scientific reports

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Detection and genetic diversity of Wolbachia and its associated prophage WO in mosquito populations from Ethiopia

Samson Leta[1,2]✉, Meskerem Mulisa Misgana[2], Bethel Befekadu Jaleta[2], Tesfaye Rufael Chibssa[3], Rob Lavigne[4] & Jan Paeshuyse[1]✉

Arboviral diseases transmitted by mosquitoes, including dengue, Chikungunya, Zika, yellow fever, West Nile virus (WNV), and Rift Valley fever (RVF), pose a significant public health challenge globally, particularly impacting populations in low and middle-income countries. Conventional mosquito control methods, which primarily rely on insecticides, face critical challenges, including the development of insecticide resistance and environmental concerns. In this context, *Wolbachia*, an endosymbiotic bacterium, presents an alternative strategy due to its ability to manipulate mosquito reproduction and impede the transmission of pathogens. This study aimed to detect and assess the genetic diversity of *Wolbachia* and prophage WO in Ethiopian mosquitoes. Mosquitoes were collected from various ecological niches in the Great Rift Valley. Molecular analyses were performed to identify the presence of *Wolbachia* using PCR targeting the 16 S rRNA and *wsp* genes. Additionally, the presence of prophage WO was assessed by detecting the conserved *orf7* capsid protein gene. To understand genetic diversity, phylogenetic and genetic diversity analyses were performed. *Wolbachia* was detected in 44.2% (34/77) of mosquitoes using the 16 S rRNA gene and 46.8% (36/77) using the *wsp* gene. The highest prevalence was observed in *Cx. pipiens* complex (100%, 11/11) and *Ma. uniformis* (92.3%, 12/13). Prophage WO was detected in 46.8% (36/77) of mosquitoes, with evidence of multiple-strain co-infections in *Cx. pipiens* complex. Phylogenetic analysis classified all isolates within *Wolbachia pipientis* Supergroup B. This study provides the first preliminary characterization of Wolbachia and prophage WO in Ethiopian mosquitoes, revealing evidence of genetic diversity. These findings lay the conceptual foundation of potential *Wolbachia*-based vector control strategies in Ethiopia and underscore the need for further studies on strain-specific impacts on vector competence and arboviral transmission dynamics.

Keywords Arboviral diseases, Ethiopian mosquitoes, Genetic diversity, Prophage WO, Vector control, *Wolbachia*

Mosquitoes serve as primary vectors for a wide range of arboviral diseases, including dengue, Chikungunya, Zika, yellow fever, and Rift Valley fever (RVF). These diseases collectively affect millions of people globally, with the burden disproportionately affecting populations in low and middle-income countries[1,2]. Globally, 146 countries have reported at least one of these arboviral diseases. The number of countries documenting the autochthonous vector-borne transmission of these diseases includes 127 for dengue, 110 for Chikungunya, 92 for Zika, 43 for yellow fever, and 39 for RVF[3–6].

Ethiopia, with its diverse ecological zones and abundant mosquito breeding sites, is at high risk for arboviral outbreaks[5,7]. Chikungunya, dengue, West Nile virus (WNV), yellow fever, Zika, and RVF[8–10] has been reported from Ethiopia. Mosquito control efforts remain heavily reliant on chemical insecticides[11]. However, this approach faces increasing challenges due to insecticide resistance[12], unintended impacts on non-target species[13], and environmental sustainability concerns[14,15]. These challenges underscore the urgent need for alternative, environmentally friendly mosquito control strategies.

[1]Laboratory of Host Pathogen Interaction in Livestock, Division of Animal and Human Health Engineering, Department of Biosystems, KU Leuven, Leuven 3001, Belgium. [2]Department of Biomedical Sciences, College of Veterinary Medicine and Agriculture, Addis Ababa University, P.O. Box 34, Bishoftu, Ethiopia. [3]Animal Health Institute (AHI), P.O. Box 04, Sebeta, Ethiopia. [4]Department of Biosystems, Laboratory of Gene Technology, KU Leuven, Leuven, Belgium. ✉email: samiwude@gmail.com; jan.paeshuyse@kuleuven.be

One promising approach lies in harnessing the potential of *Wolbachia*, a genus of intracellular bacteria that infects a broad range of arthropods, including mosquitoes[16]. *Wolbachia* influences the biology of its arthropod host through various mechanisms, including cytoplasmic incompatibility (CI) and reproductive manipulation[17,18]. *Wolbachia* is often regarded as a "master manipulator" of invertebrate biology[19], significantly influencing various aspects of mosquito physiology, including reproduction, fitness, and immunity. For instance, *Wolbachia* induces CI, a reproductive anomaly that prevents uninfected female mosquitoes from producing viable offspring when they mate with *Wolbachia*-infected males, whereas infected females are compatible with both infected and uninfected males[17]. This asymmetry gives infected females a strong reproductive advantage, facilitating the spread of *Wolbachia* in natural populations. CI is primarily driven by the activity of CI factor (*cif*) gene pairs, which encode toxin–antidote systems, whereby male expression of two genes (*cifA* and *cifB*) causes CI[20–22], while the female expression of *cifA* rescues the embryo[23]. This phenomenon, when applied through large-scale releases of infected males, can suppress naïve mosquito populations, making *Wolbachia* a powerful tool for sustainable vector control. Open-field releases of *Wolbachia*-infected male mosquitoes have successfully suppressed mosquito populations by over 95%[24].

Moreover, *Wolbachia* infections fundamentally alter the host cell environment, reducing the likelihood of arboviral replication within the vector[25–27]. The primary mechanism is thought to involve disruption of cellular resources and processes, including cholesterol and lipid homeostasis, as well as modulation of host cell cytosine methyltransferase expression, an enzyme essential for viral replication[25,27]. This intracellular modification severely limits the replication and transmission of a broad range of pathogens, including Dengue virus (DENV)[28] and Zika virus (ZIKV)[29]. This phenomenon, known as pathogen blocking, is significant for arboviral suppression. For example, a *Wolbachia* release program in Townsville, Australia (2014–2019) achieved a 65% reduction in dengue incidence during the release period and over 99% reduction within two years after the intervention[30].

The unique properties of *Wolbachia*, such as reproductive manipulation and pathogen blocking, are linked to its interactions with prophage WO[22], a temperate bacteriophage integrated into the *Wolbachia* genome. Phage WO is known to be present in the genomes of at least five *Wolbachia* supergroups, namely A, B, E, F, and S[17,19]. Prophage WO is known to play a role in the horizontal transfer of *Wolbachia* genes, affecting replication, host fitness, and spread within mosquito populations[31]. Prophage WO is believed to play a pivotal role in the induction of CI, thereby influencing host reproductive patterns[17,22].

The reproductive manipulation and pathogen-blocking properties of *Wolbachia* have made *Wolbachia* an innovative tool in integrated vector management programs, offering an environmentally friendly and effective alternative to traditional chemical methods. Different countries have successfully used *Wolbachia* to suppress arboviral diseases by releasing *Wolbachia*-infected mosquitoes into the environment[32,33]. While *Wolbachia*-based control strategies have been successfully implemented in some countries, there is limited knowledge on the prevalence and diversity of *Wolbachia* within Ethiopian mosquito populations. To date, only a single study[34] reported the presence of *Wolbachia* in Ethiopian mosquitoes, specifically in *An. stephensi*. However, preliminary data on its genetic diversity and association with prophage WO across multiple mosquito species remain lacking. Understanding the genetic variability of *Wolbachia* in Ethiopian mosquito populations is critical for assessing its feasibility as a vector control strategy and designing effective intervention programs. Therefore, this study aimed to detect and assess the genetic diversity of *Wolbachia* and prophage WO in Ethiopian mosquitoes.

Materials and methods

Study area and mosquito collection

This study was conducted in Ethiopia's Great Rift Valley, spanning from Batu (7°55'51"N, 38°42'58"E) to Gawane (10°9'59"N, 40°38'43"E), encompassing urban, peri-urban, and rural habitats (Fig. 1). The selected study areas are thought to support the oviposition and development of mosquito larvae year-round, as they contain large permanent water bodies and experience warmer temperatures. Mosquitoes were collected in August 2023 using CDC light and BG-Sentinel traps placed near water bodies, animal enclosures, and densely populated areas, as well as via hand aspirators from houses. Traps were set between 16:00 and 18:00 and retrieved the following day from 7:00 to 9:00. Captured mosquitoes were frozen at −20 °C and identified morphologically using dichotomous keys from the Walter Reed BioSystemics Unit[35,36] and molecularly as described in[37].

DNA extraction and molecular identification of mosquitoes

Genomic DNA was extracted from each mosquito using DNeasy Blood & Tissue and MACHEREY-NAGEL NucleoSpin DNA/RNA extraction kits, following the manufacturers' protocols, with minor modifications. For mosquito identification, amplification of the 710 bp fragment flanking the COI gene was performed using the primer set LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAG GGTGACCAAAAAATCA-3')[38]. Detailed methodologies for DNA extraction, molecular identification, and mosquito species identification are provided in Leta et al[37].

Wolbachia detection

To detect *Wolbachia* and prophage WO, 77 samples were randomly selected from the original 142 molecularly barcoded mosquito specimens[37]. This sample size was determined based on logistical and resource constraints. The presence of *Wolbachia* was determined using PCR assays with primers specific to the 16 S rRNA gene and the *Wolbachia* surface protein (*wsp*) gene. For the 16 S rRNA gene, the *Wolbachia*-specific primers used were WolbF (5′-GAA GAT AAT GAC GGT ACT CAC-3′) and Wspecr (5′-AGC TTC GAG TGA AAC CAA TTC-3′)[39]. For the *wsp* gene, the primers utilized were *wsp* 81 F (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and *wsp* 691R (5′-AAA AAT TAA ACG CTA CTC CA-3′)[40]. For both genes, the PCR reaction was performed in a final volume of 25 µl, containing 2.5 µl of 10× buffer, 0.5 µl of 10 mM dNTP mix, 0.5 µl each of 10 µM forward

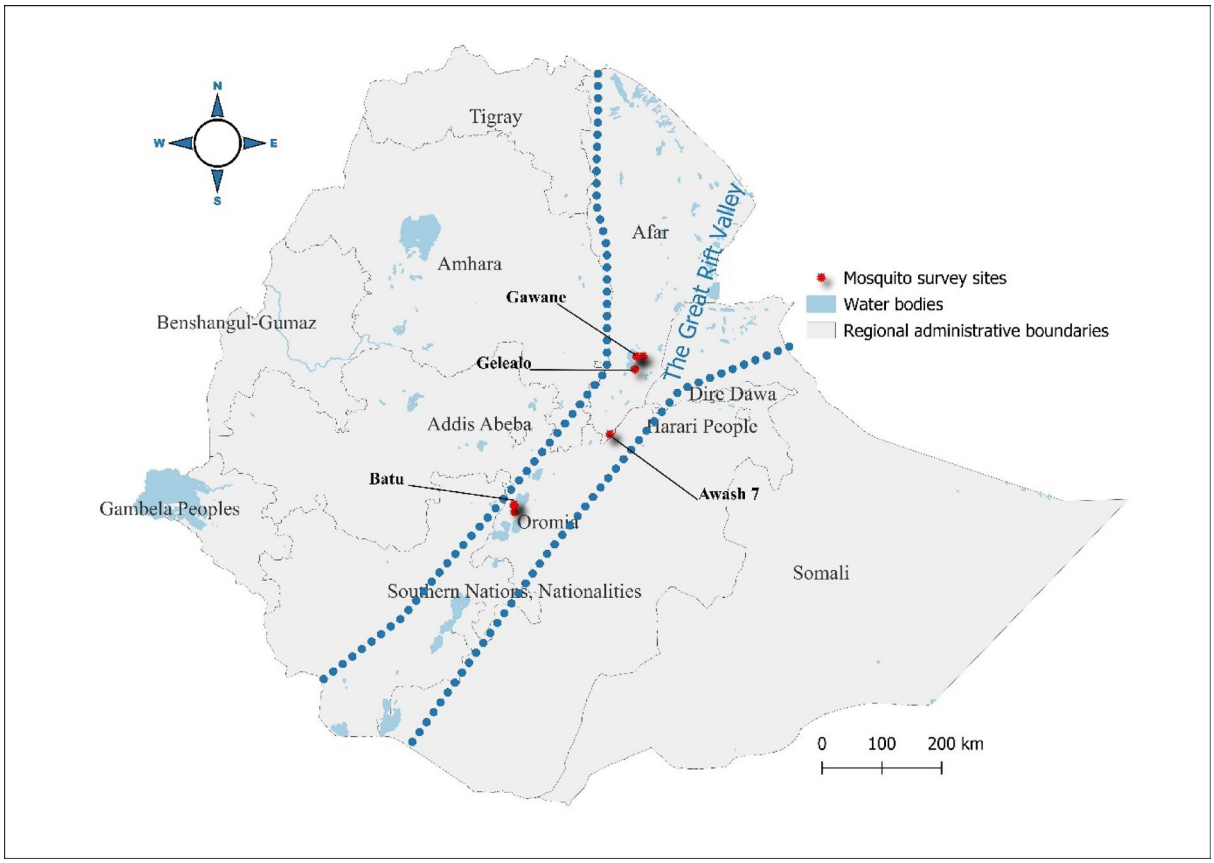

Fig. 1. Map showing the geographic distribution of mosquito collection sites across Ethiopia's Great Rift Valley. The map was generated using QGIS v3.30.0 (https://qgis.org/).

and reverse primers, 0.1 µl of Taq DNA polymerase (5.0 U/µl), 1 µl of the extracted DNA template, and 19.9 µl of ddH2O.

The PCR conditions varied between the two target genes. For the 16 S gene, the protocol included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 50 s, with a final extension step at 72 °C for 10 min. For the *wsp* gene, touchdown PCR was used. Thermal cycling conditions included an initial denaturation at 95 °C for 3 min, followed by a touchdown phase of 13 cycles consisting of denaturation at 95 °C for 45 s, annealing starting at 62 °C and decreasing by 1 °C per cycle until reaching 50 °C for 1 min, and extension at 72 °C for 50 s. This was followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 50 s, with a final extension step at 72 °C for 10 min.

Prophage WO detection

All samples were then further analyzed for the presence of prophage WO. Prophage WO detection targeted the conserved capsid protein gene *orf7*, using primers WO-F (5′-CCC ACA TGA GCC AAT GAC GTC TG-3′) and WO-R (5′-CGT TCG CTC TGC AAG TAA CTC CAT TAA AAC-3′) to amplify a 350 bp fragment[41,42]. The PCR reaction was performed in a final volume of 25 µl, comprising 2.5 µl of 10× buffer, 0.5 µl of 10 mM dNTP mix, 0.5 µl each of 10 µM forward and reverse primers, 0.1 µl of Taq DNA polymerase (5.0 U/µl), 1 µl of the extracted DNA template, and 19.9 µl of ddH_2O.

Thermal cycling conditions included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 3 min.

Sequencing and analysis

All the PCR products used for the identification of *Wolbachia* and Prophage WO were visualized on 1.5% agarose gels stained with Midori Green, purified, and sequenced via Sanger sequencing by Eurofins, using the same primers for amplification. Chromatograms were inspected, and forward and reverse sequences were assembled and edited with the BioEdit Sequence Alignment Editor v7.2.5 (https://bioedit.software.informer.com/7.2/)[43]. For all three genetic markers, sequences were compared with homologous sequences from GenBank using the BLAST (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov/BLAST) and assigned to species level based on over 99% sequence identity to GenBank sequences. Sequence alignments were performed using ClustalW in BioEdit. The phylogenetic analysis was carried out using MEGA v.12.0.9 software (https://www.m

egasoftware.net/)[44]. Using the ML model selection in MEGA, the best-fitting maximum likelihood (ML) model was identified for each dataset. Phylogenetic trees were inferred using the Maximum Likelihood (ML) method with appropriate nucleotide substitution models selected for each gene: the Kimura 2-parameter model for the *Wolbachia* 16 S rRNA gene (16 S), the Kimura 3-parameter model for the *Wolbachia* surface protein gene (*wsp*), and the Hasegawa–Kishino–Yano (HKY) model for the prophage WO capsid protein gene (*orf7*). In all models, node support was evaluated using 1,000 bootstrap replicates to ensure the robustness of the phylogenetic and genetic trees.

Several metrics were calculated for all three genetic markers to assess *Wolbachia's* genetic diversity, including the number of polymorphic sites (s), the average pairwise nucleotide differences (k), nucleotide diversity (π), the number of haplotypes (h), haplotype diversity (Hd), and neutrality tests (Fu's Fs statistic). These analyses were conducted using the DNA Sequence Polymorphism v6.12.03 (http://www.ub.edu/dnasp/)[45].

Results

Detection of *Wolbachia* and prophage WO

A total of 6,601 mosquitoes were collected, of which 4,977 were morphologically identified to the genus level. Of these, 142 mosquitoes were molecularly characterized, and 77, representing multiple genera, including *Anopheles*, *Coquillettidia*, *Culex*, and *Mansonia*, were subsequently screened for *Wolbachia* infection. *Wolbachia* has been detected among all mosquito species examined, although the detection rates differ depending on the specific mosquito species and the genetic marker employed. The detection rates of the *Wolbachia* 16 S rRNA-encoding gene (16 S), *Wolbachia* surface protein-encoding gene *(wsp)*, and prophage WO capsid protein gene (*orf7*) across 12 mosquito species are summarized in Table 1.

The 16 S rRNA gene was detected in 34 out of 77 mosquitoes (44.2%), with the highest detection rates observed in *Cx. pipiens* complex (100%, 11/11) and *Ma. uniformis* (92.3%, 12/13). In contrast, the 16 S gene could not be detected in *Ae. natronius*, *An. gambiae*, *Cx. tenagius*, and *Cx. univittatus*.

The *wsp* gene was detected in 36 out of 77 mosquitoes (46.8%), with *Cx. pipiens* complex and *Ma. uniformis* again showed the highest detection rates (100% and 92.3%, respectively). Notably, *Ae. natronius*, *An. pharoensis*, *Cq. Microannulata*, and *Cx. univittatus* exhibited 100% *wsp* gene detection, although with small sample sizes. Lower detection rates were observed in *Cx. tritaeniorhynchus* (9.5%, 2/21) and *Ma. Africana* (22.2%, 2/9).

The *orf7* gene, associated with the prophage WO, was detected in 36 out of 77 mosquitoes, representing a detection rate of 46.8%. Notably, prophage WO was also detected across all mosquito species examined in this study, although the detection rates varied among species. Similar to the *wsp* gene, the highest detection rates were observed in the *Cx. pipiens* complex and *Ma. uniformis*, with rates of 100% and 92.3%, respectively. In contrast, lower detection rates were recorded for *Cx. tritaeniorhynchus* (9.5%, 2/21) and *Ma. africana* (11.1%, 1/9). Most of the *orf7* gene sequences could not be submitted to the GenBank database due to multiple chromatogram peaks, particularly in samples from the *Cx. pipiens* complex, which suggests possible infection by multiple strains of prophage WO.

Genetic diversity of *Wolbachia* and prophage WO

Wolbachia's genetic diversity among Ethiopia's predominant mosquito species was analyzed using sequences from the 16 S rRNA, *wsp*, and *orf7*genes. The 16 S rRNA analysis uncovered 83 polymorphic sites within 888 nucleotide positions, with 86 mutations, of which 63 were singleton mutations. In the case of the *wsp* gene, 33 polymorphic sites were identified among 537 nucleotide positions, with 34 mutations, including 20 singleton

Mosquito species	Total mosquitoes processed	Wolbachia 16 S rRNA-encoding gene (16 S)	Wolbachia surface protein encoding gene (wsp)	Prophage WO capsid protein gene orf7
Ae. natronius	1	0 (0%)	1 (100%)	1 (100%)
An. coustani	6	1 (16.7%)	1 (16.7%)	1 (16.7%)
An. gambiae	1	0 (0%)	0 (0%)	1 (100%)
An. pharoensis	1	0 (0%)	1 (100%)	1 (100%)
Cq. microannulata	1	1 (100%)	1 (100%)	1 (100%)
Cx. neavei	7	1 (14%)	2 (28.6%)	2 (28.6%)
Cx. pipiens complex	11	11 (100%)	11 (100%)	11 (100%)
Cx. tenagius	4	1 (25%)	1 (25%)	1 (25%)
Cx. tritaeniorhynchus	21	4 (19%)	2 (9.5%)	2 (9.5%)
Cx. univittatus	2	0 (0%)	2 (100%)	2 (100%)
Ma. africana	9	3 (33.3%)	2 (22.2%)	1 (11.1%)
Ma. uniformis	13	12 (92.3%)	12 (92.3%)	12 (92.3%)
Total	77	34 (44.2%)	36 (46.8%)	36 (46.8%)

Table 1. Detection of 16 S rRNA, wolbachia surface protein (wsp), and prophage WO (orf7) genes in different mosquito species.

mutations. For the *orf7* gene, 47 polymorphic sites were identified among 357 nucleotide positions, with 50 mutations, including 15 singleton mutations.

Genetic diversity metrics and neutrality test results varied among mosquito species, although most differences were not statistically significant (Table 2). A significant Fu's Fs statistic was detected only in the prophage WO capsid protein gene *orf7* sequences from *Ma. uniformis*. According to the 16 S rRNA, the sequences from *Cx. pipiens* complex showed low levels of haplotype diversity (Hd = 0.56) and nucleotide diversity ($\pi = 0.001$), with an average pairwise nucleotide difference (k) of 0.62. In contrast, the sequences from *Ma. uniformis* exhibited the highest level of genetic variation (Hd = 0.97, $\pi = 0.023$), with k = 19.64, and showed the greatest number of polymorphic sites (S = 74).

For the *wsp* gene, the sequences from both *Cx. pipiens* complex and *Ma. uniformis* exhibited moderate haplotype diversity (Hd = 0.82 and 0.68, respectively) and nucleotide diversity ($\pi = 0.006$ and 0.008, respectively), with k values of 2.76 and 2.18, respectively. Even if there is a tendency towards a certain pattern, the genetic diversity results for the species analyzed using both the 16 S and *wsp* genes were not statistically significant, likely due to the small sample size.

For the *orf7* gene of prophage WO, sequences from *Ma. uniformis* exhibited low haplotype diversity (Hd = 0.42) but relatively high nucleotide diversity ($\pi = 0.034$) and an average pairwise nucleotide difference (k = 9.78), alongside a strongly positive Fu's Fs statistic (7.24), suggesting possible balancing selection or population subdivision. These results indicate that the prophage WO *orf7* gene in Ethiopian mosquitoes displays evidence of higher sequence divergence and possible historical balancing selection or population structure, reflecting complex evolutionary dynamics between *Wolbachia* and its associated prophage.

Phylogenetic analysis based on the 16 S rRNA and *wsp* genes indicates that the Ethiopian *Wolbachia* isolates cluster within the known *Wolbachia* supergroups, likely belonging to Supergroup B, which primarily associates with arthropods, including mosquitoes (Figs. 2 and 3). Phylogenetic analysis of the 16 S rRNA gene revealed that sequences from *Ma. uniformis* and *Ma. africana* formed a distinct cluster, indicating a stable *Wolbachia* association within these mosquito species. Similarly, most *Wolbachia* sequences from members of the *Cx. pipiens complex* clustered together, with Ethiopian isolates interspersed with reference strains from the United Kingdom, indicating that *Wolbachia* diversity in these mosquitoes is broadly consistent across geographic regions.

Similar to the phylogenetic analysis of the 16 S rRNA gene, the phylogenetic analysis of *wsp* gene sequences from Ethiopian *Wolbachia* isolates supports their classification within Supergroup B. The majority of sequences obtained from *Ma. uniformis* and *Cx. pipiens* complex mosquitoes grouped together forming strongly supported clades (bootstrap > 95%). In both the 16 S rRNA and *wsp* gene analyses, although some Ethiopian isolates clustered with strains from other areas, the majority formed a distinct clade, indicating the potential presence of novel strains. This study identified the *wPip* strain in *Cx. neavei* and *Cx. tritaeniorhynchus*, as well as the *wNo* strain in *Cx. tritaeniorhynchus* and *Cq. microannulata* (Fig. 3).

The phylogenetic analysis based on the *orf7* gene revealed distinct clustering patterns among mosquito-derived prophage WO sequences from Ethiopia and reference sequences from other countries (Fig. 4). All, except one prophage WO, derived from *Wolbachia*-infected *Ma. uniformis* mosquitoes from Ethiopia formed a well-supported clade, indicating a closely related prophage WO lineage circulating within this species. In contrast, the Prophage WO isolate from *Wolbachia*-infected *Ma. africana* clustered on a separate branch with moderate bootstrap support, indicating it carries a genetically distinct prophage WO sequence compared to *Ma. uniformis*. Other isolates, including from *C. microannulata*, *Cx. neavei*, and *An. coustani* also formed their separate branches with moderate to high bootstrap support, highlighting additional host-associated diversity of prophage WO within Ethiopian mosquitoes.

Overall, the phylogenetic analysis complements the genetic diversity analysis by confirming both host-associated and geographically structured patterns of prophage WO diversity in Ethiopian mosquito populations, and it supports the presence of historical diversification and possible balancing selection maintaining prophage variation across mosquito species.

Mosquito species	*N*	s	h	Hd ± SD	k	π	Fu's Fs statistic
Based on the 16 S rRNA gene							
Cx. pipiens complex	11	2	3	0.56 ± 0.13	0.62	0.001	−0.31
Ma. africana	3	5	3	1.00 ± 0.07	3.33	0.004	NA
Ma. uniformis	9	74	8	0.97 ± 0.06	19.64	0.023	0.26
Based on the *wsp* gene							
Cx. pipiens complex	11	13	7	0.82 ± 0.12	2.76	0.006	−1.90
Ma. uniformis	12	9	6	0.68 ± 0.15	2.18	0.008	−1.10
Based on the Prophage WO capsid protein gene *orf7*							
Ma. uniformis	9	44	3	0.42 ± 0.19	9.78	0.034	*7.24*

Table 2. Genetic diversity indices and neutrality tests for 16 S rRNA and Wsp genes of wolbachia and orf7 gene in prophage WO in dominant mosquito species from Ethiopia. Key: n: number of sequences considered for the analysis; S: number of polymorphic sites; k: average number of pairwise nucleotide differences; p: nucleotide diversity; h; number of haplotypes; Hd: haplotype diversity. Fu's Fs: The underlined Fu's Fs statistic values are statistically significant. Species represented by <3 specimens were not included in the analyses. NA: not applicable (at least four sequences needed).

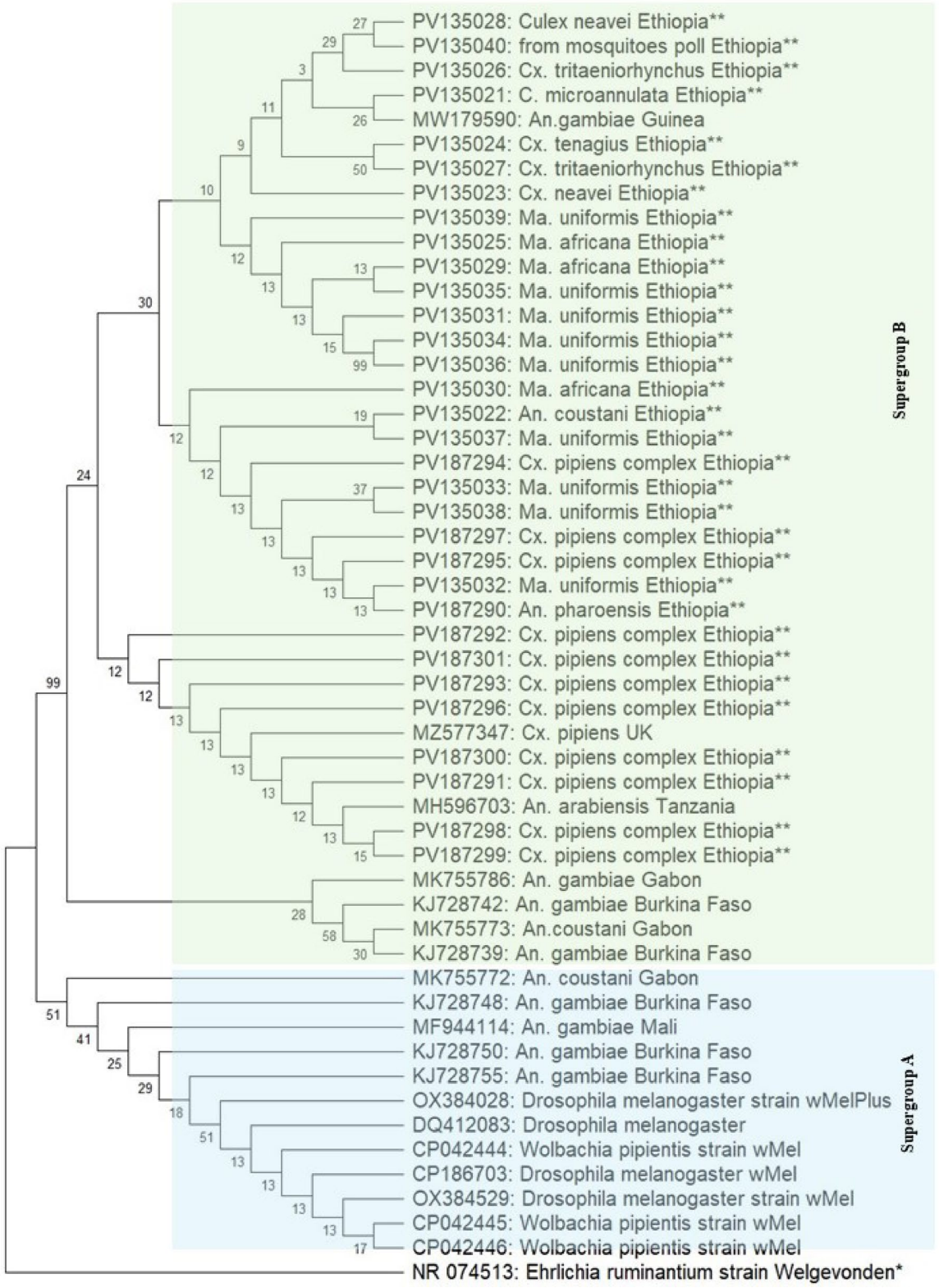

Fig. 2. Phylogenetic Analysis of *Wolbachia* 16 S rRNA Gene Sequences from Ethiopian Mosquitoes. The phylogenetic tree illustrates the evolutionary relationships among *Wolbachia* isolates based on 16 S rRNA gene sequences. It includes *Wolbachia* strains obtained from various mosquito species in Ethiopia (marked with **), as well as reference sequences from other geographic regions and hosts. The tree was constructed by the maximum likelihood method using the Kimura 2-parameter nucleotide substitution model, with *Ehrlichia ruminantium* (NR_074513) serving as the outgroup to root the tree.

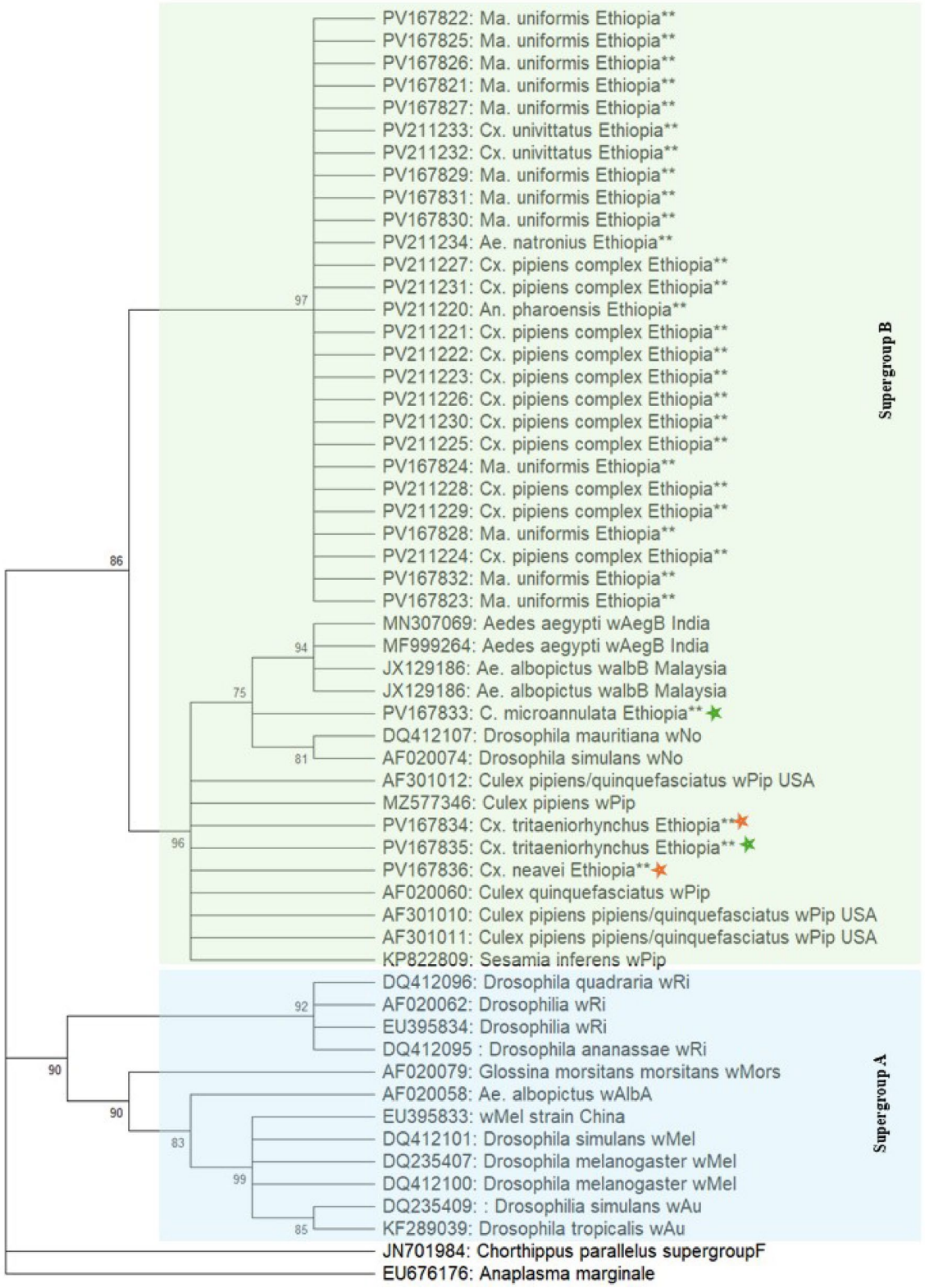

Fig. 3. Phylogenetic Analysis of *Wolbachia wsp* gene sequences from Ethiopian Mosquitoes. The phylogenetic tree illustrates the evolutionary relationships among *Wolbachia* isolates based on *wsp* gene sequences. The tree includes *Wolbachia* strains obtained from various mosquito species in Ethiopia (marked with **) and reference sequences from other geographic regions and hosts. Sequences marked by green stars are wNo strain, and sequences marked by red stars are wPip strains. The tree was constructed by the maximum likelihood method using the Kimura 3-parameter nucleotide substitution model, with *Anaplasma marginale* (EU676176) as the outgroup to root the tree.

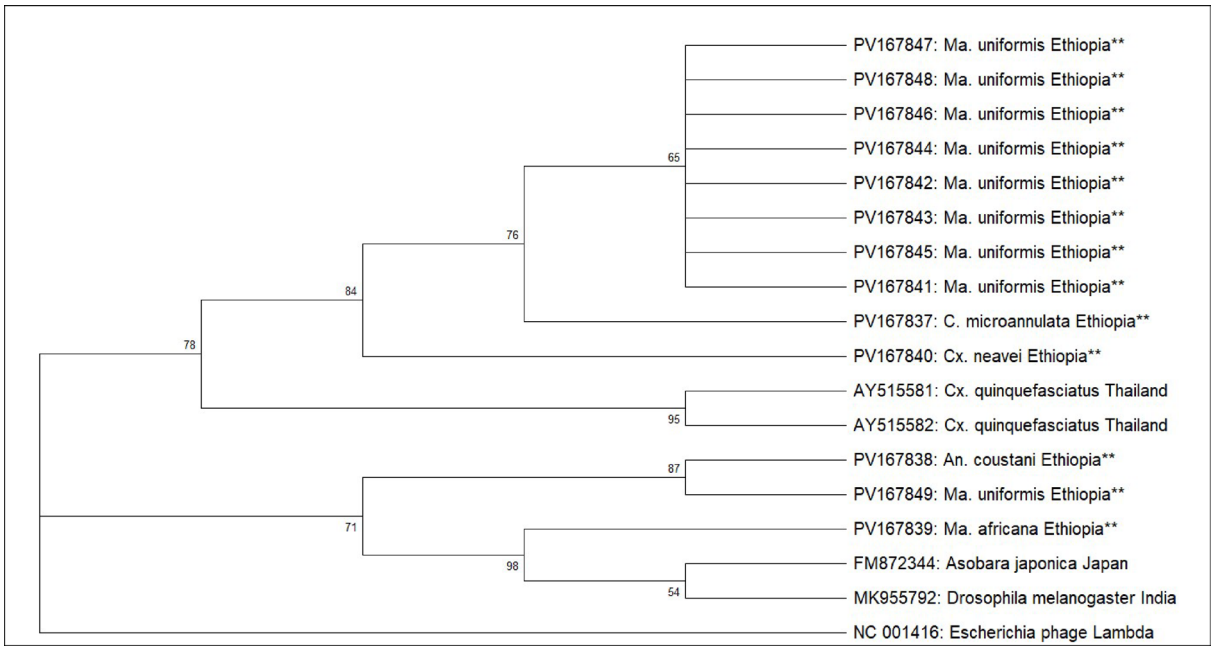

Fig. 4. Phylogenetic analysis of prophage WO capsid protein gene (*orf7*) sequences from *Wolbachia*-infected mosquitoes. The phylogenetic tree illustrates the evolutionary relationships among Prophage WO isolates based on the capsid protein gene *orf7* gene sequences. The tree includes Prophage WO isolates obtained from various *Wolbachia*-infected mosquito species in Ethiopia (marked with **) and reference sequences from other geographic areas and hosts. The tree was constructed by the maximum likelihood method using Hasegawa–Kishino–Yano (HKY) nucleotide substitution model, with *Escherichia* phage Lambda (NC_001416) as the outgroup to root the tree.

Discussion

This study reveals the prevalence, genetic diversity, and phylogenetic relationships of *Wolbachia* strains and their associated prophage WO in mosquito populations from Ethiopia. Using three genetic markers (16 S rRNA, wsp, and orf7), we characterized infection rates and genetic variability across mosquito species, despite sampling limitations and sequencing failures.

Wolbachia and their associated prophage WO were detected across all mosquito species examined. However, their prevalence varied, reflecting the differences in host susceptibility. According to recent analyses, 217 Culicidae species from 22 genera have been screened for *Wolbachia*, which constitutes only 6% of the total recognized mosquito species[46]. The *Anopheles*, *Aedes*, and *Culex* genera were particularly notable, collectively accounting for 75% of the species identified as positive for *Wolbachia*. Of the 22 genera assessed, 17 exhibited at least one species that tested positive for the endosymbiont. The study further illustrated that, among the screened species, 66 were classified as *Wolbachia*-positive, representing approximately 30% of the total examined. *Anopheles* spp. were the most frequently detected, while *Ae. albopictus* and *Ae. aegypti* have been extensively studied for their associations with *Wolbachia*[46].

In this study, the highest prevalence was observed in the *Cx. pipiens* complex, which exhibited a 100% infection rate, followed closely by *Ma. uniformis* at 92.3%. These findings align with previous research, which documented a 97% infection rate in *Cx. pipiens*[47] and a 100% infection rate in *Ma. uniformis*[48], underscoring *Wolbachia's* strong affinity for these species. The failure to amplify the 16 S rRNA gene in *Ae. natronius*, *An. gambiae*, *Cx. tenagius*, and *Cx. univittatus* indicates that these species may harbor strains of *Wolbachia* that are not detectable with the current primer sets used for 16 S rRNA amplification. Nonetheless, the detection of *Wolbachia* was successfully achieved in these species using the *wsp* gene. This highlights the necessity of utilizing multiple genetic markers to accurately evaluate *Wolbachia* infection rates and strain diversity[42]. Furthermore, it suggests that whole-genome sequencing may be necessary for a more refined resolution of *Wolbachia* strains and a better understanding of their evolutionary dynamics[49].

The *wsp* gene, recognized for its high variability and utility in strain differentiation[50], was detected in 46.8% of the analyzed mosquito samples. Consistent with findings from the 16 S rRNA marker, a high prevalence of *Wolbachia* infection was again observed in *Cx. pipiens* and *Ma. uniformis*. In contrast, both the 16 S rRNA and *wsp* markers showed lower detection rates in *Cx. tritaeniorhynchus* (9.5%) and *Ma. africana* (22.2%), suggesting either a reduced prevalence of *Wolbachia* infection or the presence of divergent *Wolbachia* strains that were not efficiently amplified by the primers used. Notably, a study from Sri Lanka reported a 0% infection rate in *Cx. tritaeniorhynchus*[48], while research from Kenya found a 27% prevalence in *Ma. africana*[51].

While some Ethiopian *Wolbachia* isolates clustered with previously characterized strains, the majority formed a distinct clade in both the 16 S rRNA and *wsp* gene phylogenies. This distinct clustering suggests potential genetic divergence and the presence of novel *Wolbachia* strains unique to Ethiopian mosquito populations.

Although confirmation with MLST and whole-genome sequencing is required, our analysis identified previously characterized Wolbachia strains, namely wPip and wNo. The *wPip* strain was detected in *Cx. neavei* and *Cx. tritaeniorhynchus*, both known vectors of arboviruses[52,53]. wNo strain was found in *Cx. tritaeniorhynchus* and *Cq. microannulata*, further expanding the documented host range of these *Wolbachia* strains. The presence of these strains in Ethiopian mosquito populations highlights their potential role in shaping vector biology and may provide valuable insights for *Wolbachia*-based biocontrol strategies, as both wPip and wNo strains are known to cause CI[54,55].

The clustering of some Ethiopian isolates with reference strains from other regions, such as *Cx. pipiens* from the UK and USA, suggests a shared evolutionary history and the potential for horizontal transmission[19]. This finding aligns with previous studies demonstrating the global distribution of *Wolbachia* strains across geographic regions and host species[16,56,57]. The close phylogenetic relationship between some Ethiopian isolates and strains from other regions underscores the ability of *Wolbachia* to infect a wide range of mosquito hosts, facilitating its spread through horizontal transmission[19].

The detection of prophage WO across all mosquito species analyzed indicates its extensive distribution among *Wolbachia* strains in Ethiopia. However, challenges in sequencing some *orf7* genes from *Cx. pipiens* complex, evidenced by multiple peaks in chromatograms, suggests potential co-infections with various prophage strains, a phenomenon that has been documented in previous research[42]. According to Bourtzis et al.[16], an extensive amount of genetic diversity is found within intergenic regions of wPip and is associated with prophage WO. To address the limitations of Sanger sequencing in resolving mixed infections, future studies could utilize PCR cloning or next-generation sequencing (NGS) methods. These approaches would enable a more accurate characterization of multiple prophage WO variants within individual mosquitoes.

While bacteriophages are generally considered rare in obligate intracellular bacteria[58], prophage WO demonstrates a broad distribution among diverse *Wolbachia*-infected insects[31,59–62]. Prophage WO has been identified within the genomes of at least five *Wolbachia* supergroups A, B, E, F, and S. Among these, the majority of known prophage WO elements are found in supergroups A and B, which are predominantly associated with arthropod hosts. Moreover, prophage WO sequences within these supergroups frequently undergo horizontal transfer and recombination, contributing to the genetic diversity and dynamic evolution observed in *Wolbachia* populations[17].

The likely presence of wPip and wNo *Wolbachia* strains in Ethiopia, alongside the detection of prophage WO among all mosquito species examined, known to contribute to reproductive manipulation and pathogen blocking, is significant. By releasing *Wolbachia*-infected mosquitoes, vector populations can be sustainably suppressed[30], reducing reliance on chemical insecticides and mitigating the environmental impacts of traditional control measures[63]. Thus, integrating *Wolbachia* into Ethiopian vector management strategies offers a sustainable alternative to conventional chemical control methods, which face challenges such as insecticide resistance and environmental concerns[12,15]. By leveraging *Wolbachia's* pathogen-blocking and reproductive manipulation capabilities, Ethiopia could enhance its capacity to control the prevalent arboviral diseases. However, further research is necessary to assess the compatibility of novel *Wolbachia* strains with local mosquito populations and their long-term stability under diverse environmental conditions. Understanding these dynamics is critical for the successful implementation of *Wolbachia*-based vector control in regions with high disease burdens[19,33].

In conclusion, this study provides a preliminary analysis of *Wolbachia* prevalence, genetic diversity, and phylogenetic relationships in Ethiopian mosquito populations. The high prevalence of *Wolbachia* in *Cx. pipiens* complex and *Ma. uniformis*, along with the potential presence of novel strains, highlights the potential for *Wolbachia*-based control strategies in Ethiopia. Future studies incorporating MLST genes and whole-genome sequencing could provide deeper insights into the genetic diversity and potential applications in the management of mosquito-borne diseases.

Data availability

All data utilized in the analyses presented in this article are accessible through public databases. The sequences have been deposited in GenBank with accession numbers PV135021-PV135040 and PV187290-PV187301 for the 16 S rRNA encoding gene, PV167821-PV167836 and PV211220-PV211234 for the Wolbachia surface protein encoding gene, and PV167837-PV167849 for the Prophage WO capsid protein gene orf7.

Received: 19 July 2025; Accepted: 9 October 2025
Published online: 17 November 2025

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Author contributions
Conceptualization, field sample collection, laboratory analysis, formal analysis, visualization, and writing the original draft were conducted by SL. Conceptualization, editing, funding acquisition, and supervision were handled by JP & RL. TRC provided supervision. Field data collection was carried out by SL, and laboratory analysis was conducted by SL, BBJ, MMM. All authors have read and agreed to the final published version of the manuscript.

Funding
KU Leuven Global Minds Doctoral Scholarship Program (project number 4520196970).

Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
This study received ethical approval from the Research Ethics Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University, certificate reference number VM/ERC/27/05/14/2022. Consent to participate does not apply to this study.

Additional information
Correspondence and requests for materials should be addressed to S.L. or J.P.

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