RESEARCH ARTICLE

Natural Wolbachia infections in malaria vectors in Kayin state, Myanmar [version 1; peer review: 2 approved with reservations]

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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 by quantitative real-time PCR.

Results: Malaria vectors were identified in the Funestus, Maculatus and Leucosphyrus Groups. Wolbachia were detected in 6/6 Anopheles species and in 5/10 villages. Mean prevalence of Wolbachia infection was 2.7% (95%CI= [1.3; 4.9]). The median Wolbachia load was seven orders of magnitude less in naturally infected malaria vectors than in artificially infected laboratory-reared Aedes aegypti. Phylogenetic analysis based on 16S rRNA sequences revealed a high diversity of Wolbachia strains and identified lineages different from those described in Africa.

Conclusion: Natural Wolbachia infections are common and widespread in malaria vectors in Kayin state, Myanmar. Their effects on Anopheles mosquitoes and malaria transmission is 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 relationships that result from infection have a broad range of phenotypic effects on the infected hosts, from mutualism (beneficial) to commensalism (neutral) and parasitism (harmful). In mosquitoes, Wolbachia invades the germline and can induce cytoplasmic incompatibilities between the sperm from infected males and oocytes from uninfected females. This type of reproductive parasitism enhances the “vertical” transmission of Wolbachia, from infected females to the progeny. In addition, Wolbachia can interfere with the development of some pathogens, including dengue virus, Plasmodium malaria parasites and filarial nematodes. Therefore, the release of mosquitoes artificially infected with Wolbachia has been proposed as an intervention to control medically important mosquito vectors and the diseases they transmit.

Natural infections may have important effects on mosquito populations and dynamics of diseases transmission but they are not well described. Wolbachia has been detected in several mosquito species including Aedes spp., Armigeres spp., Coquillettidia spp., Culex spp., Culiseta spp., Hodgesia spp., Mansonia spp., Ochlerotatus spp., Tripteroides spp. and Uranotaenia spp. 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 of the dominant African malaria vector Anopheles gambiae (s.s.) in naturally blood-fed females. Gomes et al. obtained similar results on the sporozoite stage by screening large numbers of mosquitoes. The results of Gomes et al. diverged from that of Shaw et al. regarding the effects of Wolbachia infection on the early stages of Plasmodium development. Gomes et al. infected a laboratory-adapted Anopheles 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 sporozoite development.

Natural Wolbachia infections in Southeast Asian malaria vectors have not been reported. Their effects on Anopheles mosquitoes and dynamics of malaria transmission are not known. The objective of this pilot study is therefore to assess the prevalence of natural Wolbachia infections in malaria vectors 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). Entomological surveys consisted of five consecutive nights of collection from 06:00 pm to 06:00 am in six sites per villages as described previously. In each village, five traditional houses were selected for mosquito sampling using the human-landing catch (HLC) method. Collectors were asked to collect every mosquito 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 identified at the genus level by morphology and Anopheles 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 to the Funestus, Maculatus and Leucophyirus Group were discriminated 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 II™ (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 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 GCC GGG GTA-3’, MIC 5’-GTG CAT TCA GCA ACA TCA GT-3’, MAC 5’-ACA CGG TGT ACG 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’-GTG CAT TCA GCA ACA TCA GT-3’, SAW 5’-ACA CGG TGT ACG TCC AGT-3’, K 5’-TGT ACA TCG GCC GGG GTA-3’, DRAV 5’-TTG ACC ACT TTC GAC GCA-3’ and Leucophyirus assay: D-AC 5’-CAC AGC TGT ACG TCC AGT-3’, D-B 5’-CGG GAT ATG GGT CGG CC-3’, D-D 5’-CGG CGG GAC CGT CCG TT-3’, D-F 5’-AAC GCC GGT CCC CTG TG-3’, D-AC 5’-CAC AGC GAC TCA 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 Leucophyirus 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. The PCR product was sequenced by gel electrophoresis in 2% agarose for 70 minutes at 120V.
and blasted against the National Center for Biotechnology Information nucleotide database in order to determine the corresponding species (accession numbers MK358471 - MK358807).

**Wolbachia detection**
DNA extracts were screened for the presence of *Wolbachia* using a quantitative real-time PCR (qrtPCR) assay that targets a conserved region of the 16S rRNA genes with the primer pair W-Specf/W-Specr (5’-CAT TAT TCG AAG GGA TAG-3’ and 5’- AGC TTC GAG TGA AAC CAA TTC-3’)). All experiments were performed on a CFX-96® (BioRad) machine. Reactions were conducted in 20μl of EVAGreen qPCR Mix Plus® (Euromedex); 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. Characterization of the PCR product was performed using the melt curve analysis of the amplicons (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). Specificity of the PCR was confirmed by Sanger sequencing and the sequences were used for phylogenetic analysis (Accession numbers MK336794 - MK336806).

Data analysis
For the quantitation of Wolbachia load in naturally infected samples, a standard curve was set-up by performing serial-dilution assays with laboratory-reared Aedes aegypti artificially infected with Wolbachia strain wMel as a reference material (data not shown). 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). The best-fit-line and the subsequent values of the slope and y-intercept were obtained using least-square analysis of the linear portion of each curve (Pearson’s coefficient r²=0.990). The PCR efficiency (EFF) estimate was 110% with the formula EFF = 10(-1/slope)–1. The relative quantitation of Wolbachia load in natural samples was calculated with the formula 10(ΔCP/slope), with ΔCP defined as the difference in the mean CP values measured in the natural and reference DNA extracts respectively.

For the phylogenetic analysis, sequenced PCR products were analyzed with the DECIPHER software version 2.0. Potential chimeras (4/17 samples) were removed from the dataset. Wolbachia 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 sequence alignment was performed using the CLUSTAL algorithm. A phylogenetic tree was reconstructed using a Tamura-Nei genetic distance model and neighbor joining with the ape package version 5.2 of the R software.

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
Biodiversity of Anopheles mosquitoes
A total of 4743 Anopheles was collected during 500 person-nights of collection. We report the occurrence of 12 Anopheles taxa namely the Maculatus (45.5%), Funestus (36%), Jamesii (6.5%), Leucosphyrus (5.3%), Annularis (2.2%), Barbirostris (1.4%), Kochi (1.3%), Subpictus (0.7%), Tesselatus (0.4%), Hyrcanus (0.1%) and Asiaticus Groups (<0.1%), and the currently unclassified An. karwari (0.5%). In this area, primary malaria vectors belong to the Minimus Complex (Funestus Group), Dirus Complex (Leucosphyrus Group) and Maculatus Group. Therefore, we assessed species diversity, Plasmodium and Wolbachia infection rates in these Groups.

In total 1098 mosquitoes in the Maculatus, Funestus and Leucosphyrus Groups were identified at the species level with molecular assays. The Maculatus Group was mainly composed of An. maculatus (s.s.) (range= 50 – 88%), An. sawadwongporni (range= 5 – 41%) and An. pseudowillmori (range= 0 – 19%) (Table 1). The most frequent species in the Funestus Group were Anopheles minimus (s.s.) (range= 56 - 95%), An. culicifacies B (range= 0 - 41%) and An. jeyporiensis (range= 0 – 28%) (Table 2). The Dirus Complex represented >99% of the samples in the Leucosphyrus Group with 89 to 100% of the samples identified as An. baimaii (Table 3).

Table 1. Species diversity in the Maculatus Group determined by molecular assays.

<table>
<thead>
<tr>
<th>Village</th>
<th>An. maculatus (s.s.)</th>
<th>An. pseudowillmori</th>
<th>An. rampae</th>
<th>An. sawadwongporni</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>p</td>
<td>95%CI</td>
<td>n/N</td>
</tr>
<tr>
<td>HD-3634</td>
<td>75/90</td>
<td>83.3</td>
<td>74-90.4</td>
<td>2/90</td>
</tr>
<tr>
<td>HG-369</td>
<td>42/59</td>
<td>71.2</td>
<td>57.9-82.2</td>
<td>4/59</td>
</tr>
<tr>
<td>LG-360</td>
<td>70/90</td>
<td>77.8</td>
<td>67.8-85.9</td>
<td>-</td>
</tr>
<tr>
<td>MK-3633</td>
<td>32/64</td>
<td>50.0</td>
<td>37.2-62.8</td>
<td>5/64</td>
</tr>
<tr>
<td>MK-3635</td>
<td>48/63</td>
<td>76.2</td>
<td>63.8-86.6</td>
<td>12/63</td>
</tr>
<tr>
<td>MM-3631</td>
<td>63/81</td>
<td>77.8</td>
<td>67.2-86.3</td>
<td>1/81</td>
</tr>
<tr>
<td>NT-361</td>
<td>62/81</td>
<td>76.5</td>
<td>65.8-85.2</td>
<td>1/81</td>
</tr>
<tr>
<td>TG-357</td>
<td>58/77</td>
<td>75.3</td>
<td>64.2-84.4</td>
<td>1/77</td>
</tr>
<tr>
<td>TP-339</td>
<td>68/77</td>
<td>88.3</td>
<td>79-94.5</td>
<td>-</td>
</tr>
<tr>
<td>WM-367</td>
<td>39/51</td>
<td>76.5</td>
<td>62.5-87.2</td>
<td>-</td>
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</table>
Table 2. Species diversity in the Funestus Group determined by molecular assays.

<table>
<thead>
<tr>
<th>Village</th>
<th>An. aconitus (s.s.)</th>
<th>An. culicifacies A</th>
<th>An. culicifacies B</th>
<th>An. harrisoni</th>
<th>An. jeyporiensis</th>
<th>An. minimus (s.s.)</th>
<th>An. varuna</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
</tr>
<tr>
<td>HD-3634</td>
<td>1/71 1.4 0-7.6</td>
<td>3/71 4.2 0.9-11.9</td>
<td>12/71 16.9 9-27.7</td>
<td>1/71 1.4 0-7.6</td>
<td>1/71 1.4 0-7.6</td>
<td>53/71 74.6 62.9-84.2</td>
<td></td>
</tr>
<tr>
<td>HG-369</td>
<td>2/86 2.3 0.3-8.1</td>
<td>- - -</td>
<td>11/86 12.8 6.6-21.7</td>
<td>- - -</td>
<td>2/86 2.3 0.3-8.1</td>
<td>71/86 82.6 72.9-89.9</td>
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</tr>
<tr>
<td>LK-350</td>
<td>3/82 3.7 0.8-10.3</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>482 4.9 1.3-12</td>
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<tr>
<td>MK-3633</td>
<td>2/92 2.2 0.3-7.6</td>
<td>- - -</td>
<td>25/92 27.2 18.4-37.4</td>
<td>- - -</td>
<td>2/92 2.2 0.3-7.6</td>
<td>63/92 68.5 58-77.8</td>
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<tr>
<td>MK-3635</td>
<td>2/88 2.3 0.3-8.1</td>
<td>- - -</td>
<td>7/88 8 3.3-15.7</td>
<td>- - -</td>
<td>25/88 28.4 19.3-39</td>
<td>54/88 61.4 50.4-71.6</td>
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</tr>
<tr>
<td>MM-3631</td>
<td>- - -</td>
<td>1/82 1.2 0-6.6</td>
<td>34/82 41.5 30.7-52.9</td>
<td>- - -</td>
<td>1/82 1.2 0-6.6</td>
<td>46/82 56.1 44.7-67</td>
<td></td>
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<tr>
<td>NT-361</td>
<td>- - -</td>
<td>2/83 2.4 0.3-8.4</td>
<td>32/83 38.6 28.1-49.9</td>
<td>- - -</td>
<td>1/83 1.2 0-6.5</td>
<td>48/83 57.8 46.5-68.6</td>
<td></td>
</tr>
<tr>
<td>TG-357</td>
<td>- - -</td>
<td>1/91 1.1 0-6</td>
<td>2/91 2.2 0.3-7.7</td>
<td>1/91 1.1 0-6</td>
<td>20/91 22 14-31.9</td>
<td>66/91 72.5 62.2-81.4</td>
<td>1/91 1.1 0-6</td>
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<tr>
<td>TP-339</td>
<td>- - -</td>
<td>1/84 1.2 0-6.5</td>
<td>1/84 1.2 0-6.5</td>
<td>- - -</td>
<td>2/84 2.4 0.3-8.3</td>
<td>80/84 95.2 88.3-98.7</td>
<td></td>
</tr>
<tr>
<td>WM-367</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>59/90 65.6 54.8-75.3</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Species diversity in the Leucosphyrus Group determined by molecular assays.

<table>
<thead>
<tr>
<th>Village</th>
<th>An. baimaii n/N</th>
<th>p</th>
<th>95%CI</th>
<th>An. dirus (s.s.) n/N</th>
<th>p</th>
<th>95%CI</th>
<th>An. introlatus n/N</th>
<th>p</th>
<th>95%CI</th>
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</thead>
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<tr>
<td>HD-3634</td>
<td>52/56</td>
<td>92.9</td>
<td>82.7-98</td>
<td>4/56</td>
<td>7.1</td>
<td>2-17.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HG-369</td>
<td>3/3</td>
<td>100</td>
<td>29.2-100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>LK-350</td>
<td>41/41</td>
<td>100</td>
<td>91.4-100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>65.3-98.6</td>
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<td>11.1</td>
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<td>83.2-100</td>
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<td>9.4</td>
<td>2-25</td>
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<tr>
<td>NT-361</td>
<td>24/24</td>
<td>100</td>
<td>85.8-100</td>
<td>-</td>
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<td>-</td>
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<td>TG-357</td>
<td>36/39</td>
<td>92.3</td>
<td>79.1-98.4</td>
<td>3/39</td>
<td>7.7</td>
<td>1.6-20.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TP-339</td>
<td>19/21</td>
<td>90.5</td>
<td>69.6-98.8</td>
<td>1/21</td>
<td>4.8</td>
<td>0.1-23.8</td>
<td>1/21</td>
<td>4.8</td>
<td>0.1-23.8</td>
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<td>WM-367</td>
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<td>-</td>
<td>-</td>
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</tbody>
</table>

Figure 2. Phylogenetic analysis based on the alignment of a conserved region of the 16S rRNA gene using Wolbachia-specific primers. Sequenced 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. Nodes with bootstrap support <50% were collapsed. Study samples were labeled with the host name and the study village into brackets. Formally named Wolbachia strains were labeled with their abbreviation: wPip is an endosymbiont of Culex pipiens, wNo of Drosophila simulans, wAlbB of Aedes albopictus, wAnga of An. gambiae, wMel and wMelCS of Drosophila melanogaster.
Natural *Wolbachia* infections in malaria vectors

Six *Anopheles* species were screened for *Wolbachia* infection including *An. maculatus* (s.s.), *An. sawadwongporni*, *An. pseudowillmori* (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. Six unique 16S rRNA sequences were identified. 16S rRNA sequences clustered with that of *Wolbachia* strains in the supergroups B, D and F ([Figure 2](#)). The detection of *Wolbachia* strain in the supergroup D suggests that some DNA extracts were contaminated with filarial nematodes carrying *Wolbachia* rather than actual natural *Wolbachia* infection in *Anopheles*.

After excluding *Wolbachia* that clustered in the supergroups D from the analysis, mean prevalence estimate of *Wolbachia* infections in malaria mosquitoes was 2.7% (95%CI=[1.3; 4.9]). *Wolbachia* were detected in 6/6 species and in 5/10 villages ([Figure 3](#)). CROSSING-POINT VALUES RAN 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). THE MEDIAN *Wolbachia* LOAD IN THOSE NATURALLY INFECTED SPECIMENS WAS SEVEN ORDERS OF MAGNITUDE LESS THAN THAT OBSERVED IN LABORATORY-READED *Aedes aegypti* ARTIFICIALLY INFECTED WITH *Wolbachia* STRAIN wMel.

**Discussion**

This study is the first report of natural *Wolbachia* infections in malaria vectors in Southeast Asia, including *An. minimus* (s.s.) (Minimus Complex, Funestus Group), *An. maculatus* (s.s.), *An. sawadwongporni*, *An. pseudowillmori* (Maculatus Group), *An. dirus* (s.s.) and *An. baimaii* (Dirus Complex, Leucosphyrus Group). *Wolbachia*-infected specimens were detected in 6/6 species and in 5/10 villages which suggests that *Wolbachia* infections are common and widespread in Kayin state. Our data and African studies confirm that the occurrence of natural *Wolbachia* infections has been underestimated in malaria mosquitoes.

Molecular phylogeny based on 16S rRNA sequences revealed a high diversity of *Wolbachia* strains, which belonged to different lineages than those recently reported in African malaria vectors. 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. Prevalence estimates were in the lower range of those reported in Africa (1.3 to 78%) [14-16]. We collected mosquitoes in a relatively small geographic area and over a short period, therefore we could not assess the dynamics of *Wolbachia* prevalence in the mosquito population. The bacterial loads measured in *Wolbachia*-positive samples were very low, usually close to the limit of detection of our assay. This is
consistent with previous attempts to quantify Wolbachia in naturally infected malaria vectors and probably explains why Anopheles mosquitoes were repeatedly reported not to be infected with Wolbachia in natural settings. 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, and Plasmodium infection rate are less than 1% and often nil in mosquito populations. Therefore, it was not possible to establish direct correlations between Plasmodium and Wolbachia infection rates in naturally infected malaria vectors. In this setting, the effect of Wolbachia infections on malaria transmission may be better assessed by performing experimental infections of field-collected mosquitoes with Plasmodium malaria parasites.

Conclusion

Although the prevalence and bacterial load reported in this study were low, natural Wolbachia infections seems to be common and widespread in Southeast Asian malaria vectors. Their effects on Anopheles hosts and dynamics of malaria transmission are yet to be determined.

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

This work was supported by the Wellcome Trust [101148]; the Bill and Melinda Gates Foundation [GH OPP 1081420] and the Global Fund [THA-M-DC].

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

Acknowledgments

We thank communities from the study villages for their support to the study. 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

22. Walton C, Handley JM, Kuvangkadilok C, et al. Identification of five species of


Francesco Baldini
Institute of Biodiversity Animal Health and Comparative Medicine, University of Glasgow, Glasgow, UK

This work identified Wolbachia strains in Anopheles species from Myanmar. To my knowledge this is the first evidence of identification of Wolbachia infection in Anopheles mosquito populations in Asia. This work opens novel questions on the potential role of these infections on the vectorial capacity of the vector host. Indeed, recently identified natural Wolbachia infections in Anopheles species in Africa have been shown to potentially influence the vectorial capacity of the infected vectors.

The authors state that Wolbachia infections were identified by using quantitative PCR approach followed by sequencing of the Wolbachia 16S gene, although some details are missing and the methodology should be clarified. Phylogenetic analysis is also not completely detailed. Specific comments to the manuscript are listed below.

Introduction:
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.

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.

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.

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 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-Spect/ W-Specr primers.

Competing Interests: No competing interests were disclosed.
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 references1,2,3.

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 infections4.

Methods
The primers used for Wolbachia detection W-Specf (CATACC TATTCGAAGGGATAG) and W-Specr (AGCTTCTGAGTGAAAACCAATTC) 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.5 which used a different reverse primer (5’-TTGCAGACTTAACCCACA-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) guidelines?

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

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 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.

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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**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 (CATACCTATCGAAGGGGATAG) and W-Specr (AGCTTCGAGTGAA ACCAATTC) 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.\(^5\) 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.

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, Aramrattana 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 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 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.

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### References


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