Natural Wolbachia infections in malaria vectors in Kayin state, Myanmar [version 1; referees: awaiting peer review]

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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., Coquillettia spp., Culex spp., Caliseta spp., Hodgessa 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.) (17). The authors did not observe cytoplasmic incompatibilities, difference in the number of eggs laid or progeny sex ratio, but infected females laid eggs more rapidly. Two studies demonstrated a negative effect of Wolbachia infections on the development of P. falciparum (5,17). Shaw et al. observed a negative correlation between Wolbachia infection and the development of P. falciparum oocysts 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 sporozoites 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 (20). In each village, five traditional houses were selected for mosquito sampling using human landing catch (HLC) collection 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. (21). Closely related species from to the Funestus, Maculatus and Leucophyryus Group were discriminated using allele-specific PCR assays (AS-PCR) adapted from Garros et al. and Walton et al. (22,24). 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 TissuLyser 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 CAC GGA ACG CAG TCA AT-3', MIA 5'-CCC GTG CGT CAT CCT GAC GA-3', MIC 5'-GGT CAT TCA GCA ACA TCA GT-3', ACO 5'-ACA GCG GGT AGT TCT GCC TT-3', D-AC 5'-'ACG GGT AGT TCT GCC TT-3', D-D 5'-GCG CGG AAC ATG CTT GCC TTC-3', D-F 5'-AAC ACC ACT TTC GAC GCA-3', D-B 5'-GGT CAT TCA GCA ACA TCA GT-3', D-RAV 5'-GCG CGG AAC ATG CTT GCC TTC-3', D-AC 5'-AAC ACC ACT TTC GAC GCA-3'; Maculatus assay: 5.8F 5'-TGT GAA CTG CAG GAC ACA T-3', MAC 5'-CCC GTG CGT CAT CCT GAC GA-3', PSEU 5'-GTT GAT ATG GGT CGG CC-3', D-AC 5'-CAC GAC GAC TCA ACA CG-3', D-B 5'-CGG GAT ATG GGT CGG CC-3', D-D 5'-GCG CCGG GAC CGT CGGT TT-3', D-F 5'-AAC GCC GGT CCC CTG TG-3', D-AC 5'-CAC GAC 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 Leucophyryus 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. 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. 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 Leucophyryus 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.
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’)[27,28]. 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%), Leucosiphus (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 (Leucosiphus 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 Leucosiphus 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 Leucosiphus 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-350</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</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</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-357</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>
</tr>
</tbody>
</table>

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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>
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</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>p</td>
<td>95% CI</td>
<td>n/N</td>
<td>p</td>
<td>95% CI</td>
<td>n/N</td>
</tr>
<tr>
<td>HD-3634</td>
<td>1/71</td>
<td>1.4</td>
<td>0-7.6</td>
<td>3/71</td>
<td>4.2</td>
<td>0.9-11.9</td>
<td>12/71</td>
</tr>
<tr>
<td>HG-369</td>
<td>2/86</td>
<td>2.3</td>
<td>0.3-8.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11/86</td>
</tr>
<tr>
<td>LK-350</td>
<td>3/82</td>
<td>3.7</td>
<td>0.8-10.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MK-3633</td>
<td>2/92</td>
<td>2.2</td>
<td>0.3-7.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25/92</td>
</tr>
<tr>
<td>MK-3635</td>
<td>2/88</td>
<td>2.3</td>
<td>0.3-8.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7/88</td>
</tr>
<tr>
<td>MM-3631</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/82</td>
<td>1.2</td>
<td>0-6.6</td>
<td>34/82</td>
</tr>
<tr>
<td>NT-361</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2/83</td>
<td>2.4</td>
<td>0.3-8.4</td>
<td>32/83</td>
</tr>
<tr>
<td>TG-357</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/91</td>
<td>1.1</td>
<td>0-6</td>
<td>2/91</td>
</tr>
<tr>
<td>TP-339</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/84</td>
<td>1.2</td>
<td>0-6.5</td>
<td>1/84</td>
</tr>
<tr>
<td>WM-367</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30/90</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</th>
<th></th>
<th></th>
<th>An. dirus (s.s.)</th>
<th></th>
<th></th>
<th>An. introlatus</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>n/N</td>
<td>p</td>
<td>95%CI</td>
<td></td>
<td>n/N</td>
<td>p</td>
<td>95%CI</td>
<td>n/N</td>
<td>p</td>
<td>95%CI</td>
</tr>
<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>
</tr>
<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>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MK-3633</td>
<td>16/18</td>
<td>88.9</td>
<td>65.3-98.6</td>
<td>2/18</td>
<td>11.1</td>
<td>1.4-34.7</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>MK-3635</td>
<td>20/20</td>
<td>100</td>
<td>83.2-100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<td>29/32</td>
<td>90.6</td>
<td>75-98</td>
<td>3/32</td>
<td>9.4</td>
<td>2-25</td>
<td>-</td>
<td>-</td>
<td></td>
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<td>NT-361</td>
<td>24/24</td>
<td>100</td>
<td>85.8-100</td>
<td>-</td>
<td>-</td>
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<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>
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<tr>
<td>TP-339</td>
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<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>
</tr>
<tr>
<td>WM-367</td>
<td>10/10</td>
<td>100</td>
<td>69.2-100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</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*. 

![Phylogenetic tree](image-url)
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). The bacterial loads measured in *Wolbachia*-positive samples were very low, usually close to the limit of detection of our assay. This is

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%) in African malaria vectors. 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.

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.

**Figure 3.** Prevalence of *Wolbachia* infections in naturally infected populations of malaria vectors collected in Kayin state, Myanmar. A) Prevalence estimates presented per *Anopheles* species; B) Prevalence estimates presented per study village. Error bars show 95% binomial CIs estimated with an exact method. **BAI**: *An. baimaii*, **DIR**: *An. dirus* (s.s.), **MAC**: *An. maculatus* (s.s.), **MIN**: *An. minimus* (s.s.), **PSE**: *An. pseudowillmori*, **SAW**: *An. sawadwongporni*. **Figure 3.** Prevalence of *Wolbachia* infections in naturally infected populations of malaria vectors collected in Kayin state, Myanmar. A) Prevalence estimates presented per *Anopheles* species; B) Prevalence estimates presented per study village. Error bars show 95% binomial CIs estimated with an exact method. **BAI**: *An. baimaii*, **DIR**: *An. dirus* (s.s.), **MAC**: *An. maculatus* (s.s.), **MIN**: *An. minimus* (s.s.), **PSE**: *An. pseudowillmori*, **SAW**: *An. sawadwongporni*. **Figure 3.** Prevalence of *Wolbachia* infections in naturally infected populations of malaria vectors collected in Kayin state, Myanmar. A) Prevalence estimates presented per *Anopheles* species; B) Prevalence estimates presented per study village. Error bars show 95% binomial CIs estimated with an exact method. **BAI**: *An. baimaii*, **DIR**: *An. dirus* (s.s.), **MAC**: *An. maculatus* (s.s.), **MIN**: *An. minimus* (s.s.), **PSE**: *An. pseudowillmori*, **SAW**: *An. sawadwongporni*. **Figure 3.** Prevalence of *Wolbachia* infections in naturally infected populations of malaria vectors collected in Kayin state, Myanmar. A) Prevalence estimates presented per *Anopheles* species; B) Prevalence estimates presented per study village. Error bars show 95% binomial CIs estimated with an exact method. **BAI**: *An. baimaii*, **DIR**: *An. dirus* (s.s.), **MAC**: *An. maculatus* (s.s.), **MIN**: *An. minimus* (s.s.), **PSE**: *An. pseudowillmori*, **SAW**: *An. sawadwongporni*.
consistent with previous attempts to quantify Wolbachia in naturally infected malaria vectors\textsuperscript{15,17} 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\textsuperscript{26}. 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).

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References

  Published Abstract | Publisher Full Text

  Published Abstract | Publisher Full Text

  Published Abstract | Publisher Full Text

4. Nugapola NWNP, De Silva WAPP, Karunaratne SHPP: Their effects on Plasmodium experimental infections of field-collected mosquitoes with malaria transmission may be better assessed by publishing experimental infections of field-collected mosquitoes with Plasmodium malaria parasites. 

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  Published Abstract | Publisher Full Text | Free Full Text

  Published Abstract | Publisher Full Text | Free Full Text

  Published Abstract | Publisher Full Text | Free Full Text

  Publisher Full Text

  Published Abstract

22. Walton C, Handley JM, Kuvankadiok C, et al.: Identification of five species of
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PubMed Abstract | Publisher Full Text | Free Full Text

PubMed Abstract | Publisher Full Text

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