Detection of *Wolbachia* in field-collected *Aedes aegypti* mosquitoes in metropolitan Manila, Philippines

Thaddeus M. Carvajal1,2,3, Kazuki Hashimoto1, Reza Kurniawan Harnandika1, Divina M Amalin2,3 and Kozo Watanabe1,2,3*

**Abstract**

**Background:** Recent reports reveal the presence of *Wolbachia* in *Ae. aegypti*. Our study presents additional support for *Wolbachia* infection in *Ae. aegypti* by screening field-collected adult mosquitoes using two *Wolbachia*-specific molecular makers.

**Methods:** A total of 672 *Ae. aegypti* adult mosquitoes were collected from May 2014 to January 2015 in Metropolitan Manila. Each individual sample was processed and screened for the presence of *Wolbachia* by selected markers, *Wolbachia*-specific 16S rDNA and its surface protein (wsp), under optimized PCR conditions and sequenced.

**Results:** Totals of 113 (16.8%) and 89 (13.2%) individual mosquito samples were determined to be infected with *Wolbachia* using the wsp and 16S rDNA markers, respectively. The *Ae. aegypti* wsp sample sequences were similar or identical to five known *Wolbachia* strains belonging to supergroups A and B while the majority of 16S rDNA sample sequences were similar to strains belonging to supergroup B. Overall, 80 (11.90%) individual mosquito samples showed positive amplifications in both markers and 69% showed congruence in supergroup identification (supergroup B).

**Conclusions:** By utilizing two *Wolbachia*-specific molecular makers, our study demonstrated the presence of *Wolbachia* from individual *Ae. aegypti* samples. Our results showed a low *Wolbachia* infection rate and inferred the detected strains belong to either supergroups A and B.

**Keywords:** *Wolbachia*, Metropolitan Manila, Dengue, *Ae. aegypti*
samples in Malaysia [14]; however, the sample size was too small \((n=16)\) to affirm such findings. Afterwards, metabarcoding studies by examining bacterial communities in the midgut of \(Ae. aegypti\) in the USA and Thailand reported a low presence of \(Wolbachia\) sequences [17, 18]. In 2019, evidence of natural \(Wolbachia\) infection in \(Ae. aegypti\) from India was presented, based on amplification of \(Wolbachia\)-specific 16S rRNA, \(wsp\) and \(ftsZ\) molecular markers [15]. This was followed by a report of \(Wolbachia\) presence \(Ae. aegypti\) populations in the USA, specifically from the states of New Mexico and Florida, using 16S rDNA, \(gatB, ftsZ\) and strain-specific (phosphoesterase and diaminopimelate epimerase) markers [16]. Both demonstrated the persistence of the endosymbiont across the developmental stages of \(Ae. aegypti\) through cytological examination and molecular detection. This clearly illustrates that the infection of \(Wolbachia\) in \(Ae. aegypti\) appears common than previously recognized.

This report provides additional support to the presence of \(Wolbachia\) in field-collected \(Ae. aegypti\) adult mosquitoes using \(Wolbachia\)-specific 16S rDNA and its surface protein (\(wsp\)). In comparison to previous studies, we conducted a large sampling of \(Ae. aegypti\) mosquitoes \((n=672)\) in a microgeographical area in order to discern the spatial distribution of \(Wolbachia\)-infected mosquitoes at the city scale and also to more accurately understand the infection rate in a natural \(Ae. aegypti\) population. Furthermore, our results focus primarily on the global phylogeny of \(Wolbachia\) strains within \(Ae. aegypti\). In previous studies [15, 16], the \(Wolbachia\) strain isolated belonged to supergroup B or homologous to the strain from \(Ae. albopictus\). Not only do our results conform to these previous findings, but they also reveal other prospective \(Wolbachia\) strains (e.g. supergroup A) infecting this mosquito.

**Methods**

**Study area and mosquito collection**

The study area was the National Capital Region of the Philippines, also known as Metropolitan Manila. Located on the eastern shore of Manila Bay in southwestern Luzon Island (14°35′58.2432″N, 121°59′3.1992″E), it is considered to be one of the most highly urbanized and densely populated areas in the Philippines. Dengue disease is endemic in this region where it accounted for 15–25% of the total number of reported dengue cases annually in the period 2009–2014 [19].

Adult mosquito samples were collected using a commercial branded mosquito UV-light trap (MosquitoTrap®; Jocanima Corporation, Las Piñas City, Philippines) installed in the outdoor premises of 138 residential households (sampling sites) from May 2014 to January 2015 (Fig. 1a). Collected mosquito samples were then sorted and identified as \(Ae. aegypti\) using available...
DNA extraction, PCR amplification and sequencing

Total genomic DNA of each mosquito individual was extracted using a Blood and Tissue DNAEasy Kit® (Qiagen, Hilden, Germany) following a modified protocol [21]. Our study used two molecular markers for detecting Wolbachia infection, namely wsp [22] and 16S rDNA [23]. The primer sequences were wsp 81F (5′-TGG TCC AAT TAA ACG CTA CTC-3′) and wsp 691R (5′-AAA AAT TAA ACG GAT GGT ACT CAC-3′) for the wsp marker and the 16S Wolbachia-specific primers were WolbF (5′-GAA GAT AAT GAC GGT ACT CAC-3′) and Wspecr (5′-AGC TTC GAG TGA AAC CAA TTC-3′).

For the wsp gene amplification, we followed the standard wsp protocol [11] where the suggested annealing temperature and number of cycles were 55 °C and 30 cycles, respectively. To conduct an individual-based detection, we initially performed this protocol using Culex quinquefasciatus as our positive control. Certain modifications were made in the standard protocol based on these results. The annealing temperature was increased to 57 °C and the number of cycles was increased to 35 cycles. This initial modified protocol was performed in individual Ae. aegypti samples where it yielded positive faint bands. As a result, we modified the protocol again, setting the annealing temperature at 59 °C with 40 cycles, and adding 10% DMSO (Sigma-Aldrich, St. Louis, Missouri, USA). This led to desirable results necessary for sequencing. In the end, a 10 μl final reaction volume was used consisting of 10× buffer (TaKaRa, Shiga, Japan), 25 mM MgCl₂, 10 mM of each dNTPs, 10 μM forward and reverse primers, 10% DMSO (Sigma-Aldrich) and 5.0 U/μl of Taq DNA polymerase (TaKaRa). The final thermal profile was as follows: initial denaturation at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 1 min; final extension at 72 °C for 3 min.

For the 16S rDNA gene amplification, we used a 10 μl final reaction volume consisting of 10× buffer (TaKaRa), 25 mM MgCl₂, 10 mM of each dNTPs, 10 μM forward and reverse primers, 10% DMSO (Sigma-Aldrich) and 5.0 U/μl of Taq DNA polymerase (TaKaRa). Thermal profiles followed the protocol of Simões et al. [23]: initial denaturation at 95 °C for 2 min; two cycles of denaturation at 95 °C for 2 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 45 s; final extension at 72 °C for 10 min.

All PCR amplification experiments included positive and negative controls. The positive control was a Wolbachia-infected Cx. quinquefasciatus sample while the negative control was water. The product size of each molecular marker was checked through 1.5% agarose gel electrophoresis set at 100 V for 30 min. The size of the amplified wsp gene is approximately 610 bp while the 16S rDNA gene is approximately 850 bp. The PCR amplification process underwent two replicates to validate the results (see Additional file 1: Table S1). A third screening was performed for selected individual samples that had inconsistent results based on the two prior replicates. The criteria set to confirm Wolbachia infection were based on two successful amplifications of the molecular markers. Furthermore, individual samples that met this criterion were subjected for sequencing through Eurofins Genomics, Tokyo.

Identity of Wolbachia strains and their positions in phylogroups

All sequences were subjected to the Nucleotide Basic Local Alignment Search Tool (BLAST) and compared to deposited Wolbachia sequences in GenBank. The selected sequences of Wolbachia strains (Table 1) and those obtained in the study then underwent multiple alignment using Clustal W in MEGA 6 [24]. After editing, the final lengths used for phylogenetic inference analyses were 398 and 721 bp for wsp and 16S rDNA, respectively. The identities and relationships of the Wolbachia strains obtained in our study were determined by performing Bayesian inference analysis using PhyML v.3.0 software with 1000 bootstrap replicates [25]. Smart Model Selection [26] was also utilized to set the parameters for wsp as GTR+G (number of estimated parameters k=232, Akaike information criterion (AIC)=4897.31702) and 16S rDNA as GTR+G+I (number of estimated parameters k=207, AIC=5332.86688). All newly generated sequences were submitted to the GenBank database with accession numbers MN046588–MN046789.

Statistical analysis

A Clark-Evans test was performed to determine whether the spatial distribution of Wolbachia-positive mosquito samples from each molecular marker had a pattern of complete spatial randomness. The test uses the aggregation index (R), where a value > 1 suggests an ordered distribution and a value < 1 suggests clustering. This analysis was performed using R v.3.3.5 (package spatstat) [27].

Results

Detection of Wolbachia through wsp and its phylogeny

From a total of 672 adult Ae. aegypti screened using the wsp marker, 113 (16.8%) individual adult mosquito
samples were positive for *Wolbachia* infection (Table 2) based on the study criteria (see Methods). Other than the positive individual adult mosquito samples, there were also 17 individual samples that produced one successful *wsp* amplification; however, these were excluded in reporting the prevalence and further analysis. The female/male ratio was 0.82 (Table 2). All sequenced amplicons resulted in a high degree of similarity (> 98.0%) with the *wsp* sequences in GenBank. The spatial distribution showed that 60 (43.0%) sampling sites (Fig. 1b) contained *Wolbachia* infected mosquitoes. Positive sampling sites had prevalence rates ranging between 7.69–100%. Further analysis showed that the distribution of *wsp*-positive mosquito samples was significantly clustered (*R* = 0.003, *P* < 0.001). The *wsp* phylogeny indicated that majority of the sequences belong to supergroup B (n = 84) while the remaining were in supergroup A (n = 29) (Fig. 2 and Additional file 2: Figure S1). Based on descending order of sample sizes, sample sequences from supergroup B were identical (> 99.0%) to *Wolbachia* type strains from selected hosts such as *Ae. albopictus* (*wAlbB*), *Ae. aegypti* (*wAegB*) (n = 51), *Cx. quinquefasciatus*, *Cx. pipiens* (*wPip*) (n = 23) and *Ephestia cautella* (*wCau*) (n = 10). The sample sequences from supergroup A were either similar to (98.0–99.0%) (n = 8) or identical (> 99.0%) (n = 21) with the *Wolbachia* strain (*wAlbA*) found in *Ae. albopictus*.

**Detection of Wolbachia through 16S rDNA and its phylogeny**

For the 16S rDNA, 89 (13.2%) individual adult mosquito samples were infected with *Wolbachia* (Table 2) based on

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**Table 1** Representative *Wolbachia* type sequences from different insect hosts in *wsp* and 16S rDNA molecular markers

| Molecular marker | Host                          | Wolbachia supergroup | GenBank ID |
|------------------|-------------------------------|----------------------|------------|
| *wsp*            | *Drosophila melanogaster*     | A                    | AF020072   |
|                  | *Aedes albopictus*            | A                    | AF020058   |
|                  | *Glossina morsitans*          | A                    | AF020079   |
|                  | *Drosophila simulans* (Riverside) | A                  | AF020070   |
|                  | *Muscidifurax uniraptor*      | A                    | AF020071   |
|                  | *Phlebotomus papatasii*       | A                    | AF020082   |
|                  | *Glossina austeni*            | A                    | AF020077   |
|                  | *Culex pipiens*               | B                    | AF020061   |
|                  | *Culex quinquefasciatus*      | B                    | AF020060   |
|                  | *Aedes albopictus*            | B                    | AF020059   |
|                  | *Aedes aegypti*               | B                    | MF999264   |
|                  | *Ephestia cautella*           | B                    | AF020076   |
|                  | *Dirofilaria immitis*         | C (outgroup)         | AJ252062   |
|                  | *Nasonia longicornis*         | A                    | M84691     |
|                  | *Muscidifurax uniraptor*      | A                    | L02882     |
|                  | *Aedes albopictus*            | B                    | KX155506   |
|                  | *Aedes aegypti*               | B                    | MF999263   |
|                  | *Culex pipiens*               | B                    | X61768     |
|                  | *Nasonia vitripennis*         | B                    | M84686     |
|                  | *Onchocerca volvulus*         | C                    | AF069069   |
|                  | *Dirofilaria immitis*         | C                    | Z49261     |
|                  | *Litomosoa westi*             | D                    | AJ548801   |
|                  | *Folsomia candida*            | E                    | AF179630   |
|                  | *Mansonella ozzardi*          | F                    | AJ279034   |
|                  | *Dipetalonema gracile*        | J                    | AJ548802   |
|                  | *Rickettsia sp.*              | Outgroup             | U11021     |

**Table 2** Summary of *wsp* and 16S rDNA detection results in *Ae. aegypti*

| Molecular marker | No. of individuals detected (%) | Female/(n = 379) | Male/(n = 293) | Female/male ratio |
|------------------|---------------------------------|------------------|----------------|------------------|
| *wsp*            | 113 (16.82)                     | 52               | 61             | 0.82             |
| 16S rDNA         | 89 (13.24)                      | 41               | 48             | 0.85             |
| *wsp* + 16S rDNA | 80 (11.90)                      | 36               | 44             | 0.82             |
the study criteria. In addition to these, 20 individual mosquito samples generated one successful 16S rDNA amplification, but were excluded in reporting the prevalence and further analysis. The female/male ratio was 0.85 (Table 2). Fifty (36.0%) sampling sites (Fig. 1c) contained Wolbachia-infected mosquitoes. Positive sampling sites had prevalence rates ranging from 3.9 to 100%. The distribution of 16S rDNA-positive individuals was revealed to be clustered or aggregated ($R = 0.001, P < 0.001$). All sequenced amplicons resulted in a high degree of similarity (> 98%) with 16S rDNA Wolbachia sequences in GenBank. Nearly all 16S rDNA sample sequences ($n = 84$) (Fig. 3, Additional file 3: Figure S2) belonged to supergroup B. Only one sample sequence was identical to the endosymbiont found in Nasonia vitripennis while 27 sample sequences were identical to Wolbachia isolated from Ae. aegypti. The remaining sample sequences from supergroup B were 99% similar from selected hosts of the supergroup. Five sample sequences were grouped together with Wolbachia hosts in supergroups C, D and J. Only one sample sequence was highly similar (> 99%) to Dirofilaria immitis while the remaining were 98–99% similar to the selected hosts of the supergroup.

Comparison of 16S rDNA and wsp for Wolbachia detection and phylogeny
A total of 80 (11.90%) individual samples yielded positive amplification in both markers (Table 2). In the wsp positive samples ($n = 113$), 80 had two successful amplifications of the 16S rDNA amplification while 27 had only one successful 16S rDNA amplification and the remaining 6 had no successful 16S rDNA amplification. For the 16S rDNA positive samples ($n = 89$), there were 80 individuals with two successful wsp amplification, while 9 had only one successful wsp amplification. We then focused on the supergroup classification of the 80 individual samples based on the wsp and 16S rDNA phylogeny. It was found that 55 samples (69%) belonged to supergroup B while the remaining 25 samples (31%) showed disparity. In certain instances, wsp identified an individual sample as supergroup A, but 16S rDNA revealed it as either supergroup B, C or J.

Discussion
In our study, we found a low infection rate (11%) of Wolbachia in the Ae. aegypti population studied. This finding coincides with the low infection rate
reported in Florida [16]; however, a higher infection rate (>50%) was observed in *Ae. aegypti* populations in Malaysia [14] and New Mexico [16]. It has been established that it is common to see varying *Wolbachia* infection rates of the same insect host from different geographical locations such as that observed in *Cx. quinquefasciatus* [28, 29] and *Cx. pipiens* [30–32]. The variation of infection rates could be driven by either genetic or environmental factors [16]. *Wolbachia* density in *Ae. albopictus* tends to decrease if exposed to increasing temperatures [33]. Removal of the endosymbiont from its host could be achieved by exposure to heat treatment or even antibiotics [34, 35]. The observed low infection rate could be attributed to the low density of the endosymbiont in *Ae. aegypti*. This is further supported by metabarcoding studies which yielded a low number (2–10) [16–18] of sequence reads in the midgut of *Ae. aegypti* which indicate a low probable density of the endosymbiont. Although our study did not measure the actual density, a 40-cycle PCR amplification procedure or long PCR run [36] was needed to amplify and confirm a positive infection of the endosymbiont in our *Ae. aegypti* samples.

Based on the results of our phylogenetic analysis, the *Wolbachia* strains found in our sampled *Ae. aegypti* belong to supergroups A and B. Both *wsp* and 16S rDNA phylogeny showed that the majority of the individual samples belong to supergroup B while a small number of individual samples belong to supergroup A (based on *wsp*). The same observation has been reported in previous studies [14–18]. Detecting different *Wolbachia* strains in a single mosquito species is relatively common, especially in medically-important mosquitoes such as *Ae. albopictus* [22, 37], *An. gambiae* [38] and other insect host species (e.g. *Drosophila* species [37]). Dipterans, especially mosquitoes, are commonly infected by *Wolbachia* strains from supergroups A and B. They have been shown to cause parasitism towards the insect host by producing phenotypic effects such as cytoplasmic incompatibility, male killing and feminization [39]. However, it remains unclear whether the identified *Wolbachia* strains in *Ae. aegypti* induce these phenotypic effects. Further studies are needed to confirm the pathogenic impact of this endosymbiont to the mosquito vector. It is also important to determine whether these identified *Wolbachia* strains could inhibit the replication of arboviruses such as dengue, rendering *Ae. aegypti* a less effective vector.

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Fig. 3 Phylogenetic analysis based on 16S rDNA. The alignment was analyzed in PhyML. Sample sequences of *Ae. aegypti* collected in Metropolitan Manila are in red, labeled as AAML. *Ae. aegypti* (Metropolitan Manila) and alphanumeric values indicate the unique code assigned to each *Ae. aegypti* individual sample. Merging (gray triangles) of sample and representative *Wolbachia* sequences was done to show degree of similarity (98–100%). Supergroups are indicated as A to J depending on the representative sequences used. The phylogenetic trees are re-drawn for better visualization; the expanded version is provided in Additional file 3: Figure S2. Please refer to Table 1 for the *Wolbachia* type sequences (ingroup and outgroup) for both markers.
vector. A few individual samples (n = 5) were shown to be similar to Wolbachia strains found in superfamilies C, D and J based on 16S rDNA. It is likely that our 16S rDNA amplified the Wolbachia strain found in the roundworm, Dirofilaria immitis, a parasitic nematode that Ae. aegypti mosquitoes also carry and transmit to certain mammals, such as dogs [40]. This observation was also reported in one of the metabarcoding studies that showed sequences of Wolbachia from Dirofilaria immitis. However, when these 16S rDNA results were compared to the wsp results in our study, it showed the Wolbachia wsp sample sequence of the same mosquito individuals belonged to supergroup B. We assume that this discordance may stem from the different mutation rates of the markers used. 16S rDNA is known to be a conserved gene; however, in some instances the typing system of this marker has been shown to be insufficient in establishing correct supergroup classification due to its low evolutionary rate [41]. This indicates a potential drawback of 16S rDNA as a less robust marker in estimating intraspecific phylogenetic relationship among Wolbachia supergroup members.

Previous studies reported the non-detection of Wolbachia in Ae. aegypti which is in contrast with both our results and with recent Wolbachia detection reports in this mosquito vector from India, Malaysia and the USA [14–16]. The reasons for these contrasting observations could be attributed to the following: (i) individual vis-à-vis pooled detection assays; (ii) procedural modifications; and (iii) sample size. Kulkarni et al. [16] emphasized that individual screening is more suitable in detecting Wolbachia in Ae. aegypti due to the low density load of the endosymbiont in the mosquito vector. They tested the sensitivity of a PCR assay containing a pool of 19 Wolbachia-negative individuals and one positive individual each from Ae. albopictus and Ae. aegypti. The results showed that Wolbachia could be detected in a pool containing DNA from a single positive Ae. albopictus but not in a pool containing DNA from a single Ae. aegypti specimen. These results strengthen the notion that Wolbachia prevalence studies in Ae. aegypti could be attributed due to pooled detection assays. Non-optimal DNA amplification and extraction methods could also compromise the results of detection assays. This was demonstrated and emphasized in studies detecting Wolbachia from An. gambiae [38, 42, 43].

Our study acknowledges the uncertainties associated with conventional PCR detection such as high false positive detection rates. With this in mind, we were cautious in affirming a positive infection in each Ae. aegypti adult sample. First, the selection of markers is based on the study of Simoes et al. [23] which produced low false positive and false negative rates. Secondly, our study performed replications with strict criteria for a successful Wolbachia infection in each mosquito sample. Additionally, a similar study conducted in the USA [16] identified individual mosquito samples with Wolbachia by conducting two rounds of PCR detection. Although there are several genetic markers (e.g. MLST genes) and techniques (e.g. IFA, FISH or whole-genome sequencing) available, our short report is limited in presenting the possible detection of Wolbachia using a conventional PCR-based approach. We are conducting similar experiments (see recent studies [15, 16]) to substantiate the infection status of Wolbachia in this mosquito vector. Mosquito colonies are now being reared in order to establish the maternal inheritance and persistence of Wolbachia infection through different mosquito developmental stages and generations.

**Conclusions**

The study demonstrated the detection of Wolbachia from field-collected Ae. aegypti in Metropolitan Manila, Philippines. Totals of 113 (16.8%) and 89 (13.2%) individual mosquito samples were determined to be infected with Wolbachia using the wsp and 16S rDNA markers, respectively. Overall, 80 (11.90%) individual mosquito samples showed positive amplifications in both markers, indicating a low infection rate. Our study supports previous studies that the potential Wolbachia strain in Ae. aegypti belongs to supergroup B. In addition, other Wolbachia strains (e.g. supergroup A) could potentially infect this mosquito vector.
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Authors' contributions

TMC, DMA and KW conceptualized and designed the experiment. TMC and KH directed the detection process using a molecular approach. TMC, DMA and KW performed the analysis. TMC and KW wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files. All newly generated sequences are available in the GenBank database under the Accession Numbers MN046588–MN046789.

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 Department of Civil and Environmental Engineering, Ehime University, Matsuyama, Japan. 2 Biology Department, De La Salle University, Taft Avenue, Manila, Philippines. 3 Biological Control Research Unit, Center for Natural Science and Environmental Research, De La Salle University, Taft Avenue, Manila, Philippines.

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