






RESEARCH ARTICLE

REVISÉ **Detection of diverse *Wolbachia* 16S rRNA sequences at low titers from malaria vectors in Kayin state, Myanmar [version 4; peer review: 2 approved, 1 approved with reservations]**

Previously titled: Low-density genetically diverse 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. 16S rRNA *Wolbachia* DNA sequences were detected with quantitative real-time PCR.

Results: Low titer of *Wolbachia* DNA was detected in 13/370 samples in six malaria vector species. Sequences were diverse and different from those described in the African malaria mosquitoes.

Conclusion: The detection of *Wolbachia* DNA in malaria mosquitoes from Kayin state warrants further investigations to understand better the ecology and biology of *Anopheles-Wolbachia* interactions in Southeast Asia.

Keywords

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

Open Peer Review


Reviewer Status ? ✓ ✓


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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 3

In this revised version of the manuscript, the wording “*Wolbachia* infection” was changed in the title and throughout the main text in order to address reviewer’s concern that the detection of *Wolbachia* DNA sequences with PCRs is not a demonstration of an actual infection of the insect host with the bacteria. Primer sequences and amplicon length were added in the Methods section. The possibility that *Wolbachia* DNA detected in this study may come from some sort of environmental contamination given the high number of arthropod species infected with this bacteria has been made more explicit by adding a specific statement and the suggested reference in the Discussion section.

Any further responses from the reviewers can be found at the end of the article

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)¹. In mosquitoes, *Wolbachia* can invade the germline and induce cytoplasmic incompatibilities between the sperm from infected males and oocytes from uninfected females². Hence, mass-releases of *Wolbachia*-infected male mosquitoes were attempted to extinguish mosquito populations^{3,4}. 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 virus⁵, *Plasmodium* malaria parasites⁶ and filarial nematodes⁷. Therefore, the release of *Wolbachia*-infected female mosquitoes is proposed for transmission-blocking of some mosquito-borne diseases⁸.

Most diversions of mosquito-*Wolbachia* interactions for controlling vector-borne diseases were conducted with mosquitoes artificially infected with the endosymbiont. Natural *Wolbachia* infections may have important effects on mosquito populations and dynamics of diseases transmission but they are less well described⁹. *Wolbachia* DNA was detected by PCR in 27 mosquito genera including the medically important *Aedes*, *Armigeres*, *Culex*, *Mansonia* and *Stegomyia*⁹⁻¹⁸. Interestingly, this organism was not detected in malaria mosquitoes until recent observations of naturally infected anopheline vectors in Africa^{9,13,19-24}.

Only one study assessed the effects of natural *Wolbachia* infection on the reproductive fitness anopheline mosquitoes, namely the dominant African malaria vector *Anopheles coluzzi*²². 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 *P. falciparum*^{20,22}. Shaw *et al.* observed a negative correlation between *Wolbachia* infection and the development of *P. falciparum* 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 the presence of *Wolbachia* 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 previously²⁵. 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* specimen 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.*²⁶. Closely related species in the Funestus, Maculatus and Leucopshyrus 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.*²⁷⁻²⁹. 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 water³⁰. 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 ACG TCC AGT-3', PAM 5'-TGT ACA TCG GCC GGG GTA-3', VAR 5'-TTG ACC ACT TTC GAC GCA-3'; Maculatus

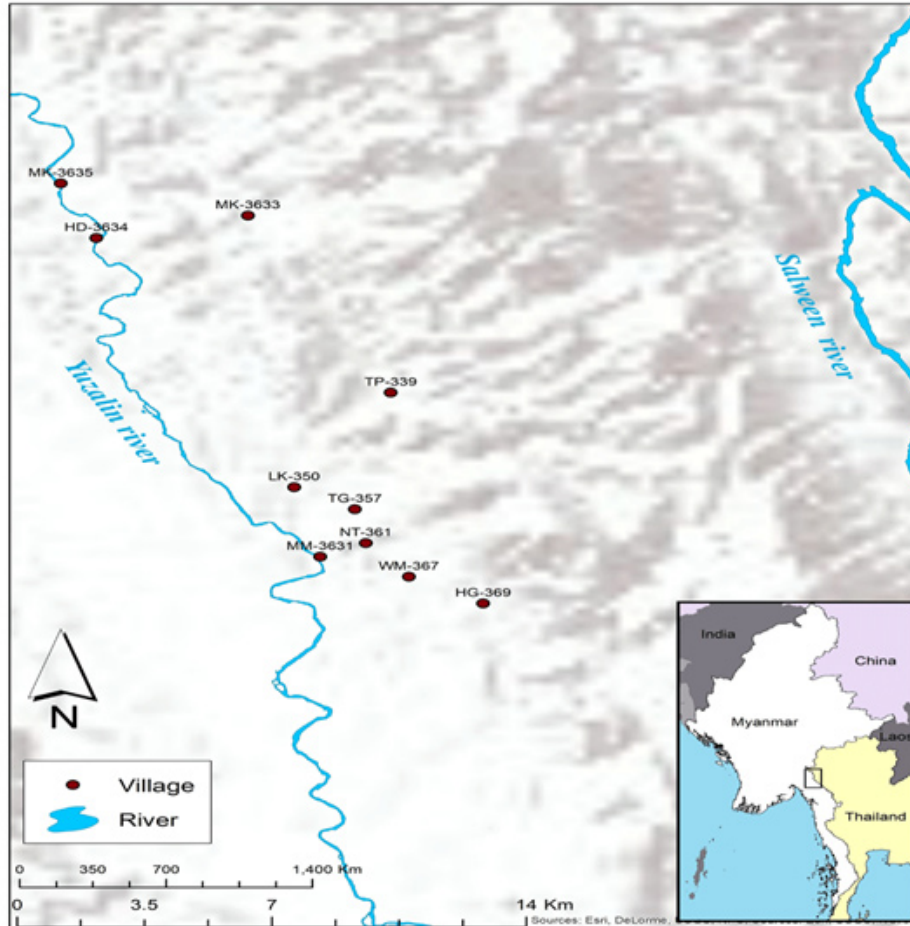


Figure 1. Map of the study area.

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 ACG TCC AGT-3', K 5'-TGT ACA TCG GCC GGG GTA-3', DRAV 5'-TTG ACC ACT TTC GAC GCA-3' and *Leucospkyrus* assay: D-AC 5'-CAC AGC GAC TCC ACA CG-3', D-B 5'-CGG GAT ATG GGT CGG CC-3', D-D 5'-GCG CGG GAC CGT CCG TT-3', D-F 5'-AAC GGC GGT CCC CTT TG-3', D-AC 5'-CAC AGC GAC TCC ACA CG-3'). The PCR was conducted in a total reaction volume of 25 μ l (1 μ l of DNA template and 24 μ l 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 *Leucospkyrus* 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

AAA TTY AGG GGG T-3') described by Beebe and Saul³¹. 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 μ l (1 μ l of DNA template and 24 μ l 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 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
Two primer sets were considered for *Wolbachia* screening in mosquito samples: W-Specf/W-Specr (5'-CAT ACC TAT TCG

AAG GGA TAG-3' and 5'- AGC TTC GAG TGA AAC CAA TTC-3') amplified a 438 bp conserved region of the 16S rRNA genes and W-Specf/W16S (5'- CAT ACC TAT TCG AAG GGA TAG -3' and 5'- TTG CGG GAC TTA ACC CAA CA -3') amplified a shorter fragment of the same region (102 bp). These two sets were selected because they were previously used by other in order to detect *Wolbachia* in *Anopheles* mosquitoes²⁰. Without *a priori* knowledge on *Wolbachia* DNA sequences detected in this study, the W-Specf/W-Specr primer set was selected for its ability to detect most *Wolbachia* strains infecting insects and to establish phylogenetic relationships among isolates³².

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 as described previously³⁰. Briefly, a published strain of laboratory-reared *Aedes aegypti* artificially infected with *Wolbachia* strain wMel were used as a reference material³³. 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 20 µl of EVAGreen qPCR Mix Plus® (Euromedex); 5 µl of DNA template was used in a total reaction volume of 25 µl. 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 amplicon 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 mosquitoes 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 *Leucopshyrus* 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^{30,34}. 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 in 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 $r^2 > 0.990$). PCR efficiency was estimated with the formula $EFF = 10^{(-1/slope)} - 1$.

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 ($r^2=0.998$ 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, $\chi^2= 2.5714$, $P=0.109$). Typical amplification and melt curves are shown in the Figure 2.

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, Jamesii, Leucosphyrus, Annularis, Barbirostris, Subpictus, Hyrcanus 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. pseudowillmori* in the Maculatus Group, *An. minimus* (*s.s.*), *An. culicifacies* B and *An. jeyporiensis* in the Funestus Group and *An. baimaii* in the Leucosphyrus Group (Table 2).

Detection of *Wolbachia* DNA in malaria vectors

The presence of *Wolbachia* DNA was assessed in six *Anopheles* species namely *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 (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).

Crossing-point values ranged from 31.0 to 40.6 amplification cycles and *Wolbachia* DNA titers were generally close or below the limit of detection of the assay (only one sample gave 3/3 positive reactions) (Table 4).

Discussion

Wolbachia DNA was detected for the first time in South-east Asian malaria vectors, including *An. maculatus* (*s.s.*), *An. sawadwongporni*, *An. pseudowillmori* (Maculatus Group), *An. dirus* (*s.s.*) and *An. baimaii* (Dirus Complex, Leucosphyrus Group).

CP values reported in this study suggest that *Wolbachia* DNA titers 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 values³⁵. Important precautions were taken to ensure the quality of our molecular data³⁶. 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

Table 1. Results of the serial dilution experiments.

Primers (%EFF, r^2) ^a	Parameter	Value of the parameter at the indicated dilution								Score (%) ^e
		Not diluted	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
W-Specf/W-Specr (108%, 0.998)	Nb. pos. / Nb. tested ^b	9/9	9/9	9/9	9/9	9/9	7/9	5/9	0/9	12/18 (66%)
	Mean CP value	18.81	21.26	24.68	28.01	30.73	34.48	34.95	-	
	Intra-assay SD ^c	0.07	0.04	0.03	0.10	0.25	1.64	0.33	-	
	Inter-assay SD ^d	0.11	0.03	0.02	0.09	0.25	1.45	0.71	-	
W-Specf/W16S (110%, 0.999)	Nb. pos. / Nb. tested	9/9	9/9	9/9	9/9	9/9	9/9	7/9	0/9	16/18 (88%)
	Mean CP value	17.80	20.71	23.98	27.29	29.90	33.40	34.86	-	
	Intra-assay SD	0.05	0.03	0.02	0.10	0.24	0.65	1.01	-	
	Inter-assay SD	0.01	0.06	0.14	0.18	0.09	0.10	1.26	-	

^a %EFF : efficiency (EFF) of the PCR was calculated with the formula $EFF = 10^{(-1/slope)} - 1$ 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; r^2 : Pearson's correlation coefficient expressing the intensity of the relationship between the logarithm of the concentration and the mean CP value. r^2 varies between 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. r^2 and EFF have been calculated on the linear dynamic of each curve (bold cells).

^b Nb. pos. / Nb. tested: number of positive reactions (amplification of the PCR DNA target) / total of reactions performed at a given dilution.

^c Intra-assay SD : intra-assay standard deviation (SD), calculated as the average SD of the mean CP value measured for each dilution during the same experiment.

^d Inter-assay SD : inter-assay standard deviation (SD), calculated as the SD of the means CP values measured during two independent experiments.

^e score of the proportion of positive reactions at low concentrations of *Wolbachia* (score was calculated on dilutions 10⁻⁵ and 10⁻⁶); an example of the calculation of the score is given here : the maximum hit for the score is 18 reactions (9 at the dilution 10⁻⁵, +9 at the dilution 10⁻⁶), the score obtained with the primer pair W-Specf/W-Specr is 66% (12/18=(7+5)/18).

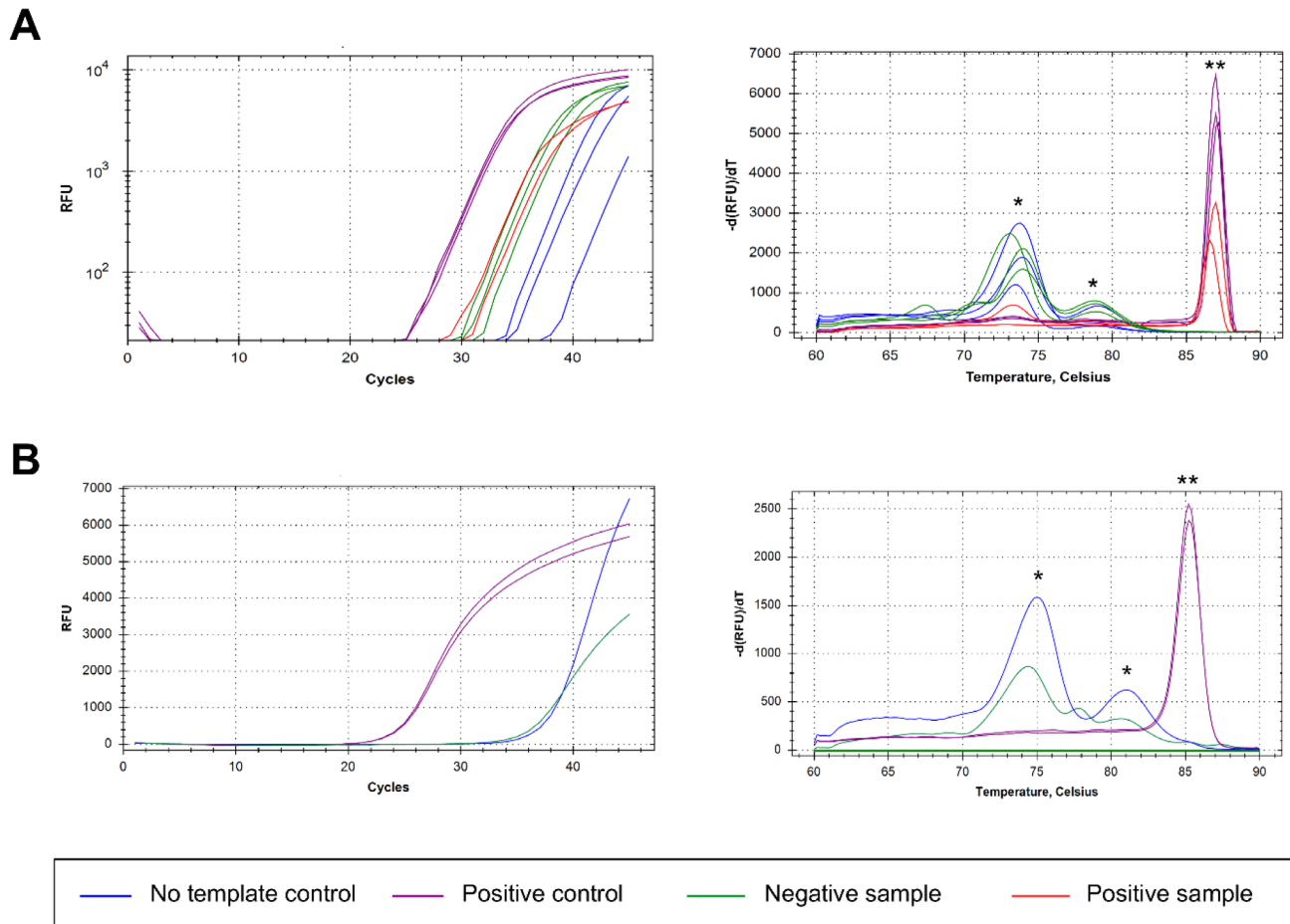


Figure 2. Typical result of the qPCR assay used for *Wolbachia* detection in mosquito samples. **A)** W-Specf/W-Specr primers; **B)** W-Specf/W16S primers. Left panels show amplification curves and right panels show the melt curve of the PCR products. (*) primer dimers, (**) PCR DNA target.

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 isolates³². 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^{19–24}. 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 significance of these findings regarding the biology and ecology of *Wolbachia-Anopheles* interactions must be interpreted cautiously as the detection of low titers of *Wolbachia* DNA by PCR is not unequivocal of an actual symbiosis between *Wolbachia* and the mosquito. 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

Table 2. Village-collated human-biting rate estimates of *Anopheles* mosquitoes.

Group	Species	Human-biting rate estimate (95%CI) in the indicated dilution, expressed in number of bites/person/month										
		HD-3634	HG-369	LK-350	MK-3633	MK-3635	MM-3631	NT-361	TG-357	TP-339	WM-367	
Annularis	<i>An. annularis</i> (s.l.) ^b	40.2 (31.2-51.1)	28.8 (21.2-38.2)	24 (17.1-32.7)	29.4 (21.8-38.9)	4.2 (1.7-8.7)	30 (22.3-39.6)	40.2 (31.2-51.1)	65.4 (53.7-78.9)	7.2 (3.7-12.6)	28.8 (21.2-38.2)	
	<i>An. asiaticus</i> (s.l.)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0.6 (0-3.3)	0 (0-2.2)	
Barbistrois	<i>An. barbistrois</i> (s.l.) ^b	27 (19.7-36.1)	2.4 (0.7-6.1)	16.8 (11.2-24.3)	3.6 (1.3-7.8)	5.4 (2.5-10.3)	10.2 (5.9-16.3)	3.6 (1.3-7.8)	24.6 (17.7-33.4)	6 (2.9-11)	2.4 (0.7-6.1)	
	<i>An. aconitus</i> ^b	3 (1-7)	1.2 (0.1-4.3)	9.6 (5.5-15.6)	6 (2.9-11)	3.6 (1.3-7.8)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	
Funestus	<i>An. culicifacies</i> A ^c	9 (5-14.8)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	3.6 (1.3-7.8)	6 (2.9-11)	4.2 (1.7-8.7)	1.8 (0.4-5.3)	0 (0-2.2)	
	<i>An. culicifacies</i> B	36.6 (28-47)	7.2 (3.7-12.6)	0 (0-2.2)	73.8 (61.3-88.1)	12.6 (7.8-19.3)	123.6 (107.3-141.7)	94.2 (80-110.1)	7.8 (4.2-13.3)	1.8 (0.4-5.3)	26.4 (19.2-35.4)	
Hyrcanus	<i>An. harrisoni</i>	3 (1-7)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	4.2 (1.7-8.7)	0 (0-2.2)	0.6 (0-3.3)	
	<i>An. jeyporiensis</i> ^c	3 (1-7)	1.2 (0.1-4.3)	12.6 (7.8-19.3)	6 (2.9-11)	44.4 (34.9-55.7)	3.6 (1.3-7.8)	3 (1-7)	78.6 (65.7-93.3)	3.6 (1.3-7.8)	0 (0-2.2)	
Jamesii	<i>An. minimus</i> ^a	161.4 (142.7-181.9)	46.8 (37-58.4)	240 (217.1-264.7)	185.4 (165.3-207.3)	96 (81.7-112.1)	167.4 (148.3-188.2)	141.6 (124.1-160.9)	259.2 (235.3-284.8)	141 (123.5-160.2)	52.2 (41.8-64.4)	
	<i>An. varuna</i>	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	4.2 (1.7-8.7)	0 (0-2.2)	0 (0-2.2)	
Kochi	<i>An. hyrcanus</i> (s.l.) ^b	0.6 (0-3.3)	0.6 (0-3.3)	2.4 (0.7-6.1)	0.6 (0-3.3)	0 (0-2.2)	0 (0-2.2)	2.4 (0.7-6.1)	0.6 (0-3.3)	0 (0-2.2)	0 (0-2.2)	
	<i>An. jamesii</i> (s.l.) ^b	140.4 (123-159.6)	27 (19.7-36.1)	9 (5-14.8)	15.6 (10.2-22.9)	8.4 (4.6-14.1)	15.6 (10.2-22.9)	46.2 (36.5-57.7)	141.6 (124.1-160.9)	119.4 (103.4-137.2)	44.4 (34.9-55.7)	
Leucosphyrus	<i>An. kochi</i>	37.8 (29-48.4)	1.8 (0.4-5.3)	3 (1-7)	0.6 (0-3.3)	55.8 (45-68.4)	6.6 (3.3-11.8)	15.6 (10.2-22.9)	36 (27.5-46.3)	54 (43.4-66.4)	2.4 (0.7-6.1)	
	<i>An. baimai</i> ^a	31.8 (23.8-41.6)	1.8 (0.4-5.3)	24.6 (17.7-33.4)	10.2 (5.9-16.3)	13.2 (8.3-20)	18.6 (12.6-26.4)	15 (9.7-22.1)	24 (17.1-32.7)	11.4 (6.9-17.8)	6.6 (3.3-11.8)	
WM-367	<i>An. dirus</i> ^a	2.4 (0.7-6.1)	0 (0-2.2)	0 (0-2.2)	1.2 (0.1-4.3)	0 (0-2.2)	1.8 (0.4-5.3)	0 (0-2.2)	1.8 (0.4-5.3)	0.6 (0-3.3)	0 (0-2.2)	
	<i>An. introlatus</i>	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0.6 (0-3.3)	0 (0-2.2)	

Group	Species	Human-biting rate estimate (95%CI) in the indicated dilution, expressed in number of bites/person/month									
		HD-3634	HG-369	LK-350	MK-3633	MK-3635	MM-3631	NT-361	TG-357	TP-339	WM-367
	<i>An. maculatus</i> ^a	241.2 (218.2-266)	40.8 (31.7-51.7)	88.2 (74.5-103.7)	26.4 (19.2-35.4)	59.4 (48.3-72.3)	61.8 (50.4-75)	226.8 (204.5-250.9)	1112.4 (1062.3-1164.2)	315 (288.6-343.1)	39 (30.1-49.7)
	<i>An. pseudowillmori</i> ^b	6.6 (3.3-11.8)	3.6 (1.3-7.8)	0 (0-2.2)	4.2 (1.7-8.7)	15 (9.7-22.1)	1.2 (0.1-4.3)	3.6 (1.3-7.8)	19.2 (13.1-27.1)	0 (0-2.2)	0 (0-2.2)
	<i>An. rampae</i>	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	0.6 (0-3.3)	0 (0-2.2)	0 (0-2.2)	0 (0-2.2)	38.4 (29.6-49)	0 (0-2.2)	0 (0-2.2)
Maculatus	<i>An. sawadwongporni</i> ^a	42 (32.7-53.1)	12.6 (7.8-19.3)	25.2 (18.2-34.1)	21.6 (15.1-29.9)	3.6 (1.3-7.8)	16.8 (11.2-24.3)	66 (54.2-79.5)	307.2 (281.2-335)	42 (32.7-53.1)	12 (7.3-18.5)
Maculatus-related	<i>An. karwarf</i> ^c	15 (9.7-22.1)	2.4 (0.7-6.1)	0 (0-2.2)	2.4 (0.7-6.1)	1.2 (0.1-4.3)	0 (0-2.2)	3 (1-7)	11.4 (6.9-17.8)	3 (1-7)	0.6 (0-3.3)
Subpictus	<i>An. subpictus</i> (s.l.) ^c	79.8 (66.8-94.6)	40.8 (31.7-51.7)	41.4 (32.2-52.4)	13.2 (8.3-20)	5.4 (2.5-10.3)	58.8 (47.7-71.7)	137.4 (120.2-156.4)	24.6 (17.7-33.4)	182.4 (162.5-204.1)	119.4 (103.4-137.2)
Tessellatus	<i>An. tessellatus</i> ^c	3.6 (1.3-7.8)	0 (0-2.2)	24 (17.1-32.7)	2.4 (0.7-6.1)	0 (0-2.2)	14.4 (9.2-21.4)	8.4 (4.6-14.1)	1.2 (0.1-4.3)	3.6 (1.3-7.8)	0.6 (0-3.3)

^a primary malaria vectors; ^b secondary malaria vectors; ^c efficient malaria vector species in some areas that were never reported infected with human malaria parasites on the Thailand-Myanmar border²⁵. Human-biting rates of *sensu stricto* anopheline species in the Funestus, Maculatus and Leucopshyrus 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.

Group	Species	Nb. pos / Nb. tested (<i>Wolbachia</i> supergroup) in the indicated village and species									
		HD-3634	HG-369	LK-350	MK-3633	MK-3635	MM-3631	NT-361	TG-357	TP-339	WM-367
Funestus	<i>An. minimus</i>	0/10	2/10 (F)	0/10		0/10	0/10 (D)	1/10 (D)	0/10	1/10 (D)	0/10
Maculatus	<i>An. maculatus</i>	0/10	0/10	0/10		2/10 (B)	0/10	0/10	1/10 (F)	0/11	1/9 (B)
	<i>An. pseudowillmori</i>	0/1	0/1		0/1	1/7 (B)			0/1		
	<i>An. sawadwongporni</i>	0/8	0/3	0/9	0/10	1/1 (B)	0/10	0/11	0/10	0/4	0/2
Leucopshyrus	<i>An. baimaili</i>	0/10	0/2	0/10	1/10 (D)	0/11	0/10	0/10	0/10	1/16 (B)	0/10
	<i>An. dirus</i>	0/4			0/2		0/2		0/3	1/1 (B)	

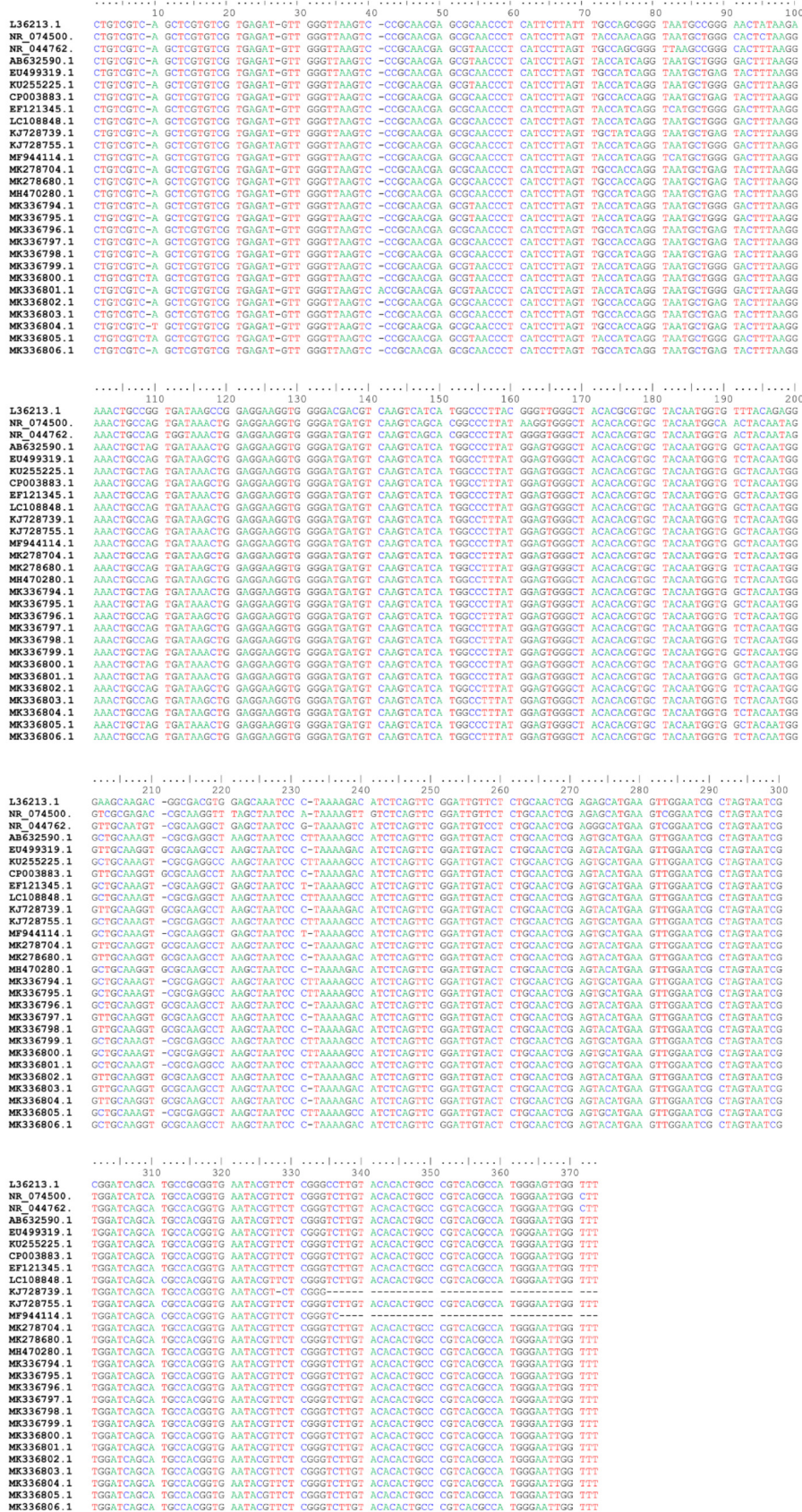


Figure 3. Multiple alignment of 16S rRNA sequences used to build the Tamara-Nei genetic distance model and neighbor-joining tree.

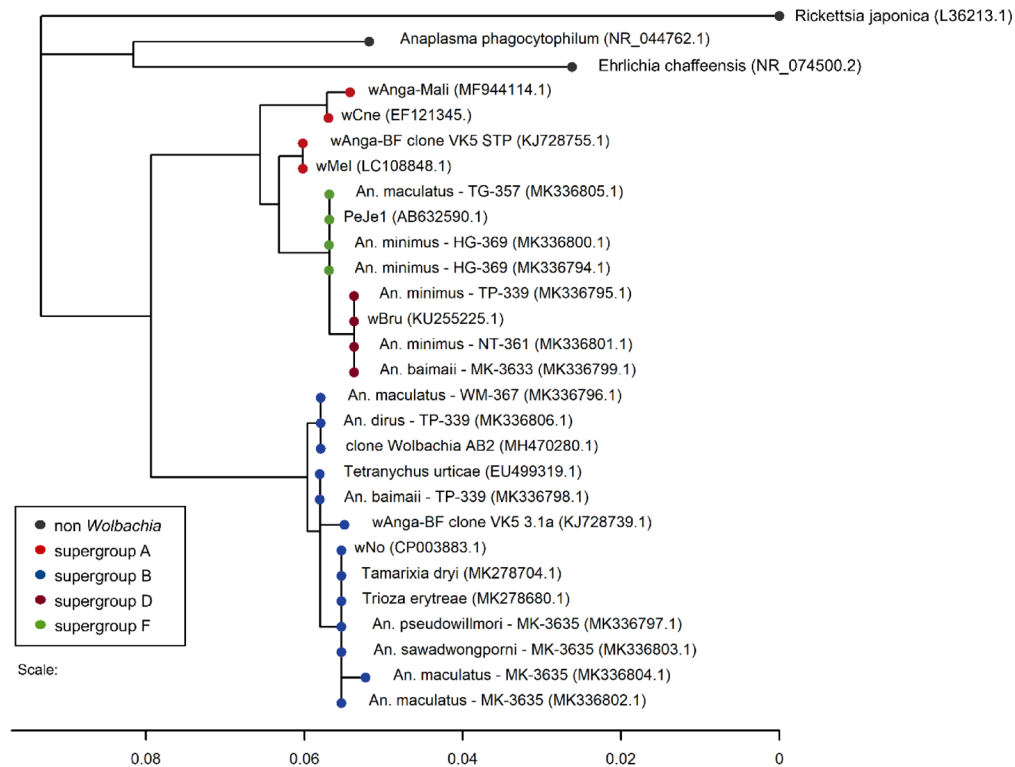


Figure 4. Phylogenetic analysis based on the alignment of a conserved region of the 16S rRNA gene using *Wolbachia*-specific primer pair W-Specf/W-Specr. Sequences of the PCR products were blasted against the NCBI nucleotide database and the most similar result was downloaded. A phylogenetic tree was 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*, wMei of *Drosophila melanogaster*, wPeJe1 of *Penicillidia jenyinsii* and wBru of *Brugia malayi*.

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

Sample ID	Village	Species	Nb pos	CP1	CP2	CP3	Supergroup
1	HG-369	<i>An. minimus</i>	1	35.8			F
2	HG-369	<i>An. minimus</i>	1	33.0			F
3	MK-3633	<i>An. baimaii</i>	1	35.6			D
4	MK-3635	<i>An. maculatus</i>	1	34.3			B
5	MK-3635	<i>An. maculatus</i>	1	34.3			B
6	MK-3635	<i>An. pseudowillmori</i>	1	37.6			B
7	MK-3635	<i>An. sawadwongporni</i>	2	34.5	32.8		B
8	NT-361	<i>An. minimus</i>	3	36.8	35.8	36.6	D
9	TG-357	<i>An. maculatus</i>	1	34.2			F
10	TP-339	<i>An. baimaii</i>	3	33.0	31.0	32.3	B
11	TP-339	<i>An. dirus</i>	1	34.1			B
12	TP-339	<i>An. minimus</i>	1	40.6			D
13	WM-367	<i>An. maculatus</i>	1	32.6			B

in mosquitoes. Chrostek and Gerth further 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*³⁷. Given that most arthropods are infected with *Wolbachia*³⁸, we cannot exclude that the DNA sequences detected in this study come from some sort of environmental contamination. An alternative explanation could be that horizontal transfers of *Wolbachia* happen at a much higher frequency than previously thought, for example via plants³⁹ or via ectoparasitic mites^{40,41}. Additional experiments would be of great interest to demonstrate actual infection, e.g. showing intracellular localization of the sequences and maternal transmission of the bacteria. Finally, we did not assess the effects of the presence of *Wolbachia* DNA on the phenotype of 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 populations²⁵. Therefore, it was not possible to establish direct correlations between *Plasmodium* and the presence of *Wolbachia* DNA in the mosquito vectors. In this setting, the effect of possible *Wolbachia* infections on malaria transmission may be better assessed by performing artificial infections of field-collected mosquitoes with *Plasmodium* malaria parasites.

Conclusion

The detection *Wolbachia* DNA in malaria vectors from Kayin state warrants 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).

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Thank you for the revisions. The problematic "Wolbachia infection" has been mostly replaced, except from one place in the discussion. Also, I disagree with calling the Wolbachia DNA from previous reports an infection in the abstract and the introduction of this paper. In my opinion, this study is not deficient compared to the previous ones on Wolbachia in Anopheles. It only has the potential to be more carefully worded.

I agree with the author's interpretation of the qPCR results. However, as primer dimers contribute to the total SYBR green fluorescence in the qPCR reaction this assay cannot be quantitative. Also, only running the qPCR products on the agarose gel can reveal if the bands can indeed be distinguished by a conventional PCR. As the authors do not aim at PCR-identification or qPCR quantification of Wolbachia here this is not crucial. It is curious though, and suggests that the qPCR assay could be optimized further.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I have been applying molecular methods to study Wolbachia symbionts of insects since 2011. In 2016-2017, I was working on putative Wolbachia infections in *Anopheles gambiae* and *Anopheles coluzzi*.

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.

Version 3

Reviewer Report 15 October 2019

<https://doi.org/10.21956/wellcomeopenres.16939.r36677>

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Ewa Chrostek 

Institute of Integrative Biology, University of Liverpool, Liverpool, UK

In this study, the authors provide evidence for qPCR amplification of a fragment of Wolbachia 16S sequence from 13 out of 370 field-collected Southeast-Asian mosquitoes. However, a single, low level, hard to amplify (despite extensive optimisation) fragment of Wolbachia rDNA does not provide sufficient evidence for the Wolbachia infection in these malaria vectors. The environment is severely contaminated with Wolbachia as most arthropods are infected with this symbiont (Weinert *et al.* 2015¹). To show an infection, additional experiments are necessary, e.g. showing intracellular localization of the sequences and maternal transmission of the bacteria (see Chrostek and Gerth 2019² for further discussion). I suggest changing the wording throughout, from “natural Wolbachia infections” to “Wolbachia 16S/wSpec sequence amplification”. I also recommend changing the title to: “Detection of low-density Wolbachia 16S sequences from malaria vectors in Kayin state, Myanmar”.

In the results section, the authors identify the supergroups D and F Wolbachia sequences as likely environmental/parasitic contaminants (6 out of 13 positive samples). It is unclear why they do not use the same caution while interpreting data on the remaining 7 sequences from supergroup B.

Figure 2 shows an interesting property of wSpec primer set. The amplification from the positive and negative samples produces similar amplification curves (Fig. 2A, green and red lines) with very similar CPs (crossing-points), that can be distinguished from each other by the shape of their melt curves (Fig. 2B). This figure shows, that when using wSpec primer set for a PCR, negative samples produce an amplicon as well. Whether the positive and negative amplicons can be distinguished by agarose gel electrophoresis, and therefore if classical PCR with wSpec has any diagnostic value when used under these conditions, remain to be determined. The comment on this in the text would help future studies embarking on Wolbachia identification projects.

Minor comments:

1. The information on the origin of the laboratory-reared wMel-infected *A. aegypti* is missing. Were they made by the authors or are they the published strains?
2. The sequence of W16S primer, the amplicon sizes for both qPCR reactions and the rationale behind trying both primer sets should be added.
3. In the discussion, p7/28, first line: the integration into the chromosome would have produced “higher CP values”, and not as it is - “lower”.

References

1. Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ: The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc Biol Sci.* 2015; **282** (1807): 20150249 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Chrostek E, Gerth M: Is *Anopheles gambiae* a Natural Host of Wolbachia?. *mBio.* 2019; **10** (3). [Publisher Full Text](#)

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?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

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?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I have been applying molecular methods to study Wolbachia symbionts of insects since 2011. In 2016-2017, I was working on putative Wolbachia infections in *Anopheles gambiae* and *Anopheles coluzzi*.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 01 Nov 2019

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

We thank to reviewer for her useful comments on the manuscript. The wording “natural infection” was changed in the title and throughout the manuscript as per reviewer’s suggestion. In addition, part of the discussion was rewritten and suggested reference was added in order to better emphasize this study limitation in the revised version of the manuscript. The possibility of environmental contamination for all sequences including that of the supergroup B was explicitly discussed in the Discussion section. The amplicon produced in no-template controls and negative samples certainly results from the formation of primer dimers, which are unlikely to reach the size of 438 bp. Therefore, it should be possible to discriminate between primer dimers and amplification of the PCR DNA target using gel electrophoresis. Nevertheless, the reviewer is right to question the value of conventional PCR in the field of molecular diagnostic given the high risk of false positive by contamination [1]. This issue has been extensively discussed in the manuscript and we do not wish to add more details in the current version of the manuscript. Point-by-point answers to specific comments are listed below:

1. *The information on the origin of the laboratory-reared wMel-infected A. aegypti is missing.*

Were they made by the authors or are they the published strains?

Wolbachia-infected reference samples were kindly provided by Dr. Lauren Carrington from the Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam. Corresponding reference [2] was added in the methods section of the revised version of the manuscript.

2. *The sequence of W16S primer, the amplicon sizes for both qPCR reactions and the rationale behind trying both primer sets should be added.*

The sequence of W16S primer, the amplicon sizes for both qPCR reactions and the rationale behind trying both primer sets were added in the Methods section in the revised version of the manuscript.

3. *In the discussion, p7/28, first line: the integration into the chromosome would have produced “higher CP values”, and not as it is - “lower”.*

The integration of *Wolbachia* genome into the mosquito chromosome would have resulted in a higher copy number of PCR DNA target in the sample (several millions versus a few dozens), and therefore in a lower CP value given the negative correlation that exists between the CP value and the logarithm of the concentration of PCR DNA target in the sample.

1. Apfalter P, Reischl U, Hammerschlag MR. In-house nucleic acid amplification assays in research: how much quality control is needed before one can rely upon the results? *J Clin Microbiol.* 2005;43 12:5835-41; doi: 10.1128/jcm.43.12.5835-5841.2005.

<http://dx.doi.org/10.1128/jcm.43.12.5835-5841.2005>.

2. Carrington LB, Tran BCN, Le NTH, Luong TTH, Nguyen TT, Nguyen PT, et al. Field- and clinically derived estimates of *Wolbachia*-mediated blocking of dengue virus transmission potential in *Aedes aegypti* mosquitoes. *Proc Natl Acad Sci U S A.* 2018;115 2:361-6; doi: 10.1073/pnas.1715788115. <http://dx.doi.org/10.1073/pnas.1715788115>.

Competing Interests: No competing interests were disclosed.

Version 2

Reviewer Report 06 September 2019

<https://doi.org/10.21956/wellcomeopenres.16771.r35969>

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Francesco Baldini 

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

The second version of this manuscript provides greater clarity and detailed methodological information. Limitations of the study are also discussed. Overall the manuscript contains novel insights on natural *Wolbachia* strains in *Anopheles* species from Myanmar, calling for further investigations on the role of these endosymbionts on the biology of malaria mosquitoes.

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

No

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

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

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

No

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

No

Are the conclusions drawn adequately supported by the results?

No

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.

Reviewer Report 22 July 2019

<https://doi.org/10.21956/wellcomeopenres.16771.r35968>

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Thomas Walker 

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

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^{-6} (34.95 +/-1.64) not being statistically higher than to the 10^{-5} 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, 2000¹) 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. baimaii*). 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

1. Werren JH, Windsor DM: *Wolbachia* infection frequencies in insects: evidence of a global equilibrium?. *Proc Biol Sci.* 2000; **267** (1450): 1277-85 [PubMed Abstract](#) | [Publisher Full Text](#)

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

No

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

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

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

No

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

No

Are the conclusions drawn adequately supported by the results?

No

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.

Author Response 18 Sep 2019

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

We thank to reviewer for his useful comments on the manuscript. Reviewer's concern on the comparison of *Wolbachia* DNA titers measured in wild *Anopheles* and laboratory-reared *Aedes aegypti* was addressed by removing the corresponding sentences in the Methods, Results and Discussion sections. Our data clearly demonstrate that wild *Anopheles* are infected with very low *Wolbachia* densities given the high CP values of the screened samples (>30 cycles) and the very low CP values of the reference *Aedes aegypti* samples (<19 cycles). The reference to "low-density infections" in the title is therefore valid, and does not overlook low densities as a specific feature of the strains detected in this study. We agree with reviewer's comment on the limitation of not having additional sequence data on other markers, and regret that it was not possible to generate such data in the current study. Although it not possible to assess accurately the phylogenic relationships between different *Wolbachia* strains only with 16S rRNA sequences, our data clearly demonstrate that the *Wolbachia* strains detected in this study are genetically diverse. Therefore, we wish to keep the title of the manuscript unchanged.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 04 March 2019

<https://doi.org/10.21956/wellcomeopenres.16370.r34658>

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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 rationale 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 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 rationale 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

<https://doi.org/10.21956/wellcomeopenres.16370.r34878>

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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^{1,2,3}.

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 (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.

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 9⁹). 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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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.

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 TATTCGAAGGGATAG) 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 in 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-time 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 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 9⁹). 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.

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Competing Interests: No competing interests were disclosed.