RESEARCH ARTICLE

Low-density genetically diverse natural Wolbachia infections in malaria vectors in Kayin state, Myanmar [version 2; peer review: 2 approved with reservations]

Previously titled: Natural Wolbachia infections in malaria vectors in Kayin state, Myanmar

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Abstract

Background: Natural Wolbachia infections in malaria mosquitoes were recently reported in Africa, and negatively correlated with the development of Plasmodium falciparum in the vectors. The occurrence and effects of Wolbachia infections outside Africa have not been described and may have been underestimated.

Methods: Mosquitoes were collected by human-landing catch during May and June 2017 in ten villages in Kayin state, Myanmar. Closely related species of malaria vectors were identified with molecular assays. Wolbachia infection rates were assessed with quantitative real-time PCR.

Results: Low titer of Wolbachia DNA was detected in 13/370 samples in six malaria vector species. Phylogenetic analysis based on 16S rRNA sequences revealed a high diversity of Wolbachia strains and identified lineages different from those described in the African malaria mosquitoes.

Conclusion: These low-density genetically diverse natural Wolbachia infections question the ecology and biology of Wolbachia-Anopheles interactions in Southeast Asia. Their effects on malaria transmission and mosquito vectors are yet to be determined.

Keywords

Wolbachia, Anopheles, Plasmodium, 16S rRNA, entomological inoculation rate, Southeast Asia, Kayin state, wAnga
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Introduction

Wolbachia are intracellular bacteria that infect a wide variety of arthropods and filarial nematodes. Symbiotic relationship that results from the infection have a broad range of phenotypic effects on the infected hosts, from mutualism (beneficial) to commensalism (neutral) and parasitism (harmful). In mosquitoes, Wolbachia can invade the germline and induce cytoplasmic incompatibilities between the sperm from infected males and oocytes from uninfected females. Hence, mass-releases of Wolbachia-infected male mosquitoes were attempted to extinguish mosquito populations. Cytoplasmic incompatibilities produce a fitness advantage of Wolbachia-infected over uninfected female mosquitoes, thereby driving the spread of Wolbachia-infected females in the population. In addition, Wolbachia can interfere with the development of some pathogens in the mosquito host, including dengue virus, Plasmodium malaria parasites and filarial nematodes. Therefore, the release of Wolbachia-infected female mosquitoes is proposed for transmission-blocking of some mosquito-borne diseases.

Most diversions of mosquito-Wolbachia interactions for controlling vector-borne diseases were conducted with mosquitoes artificially infected with the endosymbiotic Natural Wolbachia infections may have important effects on mosquito populations and dynamics of diseases transmission but they are less well described. Wolbachia DNA was detected by PCR in 27 mosquito genera including the medically important Aedes, Armigeres, Culex, Mansonia and Stegomyia. Interestingly, this organism was not detected in malaria mosquitoes until recent observations of naturally infected anopheline vectors in Africa. Only one study assessed the effects of natural Wolbachia infection on the reproductive fitness anopheline mosquitoes, namely the dominant African malaria vector Anopheles coluzzi. The authors did not observe cytoplasmic incompatibilities, differences in the number of eggs laid or progeny sex ratio, but infected females laid eggs more rapidly. Two studies demonstrated the negative effects of Wolbachia infections on the development of Plasmodium. Shaw et al. observed a negative correlation between Wolbachia infection and the development of Plasmodium in naturally blood-fed females. Gomes et al. obtained similar results on the sporozoite stage by screening large numbers of mosquitoes identified as An. gambiae sensu stricto and An. coluzzi. In addition to their field investigations, Gomes et al. infected a laboratory-adapted An. coluzzi colony with a local strain of Wolbachia, and performed artificial transmission studies with cultured gametocytes of P. falciparum strain Nf54. They observed a moderate yet significant positive correlation between Wolbachia infection and oocyst development, and a negative correlation between Wolbachia infection and the number of sporozoites that subsequently invaded the salivary glands.

Natural Wolbachia infections in Southeast Asian malaria vectors have not been reported. Their potential effects on Anopheles mosquitoes and dynamics of malaria transmission are not known. The objective of this study was therefore to assess Wolbachia infections in malaria vector populations in Kayin state, Myanmar.

Methods

Study sites and entomological collections

Entomological surveys were conducted in May and June 2017 in ten villages in Kayin state, Myanmar (Figure 1). Each survey consisted of five consecutive nights of collection from 06:00 pm to 06:00 am as described previously. In each village, five traditional houses were selected for mosquito sampling with human-landing catches. Collectors were asked to collect every mosquitoes landing on their uncovered legs for 50 min per hour and allowed to rest for 10 min per hour. Mosquitoes were shipped to Mae Sot (Thailand) at the end of each survey.

Malaria vectors identification

Mosquitoes were immediately identified at the genus level by morphology and Anopheles specimens were stored individually at -20°C in 1.5 mL plastic tubes containing silica gel. Anopheles were identified at the Group or Complex level using the key developed by Rattanarithikul et al. Closely related species in the Funestus, Maculatus and Leucosphyrus Groups were discriminated in a subsample of the total number of collected mosquito using allele-specific PCR assays (AS-PCR) adapted from Garros et al. and Walton et al. Single whole mosquitoes were crushed in 200 μl of cetyl-trimethylammonium bromide solution 2% (TrisHCl pH = 8, 20mM; EDTA 10mM; NaCl, 1.4 mM; N-cetyl-N,N,N-trimethyl ammonium bromide 2%) with a TissueLyser I™ (Qiagen) set on 29 movements/second for 3 minutes. Samples were then warmed at 65°C for 5 minutes and 200 μl of chloroform were added. The aqueous phase was collected and DNA was precipitated with 200 μl of isopropanol. After centrifugation at 20,000 g for

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15 minutes, the pellet was washed twice with 200 μl of 70% ethanol and suspended in 50 μl of PCR grade water. The PCR mix was composed of 1X Goldstar™ DNA polymerase (Eurogentec, Seraing, Belgium) and 400 nM of each primer (Funestus assay: ITS2A 5'-TGT GAA CTG CAG GAC ACA T-3', MIA 5'-CCC GTG CGA CTT GAC GA-3', MIC 5'-GTT CAT TCA GCA ACA TCA GT-3', ACO 5'-ACA GCG TGT AGC TCC AGT-3', PAM 5'-TGT ACA TCG GCC GGG GTA-3', VAR 5'-TTG ACC ACT TTC GAC GCA-3'; Maculatus assay: 5.8F 5'-TGT GAA CTG CAG GAC ACA T-3', MAC 5'-CCC GTG CGA CTT GAC GA-3', PSEU 5'-GTT CAT TCA GCA ACA TCA GT-3', SAW 5'-ACA GCG TGT AGC TCC AGT-3', K 5'-TGT ACA TCG GCC GGG GTA-3', DRAV 5'-TTG ACC ACT TTC GAC GCA-3' and Leucosphyrus assay: D-AC 5'-CAC AGC GAC TCC ACA CG-3', D-B 5'-CGG GAT ATG GGT CGG CC-3', D-D 5'-CCG GAC GCG GAC GTG CGG TT-3', D-F 5'-AAC GGC GGT CCC TTT TG-3', D-AC 5'-CAC AGC GAC TCC ACA CG-3'). The PCR was conducted in a total reaction volume of 25 μl (1 μl of DNA template and 24 μl of PCR mix). The thermocycling protocol consisted in an initial activation step of 1 minute at 94 °C, followed by 40 amplification cycles of 20 seconds at 94 °C, 20 seconds at the appropriate annealing temperature (45 °C for the Funestus assay, and 55 °C for the Maculatus and Leucosphyrus assays), and 30 seconds at 72 °C. The length of the PCR product was determined by gel electrophoresis in 2% agarose for 70 minutes at 120V. In case AS-PCR gave a negative result, amplification of ITS2 was performed using the primer pair ITS2A (5'-TGT GAA CTG CAG GAC ACA T-3') and ITS2B (5'-ATG CTT AGG GGG T-3') described by Beebe and Saul.

The PCR mix was composed of 1X Goldstar™ DNA polymerase (Eurogentec, Seraing, Belgium) and 400 nM of each primer. The PCR was conducted in a total reaction volume of 25 μl (1 μl of DNA template and 24 μl of PCR mix). The thermocycling protocol consisted in an initial activation step of 1 minute at 94 °C, followed by 40 amplification cycles of 20 seconds at 94 °C, 20 seconds at 51 °C and 30 seconds at 72 °C. PCR products were purified on site using the Illustra™ ExoStar™ PCR and Sequence Reaction Clean-Up Kit (GE Healthcare) according to manufacturer’s instruction. Macrogen (Seoul, South Korea) sequenced the purified PCR products off site with the ITS2A primer. Sequences were blasted against the

Figure 1. Map of the study area.
National Center for Biotechnology Information nucleotide database in order to determine the corresponding species (accession numbers MK358471 - MK358807).

Detection of Wolbachia DNA by quantitative real-time PCR

DNA extracts were screened for the presence of Wolbachia using an in-house quantitative real-time PCR (qPCR) assay that targets a 438 bp conserved region of the 16S rRNA genes with the primer pair W-Specf/W-Specr (5’-CAT ACC ACC TAT TCG AAG GGA TAG-3’ and 5’- AGC TTC GAG TGA AAC CAA TTC-3’). Without a priori knowledge on Wolbachia DNA sequences detected in this study, the primers were selected for their ability to detect most Wolbachia strains infecting insects and to establish phylogenetic relationships among isolates.

The performances of the primers W-Specf/W-Specr for the detection and quantitation of Wolbachia in mosquito samples were compared to that of the primers W-Specf/W16S used by Gomes et al. as described previously. Briefly, laboratory-reared Aedes aegypti artificially infected with Wolbachia strain wMel were used as a reference material. The optimal conditions for the PCR (hybridization temperature for primers annealing, and concentration of MgCl₂ and primers) were determined during a single gradient experiment in order to take into account cross-interactions between the different parameters. The range tested were 55-62 °C for the hybridization temperature, 2.5-4.5 mM of MgCl₂ and 100-400 nM of each primers. The reaction conditions that gave the smallest CP (optimal conditions) were selected for all subsequent experiments. Serial-dilution experiments were then carried out in order to verify PCR efficiency (EFF) and to estimate the standard curve parameters.

All experiments were conducted with a CFX-96® (Biorad) device. Reactions were conducted in 20μl of EvaGreen qPCR Mix Plus® (Euromexed); 5μl of DNA template was used in a total reaction volume of 25μl. The PCR mix was composed of 1X HOT FIREPol™ EvaGreen™ qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and 200 nM of each primer. The thermocycling protocol consisted in an activation step at 95°C for 15 minutes followed by 45 amplification cycles at 95°C for 15 seconds, 58°C for 15 seconds and 72°C for 20 seconds. PCR products were characterized by analyzing ampiclon melt curve (95°C for 15 seconds, 68°C for 1 minute, 80°C for 15 seconds, 60°C for 15 seconds, then 60°C to 90°C with an increment of 0.2°C per second). No template and positive controls were included in all runs. All samples and controls were tested in triplicates.

Specificity of the PCR was confirmed by Sanger sequencing with both W-Specf/W-Specr primers for all samples that give at least 1/3 positive reaction. Positive reaction was defined by the presence of a PCR product with the same melting temperature than the positive control at the end of the thermocycling.

Macrogen (Seoul, South Korea) performed both PCR product purification and sequencing off site to avoid contamination of our facilities with post-PCR amplicons. The sequences were used for phylogenetic analysis (accession numbers MK336794 - MK336806).

Data analysis

Human-biting rate was defined as the number of collected mosquitos divided by the corresponding number of collection-nights. Poisson confidence intervals were calculated using the epitools package version 0.5-10 in R software. Human-biting rate for sensu stricto species in the Funestus, Maculatus and Leucophyrs Groups was estimated using the relative proportion of the species in the corresponding group.

The limit of detection of the qPCR assay (LOD) was defined as the highest dilution (lowest concentration) that gave 100% of positive reactions. The performances of the two primer sets at low concentrations of Wolbachia were also compared by scoring the proportion of positive reactions as described previously. For the quantitation of Wolbachia load in positive samples, crossing-point (CP) values were determined using the regression algorithm of the analysis software of the PCR device (CFX Biorad Manager version 3.01, Biorad). CP values of standard samples the serial-dilution experiments were used to set-up the standard curve of the assay. The best fit-line and the subsequent values of the slope and y-intercept were estimated by performing least-square analysis of the linear portion of the curve (Pearson’s coefficient r²=0.990). PCR efficiency was estimated with the formula \( \text{EFF} = 10^{(-1/\text{slope})} - 1 \). Given that DNA was extracted from whole mosquitoes, it was possible to estimate the bacterial load in single mosquitoes without using a calibrator to normalize the signal. The relative quantitation of Wolbachia load in natural samples compared to that observed in Aedes aegypti artificially infected with Wolbachia strain wMel was calculated with the formula \( 10^{\Delta \text{CP}_{\text{target}}/\text{ref}} \), with \( \Delta \text{CP} \) defined as the difference in the mean CP values measured in the natural and reference DNA extracts respectively.

For the phylogenetic analysis, chimeric PCR products were detected with the DECIPHER software version 2.0 and excluded from subsequent analysis (4/17 samples with a positive PCR result). 16S ssuRNA sequences were blasted against the National Center for Biotechnology Information nucleotide database and the most similar sequence was downloaded. Reference Rickettsiales sequences were added and alignment was performed using the DECIPHER package version 2.10 in R software. DNA sequences were converted into RNA sequences and then aligned using the AlignSeqs() function set with default parameters in order to take into account base pairing and to use single-base and double-base substitution matrices. Tamura-Nei genetic distance model and neighbor-joining tree were computed with the ape package version 5.2 of the R software. There was 373 positions in the final dataset.

Ethical considerations

This project was approved through the ethics review committee on medical research involving human beings from Myanmar, Ministry of Health and Sports, Department of Medical Research
(lower Myanmar): 73/Ethics 2014. All participants provided their written consent to participate in this study.

Results

qPCR assay validation for the detection of Wolbachia in mosquitoes

Optimal reaction conditions were similar for both primer sets: 58°C for primer annealing (range tested= 55-62 °C), 2.5 mM of MgCl2 (range tested= 2.5-4.5 mM) and 200 nM of each primers (range tested= 100-400 nM). In these conditions, PCR efficiency was 108 and 110% with the primer sets W-Specf/W-Specr and W-Specf/W16S respectively, and the linear dynamic spanned over six orders of magnitude (r²=0.998 and 0.999) (Table 1). There was a one-log decrease in the LOD of the assay when using W16S as a reverse primer instead of W-Specr, and the assay scored better at low concentrations of Wolbachia (16/18 and 12/18 positive reactions respectively, χ²= 2.5714, P=0.109).

Biodiversity of Anopheles mosquitoes

Four thousand seven hundreds forty-three Anopheles were collected during 500 person-nights of collection. We report the occurrence of 12 Anopheles taxa among which nine were groups of closely related or sibling species (Maculatus, Funestus, Jamesi, Leucosphyrus, Annularis, Barbirostris, Subpictus, Hycanas and Asiaticus Groups) and only three were sensu stricto species (An. karwari, An. kochi and An. tessellatus). A subsample of 1098 mosquitoes in the Maculatus, Funestus and Leucosphyrus Groups were identified at the species level with molecular assays. The most frequently detected species were An. maculatus (s.s.), An. sawadwongporni and An. pseudowillmorni in the Maculatus Group, An. minimus (s.s.), An. calificacies B and An. jeyporiensis in the Funestus Group and An. baimaii in the Leucosphyrus Group (Table 2).

Natural Wolbachia infections in malaria vectors

Six Anopheles species were screened for Wolbachia infection namely An. maculatus (s.s.), An. sawadwongporni, An. pseudowillmorni (Maculatus Group), An. minimus (s.s.) (Funestus Group, Minimus Complex), An. dirus (s.s.) and An. baimaii (Leucosphyrus Group, Dirus Complex). Wolbachia DNA was detected in 13/370 samples (Table 3). Eight unique 16S rRNA sequences were identified (Figure 3). 16S rRNA sequences clustered with that of Wolbachia strains in the supergroups B, D and F (Figure 4). The detection of Wolbachia in the supergroup D and F suggests that some DNA extracts were contaminated with Wolbachia endosymbionts of filarial nematodes rather than reflecting actual Wolbachia infections in mosquitoes.

Crossing-point values ranged from 31.0 to 40.6 amplification cycles. Infected specimens were generally infected at a density close or below the limit of detection of the assay (only one sample gave 3/3 positive reactions) (Table 4). The median Wolbachia load in those naturally infected specimens was seven orders of magnitude less than that observed in laboratory-reared Aedes aegypti artificially infected with Wolbachia strain wMel.

Table 1. Results of the serial dilution experiments.

<table>
<thead>
<tr>
<th>Primers (%EFF, r²)</th>
<th>Parameter</th>
<th>Value of the parameter at the indicated dilution</th>
<th>Score (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not diluted</td>
<td>10⁻¹</td>
</tr>
<tr>
<td>W-Specf/W-Specr</td>
<td>Nb. pos. / Nb. tested</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td>(108%, 0.999)</td>
<td>Mean CP value</td>
<td>18.81</td>
<td>21.26</td>
</tr>
<tr>
<td></td>
<td>Intra-assay SD</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Inter-assay SD</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>W-Specf/W16S</td>
<td>Nb. pos. / Nb. tested</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td>(110%, 0.999)</td>
<td>Mean CP value</td>
<td>17.80</td>
<td>20.71</td>
</tr>
<tr>
<td></td>
<td>Intra-assay SD</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Inter-assay SD</td>
<td>0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>

- %EFF : efficiency (EFF) of the PCR was calculated with the formula EFF = 10^(-100/(-r² × 2.32)) × 1 and expressed as a percentage. An efficiency of 100% corresponds to a slope of -3.32 and means that the number of amplicons doubles after each cycle of amplification. *r²* : Pearson’s correlation coefficient expressing the intensity of the relationship between the logarithm of the concentration and the mean CP value. 
- χ²: Pearson's correlation coefficient (0 (no correlation) and 1 (perfect correlation), a value >0.990 testify of the linearity of the method (over a defined linear range) and allow an accurate quantification. *r²* and EFF have been calculated on the linear dynamic of each curve (bold cells).
- Nb. pos. / Nb. tested: number of positive reactions (amplification of the PCR DNA target) / total of reactions performed at a given dilution.
- Intra-assay SD : intra-assay standard deviation (SD), calculated as the average SD of the mean CP values measured for each dilution during the same experiment.
- Inter-assay SD : inter-assay standard deviation (SD), calculated as the SD of the means CP values measured during two independent experiments.
- score of the proportion of positive reactions at low concentrations of Wolbachia (score was calculated on dilutions 10⁻¹ and 10⁻²); an example of the calculation of the score is given here: the maximum hit for the score is 18 reactions (9 at the dilution 10⁻¹, +9 at the dilution 10⁻²), the score obtained with the primer pair W-Specf/W-Specr is 66% (12/18=(7+5)/18).
Figure 2. Typical result of the qPCR assay used for Wolbachia detection in mosquito samples. A) W-Specf/W-Specr primers; B) W-Specf/W16S primers. Left panels show amplification curves and right panels show the melt curve of the PCR products. (*) primer dimers, (**) PCR DNA target.

Discussion
This is the first report of natural Wolbachia infections in malaria vectors in Southeast Asia, including An. maculatus (s.s.), An. sawadwongporni, An. pseudowillmori (Maculatus Group), An. dirus (s.s.) and An. baimaii (Dirus Complex, Leucosphyrus Group). After excluding Wolbachia strains in the supergroups D and F from the dataset (which probably were endosymbionts of filarial nematodes rather than true mosquito infections), Wolbachia was detected in 5/6 species and in 3/10 villages, which suggests that natural infection in malaria mosquitoes is relatively common in Kayin state.

There were some limitations to our quantitative data: quantitation was not absolute, we did not take into account potential variations in the yield of the DNA extraction step and we did not assessed the presence of PCR inhibitors. Nevertheless, CP values reported in this study suggest that the bacterial loads in Wolbachia-infected samples were very low, usually close or below the limit of detection of our assay. This result is not compatible with the integration of Wolbachia DNA in the mosquito genome, which would have given much lower CP values\(^1\). Important precautions were taken to ensure the quality of our molecular data\(^1\). This was the first study on Wolbachia in our facilities. The 16S DNA sequences detected in the screened samples were different from that of the reference material, hence excluding cross-contaminations. In addition, all experiments were conducted with the real-time PCR technology (which allows amplification and detection of the PCR DNA target in a closed system) and great care was taken to perform all handlings of PCR products off site. These precautions, combined with the good laboratory practices relevant to molecular diagnosis (eg. dedicated facilities with unidirectional workflow, experiment conducted by qualified laboratory technicians and appropriate quality controls), drastically limited the risk of false positive by contamination. The risk of false positive results due to low specificity of the assay was ruled out by sequencing the PCR product in all positive samples. It is probable that some results were falsely negative due to limited sensitivity, given that most positive samples were infected at a density close of below the detection of the assay. In this study, we have shown that using the W16S as a reverse primer increases the analytical sensitivity of the qPCR assay in the optimal reaction conditions. However, in the absence of a priori data on the Wolbachia DNA sequences detected in this study, we selected the W-Specf/W-Specr primers to perform the screening because of their availability to detect a wide variety of Wolbachia infecting insects and to establish phylogenetic relationships among field isolates\(^1\).
## Table 2. Village-collated human-biting rate estimates of *Anopheles* mosquitoes.

<table>
<thead>
<tr>
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<td></td>
<td></td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
</tr>
<tr>
<td><strong>Annularis</strong></td>
<td><em>An. annularis</em> (s.l.)</td>
<td>40.2 (31.2-51.1)</td>
<td>28.8 (21.2-38.2)</td>
<td>24 (17.1-32.7)</td>
<td>29.4 (21.8-38.9)</td>
<td>4.2 (1.7-8.7)</td>
<td>30 (22.3-39.6)</td>
<td>40.2 (31.2-51.1)</td>
<td>65.4 (53.7-78.9)</td>
<td>7.2 (3.7-12.6)</td>
<td>28.8 (21.2-38.2)</td>
</tr>
<tr>
<td><strong>Asianus</strong></td>
<td><em>An. asiaticus</em> (s.l.)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0.6 (0-3.3)</td>
<td>0 (0-2.2)</td>
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<tr>
<td><strong>Barbirostris</strong></td>
<td><em>An. barbirostris</em> (s.l.)</td>
<td>27 (19.7-36.1)</td>
<td>2.4 (0.7-6.1)</td>
<td>16.8 (11.2-24.3)</td>
<td>3.6 (1.3-7.8)</td>
<td>5.4 (2.5-10.3)</td>
<td>10.2 (5.9-16.3)</td>
<td>3.6 (1.3-7.8)</td>
<td>24.6 (17.7-33.4)</td>
<td>6 (2.9-11.1)</td>
<td>6 (2.9-11.1)</td>
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<td></td>
<td><em>An. aconitus</em></td>
<td>3 (1-7)</td>
<td>1.2 (0.1-4.3)</td>
<td>9.6 (5.5-15.6)</td>
<td>6 (2.9-11)</td>
<td>3.6 (1.3-7.8)</td>
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<td>4.2 (1.7-8.7)</td>
<td>1.8 (0.4-5.3)</td>
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</tr>
<tr>
<td></td>
<td><em>An. culicifacies</em> A</td>
<td>9 (5-14.8)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>4.2 (1.7-8.7)</td>
<td>0.6 (0-3.3)</td>
<td>0 (0-2.2)</td>
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<td><em>An. culicifacies</em> B</td>
<td>36.6 (28-47)</td>
<td>7.2 (3.7-12.6)</td>
<td>0 (0-2.2)</td>
<td>73.8 (61.3-88.1)</td>
<td>12.6 (7.8-19.3)</td>
<td>123.6 (107.3-141.7)</td>
<td>94.2 (80-110.1)</td>
<td>7.8 (4.2-13.3)</td>
<td>1.8 (0.4-5.3)</td>
<td>26.4 (19.2-35.4)</td>
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<td><em>An. harrisoni</em></td>
<td>3 (1-7)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>4.2 (1.7-8.7)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
</tr>
<tr>
<td></td>
<td><em>An. jeyporiensis</em></td>
<td>3 (1-7)</td>
<td>1.2 (0.1-4.3)</td>
<td>12.6 (7.8-19.3)</td>
<td>6 (2.9-11)</td>
<td>44.6 (34.9-55.7)</td>
<td>3.6 (1.3-7.8)</td>
<td>3 (1-7)</td>
<td>78.6 (65.7-93.3)</td>
<td>3.6 (1.3-7.8)</td>
<td>0 (0-2.2)</td>
</tr>
<tr>
<td></td>
<td><em>An. minimus</em></td>
<td>161.4 (142.7-181.9)</td>
<td>46.8 (37-58.4)</td>
<td>240 (217.1-264.7)</td>
<td>185.4 (165.3-207.3)</td>
<td>96 (81.7-112.1)</td>
<td>167.4 (148.3-188.2)</td>
<td>141.6 (124.1-160.9)</td>
<td>259.2 (235.3-284.8)</td>
<td>141 (123.5-160.2)</td>
<td>52.2 (41.8-64.4)</td>
</tr>
<tr>
<td><strong>Funestus</strong></td>
<td><em>An. varuna</em></td>
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<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>4.2 (1.7-8.7)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
</tr>
<tr>
<td><strong>Hyrcaus</strong></td>
<td><em>An. hyrcanus</em> (s.l.)</td>
<td>0.6 (0-3.3)</td>
<td>0.6 (0-3.3)</td>
<td>2.4 (0.7-6.1)</td>
<td>0.6 (0-3.3)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>2.4 (0.7-6.1)</td>
<td>0.6 (0-3.3)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
</tr>
<tr>
<td><strong>Jamesii</strong></td>
<td><em>An. jamesii</em> (s.l.)</td>
<td>140.4 (123-159.6)</td>
<td>27 (19.7-36.1)</td>
<td>9 (5-14.8)</td>
<td>15.6 (10.2-22.9)</td>
<td>8.4 (4.6-14.1)</td>
<td>15.6 (10.2-22.9)</td>
<td>46.2 (36.5-57.7)</td>
<td>141.6 (124.1-160.9)</td>
<td>119.4 (103.4-137.2)</td>
<td>44.4 (34.9-55.7)</td>
</tr>
<tr>
<td><strong>Kochi</strong></td>
<td><em>An. kochi</em></td>
<td>37.8 (29-48.4)</td>
<td>1.8 (0.4-5.3)</td>
<td>3 (1-7)</td>
<td>0.6 (0-3.3)</td>
<td>55.8 (45-68.4)</td>
<td>6.6 (3.3-11.8)</td>
<td>15.6 (10.2-22.9)</td>
<td>36 (27.5-46.3)</td>
<td>54 (43.4-66.4)</td>
<td>2.4 (0.7-6.1)</td>
</tr>
<tr>
<td></td>
<td><em>An. baimai</em></td>
<td>31.8 (23.8-41.6)</td>
<td>1.8 (0.4-5.3)</td>
<td>24.6 (17.7-33.4)</td>
<td>10.2 (5.9-16.3)</td>
<td>13.2 (8.3-20)</td>
<td>18.6 (12.6-26.4)</td>
<td>15 (9.7-22.1)</td>
<td>24 (17.1-32.7)</td>
<td>11.4 (6.9-17.8)</td>
<td>6.6 (3.3-11.8)</td>
</tr>
<tr>
<td></td>
<td><em>An. dirus</em></td>
<td>2.4 (0.7-6.1)</td>
<td>0 (0-2.2)</td>
<td>1.2 (0-4.3)</td>
<td>0 (0-2.2)</td>
<td>1.8 (0.4-5.3)</td>
<td>0 (0-2.2)</td>
<td>1.8 (0-4.5)</td>
<td>0.6 (0-3.3)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
</tr>
<tr>
<td><strong>Leucophybus</strong></td>
<td><em>An. introlatus</em></td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-5.3)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
</tr>
</tbody>
</table>
### Human-biting rate estimate (95%CI) in the indicated dilution, expressed in number of bites/person/month

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Funestus</td>
<td>An. minimus</td>
<td>241.2 (218.2-266)</td>
<td>40.8 (31.7-51.7)</td>
<td>88.2 (74.5-103.7)</td>
<td>26.4 (19.2-35.4)</td>
<td>59.4 (48.3-72.3)</td>
<td>61.8 (50.4-75)</td>
<td>226.8 (204.5-250.9)</td>
<td>1112.4 (1062.3-1164.2)</td>
<td>315 (288.6-343.1)</td>
<td>39 (30.1-49.7)</td>
</tr>
<tr>
<td>Maculatus</td>
<td>An. maculatus</td>
<td>6.6 (3.3-11.8)</td>
<td>3.6 (1.3-7.8)</td>
<td>0 (0.2-2.2)</td>
<td>0.6 (0-3.3)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
<td>38.4 (29.6-49)</td>
<td>0 (0-2.2)</td>
<td>0 (0-2.2)</td>
</tr>
<tr>
<td>Subpictus</td>
<td>An. sawadwongporni</td>
<td>79.8 (66.8-94.6)</td>
<td>40.8 (31.7-51.7)</td>
<td>41.4 (32.2-52.4)</td>
<td>13.2 (8.3-20)</td>
<td>5.4 (2.5-10.3)</td>
<td>58.8 (47.7-71.7)</td>
<td>137.4 (120.2-156.4)</td>
<td>24.6 (17.7-33.4)</td>
<td>182.4 (162.5-204.1)</td>
<td>119.4 (103.4-137.2)</td>
</tr>
<tr>
<td>Tessellatus</td>
<td>An. tessellatus</td>
<td>3.6 (1.3-7.8)</td>
<td>0 (0-2.2)</td>
<td>24 (17.1-32.7)</td>
<td>2.4 (0.7-6.1)</td>
<td>0 (0-2.2)</td>
<td>14.4 (9.2-21.4)</td>
<td>8.4 (4.6-14.1)</td>
<td>1.2 (0.1-4.3)</td>
<td>3.6 (1.3-7.8)</td>
<td>0.6 (0-3.3)</td>
</tr>
</tbody>
</table>

* primary malaria vectors; ** secondary malaria vectors; *** efficient malaria vector species in some areas that were never reported infected with human malaria parasites on the Thailand-Myanmar border. Human-biting rates of sensu stricto anopheline species in the Funestus, Maculatus and Leucosphyrus Groups were estimates from the relative proportion of each species in the corresponding Group assessed with molecular assays.

### Table 3. Results of the screening for natural Wolbachia infections in the ten villages.

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Funestus</td>
<td>An. minimus</td>
<td>0/10</td>
<td>2/10 (F)</td>
<td>0/10</td>
<td>Q/10</td>
<td>Q/10</td>
<td>1/10 (D)</td>
<td>0/10</td>
<td>1/10 (D)</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Maculatus</td>
<td>An. maculatus</td>
<td>0/10</td>
<td>Q/10</td>
<td>0/10</td>
<td>2/10 (B)</td>
<td>Q/10</td>
<td>Q/10</td>
<td>1/10 (F)</td>
<td>0/11</td>
<td>0/11</td>
<td>1/9 (B)</td>
</tr>
<tr>
<td></td>
<td>An. pseudowillmori</td>
<td>0/1</td>
<td>Q/1</td>
<td>Q/1</td>
<td>1/7 (B)</td>
<td>Q/1</td>
<td>Q/1</td>
<td>Q/1</td>
<td>Q/1</td>
<td>Q/1</td>
<td>Q/1</td>
</tr>
<tr>
<td></td>
<td>An. sawadwongporni</td>
<td>0/8</td>
<td>Q/3</td>
<td>Q/9</td>
<td>Q/10</td>
<td>1/1 (B)</td>
<td>Q/10</td>
<td>Q/11</td>
<td>0/10</td>
<td>0/4</td>
<td>0/2</td>
</tr>
<tr>
<td>Leucopshyrus</td>
<td>An. baimaii</td>
<td>0/10</td>
<td>Q/2</td>
<td>0/10</td>
<td>1/10 (D)</td>
<td>Q/11</td>
<td>Q/10</td>
<td>0/10</td>
<td>1/16 (B)</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>An. dirus</td>
<td>0/4</td>
<td>Q/2</td>
<td>Q/2</td>
<td>Q/3</td>
<td>1/1 (B)</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>
Figure 3. Multiple alignment of 16S RNA sequences used to build the Tamura-Nei genetic distance model and neighbor-joining tree.
Figure 4. Phylogenetic analysis based on the alignment of a conserved region of the 16S rRNA gene using *Wolbachia*-specific primer pair W-Specf/W-Specr. Sequences of the PCR products were blasted against the NCBI nucleotide database and the most similar result was downloaded. A phylogenetic tree was reconstructed using a Tamura-Nei genetic distance model and neighbor joining. Sequences from other non-*Wolbachia* proteobacteria were also included, and the sequence from *Rickettsia japonica* was used as the reference outgroup. There was 373 positions in the final dataset. Nodes with bootstrap support <50% were collapsed. Study samples were labeled with the host name and the study village, and the accession number reported into the brackets. Formally named *Wolbachia* strains were labeled with their abbreviation: wNo is a symbiont of *Drosophila simulans*, wCne of *Ctenocephalides felis*, wAlbB of *Aedes albopictus*, wAnga of *An. gambiae*, wMel of *Drosophila melanogaster*, wPeJe1 of *Penicillidia jenynsi* and wBrU of *Brugia malayi*.

Table 4. qPCR results of the *Wolbachia*-infected samples detected during the screening.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Village</th>
<th>Species</th>
<th>Nb pos</th>
<th>CP1</th>
<th>CP2</th>
<th>CP3</th>
<th>Supergroup</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>HG-369</td>
<td><em>An. minimus</em></td>
<td>1</td>
<td>35.8</td>
<td></td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>2</td>
<td>HG-369</td>
<td><em>An. minimus</em></td>
<td>1</td>
<td>33.0</td>
<td></td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>3</td>
<td>MK-3633</td>
<td><em>An. baimaii</em></td>
<td>1</td>
<td>35.6</td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>4</td>
<td>MK-3635</td>
<td><em>An. maculatus</em></td>
<td>1</td>
<td>34.3</td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>5</td>
<td>MK-3635</td>
<td><em>An. maculatus</em></td>
<td>1</td>
<td>34.3</td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>6</td>
<td>MK-3635</td>
<td><em>An. pseudowillmori</em></td>
<td>1</td>
<td>37.6</td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>7</td>
<td>MK-3635</td>
<td><em>An. sawadwongporni</em></td>
<td>2</td>
<td>34.5</td>
<td>32.8</td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>8</td>
<td>NT-361</td>
<td><em>An. minimus</em></td>
<td>3</td>
<td>36.8</td>
<td>35.8</td>
<td>36.6</td>
<td>D</td>
</tr>
<tr>
<td>9</td>
<td>TG-357</td>
<td><em>An. maculatus</em></td>
<td>1</td>
<td>34.2</td>
<td></td>
<td></td>
<td>F</td>
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<tr>
<td>10</td>
<td>TP-339</td>
<td><em>An. baimaii</em></td>
<td>3</td>
<td>33.0</td>
<td>31.0</td>
<td>32.3</td>
<td>B</td>
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<tr>
<td>11</td>
<td>TP-339</td>
<td><em>An. dirus</em></td>
<td>1</td>
<td>34.1</td>
<td></td>
<td></td>
<td>B</td>
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<tr>
<td>12</td>
<td>TP-339</td>
<td><em>An. minimus</em></td>
<td>1</td>
<td>40.6</td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>13</td>
<td>WM-367</td>
<td><em>An. maculatus</em></td>
<td>1</td>
<td>32.6</td>
<td></td>
<td></td>
<td>B</td>
</tr>
</tbody>
</table>
Molecular phylogeny based on 16S rRNA sequences revealed a high diversity of Wolbachia strains, which belonged to different lineages than those recently reported in the African malaria vectors\(^{15-24}\). Eight out of thirteen sequences reported in this study were unique. The DNA extracts were also used to assess Plasmodium infection rates in the mosquito population (data not shown), precluding multi locus sequence typing of the Wolbachia strains because there was no material remaining after the screening. The detection of low titers of Wolbachia DNA by PCR is not unequivocal of an actual symbiosis between Wolbachia and the mosquito. Chrostek and Gerth argued that the high diversity of Wolbachia sequences combined with the very low titers detected was incompatible with the notion of a stable, intraovarially-transmitted Wolbachia symbiont in An. gambiae\(^6\). An alternative explication could be that horizontal transfers of Wolbachia happens at a much higher frequency than previously thought, for example via plants\(^{37}\) or via ectoparasitic mites\(^{18,19}\). High-resolution imaging of wild-caught specimen should be attempted in order to better characterize Wolbachia-mosquito interactions in this setting.

Finally, we did not assess the effects of Wolbachia infection on the phenotype of infected mosquitoes and dynamics of malaria transmission. In Kayin state, malaria transmission is low, seasonal and unstable. Plasmodium infection rate is usually less than 1% and often nil in the mosquito populations\(^{36}\). Therefore, it was not possible to establish direct correlations between Plasmodium and Wolbachia infection rates in the mosquito vectors. In this setting, the effect of Wolbachia infections on malaria transmission may be better assessed by performing artificial infections of field-collected mosquitoes with Plasmodium malaria parasites.

Conclusion
The low-density genetically diverse natural Wolbachia infections reported in this study warrant further investigations to understand better the ecology and biology of Anopheles-Wolbachia interactions in Southeast Asia.

Data availability
The data is available upon request to the Mahidol Oxford Tropical Medicine Research Unit Data Access Committee (http://www.tropmedres.ac/data-sharing) and following the Mahidol Oxford Tropical Medicine Research Unit data access policy (http://www.tropmedres.ac/_asset/file/data-sharing-policy-v1-0.pdf).

Grant information
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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments
We thank to the communities from the study villages for their support to the study, and to the SMRU Entomology Department for their work. Wolbachia-infected reference samples were kindly provided by Dr. Lauren Carrington from the Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam. SMRU is part of the Mahidol Oxford University Research Unit, supported by the Wellcome Trust of Great Britain.

References

15. Kitayayong P, Baitsle KJ, Baimai V, et al.: Distribution and diversity of Wolbachia...
Figure 2 has been added to show the typical result of the qPCR assay used for Wolbachia detection in mosquito samples. The Wspec results (panel A) show that CP values were generated from no template controls (some below 35) and melt curve analysis was required to differentiate primer dimers from genuine target amplification. With Wolbachia gene qPCR I would not have expected no template control CPs to come up at below 35 cycles and this could be due to trying to amplify a 16S gene fragment >400 base pairs (the first report of this to my knowledge). According to Table 1, the threshold CP value for using the Wspec primer set appears to be between 30-33 cycles given only 7/9 of the 10^-5 dilution amplified. This would be further supported by the CP of 10^-5 (34.95 +/- 1.64) not being statistically higher that to the 10^-6 dilution (34.48 +/- 0.33). The authors rightly conclude that all of the samples in this study are at threshold detection (CP values >31 cycles) and the authors correctly used both melt curve analysis and sequencing to confirm this was genuine amplification. Further details of the qPCR analysis strengthens the manuscript but I’m not entirely sure how the authors estimated bacterial load. The sentence ‘Given that DNA was extracted from whole mosquitoes, it was possible to estimate the bacterial load in single mosquitoes without using a calibrator to normalize the signal’ needs further clarification, particularly given the title has changed to include reference to ‘low density strains’. The CP values would suggest low density infections but in order to make the comparison to wMel in Aedes aegypti you would need to account for body size and/or extraction efficiency as these factors will influence Wolbachia CP values and therefore estimating bacterial load. Lab-reared Aedes aegypti adults will likely be larger in size than wild caught Anopheles species so that comparison is not possible without either normalising to host genes or measuring the total DNA extracted. I also would suggest a better measure of prevalence rates would have resulted from using the Wspec primer set in the conventional PCR format (Werren & Windsor, 2000) which has been routinely used to screen mosquito populations.

Figure 4 shows the phylogeny of the Wolbachia 16S gene and it appears that the sequences are quite diverse within individual species (eg. An. maculates appearing to have four different strains) and the same strains appear across multiple species (eg. An. minimus and An. baimai). Although Wolbachia superinfections exist in mosquito species, having the same strain of Wolbachia (based on 16S sequences) in multiple species would need confirmation from additional gene sequencing as this seems
unlikely for endosymbiotic *Wolbachia* bacteria. The discussion does now contain an explanation that no material was left for MLST due to assessing *Plasmodium* infection rates despite having 50 μL of eluted DNA. Overall the reliance on only 16S sequences (some of which appear to have identical sequences across multiple *Anopheles* species) is still problematic in my opinion and I would think a title that contains ‘genetically diverse’ is inappropriate based on sequencing of only one *Wolbachia* gene fragment.

**References**


**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Medical entomology, Wolbachia, Anopheles

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
development of *P. falciparum* oocysts in naturally blood-fed females’. This is not completely correct, as in this work the authors have quantified the prevalence of *P. falciparum* in resting blood fed females inside house 5 days after collection/blood feeding, without any prior knowledge on the infectious status of the mosquitoes; thereby, the stage of parasite infection (oocyst or sporozoite) was not investigated.

**Methods:**
Authors indicate Shaw et al. and Gomes et al. as references for the qPCR using W-Spec primers against Wolbachia 16S. This is where I am getting confused, as Shaw et al. did not use qPCR and Gomes et al. use a different primer set for qPCR. The methodology should be clarified.

If W-Spec primers were used, the expected product size is >400 base pairs (bp); this bp size is often too large for qPCR, as large amplicons tend to produce secondary structures during the dissociation steps, thus resulting in multiple melting peaks. If possible, it would be informative to provide more details on the optimization of this assay (in case additional reagents were added, for example) and to show the dissociation profile of the obtained amplicons, as this would enable troubleshooting of the technique if others will try to replicate the work and/or use the same methodology. Also, it should be specified how sequencing was performed, e.g. direct purification after qPCR (how?), which primer was used, etc.

In the phylogenetic analysis the authors should state what was the sequence size used to build the tree. Novel obtained sequences should also be uploaded and their unique identifier indicate in the article.

Statistical analysis used in Table 1-3 should be indicated (if any).

**Results:**
The authors state the ‘we assessed species diversity, Plasmodium and Wolbachia infection rates in these Groups’. This is incorrect as Plasmodium infection rates are not shown.

Captions in Table 1-3 are missing information on what each column indicates. Although these tables can be generally informative, I wonder if showing species diversity using pie-charts (for each species group) over imposed on the map in Figure 1 would provide a more direct illustration of the species composition and abundances of the Anopheles species in the study area.

As indicated in the methods, the size of the sequence used for Figure 2 should be indicated. It would be informative to include the alignment use for the tree figure.

Figure 3b shows the overall prevalence of Wolbachia in different villages without specifying the species, so I am not sure what is the purpose of illustrating the result in this way. If this is too show that some villages have higher prevalence over others this should be indicated only if statistical analysis supports it (although I doubt this is the case if species distribution is included as a variable).

The authors state that ‘Crossing-point values ranged from 31.0 to 40.6 amplification cycles. Infected specimens were generally infected at a density close or below the limit of detection of the assay (only one sample gave 3/3 positive reactions).’ More details should be given regarding the rational for inclusion (or exclusion) of an infected/amplified sample; it is not clear to me if ‘reactions’ refers to technical replicates in the same qPCR run or in different qPCR assays. This should be described with more details. It would also be informative to normalize the quantity of the amplified Wolbachia 16S using a mosquito housekeeping gene, for example. Indeed, as ‘density [was] close or below the limit of detection of the assay’ normalization would provide information on the likelihood of false negatives in samples, as you would expect if the total DNA is very low (for example due to inefficient DNA extraction).
Discussion:
The authors state that ‘Our data and African studies confirm that the occurrence of natural Wolbachia infections has been underestimated in malaria mosquitoes.’ As direct assessment of Wolbachia prevalence on samples previously identified as uninfected was not performed here (nor in African samples) it cannot be ruled out that previous Wolbachia negative samples were not true negatives, so this work (and others) only suggest possible underestimation in previous works, as they have not directly confirmed it.

The authors state that ‘It was not possible to study more in detail the phylogeny of Wolbachia strain detected in this study by multi locus sequence typing because of the lack of DNA extracts after the screening.’ It is not clear to me if any attempts were made at all or not. If so, please give more details on the targeted genes and discuss why these could not be amplified.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** I believe that I have an appropriate level of expertise to assess the submitted article. I have expertise in identification of natural Wolbachia infections in natural populations of Anopheles and other vectors; I have also expertise in the methods used, mainly quantitative PCR and phylogenetic analysis.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
We thank to the reviewer for his useful feedback on the manuscript. Additional details on the qPCR assay used for Wolbachia detection and on the phylogenetic analysis were added to the revised manuscript. Answer to point-specific comments are given below:

- The authors state that cytoplasmic incompatibility (CI) ‘enhances the “vertical” transmission of Wolbachia’. From my knowledge this is incorrect, as CI does not directly affect “vertical” transmission, but rather increases ‘indirectly’ the fitness of the progeny of Wolbachia infected mothers.

Reviewer’s comment has been addressed in the revised version of the manuscript.

- The authors state that ‘Shaw et al. observed a negative correlation between Wolbachia infection and the development of P. falciparum oocysts in naturally blood-fed females’. This is not completely correct, as in this work the authors have quantified the prevalence of P. falciparum in resting blood fed females inside house 5 days after collection/blood feeding, without any prior knowledge on the infectious status of the mosquitoes; thereby, the stage of parasite infection (oocyst or sporozoite) was not investigated.

Reviewer’s comment has been addressed in the revised version of the manuscript.

- Authors indicate Shaw et al. and Gomes et al. as references for the qPCR using W-Spec primers against Wolbachia 16S. This is where I am getting confused, as Shaw et al. did not use qPCR and Gomes et al. use a different primer set for qPCR. The methodology should be clarified.

In this study, we adapted an in-house real-time PCR assay with legacy primers describe previously (W-Spec forward and reverse primers). More details and appropriate reference were added in the paragraph on Wolbachia detection in the Methods in section.

- If W-Spec primers were used, the expected product size is >400 base pairs (bp); this bp size is often too large for qPCR, as large amplicons tend to produce secondary structures during the dissociation steps, thus resulting in multiple melting peaks. If possible, it would be informative to provide more details on the optimization of this assay (in case additional reagents were added, for example) and to show the dissociation profile of the obtained amplicons, as this would enable troubleshooting of the technique if others will try to replicate the work and/or use the same methodology. Also, it should be specified how sequencing was performed, e.g. direct purification after qPCR (how?), which primer was used, etc.

Additional information on the validation of the assay and sequencing of the PCR products were added to the revised version of the manuscript as per reviewer’s suggestion.

- In the phylogenetic analysis the authors should state what was the sequence size used to build the tree. Novel obtained sequences should also be uploaded and their unique identifier indicate in the article.

The sequence size used to build the tree was added in the figure legend in the revised version of the manuscript. Accession number are given in the method section.

- Statistical analysis used in Table 1-3 should be indicated (if any).

Tables 1-3 were replaced by a single table presenting human-biting rate instead of relative proportion of each mosquito species in the corresponding group. Appropriate definition of the statistics used to calculate human-biting rate are given in the Methods section.

- The authors state the ‘we assessed species diversity, Plasmodium and Wolbachia infection rates in these Groups’. This is incorrect as Plasmodium infection rates are not shown.

The inaccurate statement was removed from the revised version of the manuscript.
Captions in Table 1-3 are missing information on what each column indicates. Although these tables can be generally informative, I wonder if showing species diversity using pie-charts (for each species group) over imposed on the map in Figure 1 would provide a more direct illustration of the species composition and abundances of the Anopheles species in the study area.

Table 1-3 were removed from the manuscript and replaced by a single table showing human-biting rate estimates collated by village and species. Given the number of study villages and diversity of Anopheles mosquitoes, we do not think that figuring multiple pie-charts on the map will improve the readability of the data.

As indicated in the methods, the size of the sequence used for Figure 2 should be indicated. It would be informative to include the alignment use for the tree figure.

The sequence alignment was added to the revised version of the manuscript and the number of positions in the final dataset used to build the tree was added to the figure legend of the revised version of the manuscript.

Figure 3b shows the overall prevalence of Wolbachia in different villages without specifying the species, so I am not sure what is the purpose of illustrating the result in this way. If this is too show that some villages have higher prevalence over others this should be indicated only if statistical analysis supports it (although I doubt this is the case if species distribution is included as a variable).

The reviewer is right to question the relevance of our prevalence data. Given the low sample size and the diversity of Wolbachia strains and Anopheles species, prevalence data were removed from the revised version of the manuscript.

The authors state that ‘Crossing-point values ranged from 31.0 to 40.6 amplification cycles. Infected specimens were generally infected at a density close or below the limit of detection of the assay (only one sample gave 3/3 positive reactions).’ More details should be given regarding the rational for inclusion (or exclusion) of an infected/amplified sample; it is not clear to me if ‘reactions’ refers to technical replicates in the same qPCR run or in different qPCR assays. This should be described with more details. It would also be informative to normalize the quantity of the amplified Wolbachia 16S using a mosquito housekeeping gene, for example. Indeed, as ‘density [was] close or below the limit of detection of the assay’ normalization would provide information on the likelihood of false negatives in samples, as you would expect if the total DNA is very low (for example due to inefficient DNA extraction).

It is common that at low parasite concentration, only some replicates give a positive result because the distribution of the DNA template in the reaction tube follow a Poisson distribution (Sterkers, Varlet-Marie et al. 2010, Stahlberg and Kubista 2014, Chaumeau, Andolina et al. 2016). This observation does not challenge the validity of our results. A clear statement that some Wolbachia infected sample have probably been missed because bacterial density observed in Anopheles are close or below the limit of detection of the assay that give 95% positive reaction. Misleading interpretations on the prevalence of Wolbachia infection were removed given the small sample size and the possibility of false negative.

The authors state that ‘Our data and African studies confirm that the occurrence of natural Wolbachia infections has been underestimated in malaria mosquitoes.’ As direct assessment of Wolbachia prevalence on samples previously identified as uninfected was not performed here (nor in African samples) it cannot be ruled out that previous Wolbachia negative samples were not true negatives, so this work (and others) only suggest possible underestimation in previous works, as they have
not directly confirmed it. The authors state that ‘It was not possible to study more in
detail the phylogeny of Wolbachia strain detected in this study by multi locus
sequence typing because of the lack of DNA extracts after the screening.’ It is not
clear to me if any attempts were made at all or not. If so, please give more details on
the targeted genes and discuss why these could not be amplified.

The methodology used for Wolbachia detection in this study was described into detail in the
Methods section and we did not attempt additional experiments on Wolbachia than that described
in the manuscript. In addition to Wolbachia detection and molecular identification of the mosquito
species, sample were also screened for Plasmodium infection. There was not DNA material to
perform additional experiment after the screening with the W-Specf/ W-Specr primers.

Competing Interests: No competing interests were disclosed.

Reviewer Report 28 February 2019

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Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original
work is properly cited.

Thomas Walker
Department of Disease Control, London School of Hygiene & Tropical Medicine, London, UK

The authors present an interesting study in which Wolbachia strains were detected in Anopheles species
from Myanmar. This study is particularly timely given the recent discoveries of natural Wolbachia strains
in Anopheles malaria vectors in Sub-Saharan Africa and evidence that these natural endosymbiotic
bacteria could be influencing malaria parasite infection prevalence in wild mosquito populations. The
study provides evidence for Wolbachia infections using amplification and sequencing of the Wolbachia
16S gene although more clarity is needed on which primer set was used given the authors report
undertaking qrtPCR and sequencing of PCR products. The manuscript would be significantly improved
with additional Wolbachia gene analysis and to provide the quantitative PCR data. This would provide
more information on the Wolbachia strain infections being presented and allow these strains to be put into
context with recent discoveries in other Anopheles species.

Introduction
The introduction needs significant improvement in the referencing. For example, the sentence ‘In addition,
Wolbachia can interfere with the development of some pathogens, including dengue virus’ contains a
reference to a publication that only describes Wolbachia establishment and invasion in an Aedes aegypti
laboratory population (not virus inhibition).

Furthermore, including references 5 & 6 in the context of pathogen blocking is not appropriate given this
was work which was proposing to use cytoplasmic incompatibility to reduce Culex mosquito populations
and artificial Wolbachia-infected mosquito lines were only established in the mid 2000s.

The paragraph describing natural Wolbachia infections in mosquitoes also needs further references.
The final paragraph in the introduction presents the fact that Natural Wolbachia infections in Southeast Asian malaria vectors have not been reported. However, the authors should reference the studies in which screening of Anopheles species for Wolbachia was undertaken despite finding no evidence of natural infections.

Methods
The primers used for Wolbachia detection W-Specf (CATACC TATTCGAAGGGATAG) and W-Specr (AGCCTGAGTGAACCAATTC) produce a product size of 438 bp and this (to my knowledge) would not be possible or has not been reported using a qrtPCR format. The authors also reference Gomes et al. which used a different reverse primer (5′-TTGCGGGACTTAACCCAACA-3′) that results in amplification of a smaller fragment of the 16S rRNA gene for qrtPCR. The accession numbers MK336794 - MK336806 refer to sequences with >400 bases indicating W-Specf/W-Specr was used. The authors need to clarify if W-Specf/W-Specr was used on a qrtPCR format or if both were used independently and report the differences in prevalence rates using these two primer sets.

Results
The inclusion for analysis of only what would be considered ‘primary malaria vectors’ needs more explanation if the authors overall aim was to provide evidence for natural Wolbachia infections in Anopheles species given the mosquitoes were not screened for Plasmodium infection.

Tables 1-3 provide a breakdown of the species composition collected at the different villages but I think it would be more informative to have all the different species grouped according to villages. Currently it’s difficult to determine mosquito species prevalence on a village level.

The statistics used in tables 1-3 don’t appear to be explained either in the manuscript methods or in the table legends. For example, I am assuming ‘n/N’ means the species/total number collected but again this would be much easier to understand if species were grouped by villages.

Wolbachia infections
With reference to my previous point raised in the methods, which 16S PCR primer set and format was used to determine the prevalence rates and to generate sequences for Figure 2?

Figure 2 needs to have more details included such as the number of nucleotide sequences used in the analysis and the total positions in the final dataset.

The authors provide the overall prevalence rate (13/370) and then have Figure 3 to show the individual species. Figure 3a I don’t feel is needed because plotting 1/11 (PSE) and 1/12 (DIR) seems unnecessary and could be in a table that incorporates prevalence rates by species and village. Having an overall village prevalence rate (Fig 3b) has little biological relevance given you have variable Anopheles species containing what appears to be different Wolbachia strains based on 16S analysis.

A major limitation of the phylogenetic analysis (and even the prevalence rates) is only using a single Wolbachia gene (16S) but I appreciate that Cp values ranging from 31-40.6 are at the limit of detection. The authors should provide these 16S Cp values to allow the reader to see the variation both between technical and biological replicates.

Could the authors also provide the rationale for concluding that samples were positive where not all technical replicates produced positive amplification given ‘only one sample gave 3/3 positive reactions’? How do these results fit with the Minimum Information for Publication of Quantitative Real-Time PCR
Experiments (MIQE) guidelines⁶?

Could the authors not have used another Wolbachia qPCR assay based on a second gene that targets a broad range of strains⁷?

The density comparison to laboratory-reared Aedes aegypti artificially-infected with the wMel Wolbachia strain is not particularly informative for several reasons. Firstly, quantifying Wolbachia density without accounting for mosquito body size and/or DNA extraction efficiency is problematic. Secondly, the wMel is a strain that naturally infects Drosophila melanogaster so a better comparison would be to natural infections in mosquito species (such as wPip in Culex quinquefasciatus or even the wAlbA/wAlbB strains in Aedes albopictus). Therefore, I would question the inclusion of this density data given wMel in Ae. aegypti is an artificial infection.

Discussion

The sentence ‘It was not possible to study more in detail the phylogeny of Wolbachia strain detected in this study by multi locus sequence typing because of the lack of DNA extracts after the screening’ needs clarification. Do the authors mean that they were unable to amplify any of the Wolbachia MLST genes? Did they try using degenerate primer protocols or nested PCR given the qPCR data would indicate low density infections?

The statement “This is consistent with previous attempts to quantify Wolbachia in naturally infected malaria vectors” is incorrect and refers to some (An. gambiae complex) but not all species analysed in Sub-Saharan Africa⁸. The authors should expand this discussion as the low density infections presented in this study are comparable to those strains detected in An. gambiae mosquitoes from Sub-Saharan Africa. Some of these studies have only resulted in 16S gene amplification and sequencing resulting in conflicting phylogenetics which appear incompatible with the traditional criteria for vertically transmitted endosymbionts (reviewed in reference ⁹). The authors should provide some further discussion points on whether their results only amplifying 16S could have resulted from either 1) integration into the mosquito genome or 2) some form of contamination. However, additional Wolbachia gene analysis would allow more confidence in these detected strains given the high 16S qPCR Cp values are at the limit of detection.

References


**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
No

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** medical entomology, Wolbachia, Anopheles

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Author Response 03 Jul 2019**

**Victor Chaumeau**, Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK

We thank to the reviewer for his useful feedback on the manuscript. More details on the qPCR assay used to detect *Wolbachia* in this study were provided in the revised version of the manuscript, including the raw quantitative data as per reviewer’s suggestion. We also agree that additional *Wolbachia* genotyping would have been an important added value to the manuscript. However, there was not enough DNA material to attempt additional genotyping of the *Wolbachia*
strains detected in this study given that mosquito samples were also screened for *Plasmodium* infection and identified at the species level with molecular assays.

Response to point specific comments are listed below:

- **The introduction needs significant improvement in the referencing.** For example, the sentence ‘In addition, Wolbachia can interfere with the development of some pathogens, including dengue virus’ contains a reference to a publication that only describes Wolbachia establishment and invasion in an Aedes aegypti laboratory population (not virus inhibition). Furthermore, including references 5 & 6 in the context of pathogen blocking is not appropriate given this was work which was proposing to use cytoplasmic incompatibility to reduce Culex mosquito populations and artificial Wolbachia-infected mosquito lines were only established in the mid 2000s. The paragraph describing natural Wolbachia infections in mosquitoes also needs further references. The final paragraph in the introduction presents the fact that Natural Wolbachia infections in Southeast Asian malaria vectors have not been reported. However, the authors should reference the studies in which screening of *Anopheles* species for Wolbachia was undertaken despite finding no evidence of natural infections.

The referencing of the introduction was improved as per reviewer’s suggestions.

- **The primers used for Wolbachia detection W-Specf (CATACC TATTCAAGGGATAG) and W-Specr (AGCTTGAGTAAACCACCAC) produce a product size of 438 bp and this (to my knowledge) would not be possible or has not been reported using a qrtPCR format.** The authors also reference Gomes et al. which used a different reverse primer (5’-TTGCGGGACTTAACCCCA-3’) that results in amplification of a smaller fragment of the 16S rRNA gene for qrtPCR. The accession numbers MK336794 - MK336806 refer to sequences with >400 bases indicating W-Specf/W-Specr was used. The authors need to clarify if W-Specf/W-Specr was used on a qrtPCR format or if both were used independently and report the differences in prevalence rates using these two primer sets.

Amplification of fragments much longer than 438 bp with real-time PCR technology has been reported previously (Rothfuss, Gasser et al. 2010). Without a priori knowledge on the DNA sequences of the *Wolbachia* strains detected in this study, the primer W-Specf and W-Specr were chosen for their ability to detect most *Wolbachia* strains infecting insects and to establish phylogenetic relationship among isolates (Werren and Windsor 2000). The results of additional assay optimization and serial dilution experiments with the W-Specf/W16S primers used by Gomes et al. were added to the revised version of the manuscript, although we did not use these primers for the screening of *Wolbachia* in field mosquito samples.

- **The inclusion for analysis of only what would be considered ‘primary malaria vectors’ needs more explanation if the authors overall aim was to provide evidence for natural *Wolbachia* infections in *Anopheles* species given the mosquitoes were not screened for *Plasmodium* infection.**

The vector status of *Anopheles* species in the Thailand-Myanmar border area has been determined previously (Somboon, Aaramrattana et al. 1998, Chaumeau, Fustec et al. 2018). Primary vectors in the study area are *An. minimus* s.s. (Minimus Complex, Funestus Group), *An. maculatus* s.s., *An. sawadwongporni* (Maculatus Group), *An. dirus* s.s. and *An. baimaii* (Dirus Complex, Leucosphyrus Group). Proper referencing was added in the revised version of the manuscript.

- **Tables 1-3 provide a breakdown of the species composition collected at the different villages but I think it would be more informative to have all the different**
species grouped according to villages. Currently it’s difficult to determine mosquito species prevalence on a village level. The reviewer is right to question the relevance of presenting specific diversity as a proportion of a given species in the corresponding group. In the revised manuscript, human-biting rates were reported is a single table instead of the relative proportions.

- The statistics used in tables 1-3 don’t appear to be explained either in the manuscript methods or in the table legends. For example, I am assuming ‘n/N’ means the species/total number collected but again this would be much easier to understand if species were grouped by villages.

In the revised version of the manuscript, table 1-3 were merged in a single table and appropriate description of the statistics used in this table were added to the Methods section.

- With reference to my previous point raised in the methods, which 16S PCR primer set and format was used to determine the prevalence rates and to generate sequences for Figure 2?

The primer W-Specf and W-Specr were used in a real-team PCR format for both estimation of the prevalence rates and phylogenetic analysis as described in the Methods section.

- Figure 2 needs to have more details included such as the number of nucleotide sequences used in the analysis and the total positions in the final dataset.

More details were added in the revised phylogenetic tree and the total number of position in the final dataset was stated in the figure legend.

- The authors provide the overall prevalence rate (13/370) and then have Figure 3 to show the individual species. Figure 3a I don’t feel is needed because plotting 1/11 (PSE) and 1/12 (DIR) seems unnecessary and could be in a table that incorporates prevalence rates by species and village. Having an overall village prevalence rate (Fig 3b) has little biological relevance given you have variable Anopheles species containing what appears to be different Wolbachia strains based on 16S analysis.

The reviewer is right to question the biological significance of plotting prevalence estimates per species and per village. In the revised manuscript, the screening results collated by village and species are presented in a table, and the two plots were removed.

- A major limitation of the phylogenetic analysis (and even the prevalence rates) is only using a single Wolbachia gene (16S) but I appreciate that Cp values ranging from 31-40.6 are at the limit of detection. The authors should provide these 16S Cp values to allow the reader to see the variation both between technical and biological replicates.

We agree with the reviewer the analyzing only 16S ssuRNA genes is a limitation of our study. Raw quantitative data were added to the revised version of the manuscript as per reviewer suggestion.

- Could the authors also provide the rationale for concluding that samples were positive where not all technical replicates produced positive amplification given ‘only one sample gave 3/3 positive reactions’? How do these results fit with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines?

It is common that at low parasite concentration, only some replicates give a positive result because the distribution of the DNA template in the reaction tube follow a Poisson distribution (Sterkers, Varlet-Marie et al. 2010, Stahlberg and Kubista 2014, Chaumeau, Andolina et al. 2016). This observation does not challenge the validity of our results. A clear statement that some Wolbachia infected sample have probably been missed because bacterial density observed in Anopheles are close or below the limit of detection of the assay that give 95% positive reaction. Misleading interpretations on the prevalence of Wolbachia infection were removed given the small sample size and the possibility of false negative.
Could the authors not have used another Wolbachia qPCR assay based on a second gene that targets a broad range of strains.

In the absence of a priori knowledge on the Wolbachia strains infecting Anopheles samples, the W-Specf/ W-Specr primers were chosen for their ability to detect a broad range of strains infecting insects (Werren and Windsor 2000). There was not enough DNA material remaining after the screening with the W-Specf/ W-Specr primers to use another assay. However, the specificity of the PCR was confirmed in all positive sample by Sanger sequencing of the PCR product.

The density comparison to laboratory-reared Aedes aegypti artificially-infected with the wMel Wolbachia strain is not particularly informative for several reasons. Firstly, quantifying Wolbachia density without accounting for mosquito body size and/or DNA extraction efficiency is problematic. Secondly, the wMel is a strain that naturally infects Drosophila melanogaster so a better comparison would be to natural infections in mosquito species (such as wPip in Culex quinquefasciatus or even the wAlbA/wAlbB strains in Aedes albopictus ). Therefore, I would question the inclusion of this density data given wMel in Ae. aegypti is an artificial infection.

We agree with the reviewer that there is little biological relevance in comparing the density of Wolbachia infection in artificially infected Aedes aegypti and naturally infected Anopheles. We think that presenting those quantitative data is an added value to support that natural Wolbachia infection in this study actually occur at low density rather than resulting from of a low assay sensitivity. We believe that a calibrator to normalize the signal is not necessary as DNA was extracted from whole mosquitoes and bacterial loads expressed as an (arbitrary) number of bacteria per mosquito rather the a number of bacteria per weight-unit of mosquito body (Varlet-Marie, Sterkers et al. 2014, Chaumeau, Andolina et al. 2016).

The statement 'It was not possible to study more in detail the phylogeny of Wolbachia strain detected in this study by multi locus sequence typing because of the lack of DNA extracts after the screening' needs clarification. Do the authors mean that they were unable to amplify any of the Wolbachia MLST genes? Did they try using degenerate primer protocols or nested PCR given the qPCR data would indicate low density infections?

The methodology used for Wolbachia detection in this study was described into detail in the Methods section and we did not attempt additional experiments on Wolbachia than that described in the manuscript. In addition to Wolbachia detection and molecular identification of the mosquito species, samples were also screened for Plasmodium infection (data not shown). There was no DNA material to perform additional experiment after the screening with the W-Specf/ W-Specr primers.

The statement “This is consistent with previous attempts to quantify Wolbachia in naturally infected malaria vectors” is incorrect and refers to some (An. gambiae complex) but not all species analysed in Sub-Saharan Africa. The authors should expand this discussion as the low density infections presented in this study are comparable to those strains detected in An. gambiae mosquitoes from Sub-Saharan Africa. Some of these studies have only resulted in 16S gene amplification and sequencing resulting in conflicting phylogenetics which appear incompatible with the traditional criteria for vertically transmitted endosymbionts (reviewed in reference 9). The authors should provide some further discussion points on whether their results only amplifying 16S could have resulted from either 1) integration into the mosquito genome or 2) some form of contamination. However, additional Wolbachia gene analysis would allow more confidence in these detected strains given the high 16S qPCR Cp values are at the limit of detection.
We thank to the reviewer for his useful feedback on the interpretation of our quantitative data. Suggested edits and references were added in the revised version of the manuscript.

References


Competing Interests: No competing interests were disclosed.