**Wolbachia 16S rRNA haplotypes detected in wild *Anopheles stephensi* in eastern Ethiopia**

Elizabeth Waymire¹, Sowmya Duddu¹, Solomon Yared², Dejene Getachew³, Dereje Dengela⁴, Sarah R. Bordenstein⁵, Meshesha Balkew⁴, Sarah Zohdy⁶, Seth R. Irish⁶ and Tamar E. Carter¹*

**Abstract**

**Background:** About two out of three Ethiopians are at risk of malaria, a disease caused by the parasites *Plasmodium falciparum* and *Plasmodium vivax*. *Anopheles stephensi*, an invasive vector typically found in South Asia and the Middle East, was recently found to be distributed across eastern and central Ethiopia and is capable of transmitting both *P. falciparum* and *P. vivax*. The detection of this vector in the Horn of Africa (HOA) coupled with widespread insecticide resistance requires that new methods of vector control be investigated in order to control the spread of malaria. *Wolbachia*, a naturally occurring endosymbiotic bacterium of mosquitoes, has been identified as a potential vector control tool that can be explored for the control of malaria transmission. *Wolbachia* could be used to control the mosquito population through suppression or potentially decrease malaria transmission through population replacement. However, the presence of *Wolbachia* in wild *An. stephensi* in eastern Ethiopia is unknown. This study aimed to identify the presence and diversity of *Wolbachia* in *An. stephensi* across eastern Ethiopia.

**Methods:** DNA was extracted from *An. stephensi* collected from eastern Ethiopia in 2018 and screened for *Wolbachia* using a 16S targeted PCR assay, as well as multilocus strain typing (MLST) PCR assays. Haplotype and phylogenetic analysis of the sequenced 16S amplicons were conducted to compare with *Wolbachia* from countries across Africa and Asia.

**Results:** Twenty out of the 184 mosquitoes screened were positive for *Wolbachia*, with multiple haplotypes detected. In addition, phylogenetic analysis revealed two superclades, representing *Wolbachia* supergroups A and B (bootstrap values of 81 and 72, respectively) with no significant grouping of geographic location or species. A subclade with a bootstrap value of 89 separates the Ethiopian haplotype 2 from other sequences in that superclade.

**Conclusions:** These findings provide the first evidence of natural *Wolbachia* populations in wild *An. stephensi* in the HOA. They also identify the need for further research to confirm the endosymbiotic relationship between *Wolbachia* and *An. stephensi* and to investigate its utility for malaria control in the HOA.

**Keywords:** *Anopheles stephensi*, *Wolbachia*, Horn of Africa, Malaria, Vector control, Disease control
across eastern Ethiopia [5]. It has been demonstrated that \textit{An. stephensi} is capable of transmitting both \textit{P. falciparum} and \textit{P. vivax} in eastern Ethiopia and the establishment of this vector could increase the prevalence of \textit{P. falciparum} cases by 50\% in areas found to be suitable if no additional intervention occurs [6, 7]. With the knowledge that \textit{An. stephensi} can transmit both \textit{Plasmodium} species in eastern Ethiopia, strategies for the control or elimination of \textit{An. stephensi} in the HOA are being investigated. Insecticide resistance has already been identified in \textit{An. stephensi} in eastern Ethiopia [8, 9], requiring alternative methods of vector control such as the use of \textit{Wolbachia}.

\textit{Wolbachia pipiens} is an endosymbiotic bacterium of invertebrates that is classified into different supergroups based on major phylogenetic diversions. All \textit{Wolbachia} in mosquitoes belong to supergroups A and B [10]. \textit{Wolbachia} has been identified as a resource to address vector-borne diseases in \textit{Aedes} mosquitoes through two main methods: population suppression [11] and population replacement [12, 13]. Both methods are based on the mechanism of cytoplasmic incompatibility (CI). When an uninfected female mosquito mates with a male mosquito infected with \textit{Wolbachia}, embryonic death occurs, and the female's larvae are inviable. In population suppression, male mosquitoes infected with \textit{Wolbachia} are released on a large scale to decrease the population size, thus decreasing the transmission of vector-borne diseases. Population replacement involves releasing both male and female mosquitoes with \textit{Wolbachia} infection into a population of \textit{Wolbachia}-free, disease-carrying mosquitoes [14].

Generally, the relationship between \textit{Wolbachia} infection and \textit{Plasmodium} infection in field \textit{Anopheles} species is not well studied. Kambris et al. [15] and Hughes et al. [16] have shown that in the lab, \textit{Anopheles gambiae} can be infected with \textit{Wolbachia} pipientis, and that \textit{Plasmodium berghei} and \textit{P. falciparum} development is inhibited with both \textit{Wolbachia} strains. In field populations of \textit{Anopheles coluzzii} and \textit{An. gambiae}, Shaw et al. [17] and Gomes et al. [18] demonstrated that \textit{Wolbachia} infection correlated negatively with \textit{Plasmodium} development. This is indicative of potential for \textit{Wolbachia}-based interventions, but more research is needed.

In \textit{An. stephensi} specifically, \textit{Wolbachia} infections in lab populations have been briefly studied. Bian et al. [19] were able to stably infect \textit{An. stephensi} with \textit{Wolbachia} pipientis. They showed that maternal transmission and CI induction were successful, and observed resistance to \textit{P. falciparum} infection in the lab strain of \textit{An. stephensi}. Chen et al. [20] showed that \textit{An. stephensi} can be stably infected by \textit{Wolbachia} pipientis and that there was no change in the microbiome of the mosquitoes upon \textit{Wolbachia} infection. These results indicate that lab strains of \textit{An. stephensi} can hold stable infections of \textit{Wolbachia}, which may mean that field populations are susceptible to infection, although further research is needed [18].

The limited study of \textit{Wolbachia} infection in \textit{An. stephensi} has mainly been in lab populations. We need to determine whether \textit{Wolbachia} infection is present in order to begin evaluating the potential of \textit{Wolbachia}-based control strategies for malaria transmission in \textit{An. stephensi} in eastern Ethiopia. Furthermore, the strain of \textit{Wolbachia} in the wild host population needs to be determined, to establish which population control strategy would function best, should a validated \textit{Wolbachia}-based malaria control tool become achievable [13]. \textit{Wolbachia} is typically detected via DNA or RNA extraction from the host invertebrate and targeting multiple genes to determine infection. These genes include the 16S-encoding gene, the surface protein-encoding gene (\textit{Wolbachia} surface protein \texttt{[wsp]}), and the five multilocus strain typing (MLST) genes (\texttt{ftsZ}, \texttt{fbpA}, \texttt{hcpA}, \texttt{coxA}, and \texttt{gatB}) [10]. Previous studies have depended solely on \textit{Wolbachia} 16S for confirming infection [18, 19]; however, more recent research has indicated that multiple genes must be detected in order to confirm infection [20]. Very few \textit{Anopheles} species have been screened for \textit{Wolbachia}, with proven infection occurring only in two highly diverged species in sub-Saharan Africa [21]. There has also been significant difficulty in many studies with amplifying the genes in MLST. Gomes et al. were only able to amplify three out of five genes, and Jeffries et al. were unable to amplify any MLST genes in multiple sample groups [18, 20]. This may indicate that environmental contamination is present or that divergence among these genes has emerged, precluding the use of the standard MLST primer set [18, 22, 23].

Since the detection of \textit{An. stephensi} in the HOA, no investigation regarding the possibility of \textit{Wolbachia} endosymbiosis has been conducted in this population. One study surveyed \textit{An. stephensi} for \textit{Wolbachia} in Tamil Nadu, India, and reported the detection of \textit{16S Wolbachia} DNA in a portion of their mosquitoes [24]. Still, more information is needed to confirm whether \textit{Wolbachia} is a true endosymbiont of \textit{An. stephensi}, as \textit{Wolbachia} in \textit{An. stephensi} in the Middle East and the rest of South Asia has not been investigated.

In order to evaluate \textit{Wolbachia} as a potential control method for malaria transmission, we first need to know whether \textit{Wolbachia} is present in \textit{An. stephensi} in eastern Ethiopia. In addition, the detection of \textit{Wolbachia} DNA could provide a basis for using it in the complementary phylogeographic analysis of the \textit{An. stephensi} invasion. In this study, we used \textit{Wolbachia} 16S ribosomal RNA
(rRNA)-, wsp-, and MLST-targeted polymerase chain reaction (PCR) assays to detect Wolbachia in An. stephensi from eastern Ethiopia.

Methods

Sampling of An. stephensi

Anopheles stephensi surveys were conducted from August through November 2018 in Semera, Godey, Kebridehar, Erer-Gota, and Dire Dawa. Details about the collection sites and approach have been described previously [5]. Briefly, adult mosquitoes were collected via pyrethrum spray catch (PSC) and Centers for Disease Control and Prevention (CDC) light traps. The collected mosquitoes were sorted between culicines and Anoph eles, and the latter were further sorted to distinguish An. stephensi from other Anopheles species using a standard morphological key [25] and a key prepared by Coetzee (subsequently published in 2020 [26]). Analysis of Plasmodium detection was conducted previously, and no wild-caught adult mosquitoes contained Plasmodium falciparum or P vivax DNA [5]. Larvae and pupae of Anopheles were also collected from larval habitats including artificial water storage containers, freshwater pools, discarded tires, and plastic containers. Larvae were reared in field insectaries using water taken from larval habitats, feeding them with baker’s yeast and exposing them to sunlight during the day. Pupae were transferred into adult emergence cages and adults were morphologically identified using the same keys [25, 26]. The numbers of wild-caught adults and reared larvae are documented in Additional file 1: Table S1.

DNA extraction and molecular identification of mosquitoes

DNA was extracted from either the abdomen or whole bodies of An. stephensi mosquitoes, selected at random, using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). PCR was performed for each individual mosquito, targeting the nuclear internal transcribed spacer 2 (ITS2) region and the mitochondrial cytochrome c oxidase subunit 1 (COI). The reagent components and final concentrations for the PCR assays were 1× Promega Hot Start Master Mix (Promega, Madison, WI, USA) and 0.5 mM for both primers, plus 1 µl of isolated DNA template. A region including the ITS2 gene was amplified via PCR using universal primers as described previously [3]. The PCR temperature protocol consisted of 95 °C for 1 min, 30 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min; followed by 72 °C for 10 min.

For the wsp assay, a 546 bp region including the gene was amplified using primers as described in Zhou et al. [30]. The PCR temperature protocol was as follows: 94 °C for 2 min, then 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; followed by 72 °C for 5 min. All primers used are listed in Additional file 1: Table S2. All PCR products were run on a 2% agarose gel, and the gel was visualized using a Gel Doc EZ imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA) prior to Sanger sequencing by a commercial laboratory (Eurofins Genomics LLC).

Multilocus strain typing

In addition to amplifying the 16S and wsp genes, MLST was performed on each 16S-positive sample to determine whether the Wolbachia infection was present. Five loci, including ftsZ, fbpA, hcpA, coxA, and gatB, were tested,
using region-specific primers. The *ftsZ* universal assay amplified a 775 bp region [31], the *fbpA* assay amplified 429 bp, the *hcpA* assay amplified a 444 bp region, the *coxA* assay amplified a 402 bp region, and the *gatB* assay amplified a 369 bp region [10]. The final concentrations for the PCR assays were 1X Promega Hot Start Master Mix (Promega, Madison, WI, USA), 0.4 mM for each primer, plus 2 µl of isolated DNA template for each reaction.

The PCR cycling protocol for *coxA*, *hcpA*, and *gatB* consisted of 94 °C for 2 min, 36 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 1 min and 30 s; followed by 72 °C for 10 min. The PCR cycling protocol for *fbpA* consisted of 94 °C for 2 min, 36 cycles of 94 °C for 30 s, 59 °C for 45 s, and 72 °C for 1 min and 30 s; followed by 72 °C for 10 min. Lastly, the PCR cycling protocol for *ftsZ* universal consisted of 95 °C for 2 min, 35 cycles of 94 °C for 30 s, 58.2 °C for 1 min, and 72 °C for 1 min; followed by 72 °C for 5 min.

A nested protocol was used for all five MLST genes. The *hcpA*, *fbpA*, and *gatB* nested assays were used as previously described on the PubMed website [32]. Two microliters of PCR product from the un-nested reaction was used in this reaction as template DNA. Nested PCR cycling conditions were as follows: 95 °C for 15 min, 50 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min; followed by 72 °C for 5 min. The *ftsZ* and *coxA* nested assays were used as described previously and amplified products 424 bp and 357 bp in length, respectively [33]. The nested PCR protocol for *ftsZ* and *coxA* consisted of 94 °C for 5 min, 36 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; followed by 72 °C for 10 min. All primers used are listed in Additional file 1: Table S2. All PCR products were run on a 2% agarose gel, and the gel was visualized using a Gel Doc EZ imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Sequence and phylogenetic analysis**

Sequences were cleaned and analyzed using CodonCode (CodonCode Corporation, Centerville, MA, USA). *Wolbachia 16S*, *Wolbachia coxA*, and *An. stephensi COI* sequences were submitted as queries to the National Center for Biotechnology Information’s (NCBI) Basic Local Alignment Search Tool (BLAST) against the nucleotide collection under standard parameters (100 max target sequences, expect threshold 0.05, word size 28, optimized for highly similar sequences, not specific to any organism). This information was used to confirm that the amplicons produced were the *Wolbachia 16S* gene, *Wolbachia coxA* gene, or *An. stephensi COI*. An alignment of observed haplotypes and the nucleotide conservation in the *An. stephensi Wolbachia 16S* sequences were visualized on CLC Sequence Viewer 7.6 (CLC Bio, Aarhus, Denmark).

Phylogenetic analysis was performed with the representative 16S sequences collected from this study, as well as peer-reviewed, published *Wolbachia 16S* sequences from NCBI GenBank in anopheline mosquitoes with an outgroup of *Rickettsia japonica* (Additional file 1: Table S3) [18, 19, 33–38]. Phylogenetic analysis was also performed with the *coxA* sequences collected from this study, as well as peer-reviewed, published *Wolbachia coxA* sequences from NCBI GenBank in anopheline mosquitoes with an outgroup of the *Wolbachia* endosymbiont of *Dirofilaria immitis* (Additional file 1: Table S3) [18, 33, 36]. An additional phylogenetic analysis was performed with the *Wolbachia 16S* sequences from sub-Saharan Africa used in the first analysis, the three 16S haplotypes collected in this study, and four 16S sequences from a study in pre-print from Tamil Nadu, India (Additional file 1: Table S3) [18, 24, 33, 36]. Lastly, phylogenetic analysis was performed on the COI genes from the *An. stephensi* that were *Wolbachia 16S*-positive, with an outgroup of *Anopheles maculatus* (Additional file 1: Table S3). Representative sequences from each location were determined by aligning sequences and eliminating non-distinct haplotypes. Alignments were created with MAFFT version 7 [39] and trimmed to 283 bp (16S; Fig. 2), 323 bp (16S; Additional file 1: Fig. S1), 357 bp (*coxA*; Fig. 3), and 321 bp (COI; Fig. 4) using Mesquite version 3.61 [40]. Phylogenetic associations between all sequences were estimated using a maximum likelihood approach with RAxML [41]. The GTR+GAMMA option that utilizes the general time-reversible (GTR) model of nucleotide substitution with the gamma model of the rate of heterogeneity was used. A total of 1000 runs were completed using the maximum likelihood criteria with rapid bootstrap analysis. The RAxML output was viewed in FigTree [42] with a root at the outgroup and transformed branches, and a phylogenetic tree image was made.

**Results**

*Wolbachia detected in four of five survey sites*

A total of 184 mosquito samples were tested, with 20 samples testing positive for *Wolbachia 16S* DNA in the nested PCR (Table 1). Two of these 20 samples were from wild-caught adult mosquitoes, whereas 18 were from laboratory-reared adults. Out of the 20 samples, seven samples were also positive in the un-nested PCR reaction (Table 2). The highest prevalence was found in Godey and Semera. One sample tested positive for *Wolbachia coxA* in the nested PCR reaction. No positives were detected using the *wsp* gene or the four other MLST genes.
Multiple 16S haplotypes detected

After performing sequence analysis, three separate haplotypes of *Wolbachia 16S* were identified, designated as 1, 2, or 3. The most predominant haplotype was 2 (n=11), followed by 1 (n=8) and 3 (n=1). Haplotype 1 was observed in Dire Dawa, Godey, Kebridehar, and Semera, with the highest number detected in Godey. Haplotype 2 was observed in Dire Dawa, Godey, Kebridehar, and Semera, with the highest number in Semera. Haplotype 3 was only present in one sample from Semera (Fig. 1).

Haplotypes 1 and 2 are the most similar, with 3 being more differentiated (16 nucleotide differences). Haplotype 3 has the highest number of single nucleotide substitutions or deletions in comparison with haplotypes 1 and 2. Even though there is a significant difference between haplotype 3 and haplotypes 1 and 2, most (94.33%) of the 16S gene is conserved (Fig. 1a).

**Table 1** Prevalence of Wolbachia 16S sequences in all *An. stephensi* samples. Samples were considered positive if a band appeared from the nested PCR and a high-quality sequence was produced

| Site          | No. | 16S-positive samples | Prevalence (%) |
|---------------|-----|----------------------|----------------|
| Erer-Gota     | 20  | 0                    | 0.0            |
| Dire Dawa     | 50  | 4                    | 8.0            |
| Godey         | 46  | 7                    | 15.2           |
| Kebridehar    | 22  | 2                    | 9.1            |
| Semera        | 46  | 7                    | 15.2           |
| Total         | 184 | 20                   | 10.9           |

**Wolbachia 16S in An. stephensi falls into multiple clades**

Phylogenetic analysis revealed two superclades with bootstrap values of 81 and 72 representing *Wolbachia* supergroups A and B, respectively. Haplotype 3 only occurs in supergroup B, while haplotypes 1 and 2 occur in supergroup A. One of these superclades had a sub-clade composed of just haplotype 2 sequences from Ethiopia and received significant support separating this clade from the rest of the superclade (bs=89). The *Wolbachia 16S* sequences from Ethiopia were in clades with sequences from both Africa and SE Asia and multiple *Anopheles* species. Overall, there was no clear clustering by geography or species (Fig. 2).

**Wolbachia coxA in eastern Ethiopia confirms the presence of Wolbachia in supergroup B**

Out of the five MLST genes, cytochrome c oxidase subunit 1 (*coxA*) was the only gene to be amplified, in the sample SM006. This sample is the only sample we found in supergroup B, and is the most distinguished haplotype, haplotype 3. We were able to amplify this

**Table 2** 16S-positive *An. stephensi* from each site, number of samples with a positive band in the un-nested PCR reaction, and number of samples with a positive band in the 16S nested PCR reaction

| Site          | Number of *An. stephensi* samples with a band in un-nested 16S PCR | Number of *An. stephensi* samples with a band in nested 16S PCR |
|---------------|---------------------------------------------------------------------|----------------------------------------------------------------|
| Erer-Gota     | 0                                                                   | 0                                                               |
| Dire Dawa     | 1                                                                   | 4                                                               |
| Godey         | 4                                                                   | 7                                                               |
| Kebridehar    | 0                                                                   | 2                                                               |
| Semera        | 2                                                                   | 7                                                               |
| Total         | 7                                                                   | 20                                                              |

**Fig. 1** a Alignment of the three haplotypes observed from the Wolbachia 16S gene in eastern Ethiopia. Haplotypes were categorized as 1, 2, and 3. Nucleotide conservation is indicated by a gray bar below the alignment. b Haplotype frequency per collection site
gene three times from the same sample. Phylogenetic analysis showed that the sample from Ethiopia is significantly differentiated from the rest of the tree and clusters with samples known to be in supergroup B (Fig. 3).

**Wolbachia in An. stephensi from India falls into the same clade as haplotype 3 from eastern Ethiopia**

To investigate whether *Wolbachia* in *An. stephensi* in eastern Ethiopia is similar to that found in the Middle East and South Asia, we performed a phylogenetic analysis of *Wolbachia 16S* sequences from Tamil Nadu, India, to those of eastern Ethiopia and sub-Saharan Africa. We again see separation into supergroups A and B. Supergroup B is represented in the top superclade and supergroup A is represented in the bottom superclade. The sample that is the strongest candidate for infection clusters with the sequences from India in supergroup B, but the sequences were not the same (Additional file 1: Fig. S1).

**Wolbachia 16S diversity and An. stephensi COI diversity**

To investigate any correlation between *Wolbachia* diversity and *An. stephensi* diversity, we amplified the *COI* gene in all of our *Wolbachia 16S*-positive mosquitoes. The sample from Semera presents both the most *COI* diversity and the most *Wolbachia 16S* diversity. This is consistent with previous reports of mitochondrial diversity in the *An. stephensi* in eastern Ethiopia [5]. Samples from Semera (SM) occur in all three major clades in this tree. More southern sites, such as Godey (GD) and Kebridehar (KB), are in the topmost clade, and more northern and central sites such as Dire Dawa (DD) and Semera are mostly in the bottom two clades (Fig. 4). While we are unable to say that this pattern is
definitive based on our sample size, we predict that if we were to obtain a larger sample set, we would see this trend separate our samples out more distinctively.

Discussion
The data presented here confirm Wolbachia 16S rRNA molecular detection in An. stephensi and provide preliminary evidence of Wolbachia infection in this species. Wolbachia 16S DNA was detected in about 10% of samples across all collection sites. Three haplotypes were detected across all the sites with Wolbachia 16S-positive samples. Haplotypes 1 and 2 (supergroup A) were the most similar, with haplotype 3 (supergroup B) being the most dissimilar. Haplotype 2 was the most predominant haplotype, with 11 samples. Semera presented with the most diversity, exhibiting all three haplotypes. It should be noted that the number of positive Wolbachia samples was relatively low at each site, thus conclusions about the correlation between geographic location in Ethiopia and Wolbachia 16S diversity should be followed up with a larger sample set. Detection of Wolbachia coxA confirmed one of our samples, SM006, as a part of supergroup B. This provides an additional line of evidence of infection in the case of sample SM006, as most contamination scenarios can be eliminated.

Phylogenetic analysis revealed no significant clustering of Wolbachia 16S rRNA sequences with host Anoph eles species or geographical regions. We predicted that there may be clustering of Wolbachia 16S based on geographic region or relatedness of the vector, but this was not observed. Phylogenetic analysis also revealed no significant relationship between Wolbachia 16S in India and that of eastern Ethiopia. Thus, there does not appear to be any specificity of 16S haplotype to Anopheles species or geographic region. Lastly, phylogenetic analysis of An. stephensi COI colored by Wolbachia 16S haplotype revealed possible patterns of Wolbachia diversity. There is separation between southeastern Ethiopia and central/northeastern Ethiopia. Most of the sequences in Wolbachia 16S haplotype 1 appear in southeastern Ethiopia and most of the sequences in Wolbachia 16S haplotype 2 appear in central/northeastern Ethiopia. More samples would be needed in order to confirm any patterns observed.

The prevalence of detection found here is similar to that found in a study of the An. gambiae complex in Guinea, which was around 11% in Anopheles melas via MLST [20]. The authors of that study suggested that using a nested 16S approach is problematic, citing a study investigating the presence of Wolbachia 16S in the An.
The authors discussed the establishment of the nested 16S PCR protocol, and how it produces unreliable and non-replicable results [22]. We see in our study that only seven out of 20 total 16S-positive samples showed a band in the un-nested PCR. All quality sequences were produced from nested PCR products, which raises the question of whether nested PCR should be used to determine Wolbachia infection.

The density of infection is currently unknown in Anopheles species. In a previous study, infections found in An. demeilloni and An. moucheti were described as high-density; however, corresponding data show Wolbachia 16S representing from less than 20% to just over 80% of the total microbiome. This indicates that densities in a species could vary, which would mean that the nested protocol may need to be used in the case of lower-density infections or co-infection with another bacterium such as Asaia [20]. As this method does not provide a clear measure of the number of bacteria, we suggest incorporating quantitative PCR (qPCR) and fluorescence in situ hybridization (FISH) in the future.

Questions about whether the detection of Wolbachia DNA in An. stephensi populations from this study is indicative of an actual infection can be further examined by comparing the diversity of the Wolbachia detected in the recently introduced population of An. stephensi in Ethiopia to Wolbachia detected in long-established An. stephensi populations. As an endosymbiont of invasive mosquitoes, the Wolbachia population should mirror a lower level of diversity in its invasive host population.

**Fig. 4** Phylogenetic analysis of An. stephensi COI in the Wolbachia 16S-positive An. stephensi with transformed branches. Each sample is colored by Wolbachia haplotype, where haplotype 1 is colored in blue, haplotype 2 is colored in red, and haplotype 3 is colored in yellow. Haplotypes 1 and 2 are representative of Wolbachia supergroup A, and haplotype 3 is representative of supergroup B. Anopheles stephensi COI haplotypes are designated on the clades that they represent according to Carter et al. [45]. Anopheles maculatus was used as an outgroup (KT382822). The sample sites are abbreviated as follows: Semera (SM), Dire Dawa (DD), Godey (GD), and Kebridehar (KB). Only 19 An. stephensi samples amplified COI, which was likely due to sequence divergence in the COI region in that mosquito.
relative to the corresponding host source population [43, 44]. A study in pre-print from the state of Tamil Nadu, India, detected four haplotypes of Wolbachia 16S, and in eastern Ethiopia we detected three haplotypes. While the SM006 sequence was found in supergroup B that also included the India sequences, we did not have enough sequence resolution to provide definitive evidence of a shared recent ancestor between An. stephensi Wolbachia from Ethiopia and India. Further testing would require additional sampling and congruent multilocus genetic data.

There are limitations to using only a PCR assay for detection. Multiple studies have been published regarding Wolbachia infection and the detection of 16S, wsp, and MLST. Jeffries et al. [20] discussed how extracting RNA from the mosquitoes would increase the chance of detecting actively expressed Wolbachia genes. This could increase the effectiveness of the PCR assays, as they would amplify actively expressed genes rather than the scenarios below. A recent systematic review discussed the different scenarios in which Wolbachia could be detected in a mosquito [23]. The authors pointed out that under scenarios such as a mosquito with an endogenized gene from Wolbachia, environmental contamination, or a mosquito infected with a nematode infected with Wolbachia, Wolbachia would be detectable by PCR assay, nested PCR assay, qPCR, and possibly MLST. Additional scenarios were discussed in Chrostek and Gerth [22], where Wolbachia sequences could be detected in the gut as a result of the consumption of Wolbachia-infected food. In this study, we are able to rule out the scenario in which the mosquitoes were infected with a nematode that was infected with Wolbachia, as filarial worms are infected with Wolbachia in supergroups C and D. Environmental contamination is a possibility, as Aedes spp. share similar breeding habits with An. stephensi in eastern Ethiopia. However, other mosquitoes have not been tested for Wolbachia in eastern Ethiopia, so we cannot rule this out. For sample SM006, the integration of a Wolbachia gene into the host genome is unlikely, as it is not likely that multiple genes would integrate into the host genome [23]. In the scenario that a mosquito is infected with Wolbachia, methods such as FISH, transmission electron microscopy (TEM), and Giemsa staining (GIEMSA) must be used to detect the active Wolbachia in the ovaries [23]. This suggests that future studies aiming to detect Wolbachia in mosquitoes should aim to perform one of these visual methods in addition to molecular assays.

If the detection of Wolbachia 16S and coxA represents true infection, then our findings provide helpful information about the strains present in An. stephensi in eastern Ethiopia. These are helpful data that can inform the approach to malaria control, should Wolbachia-based strategies in Anopheles become a reality [13]. Insecticide resistance has been identified in An. stephensi to pyrethroids, carbamates, and organophosphates, suggesting the need for alternative methods of vector control to prevent the prevalence of malaria from increasing [8, 9]. Identifying the presence of Wolbachia 16S and coxA DNA in this invasive vector in eastern Ethiopia warrants further studies, including methods such as reverse transcription qPCR and FISH.

Conclusions

In this study, we present the first evidence suggesting the presence of a Wolbachia population in An. stephensi in eastern Ethiopia. The detection of Wolbachia 16S and coxA DNA in An. stephensi in Ethiopia supports the need for further investigation of natural Wolbachia infections in this species. This could help inform the development of novel approaches for preventing the spread of malaria resulting from this invasive species in the HOA.

Abbreviations

HOA: Horn of Africa; MLST: Multilocus strain typing; CI: Cytoplasmic incompatibility; wsp: Wolbachia surface protein; FISH: Fluorescence in situ hybridization; PSC: Pyrethrum spray catch; CDC: Centers for Disease Control and Prevention; PCR: Polymerase chain reaction; ITS2: Internal transcribed spacer 2; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; qPCR: Quantitative PCR; TEM: Transmission electron microscopy; GIEMSA: Giemsa stain.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05299-9.

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Disclaimer

The findings and conclusions in this report are those of the author and do not necessarily represent the official position of the Centers for Disease Control and Prevention.
Author contributions

EW and TEC contributed to the conceptualization of the project. EW, SD, SRB, and TEC contributed to the design of the study. MB, DD, and SRI designed the mosquito collection protocol. SY and DG collected the mosquitoes. EW, SD, and TEC contributed to the molecular analysis. SR assisted with the supply of Wolbachia-positive control DNA. EW, SD, and TEC contributed to the writing of the manuscript. SY, DG, DD, SRB, MB, SRI, and SZ contributed to manuscript revision. DD and MB provided a PMI VectorLink overview of the mosquito collection. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. Sequences have been deposited in the NCBI GenBank database. The accession numbers for Wolbachia coxA are OM751324-OM751326, for Wolbachia cox1 OM811269, and for An. stephensi COI OM801690-OM801708.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

\(^1\) Department of Biology, Baylor University, Waco, TX, USA. \(^2\) Jigjiga University, Jigjiga, Ethiopia. \(^3\) Dire Dawa University, Dire Dawa, Ethiopia. \(^4\) PMI VectorLink: Ethiopia Project, Abt Associates, Addis Ababa, Ethiopia. \(^5\) Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA. \(^6\) U.S. President’s Malaria Initiative and Entomology Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA.

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