences were subjected to multiple sequence alignment using the ClustalW algorithm in MEGA 7.0. The Gamma distributed, Tamura 3-parameter substitution model was chosen based on the lowest Bayesian information criterion. A Neighbor-Joining tree was constructed using 1000 bootstraps in MEGA 7.0 [66].

Wolbachia strains were characterized by concatenating the coxA, gatB,ftsZ, hcpA and fbpA gene sequences from each sample in Geneious. Following concatenation, each sequence was exported in FASTA format and queried against the Wolbachia MLST database (https://pubmlst.org/Wolbachia/) to determine allelic profiles [57, 67]. An exact match with the queried database was necessary to distinguish profile composition. All sequences were submitted to Genbank.

Results
Mosquito collections, identification and abundance
In total, 12 mosquito species from 4 genera were collected from 85 sites within Merced county in 2017 and 2018 (Table 2, Additional file 1: Table S1). There was a total of 7150 mosquitoes identified to species. The species collected were the following: Aedes melaninon, Aedes vexans, Aedes nigromaculis, Aedes aegypti, Culex stigmatosoma, Culex pipiens, Culex tarsalis, Anopheles franciscanus, Anopheles freeborni, Anopheles punctipennis, Culiseta incidens, Culiseta inornata (Table 2). These species represent the diversity of nearly every mosquito from the region where trapping occurred [59]. The 85 trap sites were in the vicinity of 8 cities within Merced county: Atwater, Ballico, Hilmar, Le Grand, Los Banos, Merced, Snelling and Winton (Fig. 1, Table 2). Each mosquito species was trapped from two to five different regions of the county (Table 2, Additional file 1: Table S1), to provide geographic diversity in samples which were tested. Some mosquito species were more abundant than others. For example, Cx. pippens and Cx. tarsalis were trapped in cities as well as in rural sites (Additional file 1: Table S1). Ae. melaninon and Ae. vexans adults were most abundant within rural wetland habitats. Aedes aegypti was found in several Merced neighborhoods and near the Merced Zoo. Anopheles franciscanus, An. freeborni and An. punctipennis were found at rural riparian sites. Culex stigmatosoma were numerous at a semi-natural rural site near dairy runoff. Aedes nigromaculis, Cs. incidens and Cs. inornata were collected from rural and residential properties.

Wolbachia screening with qPCR
For each species, 30–50 mosquitoes were typically screened for the presence or absence of Wolbachia, except for a few species which had smaller numbers of individuals collected (Table 2). A total of 406 mosquitoes were screened for Wolbachia prevalence using qPCR, and all mosquitoes screened were females. Wolbachia was detected within 73 of the 406 samples tested, and sites with mosquitoes positive for Wolbachia were found throughout the county (Table 2, Additional file 1: Table S1). Eight species within four genera tested positive for Wolbachia (Table 2). The frequency and percent of samples positive for each species from highest to lowest was the following: Cx. stigmatosoma (30/34; 88.2%), Cx. pippens (31/37; 83.8%), Cs. incidens (1/19: 5.3%), Cs. inornata (1/7; 14.3%), Ae. melanimon (6/55: 10.9%), An. punctipennis (1/19: 5.3%), Cx. tarsalis (1/26: 3.9%), Ae. vexans (2/52; 3.9%) and Cs. inornata (1/42; 2.4%) (Table 2). Species where no Wolbachia was detected were An. freeborni, An. franciscanus, Ae. nigromaculis and Ae. aegypti (Table 2).

Each species was screened by qPCR for Wolbachia with two primers. For Cx. pippens and Cx. stigmatosoma, all individuals were positive for Wolbachia when tested with both genes (16S rRNA and wsp) (Table 2). In a few cases, one primer would detect Wolbachia, while another would not (Table 2). For Ae. melanimon, Cx. tarsalis, Cs. incidens, Cs. inornata, An. punctipennis and Ae. vexans, Wolbachia was detected in very few individuals (Table 2). For Cs. incidens and Cs. inornata, both primers detected only one positive individual (Table 2). Six individuals were positive detections with the 16S rRNA primer set but were negative with wsp (one An. punctipennis, one Cx. tarsalis, two Ae. vexans and two Ae. melanimon). Only one sample was negative with 16S rRNA but positive for wsp (Ae. melanimon) (Table 2, Additional file 1: Table S1).

To evaluate the relative Wolbachia density of the two Culex spp., we conducted a relative comparison using qPCR for 30 individuals each of Culex stigmatosoma and Cx. pippens, the later which was used as a control. The relative Wolbachia density comparison indicated no significant difference between the two species (16S, t-test, t = 0.80, df = 48, P = 0.43; wsp, t-test, t = −1.34, df = 48, P = 0.18).
**Wolbachia supergroup identification**

Wolbachia supergroup identification was carried out by PCR of samples using general wsp supergroup A and supergroup B primers. A total of 18 Wolbachia surface protein sequences were generated from three species, Cx. pipiens (n = 8), Cx. stigmatosoma (n = 9) and Cs. incornata (n = 1). Wsp sequences were not successfully obtained from the other species with low frequency Wolbachia detections (Table 2). The sequences produced in this study were combined with an additional 15 wsp sequences from GenBank for supergroup comparison (described above). The Cx. pipiens and Cx. stigmatosoma individuals grouped with the reference supergroup B samples, and Cs. incornata grouped with supergroup A reference samples (Fig. 2).

**Wolbachia strain characterization**

There were five individual Cx. pipiens which had 5 MLST genes (coxA, gatB, ftsZ, hcpA and fbpA) successfully sequenced (Cx. pipiens nos. 29, 31, 32, 34 and 35) and they were matches with strain type 9, wPip supergroup B Wolbachia in the MLST database (Table 3). Four additional Cx. pipiens were similar at 3 or 4 of the five gene sequences to strain type 9 wPip; however, these had a low quality hcpA sequences and exact match of that allele could not be confirmed.

For Cx. stigmatosoma, two samples had complete gene sequences for the five MLST genes (coxA, gatB, ftsZ, hcpA and fbpA); the allelic profile for Cx. stigmatosoma samples 10 and 15 from Ballico were a match for the five sequences retrieved from several Cx. pipiens samples (nos. 29, 31, 32, and 35), and these were characterized as Wolbachia wPip supergroup B strain-type 9 (Table 3).

The two Cx. stigmatosoma individuals had four of the five MLST genes sequenced and also matched strain type 9, but only partial sequences were obtained for the hcpA gene. The hcpA locus has been observed with variable sequence lengths, ranging from 435 to 477 bp (pubmlst.org/Wolbachia). Five additional samples (Cx. stigmatosoma nos. 16,17, 20, 25, 38) were sequenced at 3 or 4 of the 5 genes, which also had matching profiles to Wolbachia housekeeping genes (coxA, ftsZ and fbpA) from this study.

One Cs. incornata sample had a detection of Wolbachia with 16S rRNA gene, and this individual was used to generate sequence data for the five MLST genes. The one Cs. incornata had four sequences (fbpA, gatB, coxA, ftsZ) which had mlst allele matches; these sequences matched fbpA allele 277, gatB 312, coxA 236, and ftsZ 154, while hcpA had no match [67]. For Cs. incornata, the wsp sequence grouped with others in supergroup A.

**Discussion**

This study screened 12 field-collected mosquito species in the Central Valley of California for the presence or absence of Wolbachia, and for species with Wolbachia detections, attempted to characterize the supergroup and strain type. The 12 mosquito species identified and screened were the following: Ae. melanimon, Ae. nigromaculis, Ae. vexans, Ae. aegypti, Cx. pipiens, Cx. stigmatosoma, Cx. tarsalis, An. franciscanus, An. freeborni, An. punctipennis, Cs. incidens and Cs. incornata. Wolbachia was detected in eight of the mosquito species. To our knowledge, this study is the first to report Wolbachia detection in five of these species (Ae. melanimon, Cx. stigmatosoma, Cx. tarsalis, Cs. incidens and Cs. incornata), while three species which were positive in

| Table 2 | Mosquito species collected and screened for Wolbachia by qPCR of 16S rRNA gene and WSP |
|---------|-------------------------------------------------------------------------------------|
| Mosquito species | Total trapped | Atwater | Ballico | Hilmar | Le Grand | Los Banos | Merced | Snelling | wsp | 16S | Total |
| Ae. melanimon | 1827 | – | – | 5/26 | – | 1/20 | – | 0/9 | 4/55 | 5/55 | 6/55 (10.9%) |
| Ae. nigromaculis | 12 | – | 0/1 | – | – | 0/8 | 0/3 | – | – | – | 0/12 (0%) |
| Ae. vexans | 488 | – | – | 2/36 | – | 0/16 | – | – | 0/52 | 2/52 | 2/52 (3.9%) |
| Ae. aegypti | 60 | – | – | – | – | 0/60 | – | – | – | 0/60 (0%) |
| Cx. pipiens | 994 | 5/5 | 15/15 | – | – | – | 10/10 | 1/7 | 31/37 | 31/37 | 31/37 (83.8%) |
| Cx. stigmatosoma | 36 | 2/2 | 28/28 | – | – | – | 0/1 | 0/3 | 30/34 | 30/34 | 30/34 (88.2%) |
| Cx. tarsalis | 3878 | – | 1/15 | – | 0/5 | – | 0/4 | 0/2 | 0/26 | 1/26 | 1/26 (3.9%) |
| An. franciscanus | 2 | – | – | – | – | – | 0/2 | – | – | 0/2 (0%) |
| An. freeborni | 221 | – | 0/29 | 0/1 | 0/22 | – | 0/1 | 0/7 | – | – | 0/60 (0%) |
| An. punctipennis | 19 | – | – | – | – | – | 0/1 | 1/18 | 0/19 | 1/19 | 1/19 (5.3%) |
| Culiseta incidens | 94 | – | – | – | 0/1 | – | 1/35 | 0/6 | 1/42 | 1/42 | 1/42 (2.4%) |
| Total | 7150 | 7/7 | 44/88 | 7/63 | 0/28 | 1/45 | 11/114 | 3/60 | 67/406 | 72/406 | 73/406 |

*a Number positive/Number tested

*b Percent of samples screened positive for Wolbachia by either wsp or 16S rRNA. Collections details for all mosquitoes are detailed in Additional file 1: Table S1
this study have been previously reported in the literature (Ae. vexans, Cx. pipiens and An. punctipennis). The Wolbachia supergroup was determined for two of these new records (Cx. stigmatosoma and Cs. inornata), and the strain was characterized for Cx. stigmatosoma using MLST. The other species with detections of Wolbachia had a very low prevalence (frequency) and could not be sequenced.

The two mosquito species which were positive for Wolbachia at high frequencies (prevalence) were Cx. pipiens and Cx. stigmatosoma. The other six species showed detections of Wolbachia at low prevalence (< 13%). Furthermore, when the relative Wolbachia density was compared between Cx. pipiens and Cx. stigmatosoma, there was no statistical difference indicating that these two species potentially hold similar Wolbachia densities. Further assessment via absolute quantification of Wolbachia would further confirm this finding. In addition, future work with Cx. stigmatosoma could investigate maternal transmission to provide supporting evidence for Wolbachia infection. The inability to sequence Wolbachia in the species with low Wolbachia prevalence could be due to a low Wolbachia density. One species, Cs. inornata, had a low Wolbachia prevalence (13%), yet the wspA sequence was obtained which allowed it to be tentatively classified into supergroup A. Four of five MLST genes were sequenced for Cs. inornata in this study. This Wolbachia isolate may potentially represent a new Wolbachia strain, but further research would be needed with additional samples collected to replicate detection of Wolbachia.

Wolbachia infections were previously reported in Cx. pipiens [58, 68], An. punctipennis [69], and Ae vexans [70]. Although Wolbachia has been previously detected in An. punctipennis and Ae. vexans, currently there is no description of a strain type for these mosquitoes.
Our study did not detect *Wolbachia* in several mosquito species including *An. freeborni*, *An. franciscanus*, *Ae. nigromaculis* and *Ae. aegypti*. Although a few studies have indicated *Wolbachia* detection in *Ae. aegypti* [36–39], others found absence of infection in this species [40, 71] and suggest that the variability of strains found in previous studies on *Ae. aegypti* may indicate environmental contamination rather than a true *Wolbachia* infection. Ross et al. [40] recommend that to confirm *Wolbachia* infection, experiments should be run to demonstrate maternal transmission or to visualize *Wolbachia* in the mosquito using a method such as florescent in situ hybridization (FISH), in addition to determining sequences. *Culex pipiens* is well known for its infection with *Wolbachia*, as *Wolbachia pipiensis* was first described from this mosquito species [1, 2]. Previous research identified wPip supergroup B infections in the *Cx. pipiens* species complex in five California populations [58]. Since then more than 60 wPip haplotypes have been identified [31, 72]. Our study screened *Cx. pipiens* from four sites and found individuals from all sites carrying *Wolbachia*. In the present study, the MLST results for *Cx. pipiens* found strain type 9 supergroup B among samples with complete allelic profile data. These were all acquired from the Ballico collection site. Isolates of strain type 9 have been documented in *Cx. pipiens* and *Cx. quinquefasciatus* [67]. Other studies have found *Cx. pipiens* with strain type 9 in Placer County, California and Tompkins County, New York; while *Wolbachia*-infected *Cx. quinquefasciatus* were found in Hawaii, Midway and Kenya [57, 73].

Interestingly, our study also found a new *Wolbachia* detection record for *Cx. stigmatosoma*. This species is highly ornithophilic [74] and often found in urban residential areas and near farms. It prefers foul water sources like street drains and dairy lagoons for oviposition [75].
| Species            | Sample # | MLST gene | Strain | SG \(^a\) | Strain # |
|--------------------|----------|-----------|--------|-----------|----------|
| *Cx. pipiens*      | Cxpip 29 | 4         | 3      | 354       | 22       | 444      | wPip | B   | 9      |
|                    | Cxpip 31 | 4         | 3      | 354       | 22       | 444      | wPip | B   | 9      |
|                    | Cxpip 32 | 4         | 3      | 354       | 22       | 444      | wPip | B   | 9      |
|                    | Cxpip 34 | 4         | 227    | 354       | 22       | 444      | wPip | B   | 9      |
|                    | Cxpip 35 | 4         | 3      | 354       | 22       | 444      | wPip | B   | 9      |
| *Cx. stigmatorum*  | Cxstig 15| 4         | 3      | 354       | 22       | 444      | wPip | B   | 9      |
|                    | Cxstig 10| 148       | 3      | 354       | 22       | 444      | wPip | B   | 9      |

\(^a\) Supergroup
These types of habitat are similar to those where *Cx. pipiens* can also be found. This species is known to occur throughout the western USA to Mexico, Central America and northern South America [75, 76]. *Culex stigmatosoma* is a competent vector of West Nile virus, and is capable of transmitting St Louis encephalitis and avian malaria [76, 77].

*Culex stigmatosoma* had MLST sequences produced from two different collection sites. One site was a rural semi-natural habitat near a dairy (Ballico), and another was a rural farm in Atwater. At the first site, *Cx. stigmatosoma* had sequences from the five MLST genes that were an identical match for those from *Wolbachia* strain type 9 (ST-9) *wPip* infection in the MLST database, sequences which were identical to those characterized from *Cx. pipiens* tested in this study (Table 3). Although the five MLST housekeeping genes sequenced from *Cx. stigmatosoma* matched those of *Cx. pipiens* for strain type 9 *wPip*, it would be worthwhile to examine differences in *Wolbachia* from these two species using a more comprehensive method such as comparative genomics before concluding the two species harbor the same strain [78]. Bleidorn & Gerth (2018) discussed the limits of the MLST for *Wolbachia* strain characterization; one of these is that several of the MLST genes used to characterize *Wolbachia* strains evolve slowly, and may not sufficiently differentiate among strains where significant biological differences may exist. In this study, *Cx. stigmatosoma* was not likely to be misidentified as adult *Cx. pipiens*. Adult *Cx. stigmatosoma* more closely resemble *Cx. tarsalis*, but the two species are distinguished by distinct markings on ventral abdominal segments [59]. *Culex stigmatosoma* had a high prevalence (frequency) of individuals with detections of *Wolbachia*. The second collection site (Atwater) where *Cx. stigmatosoma* was positive for *Wolbachia* in this study also had an individual with MLST alleles match those of *Cx. pipiens* *Wolbachia* strain type as well (strain 9). This species could represent a new *Wolbachia* infection, not just a detection of *Wolbachia*. However, further studies would be needed to provide evidence of infection which are complementary to sequencing, such as FISH or loop mediated isothermal amplification (LAMP) [40].

The *Wolbachia* similarity observed between the two species above (*Cx. pipiens* and *Cx. stigmatosoma*) is not unusual. In fact, several studies have documented high similarity among some *Wolbachia*-infections in hosts within the same genus [57, 79, 80]. In Italy, evidence of natural *wPip* *Wolbachia* infections have been identified within *Culex modestus* and *Culex torrentium* mosquito species, and there was no observable divergence in wsp sequences when compared to field collected *Cx. pipiens* [79]. Another example was documented in Portugal, where low prevalence *Wolbachia*-infections were found in *Culex theileri* and indistinguishable from *Cx. pipiens* by 16S rRNA, *ank2* and *pk1* genes [80]. Furthermore, the results of restricted fragment length polymorphisms (RFLP) suggested a shared *wPip* haplotype I infection among both *Cx. theileri* and *Cx. pipiens*. Thus, it is not surprising that two closely related *Culex* species in the present study could harbor very similar or closely related *Wolbachia* strains.

*Culisetia inornata* had several MLST genes match those in the MLST database. When grouped in the supergroup phylogeny with other vector species, *Cs. inornata* was closely related to supergroup A infections previously reported in a dipteran, *Phlebotomos papatasi* (sand fly) and an orthopteran (*Laxoblemmus* spp.) (Fig. 2). *Culisetia inornata* in this study was collected in a semi-natural riparian habitat along the Merced River. This species is predominant in rural areas, and is capable of vectoring West Nile virus, western equine encephalitis, St Louis encephalitis, Japanese encephalitis, California encephalitis and avian malaria [76, 81, 82]. This species occurs throughout the United States, with a known presence in 46 states from California to New York and the range also expands north into Canada [75, 83]. *Culisetia inornata* persists through the winter months, which could have implications for the seasonality of arbovirus transmission. Given that *Cs. inornata* transmits a number of vector-borne diseases, further study to investigate *Wolbachia* within this species could be worthwhile, since *Wolbachia* can influence vector competence. Moreover, future research could investigate whether *Wolbachia* in this species persist within other populations in California or other regions.

Several other mosquito species had *Wolbachia* at a low frequency or density. Some of these species have been previously tested through traditional PCR, but perhaps escaped detection due to the lower sensitivity of traditional PCR compared to qPCR [58]. Our study detected *Ae. melanimon* with *Wolbachia* at a low frequency. This study is the first record of *Ae. melanimon* with detection of *Wolbachia*, but additional tests as previously described would be needed to confirm infection [40]. *Aedes melanimon* are widely distributed throughout western and southwestern USA and Canada [75, 84, 85]. This species prefers to oviposit in or around irrigated pastures, ponds and fields. *Aedes melanimon* is the primary vector of California encephalitis and is capable of transmitting western equine encephalitis and West Nile virus [76, 86, 87]. Past literature has identified *Ae. melanimon* to have a secondary role in maintenance of western equine encephalitis virus within the Central Valley of California, and has identified this mosquito as preferentially feeding on humans and other mammals [88, 89]. Along with *Ae.
melanomlon, several other species (An. punctipennis, Cx. tarsalis and Cs. incidunt) had very low prevalence (all less than 10%), perhaps due to horizontal transmission. Recently, Shaikievich et al. [90] suggested that Wolbachia diversity is likely attributed to horizontal transfer and strain recombination. By utilizing one-allele-criterion (OAC) phylogenetic networks, the authors suggest a link between the Ae. albopictus (wAlbB) Wolbachia strain and Wolbachia from ants; furthermore, that supergroup B strains from mosquitoes are linked with Wolbachia from Lepidoptera. Routes of horizontal transmission have been shown to occur through parasitism, shared habitats, and predation [11, 91–93].

Conclusions
Our survey of Wolbachia infections in Merced county mosquitoes identified new Wolbachia detections, providing information to support current and future Wolbachia-mediated vector control applications. As noted, it will be important to confirm Wolbachia detections are true infections by providing evidence in addition to Wolbachia sequences. Wolbachia-based approaches have been implemented within vector control strategies by propagation of a desired strain within an uninfected population, or by inducing cytoplasmic incompatibility through mating incompatibility. Successful integration depends on the strain chosen for its effects on the novel host [94]. Characterizing new Wolbachia strains and determining their mosquito host species are critical to efforts to further develop Wolbachia-mediated vector control applications.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04429-z.

Additional file 1: Table S1. Mosquito collections in Merced county.

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Availability of data and materials
The datasets generated during this study consist of sequences submitted to GenBank under the accession numbers MW125593- MW125610 (wsp), MW133153-MW133170 (ftsZ), MW133171-MW133187 (coxA), MW133188-MW133204 (fbpA), MW133205-MW133220 (garB), and MW133221-MW133228 (hspA).

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 Public Health, University of California, 5200 North Lake Road, Merced, CA 95343, USA. 2 USDA-ARS, NCAUR, Crop Protection Research, 1815 N. University, Peoria, IL 61604, USA.

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