Evidence for natural hybridization and novel Wolbachia strain superinfections in the Anopheles gambiae complex from Guinea

Claire L. Jeffries¹, Cintia Cansado-Utrilla¹,†, Abdoul H. Beavogui², Caleb Stica¹, Eugene K. Lama³, Mojca Kristan¹, Seth R. Irish⁴ and Thomas Walker¹

¹Department of Disease Control, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK
²Centre National de Formation et de Recherche en Santé Rurale de Mafèrinyah B.P. 2649, Conakry, Guinea
³Programme National de Lutte contre le Paludisme, Guinée, B.P. 6339 Conakry, Guinea
⁴The US President’s Malaria Initiative and Entomology Branch, Centers for Disease Control and Prevention, Atlanta, GA 30329-4027, USA

Wolbachia, a widespread bacterium which can influence mosquito-borne pathogen transmission, has recently been detected within Anopheles (An.) species that are malaria vectors in Sub-Saharan Africa. Although studies have reported Wolbachia strains in the An. gambiae complex, apparent low density and prevalence rates require confirmation. In this study, wild Anopheles mosquitoes collected from two regions of Guinea were investigated. In contrast with previous studies, RNA was extracted from adult females (n = 516) to increase the chances for the detection of actively expressed Wolbachia genes, determine Wolbachia prevalence rates and estimate relative strain densities. Molecular confirmation of mosquito species and Wolbachia multilocus sequence typing (MLST) were carried out to analyse phylogenetic relationships of mosquito hosts and newly discovered Wolbachia strains. Strains were detected in An. melas (prevalence rate of 11.6%–16/138) and hybrids between An. melas and An. gambiae sensu strico (prevalence rate of 40.0%–6/15) from Senguelen in the Maferinyah region. Furthermore, a novel high-density strain, termed wAnsX, was
found in an unclassified Anopheles species. The discovery of novel Wolbachia strains (particularly in members, and hybrids, of the An. gambiae complex) provides further candidate strains that could be used for future Wolbachia-based malaria biocontrol strategies.

1. Introduction

Wolbachia endosymbiotic bacteria are estimated to infect approximately 40% of insect species [1] and natural infections have been shown to have inhibitory effects on human arboviruses in mosquitoes [2–4]. High-density Wolbachia strains have been used for mosquito biocontrol strategies targeting arboviruses as they induce synergistic phenotypic effects. Wolbachia strains that have been transinfected into Aedes (Ae.) aegypti and Ae. albopictus induce inhibitory effects on arboviruses, with maternal transmission and cytoplasmic incompatibility (CI) enabling introduced strains to spread through populations [5–13]. The successful release and establishment of Wolbachia-transinfected Ae. aegypti populations in Cairns, Australia [14] was followed by further evidence of strong inhibitory effects on arboviruses from field populations [15]. Further studies in Australia [16,17] and Kuala Lumpur, Malaysia [18] have now shown that Wolbachia frequencies have remained stable since initial releases, and there is a reduction in human dengue incidence (case notifications) in the release sites.

The potential for Wolbachia to be used for biocontrol strategies targeting malaria transmission by Anopheles (An.) species has also been postulated [19] and initial laboratory experiments demonstrated that transient infections in An. gambiae reduce the density of Plasmodium (P.) falciparum parasites [20]. However, as with arboviruses, there is variability in the level of inhibition of malaria parasites for different Wolbachia strains in different mosquito species [21–23]. A major step forward was achieved through the transinfestation of a Wolbachia strain from Ae. albopictus (wAlbB) into An. stephensi and the confirmation of P. falciparum inhibition [24]. The interest in using Wolbachia for biocontrol strategies targeting malaria transmission in Anopheles mosquitoes has further increased due to the detection of natural strains of Wolbachia residing in numerous malaria vectors of Sub-Saharan Africa [25–29]. The An. gambiae complex, which consists of multiple morphologically indistinguishable species including several major malaria vector species, appears to contain diverse Wolbachia strains (collectively named wAnGa) at both low prevalence and low infection densities [25,26,28–31]. As the majority of studies have used nested-PCR for detection, more robust evidence is required to determine whether Wolbachia strains are established as endosymbionts in Anopheles species [31]. The majority of these studies are limited to amplification of only a few genes (particularly 16S rRNA), and this is problematic given the possibility of amplifying prokaryotic 16S rRNA genes from non-living cells [32]. By contrast, the recently discovered wAnM and wAnSA strains, found in An. moucheti and An. species A, respectively, are higher density infections that dominate the mosquito microbiome [26,33].

Interestingly, the presence of Wolbachia strains in Anopheles was inversely correlated to other bacterial species such as Asaia that are stably associated with several species [34–37]. Evidence for this ‘mutual exclusion’ between bacterial species in Anopheles was also present from analysis of field-collected mosquitoes from multiple countries in Sub-Saharan Africa [26]. In this study, we collected wild Anopheles mosquitoes from two regions of Guinea in June–July 2018 and characterized the natural Wolbachia strains to provide further evidence for the presence of these endosymbionts in malaria vectors. In contrast with previous studies, we extracted RNA to make any detection of Wolbachia more likely to be from actively expressed Wolbachia genes and undertook qRT-PCR analysis to compare Wolbachia densities. Phylogenetic analysis revealed the presence of novel strains in An. melas, An. gambiae sensu stricto (s.s.)–melas hybrids (including Wolbachia superinfections within individual mosquitoes) and an unclassified Anopheles species.

2. Material and methods

2.1. Study sites and collection methods

Anopheles adult mosquitoes were collected in 2018 from two regions (sub-prefectures) in Guinea: Faranah and Maferinyah. Human landing catches (HLCs) and larval dipping were conducted in three villages in the Faranah Prefecture: Balayani (10.1325, −10.7443), Foulaya (10.14463, −10.749717) and Tindo (9.9612230, −10.7016560) [38]. Three districts were selected for mosquito collections in the Maferinyah
sub-prefecture using a variety of traps [39]. BG-Sentinel 2 traps (BG2) (Biogents), CDC light traps (John W. Hock), gravid traps (BioQuip) and stealth traps (John W. Hock) were used to sample adult mosquitoes in Maferinyah Centre I (9.54650, −13.28160), Senguelen (9.41150, −13.37564) and Fandie (9.53047, −13.24000). Mosquitoes collected from traps and HLCs were morphologically identified using keys and stored in RNAlater® (Invitrogen) at −70°C [38,39].

2.2. RNA extraction and generation of complementary DNA

RNA was extracted from individual whole female mosquitoes using Qiagen 96 RNeasy Kits according to manufacturer’s instructions and a Qiagen Tissue Lyser II (Hilden, Germany) with a 5 mm stainless steel bead (Qiagen) to homogenize mosquitoes. RNA was eluted in 45 µl of RNase-free water and stored at −70°C. RNA was reverse transcribed into complementary DNA (cDNA) using an Applied Biosystems High Capacity cDNA Reverse Transcription kit. A final volume of 20 µl contained 10 µl RNA, 2 µl 10X RT buffer, 0.8 µl 25X dNTP (100 mM), 2 µl 10X random primers, 1 µl reverse transcriptase and 4.2 µl nuclease-free water. Reverse transcription was undertaken in a Bio-Rad T100 Thermal Cycler as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min and cDNA stored at −20°C.

2.3. Molecular mosquito species identification

Species identification of the An. gambiae complex was initially undertaken using diagnostic species-specific PCR assays targeting the ribosomal intergenic spacer (IGS) [40] and SINE200 insertion [41] to distinguish between the morphologically indistinguishable sibling species. To confirm species identification for samples of interest and samples that could not be identified by species-specific PCR, Sanger sequencing and phylogenetic analysis was performed for PCR products from a range of gene targets including ribosomal IGS and internal transcribed spacer 2 (ITS2) [42] and mitochondrial cytochrome c oxidase subunit 1 (COI) [43], cytochrome c oxidase subunit 2 (COII) [44] and NADH dehydrogenase subunits 4 and 5 (ND4-ND5) [45]. Where ITS2 PCR products for a particular sample were not successfully generated, or the sequencing generated was not of sufficient quality for onward analysis, a slight modification to the ITS2 primers was used to attempt to increase the success of amplification and sequencing. Alternative ITS2 primers adjusted from those published [42] were ITS2A-CJ: 5'-TA TGCTTAAA TTYAGGGGGT-3' and ITS2B-CJ: 5'-TATGCTTAAAAATTTAGGCGGT-3'. For confirmation of Culex (Cx.) waltti—a species collected in the same location and used for comparative Wolbachia density analysis—a different fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene [46] was sequenced given the lack of available sequences in certain regions for this species and to optimize sequencing quality and species discrimination. PCR reactions for IGS, SINE200, ITS2 and COI were prepared as previously described [39]. For COII amplification, PCR reactions were prepared using 10 µl of Phire Hot Start II PCR Master Mix (Thermo Scientific™) with a final concentration of 1 µM of each primer, 1 µl of PCR grade water and 2 µl template cDNA, to a final reaction volume of 20 µl. PCR reactions were carried out in a Bio-Rad T100 Thermal Cycler and cycling was 98°C for 30 s followed by 34 cycles of 98°C for 5 s, 55°C for 5 s, 72°C for 30 s followed by 72°C for 1 min. For ND4-ND5 PCR, reactions were prepared using 10 µl of HotStart Taq 2x Master Mix (New England BioLabs®) with a final concentration of 2 µM of each primer, 1 µl of PCR grade water and 2 µl template cDNA, to a final reaction volume of 20 µl. PCR reactions were carried out in a Bio-Rad T100 Thermal Cycler and cycling was 95°C for 30 s followed by 35 cycles of 95°C for 30 s, 53°C for 60 s, 68°C for 90 s followed by 68°C for 5 min. PCR products were separated and visualized using 2% E-Gel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-Gel iBase Real-Time Transilluminator.

2.4. Wolbachia detection and amplification of Wolbachia genes

Wolbachia detection was first undertaken on cDNA targeting the conserved Wolbachia genes previously shown to amplify a wide diversity of strains; 16S rRNA gene using primers W-Spec-16S-F: 5'-CATACTATTGCAAAGGATTA-3' and W-Spec-16S-R: 5'-AGCTTCAGTGAAACCAA TTC-3' [47] and Wolbachia surface protein (wsp) gene using primers wsp81F: 5'-TGTTCAATAAGTGA AGGAAAC-3' and wsp691R: 5'-AAAAATAAAACGCTACTCCA-3' [48]. Multilocus strain typing (MLST) was undertaken to characterize Wolbachia strains using the sequences of five conserved genes as molecular markers to genotype each strain. In brief, 450–500 base pair fragments of the gatB, coxA, hcpA, ftsZ and fbpA Wolbachia genes were amplified from individual Wolbachia-infected mosquitoes using previously optimized protocols [49,50]. Primers used were as follows: gatB_F1: 5'-GAKTTAAAYCGYGCAGGBGTT-

Downloaded from https://royalsocietypublishing.org/ on 07 April 2021
3', gatB_R1: 5'-TGGYAYATCRGGYAAAGATG-3', coxA_F1: 5'-TTGGRGCRATYAAACTTTATAG-3', coxA_R1: 5'-CTAAAGACTTTCRCCCATG-3', hcpA_F1: 5'-GAAATARCGTGGCTGCAA-3', hcpA_R1: 5'-GAAGATRYACGAAGTCTG-3', ftsZ_F1: 5'-ATYATGCGARCTATAAARCGAT-3', ftsZ_R1: 5'-TCRAGYATGGAATRGTAT-3', fbpA_F1: 5'-GCTGCTCCRCTTGGYWTGAT-3' and fbpA_R1: 5'-CCRCGAGAAAAYACTATTC-3' with the addition of M13 adaptors. If no amplification was detected using standard primers, further PCR analysis was undertaken using degenerate primer sets, with or without M13 adaptors [49]. In selected *An. melas* specimens where *Wolbachia* 16S rRNA Sanger sequencing (detailed below) indicated the possibility of superinfections, further MLST testing was carried out using *Wolbachia* Supergroup A and B strain-specific primers [49]. PCR reactions were prepared using 10 µl of Phire Hot Start II PCR Master Mix (Thermo Scientific™) with a final concentration of 1 µM of each primer, 1 µl of PCR grade water and 2 µl template cDNA, to a final reaction volume of 20 µl. PCR reactions were carried out in a Bio-Rad T100 Thermal Cycler using variable optimized cycling conditions. For *gatB*, *hcpA* and *fbpA* genes, cycling was 98°C for 30 s followed by 34 cycles of 98°C for 5 s, 65°C for 5 s, 72°C for 10 s followed by 72°C for 1 min. For *coxA* and *ftsZ* genes, cycling was 98°C for 30 s followed by 34 cycles of 98°C for 5 s, 55°C for 5 s and 72°C for 30 s followed by 72°C for 1 min. PCR products were separated and visualized using 2% E-Gel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-Gel iBase Real-Time Transilluminator.

### 2.5. Sanger sequencing

PCR products were submitted to Source BioScience (Source BioScience Plc, Nottingham, UK) for PCR reaction clean-up, followed by Sanger sequencing to generate both forward and reverse reads. Where *Wolbachia* PCR primers included M13 adaptors, just the M13 primers alone (M13_adaptor_F: 5'-TGAAACGGCCGCTG-3' and M13_adaptor_R: 5'-CAGAAAACGCATCTGACC-3') were used for sequencing; otherwise, the same primers as used for PCR were used. Sequencing analysis was carried out in MEGAX [51]. Both chromatograms (forward and reverse traces) from each sample were manually checked, edited and trimmed as required, followed by alignment by ClustalW and checking to produce consensus sequences. Consensus sequences were used to perform nucleotide BLAST (NCBI) database queries and for *Wolbachia* genes searches against the *Wolbachia* MLST database (https://pubmlst.org/organisms/wolbachia-spp/). If a sequence produced an exact match in the MLST database we assigned the appropriate allele number; otherwise, we obtained a new allele number for each novel gene locus sequence for *Anopheles Wolbachia* strains through submission of the FASTA and raw trace files on the *Wolbachia* MLST website for new allele assignment and inclusion within the database. Full consensus sequences were also submitted to GenBank and assigned accession numbers. The Sanger sequencing traces from the *wsp* gene were also treated in the same way and analysed alongside the MLST gene locus scheme, as an additional marker for strain typing. Where potential mixed strains were detected (in *An. melas* and *An. gambiae s.s.–melas* hybrid individuals) and any further Supergroup A or B specific testing was exhausted, it was not possible to submit these sequences to the MLST database for a new allele to be assigned; however, clean 16S consensus sequences from representative individuals for each of the Supergroup A and B strains characterized were submitted to GenBank, in addition to the full MLST profile of one individual demonstrating one of the Supergroup A strain infections.

### 2.6. Phylogenetic analysis

Alignments were constructed in MEGAX by ClustalW to include all relevant and available sequences highlighted through searches on the BLAST and *Wolbachia* MLST databases. Maximum-likelihood (ML) phylogenetic trees were constructed from Sanger sequences as follows. The most appropriate nucleotide substitution model for each phylogenetic analysis was selected through the use of the ‘Find Best-Fit Substitution Model (ML)’ option within the MEGAX software. The model with the lowest Bayesian information criterion (BIC) score from this analysis is considered to describe the substitution pattern the best. Options to model non-uniformity of evolutionary rates among sites using a discrete Gamma distribution (+G) with five rate categories and by assuming that a certain fraction of sites is evolutionary invariable (+I) were also evaluated during this analysis to highlight the most appropriate model and options to use for construction of each phylogenetic tree. The evolutionary history was then inferred by using the ML method with the most appropriate model and options for each respective tree selected, with details of the methods used for each specific tree included in the figure legends. The models used in the analysis included the Jukes–Cantor model [52], the Kimura two-parameter model [53], the general time reversible model [54], the Hasegawa–Kishino–Yano model [55] and the Tamura three-parameter model.
The tree with the highest log likelihood in each case is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The phylogeny test was by the bootstrap method with 1000 replications. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGAX [51].

2.7. Wolbachia quantification

To estimate Wolbachia density across multiple mosquito species, RNA extracts were added to Qubit™ RNA High-Sensitivity Assays (Invitrogen) and total RNA measured using a Qubit 4 Fluorometer (Invitrogen). All RNA extracts were then diluted to produce extracts that were 2.0 ng μl⁻¹ prior to being used in quantitative reverse-transcription PCR (qRT-PCR) assays targeting the Wolbachia 16S rRNA gene [28]. A synthetic oligonucleotide standard (Integrated DNA Technologies) was designed to calculate 16S rRNA gene copies per μl using a 10-fold serial dilution (electronic supplementary material, figure S1). 16S rRNA gene real-time qRT-PCR reactions were prepared using 5 μl of Quantitec SYBR® Green RT-PCR Kit (Qiagen), a final concentration of 1 μM of each primer, 1 μl of PCR grade water and 2 μl template RNA, to a final reaction volume of 10 μl. Prepared reactions were run on a Roche LightCycler® 96 System for 15 min at 95°C, followed by 40 cycles of 95°C for 15 s and 58°C for 30 s. Amplification was followed by a dissociation curve (95°C for 10 s, 65°C for 60 s and 97°C for 1 s) to ensure the correct target sequence was being amplified. Each mosquito RNA extract was run in triplicate alongside standard curves and no template controls (NTCs) and PCR results were analysed using the LightCycler® 96 software (Roche Diagnostics).

2.8. Asaia detection

Asaia PCR screening was undertaken by targeting the Asaia 16S rRNA gene using primers Asafor: 5'-GCGCGTAGGCGGTTTACAC-3' and Asarev: 5'-AGCGTCAGTAATGAGCCAGGTT-3' [35,57]. Asaia 16S rRNA gene real-time qRT-PCR reactions were prepared using 5 μl of Quantitec SYBR® Green RT-PCR Kit (Qiagen), a final concentration of 1 μM of each primer, 1 μl of PCR grade water and 2 μl template DNA, to a final reaction volume of 10 μl. Prepared reactions were run on a Roche LightCycler® 96 System for 15 min at 95°C, followed by 40 cycles of 95°C for 15 s and 58°C for 30 s. Amplification was followed by a dissociation curve (95°C for 10 s, 65°C for 60 s and 97°C for 1 s) to ensure the correct target sequence was being amplified.

2.9. Statistical analysis

Normalized qRT-PCR Wolbachia 16S rRNA gene copies per μl were compared using unpaired t-tests in GraphPad Prism 7.

3. Results

3.1. Mosquito species and Wolbachia strain prevalence rates

In addition to confirmation of species for the morphologically indistinguishable individuals within the An. gambiae complex, initial screening using diagnostic species-specific PCRs highlighted the presence of some naturally occurring hybrids between members of the An. gambiae complex. Concomitant PCR screening demonstrated the presence of Wolbachia within individuals of the An. gambiae complex, including a number of the hybrid specimens (electronic supplementary material, table S1). The composition of these hybrids was further investigated and confirmed through a repeat of the normally multiplex ribosomal IGS PCR [40] in single-plex format, separating the An. gambiae s.s./coluzzii primer set from the An. melas primer set, achieving strong amplification for both target sequences (figure 1a) and confirmed for some representative samples through Sanger sequencing and phylogenetic analysis of both IGS PCR products from the same individuals (figure 1b). The further use of PCR amplification, Sanger sequencing and phylogenetic analysis of the ribosomal ITS2
Figure 1. *Anopheles gambiae* complex PCR and phylogenetic analysis of the ribosomal IGS and ITS2 gene fragments. (a) Gel electrophoresis analysis of IGS *An. gambiae/melas* primer split down PCR products from two representative Wolbachia positive *An. gambiae* s.s.–*melas* hybrids. (b) Maximum-likelihood molecular phylogenetic analysis of sequences from IGS *An. gambiae/melas* primer split down PCR products for representative Wolbachia positive (W+) hybrid samples. Sequences from IGS *An. melas* specific primer set PCR products (blue) are shown alongside IGS *An. gambiae* primer set PCR products (red). The Jukes–Cantor model was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.6126)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], parameter = 0.010). Interestingly, the tree with the highest log likelihood (−1952.82) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 nucleotide sequences. There was a total of 901 positions in the final dataset. Sequences obtained from GenBank for comparison are shown with their accession numbers. Relevant subtrees are compressed, labelled with the species and location (table 1). Wolbachia strains were detected in *An. gambiae* s.s. mosquitoes from the Faranah region with prevalence rates ranging from 0.0 to 2.8%. In the Maferinyah region, from individuals collected in Senguelen, Wolbachia strains were detected in *An. melas* (11.6%—16/138) and in *An. gambiae* s.s.–*melas* hybrids (40.0% prevalence—6/15). Interestingly, *Wolbachia* was not found in any of the 4 *An. gambiae* s.s., 18 *An. coluzzii*, 2 *An. coluzzii–gambiae* s.s. hybrids or an *An. coluzzii–melas* hybrid collected from Senguelen, suggesting *Wolbachia* strains are not currently widespread across all members of the *An. gambiae* complex in this location. Phylogenetic hybrid composition analysis combined with Wolbachia screening highlighted the majority of *An. gambiae* s.s.–*melas* hybrids collected from Senguelen had *An. melas* mothers (8/12 *An. melas* by mitochondrial analysis), with 4/6 *Wolbachia* positive hybrids having *An. melas* as the maternal species. These results, combined with the prevalence of maternally inherited *Wolbachia* in the *An. melas* individuals and not in the *An. gambiae* s.s. individuals from this location, suggests the *Wolbachia* in this population has most likely originated from *An. melas*. 

...
Wolbachia-negative An. *gambiae s.s.*–melas hybrids were also confirmed for two specimens from Fandie (with An. *gambiae s.s.* mitochondrial results) and a Wolbachia-negative An. *coluzzii–gambiae s.s.* hybrid (maternally An. *coluzzii*) from Maferinyah.

**Table 1.** Wolbachia prevalence rates in Anopheles species collected in two regions of Guinea in 2018. Species containing Wolbachia-infected individuals are denoted in bold.

| region          | location  | species                  | Wolbachia+ individuals | total individuals | prevalence (%) |
|-----------------|-----------|--------------------------|------------------------|------------------|----------------|
| Faranah Balayani|           | An. *gambiae* s.s.       | 4                      | 143              | 2.80           |
| Faranah Balayani|           | An. *coluzzii*           | 0                      | 1                | 0.00           |
| Faranah Balayani|           | An. *coluzzii-gambiae s.s.* hybrid | 0 | 1 | 0.00 |
| Faranah Balayani|           | species unknown          | 0                      | 1                | 0.00           |
| Faranah Balayani|           | An. *gambiae* s.s.       | 0                      | 26               | 0.00           |
| Faranah Balayani|           | An. *coluzzii*           | 0                      | 1                | 0.00           |
| Faranah Foulaya |           | An. *gambiae* s.s.       | 0                      | 63               | 0.00           |
| Faranah Foulaya |           | An. *coluzzii-gambiae s.s.* hybrid | 0 | 1 | 0.00 |
| Faranah Tindo   |           | An. *gambiae* s.s.       | 1                      | 48               | 2.08           |
| Faranah Tindo   |           | An. *coluzzii*           | 0                      | 1                | 0.00           |
| Faranah Tindo   |           | An. *coluzzii-gambiae s.s.* hybrid | 0 | 2 | 0.00 |
| Maferinyah      | Fandie    | An. *coluzzii*           | 0                      | 20               | 0.00           |
| Maferinyah      | Fandie    | An. *gambiae* s.s.       | 0                      | 1                | 0.00           |
| Maferinyah      | Fandie    | An. *melas*              | 0                      | 2                | 0.00           |
| Maferinyah      | Fandie    | An. *gambiae s.s.*-melas hybrid | 0 | 4 | 0.00 |
| Maferinyah      | Fandie    | An. *coustani*           | 0                      | 1                | 0.00           |
| Maferinyah      | Maferinyah| An. *coluzzii*           | 0                      | 6                | 0.00           |
| Maferinyah      | Maferinyah| An. *coustani*           | 0                      | 3                | 0.00           |
| Maferinyah      | Maferinyah| An. *gambiae* s.s.       | 0                      | 1                | 0.00           |
| Maferinyah      | Maferinyah| An. *coluzzii-gambiae s.s.* hybrid | 0 | 1 | 0.00 |
| Maferinyah      | Maferinyah| An. *squamosus*          | 0                      | 8                | 0.00           |
| Maferinyah      | Senguelen | An. *coluzzii*           | 0                      | 18               | 0.00           |
| Maferinyah      | Senguelen | An. *coluzzii-melas*     | 0                      | 1                | 0.00           |
| Maferinyah      | Senguelen | An. *coluzzii-gambiae s.s.* hybrid | 0 | 2 | 0.00 |
| Maferinyah      | Senguelen | An. *gambiae s.s.*-melas hybrid | 6 | 15 | 40.00 |
| Maferinyah      | Senguelen | An. *coustani*           | 0                      | 1                | 0.00           |
| Maferinyah      | Senguelen | An. *gambiae* s.s.       | 0                      | 4                | 0.00           |
| Maferinyah      | Senguelen | An. *melas*              | 16                     | 138              | 11.59          |
| Maferinyah      | Senguelen | An. *species X*          | 1                      | 1                | 100.00         |
| Maferinyah      | Senguelen | An. *squamosus*          | 0                      | 1                | 0.00           |
A **Wolbachia** strain was also found in a single female of an unclassified *Anopheles* species from Senguelen. Sanger sequencing and BLAST analysis of the ITS2 region revealed this *Anopheles* sp. ‘X’ was most similar to *Anopheles* sp. 7 BSL-2014 (GenBank accession number KJ522819.1) but at only 93.2% sequence identity, and *An. theileri* (GenBank accession number MH378771.1) with 90.9% sequence identity (both full query coverage). Phylogenetic analysis of the ribosomal ITS2 region and COI and ND4-ND5 regions for *An. sp. ‘X’ (figure 2) revealed that this species is from the Myzomyia Series, within the *Cellia* subgenus of *Anopheles*, with the agreement for this placement across all three phylogenies. The ITS2 region gave the greatest discrimination for this species; however, currently, no other sequences from this species are available in order to classify it any further than to Series level and closest to, but distinct from, sequences denoted *Anopheles* sp. 7, another as yet undetermined *Anopheles* species [88]. Mosquito ribosomal and mitochondrial gene sequences were deposited in GenBank and accession numbers obtained (electronic supplementary material, table S2).
Although amplification of the *Wolbachia* 16S rRNA fragments of the natural strain in *An. gambiae* s.s. from the Faranah region was possible, sequences obtained were of insufficient quality for further analysis. Furthermore, no *wsp* gene amplification was possible from *An. gambiae* s.s. from the Faranah region. By contrast, *Wolbachia* 16S rRNA (figure 3) and *wsp* sequences (electronic supplementary material, figure S4)
Table 2. Novel resident Wolbachia strain WSP typing and multilocus sequence typing (MLST) gene allelic profiles. Newly assigned novel alleles for wAnSX are shown in bold. *wAnga-Guinea-A1 hcpA could not be assigned a novel allele number due to a possible double infection which was unresolvable, therefore the allele number of the closest match (CM) is shown with the number of single nucleotide differences to the closest match in brackets.

| mosquito species | Wolbachia strain | WSP typing allele numbers | MLST gene allele numbers |
|------------------|------------------|---------------------------|--------------------------|
|                  |                  | wsp | HVR1 | HVR2 | HVR3 | HVR4 | gatB | coxA | hcpA | ftsZ | fbpA |
| An. melas        | wAnga-Guinea-A1  | 23  | 1    | 12   | 21   | 19   | 1    | 1    | CM1  (2)* | 3 | 1 |
| An. sp. X        | wAnSX           | 737 | 264  | 297  | 3    | 323  | 285  | 282  | 310  | 246  | 454 |

were generated from both An. melas/An. gambiae s.s.–melas hybrids and An. sp. X collected from Senguelen in the Maferinyah region. Analysis of Wolbachia 16S rRNA sequences obtained from An. melas and An. gambiae s.s.–melas hybrid individuals highlighted the occurrence of superinfections within this population, with the presence of multiple Wolbachia strains being indicated. The Wolbachia 16S sequences from some An. melas and hybrid individuals produced consensus sequences which were most closely related to Wolbachia strains of Supergroup A (such as wMel, wAlbA and wAu) (therefore named wAnga-Guinea-A), of which two different A strains (named A1 and A2) could be determined in different individuals. By contrast, other An. melas and hybrid specimens produced Wolbachia 16S consensus sequences which grouped clearly with Supergroup B strains (wAnga-Guinea-B), also with two differing B strains able to be determined (B1 and B2) (figure 3). In addition, the sequence chromatograms from other An. melas and hybrid individuals consistently demonstrated mixed bases in the positions of variation between the wAnga-Guinea-A and wAnga-Guinea-B strains, with agreement both between forward and reverse sequence traces from the same individuals, as well as across multiple individuals, suggesting the presence of superinfections of both Supergroup A and B Wolbachia strains within these individuals. Repeat Wolbachia 16S sequencing for samples suggesting the presence of a wAnga-Guinea A and B strain superinfection did not allow confident separation of An. melas and hybrid individuals into wAnga-Guinea-A only, wAnga-Guinea-B only, or superinfected groups.

This, combined with the overall results from the Wolbachia 16S analysis from all infected individuals, suggested superinfections were widespread in Wolbachia positive individuals but there did not currently appear to be a clear dominant strain, or strain variant, which could be identified with the greater relative occurrence or apparent density (through consistent stronger sequencing signal strength) to the other strain(s) present in individuals from this population. This complexity was also mirrored when looking between An. melas and the hybrid specimens, with no clear distinction in the Wolbachia strain variants apparent in each group. Unfortunately, further comparative analysis of differing strains in An. melas and hybrid individuals using the Wolbachia wsp gene locus was not possible, as wsp sequence could only be successfully obtained from one An. melas individual, where 16S analysis had indicated the presence of wAnga-Guinea-A1 only. This wsp sequence matched allele 23 within the Wolbachia MLST database (table 2), demonstrating that it is identical to the wsp sequences obtained from 20 other Supergroup A Wolbachia isolates contained within the database.

Phylogenetic analysis of both Wolbachia 16S and wsp gene fragments from An. sp. X indicated that the wAnSX strain is most closely related to Wolbachia strains of Supergroup B (such as wPip, wAlbB, wAnS, wAnM, wMa and wNo). Typing of the wAnSX wsp nucleotide sequence highlighted that there were no exact matches to wsp alleles currently in the Wolbachia MLST database (https://pubmlst.org/organisms/wolbachia-spp/), and only one of the four hypervariable regions (HVRs) matched a known sequence (HVR3: allele 3). All Wolbachia gene sequences of sufficient quality to generate a consensus were deposited into GenBank and accession numbers obtained (electronic supplementary material, tables S3 and S4).

Wolbachia MLST was undertaken to attempt to provide more accurate strain discrimination and phylogenies. This was successfully done for the novel Anopheles Wolbachia strains wAnga-Guinea-A1 and wAnSX although MLST gene fragment amplification was variable for wAnga-Guinea strains found in An. melas and An. gambiae s.s.–melas hybrids. The resultant MLST allelic profile for wAnga-Guinea-A1 (table 2) was closest to the profile for strain type 13, with the variation occurring in the two positions of mixed bases in the hcpA locus, being closest to hcpA allele 1, except for a change from G to A at position 313 and from A to G at position 319 on this locus (table 2). This may indicate the presence of both hcpA allele 1 and an hcpA variant. Even if wAnga-Guinea-A1 were identical to strain type 13, of the 19 records
Figure 4. Wolbachia multilocus sequence typing (MLST) phylogenetic analysis of wAnga-Guinea-A1 and wAnsX. Maximum-likelihood molecular phylogenetic analysis from concatenation of all five MLST gene loci for resident Wolbachia strains wAnga-Guinea-A1 (in purple) and wAnsX (in green). Concatenated sequences obtained in this study are highlighted in bold with a filled circle node marker. The general time reversible model was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2484)). The rate variation model allowed for some sites to be evolutionarily invariable (I+I), 36.51% sites. The tree with the highest log likelihood (−10595.38) is shown and drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 102 nucleotide sequences. There was a total of 2079 positions in the final dataset. The concatenated MLST sequence data from wAnga-Ghana obtained from An. coluzzii in a previous study [26] is shown in orange. Concatenated sequence data from Wolbachia strains downloaded from the MLST database for comparison are shown with isolate numbers in brackets (IsoN). Wolbachia strains isolated from mosquito species are shown in blue, with those strains from other Anopheles species highlighted in bold. Strains isolated from other Dipteran species are shown in navy blue, from Coleoptera in olive green, from Hemiptera in purple, from Hymenoptera in teal blue, from Lepidoptera in maroon and from other, or unknown orders in black.

available on the MLST database (all Supergroup A), no other isolates with this strain type, where host information had been provided, were found in mosquito species. Concatenation of the MLST loci and phylogenetic analysis also confirms wAnga-Guinea-A1 is closest to strains belonging to Supergroup A, including wMel and wAlbA (as also suggested by 16S and wsp gene phylogenies). For wAnsX, new alleles for all five MLST gene loci (sequences differed from those currently present in the MLST database), and the therefore novel allelic profile, confirms this is a divergent novel Wolbachia strain (table 2). The phylogeny of wAnsX based on concatenated sequences of all five MLST gene loci confirms this strain clusters within Supergroup B and further demonstrates that it is distinct from other currently
available strain profiles (figure 4). Consistent with previous studies looking at novel Wolbachia strains in Anopheles species using MLST [24], these results highlight the lack of concordance between Wolbachia strain phylogeny and their insect hosts across diverse geographical regions.

### 3.3. Wolbachia strain densities and relative abundance

The relative densities of Wolbachia strains were estimated using qRT-PCR targeting the 16S rRNA gene after first standardizing total RNA (ng per reaction). This allowed direct comparisons between phylogenetically diverse Anopheles species and accounts for variation in mosquito body size and RNA extraction efficiency between samples. This also allows a comparison with another novel natural Wolbachia strain present in Cx. watti (termed wWat strain) collected in Maferinyah, contemporaneously with the Anopheles specimens. 16S rRNA qRT-PCR analysis revealed a mean of $1.50 \times 10^4 (\pm 4.37 \times 10^3)$ 16S rRNA copies $\mu l^{-1}$ for the wAnsX strain in the single individual (figure 5, electronic supplementary material, electronic supplementary material table S5). Lower mean densities were found for the wAnga-Guinea strains in An. melas individuals ($n = 14$) and An. gambiae s.s.–melas hybrids ($n = 4$) with $8.20 \times 10^2 (\pm 2.90 \times 10^2)$ and $1.41 \times 10^2 (\pm 3.95 \times 10^1)$ 16S rRNA copies $\mu l^{-1}$, respectively. The densities were compared with the wWat strain in Cx. watti females also collected in the Maferinyah region with a mean density of $2.37 \times 10^4 (\pm 5.99 \times 10^3)$. The density of the wWat strain was significantly higher than the wAnga-Guinea strains found in An. melas and hybrids (unpaired t-test, $p = 0.002$). Individual An. gambiae s.s. extracts from the Faranah region that were identified as Wolbachia-infected by amplification of the 16S rRNA gene [47] did not result in any 16S rRNA qRT-PCR amplification, suggesting a very low density Wolbachia strain present in these individuals.

### 3.4. Wolbachia and Asaia co-infections

Individual mosquitoes shown to be infected with the wAnsX or wAnga-Guinea strains were screened for the presence of Asaia bacteria using qRT-PCR. Co-infections were detected in all An. melas ($n = 14$, mean Asaia 16S rRNA Ct value = $30.60 \pm 2.02$), all An. gambiae s.s.–melas hybrids ($n = 4$, mean Asaia 16S rRNA Ct value = $26.32 \pm 3.54$) and in the single An. species X (Asaia 16S rRNA Ct value = $34.92$) (electronic supplementary material, table S5).
4. Discussion

Endosymbiotic Wolbachia bacteria are particularly widespread through insect populations but were historically considered absent from the Anopheles genera [19]. The discovery of additional novel natural strains of Wolbachia in Anopheles species suggests that the prevalence and diversity has been significantly under-reported to date. Since 2014, there have been several reports of detection of Wolbachia strains in major malaria vectors, such as sibling species in the An. gambiae complex [25,26,28–30] and An. moucheti [26]. This study provides evidence for Wolbachia strains in An. melas, a species within the An. gambiae complex, which can be an important local vector of malaria in West-African coastal areas where it breeds in brackish water, mangrove forests and salt marshes [59,60]. Its importance as a local malaria vector was shown in Equatorial Guinea where the average number of malaria infective An. melas bites/person/year was recorded at up to 130 [61]. The finding of natural An. gambiae s.s.–melas hybrids in this study appears highly unusual, with published accounts of hybridization between members of the An. gambiae complex seeming to agree that detection of hybrids in wild populations is relatively rare [62], and when it does occur, seems most often to be a combination of hybrids between An. gambiae s.s., An. coluzzii or An. arabiensis. Historical reports of An. gambiae s.s.–melas hybrids were also in West Africa but with laboratory colonies, giving variable results for ongoing success of hybrid colonies [63–65]. Interestingly colonized An. melas and F1 hybrid larvae were able to be reared in distilled water in the laboratory, rather than requiring a higher salinity content as might be expected from the natural ecology of An. melas [65]. As An. melas is more geographically restrained and has a more defined ecological niche than other members of the An. gambiae complex, natural hybrids composed of these constituent species are arguably less likely to occur, with fewer areas of sympathy. Natural hybrids may also be underestimated [66] due to sampling bias with a greater proportion of studies focusing on the more widely distributed major anthropic malaria vectors, An. gambiae and An. arabiensis [67].

Hybrid detection is also dependent on the methodology used for species identification and the format of species-specific diagnostic assays [40,62]. Our testing highlighted that amplification and clarity of hybrid detection was improved with the use of the ribosomal IGS PCR primers [40] for each species in single-plex format, rather than the standard higher throughput multiplex format, where primers for multiple members of the An. gambiae complex are included at the same time, with different product sizes for species discrimination. This is unsurprising due to the designed aims of the multiplex assay, and potential variations in reaction efficiency between species, particularly when hybridized, which were highlighted in the original publication [40]. However, this could potentially result in reduced detection of natural hybrids, compared with the apparent detection of individual species, when used for widespread screening and species identification. Sanger sequencing of the single-plex species-specific IGS PCR products for representative hybrid samples enabled confirmation of the hybridization and the avoidance of doubt from any possibility of specificity problems [40,62], before further confirmation was obtained through subsequent sequencing and phylogenetic analysis of other gene fragments.

Genetic divergence also probably affects interspecific hybrids, and the original delineation of the member species within the An. gambiae complex was concluded on the basis of hybrid male sterility from early crossing experiments [67]. However, the full extent and impacts of interspecific hybridization between members of the complex is still under investigation and debate [66]. Anopheles gambiae s.s. and An. melas have a greater degree of genetic divergence from one another when compared with other members of the complex (such as An. gambiae s.s., An. coluzzii and An. arabiensis) and An. melas groups separately and more closely to An. merus and An. quadriannulatus sequences. Even within An. melas, species-specific microsatellite markers and mitochondrial genetic analysis of geographically distinct populations suggested there was species-level divergence between different populations, resulting in three distinct major clusters; Bioko Island, Western mainland and Southern mainland African populations (with mainland population division occurring in Cameroon) [67]. In the context of the results of this study, our An. melas would be included in the Western mainland cluster (this is supported by our phylogenetic analysis). Following the discovery of Wolbachia in this population, it would be interesting to investigate whether Wolbachia strains were also present in other An. melas geographic clusters, and whether the CI phenotype was evident in some or all of these strains. If stable Wolbachia infections were present in some populations but not others, it also raises the question of the length of time Wolbachia may have been present in this species and whether Wolbachia infections may be having an influence on the host population genetics and affecting genetic divergence and speciation over time.
This study and previous studies have highlighted how thorough and accurate molecular identification of mosquito specimens is important given the difficulties of morphological identification, the potential for currently unrecognized cryptic species [58,68] and potential for inaccuracies for certain species where only diagnostic species PCR-based methods are used for molecular identification [69]. The discovery of the wAnsX strain through PCR screening and Sanger sequencing led to the retrospective confirmation of the host mosquito species using Sanger sequencing as all individuals that were Wolbachia positive by PCR were initially morphologically identified as members of the An. gambiae complex. Phylogenetic analysis and confident species discrimination is dependent on the sequences available for comparison at the time. Sequencing and phylogenetic analysis of all three regions for this specimen indicated placement within the Cellia subgenus and Myzomyia Series of Anopheles, with the greatest number of closely related comparative sequences available for comparison in the ITS2 region. Our analysis revealed that this species is closest to Anopheles sp. 7, followed by An. thelleri from sequences currently available. Anopheles sp. 7 BSL-2014 was collected in the western Kenyan Highlands, with 1 of 23 specimens P. falciparum ELISA sporozoite and PCR positive [58]. Anopheles thelleri was collected in the Democratic Republic of Congo [70] and was found to be infected with Plasmodium sporozoites in eastern Zambia [71].

The results of this study also highlight the requirement to provide as much genetic information and confirmation as possible for a newly discovered strain of Wolbachia (particularly low-density infections). The first discovery of Wolbachia strains in wild An. gambiae populations in Burkina Faso resulted from sequencing of the 16S rRNA gene rather than screening using Wolbachia-specific genes [25]. A more recent comprehensive analysis through screening of An. gambiae genomes (Ag1000G project) concluded that determining whether a Wolbachia strain is present in a given host based on the sequencing of one gene fragment (often 16S rRNA) is problematic and caution should be taken [31]. In this study, from An. gambiae s.s., we were only able to amplify a Wolbachia 16S rRNA gene fragment, which is consistent with numerous recent studies in which low-density strains have been detected [27,30]. As a result, caution must be taken in drawing conclusions on the stability of infection and biological significance. Other explanations for the amplification of 16S rRNA gene fragments include Wolbachia DNA insertions into an insect chromosome or contamination from non-mosquito material such as ectoparasites or plants [31]. In contrast with previous studies, we extracted RNA, increasing the chances that detection of the 16S rRNA gene is from actively expressed Wolbachia and indicating amplification is more likely of bacterial gene origin (rather than through integration into the host genome). Although RNA extraction kits are optimized for RNA and we measured high levels of total RNA using a fluorometer, there is a small possibility that our amplification and sequencing could result from co-extracted Wolbachia gDNA. Detection and sequencing of Wolbachia gDNA have been used previously in numerous studies to characterize strain phylogenies [25–29]. However, to provide greater confidence in the expression of Wolbachia genes in future work, a DNase treatment could be undertaken to ensure amplification is only resulting from the cDNA. Regardless, these results are consistent with previous studies in which every Wolbachia 16S rRNA amplicon and sequence attributed to An. gambiae s.s. is unique and appears at very low density [31]. Therefore, further experiments are needed to confirm these strains are genuine endosymbionts in their hosts such as microscopy and genome sequencing [33].

The densities of the wAnga-Guinea and wAnsX strains detected in Senguelen (measured using qRT-PCR) are significantly higher than Wolbachia detected in An. gambiae s.s. from Faranah (which were not detectable using this qRT-PCR assay targeting the 16S rRNA gene). The wAnga-Guinea strains appear to have both an intermediate prevalence rate and density and further studies are required to elucidate the relative density contribution and possible differential localization of these Wolbachia strains within the mosquito host, whether these strains may be influencing host population genetics (including the occurrence of natural hybrids and the intraspecific diversity within An. melas) and investigate these strains across more diverse geographical areas. Unfortunately, more extensive separate characterization and determination of relative densities of the Wolbachia strains within superinfected An. melas and An. gambiae s.s.–melas hybrids was not possible in this study. Further work, through the design of strain-specific PCRs and cloning followed by sequencing of genes from the separate strains, would help to expand knowledge on the characteristics and possibilities for the further potential use of these strains. Caution and further investigation is also required for the wAnsX strain, as this was detected from the only collected individual of this unclassified Anopheles species. The detection of Wolbachia-Asaia co-infections in all individuals was in contrast with our previous study [26] but Asaia can be environmentally acquired at different mosquito life stages and the prevalence and density were significantly variable across different Anopheles species and locations [26]. These contrasting results suggest a complex association between these two bacterial species in wild Anopheles mosquito populations, and given that Asaia is environmentally acquired, this association will be highly location dependent.
Wolbachia strains in *An. species A* (*wAnsA*) and *An. moucheti* (*wAnM*) [26], and now *An. melas* (*wAnga-Guinea-A1*) and *An. sp. X* (*wAnsX*), have complete MLST and *wsp* profiles and are at significantly higher densities when compared with strains detected in *An. gambiae* s.s. from the same countries. As Wolbachia density is strongly correlated with arbovirus inhibition in *Aedes* mosquitoes [5,7,11,12], higher density strains in *Anopheles* species would be predicted to have a greater impact on malaria transmission in field populations. In this study, we screened for *P. falciparum* infection and found very low prevalence rates (less than 1%; data not shown) preventing any statistical analysis on *Wolbachia-Plasmodium* interactions. This study and previous studies measuring a direct impact on *Plasmodium* infection in wild populations are dependent on parasite infection rates which can be low even in malaria-endemic areas [26] and particularly for the infective sporozoite stage [72]. Low pathogen prevalence rates are also limiting factors in assessing the effect of natural strains of *Wolbachia* on arboviruses in wild mosquito populations [73]. In addition to looking at effects on *Plasmodium* prevalence in field populations, further work should look to undertake vector competence experiments with colonized populations and to determine if these *Wolbachia* strains are present in tissues such as the midgut and salivary glands which are critical to sporogony. Further studies are also needed to determine if the *wAnga-Guinea* strains are maternally transmitted given our results would suggest they are likely to be from the *An. melas*, rather than from *An. gambiae* s.s. Furthermore, an assessment of how these *Wolbachia* strains are being maintained in field populations is needed, and to determine if the CI reproductive phenotype can be induced by these strains (and if it affects the viability of subsequent generations). As the chances of success of *Wolbachia* transinfection experiments can be improved by the adaptation of the *Wolbachia* strain to the target host genetic background [74], this may imply a favourable potential for *wAnga-Guinea* transinfection experiments and successful establishment of a stable *Wolbachia* infection within *An. gambiae* s.s. colonies, in addition to *An. melas*. If achievable, this would be a big step forward in determining whether these strains (which appear relatively higher density than *Wolbachia* previously detected in the *An. gambiae* complex) could reduce malaria transmission through *Wolbachia*-based biocontrol strategies.

5. Conclusion

Although the debate continues over the biological significance (or even presence) of natural strains in the *An. gambiae* complex, this study provides strong evidence of additional novel strains with relatively higher density infections, in addition to *Wolbachia* positive natural hybrids in the *An. gambiae* complex, and may reflect the under-reporting of natural strains in the *Anopheles* genus. The presence of *Wolbachia* superinfections increases the complexity of phylogenetic characterization of individual strains and the determination of the relative contribution of each strain to the overall density. There are previous studies showing natural *Wolbachia* superinfections in wild mosquito populations such as *Ae. albopictus* [75] and superinfections have been generated in mosquitoes used for biocontrol strategies [7,76], indicating superinfections can form stable associations with mosquito hosts. Candidate *Wolbachia* strains for mosquito biocontrol strategies require synergistic phenotypic effects to impact the transmission of mosquito-borne pathogens and further studies are needed to determine if these strains would induce CI and what effects they may have on host fitness. Whether these *Wolbachia* superinfections can inhibit *Plasmodium* parasites [28,29] or influence the ability to transinfect other *Wolbachia* strains for population suppression and replacement strategies [77] remains to be determined, but further investigation is warranted.

Ethics. Mosquito collection protocols were reviewed and approved by the Comité National d’Ethique pour la Recherche en Sante (030/CNERS/17) and the institutional review boards (IRB) of the London School of Hygiene and Tropical Medicine (nos. 14798 and 15127) and the Centers for Disease Control and Prevention, USA (2018-086); all study procedures were performed in accordance with relevant guidelines and regulations. Fieldworkers participating in human landing catches were provided with malaria prophylaxis for the duration of the study.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material and all the sequencing data generated is available in GenBank with accession numbers as shown in the relevant electronic supplementary material, tables.

Authors’ contributions. C.L.J. designed the study, carried out the analyses and drafted the manuscript. C.C.-U. performed fieldwork, carried out preliminary laboratory analysis and reviewed the manuscript. A.H.B. supervised fieldwork and reviewed the manuscript. C.S. performed fieldwork and reviewed the manuscript. E.K.L. supervised fieldwork and reviewed the manuscript. M.K. performed fieldwork and reviewed the manuscript. S.R.I. supervised fieldwork and reviewed the manuscript. T.W. designed the study, carried out analyses, drafted the manuscript and provided overall supervision.
References

1. Zug R, Hammerstein P. 2012 Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS ONE 7, e38544. (doi:10.1371/journal.pone.0038544)

2. Glaser RL, Meola MA. 2010 The native Wolbachia endosymbionts of Drosophila melanogaster and Culex quinquefasciatus increase host resistance to West Nile virus infection. PLoS ONE 5, e11977. (doi:10.1371/journal.pone.0011977)

3. Mousson L, Zouache K, Arias-Goeta C, Failloux AB, Sinkins SP. 2012 The native Wolbachia symbiont limits transmission of dengue virus in Aedes mosquitoes. PLoS Negl. Trop. Dis. 6, e1989. (doi:10.1371/journal.pntd.0001989)

4. Silva JBL, Magalhaes Alves D, Bottonio-Rojas V, Pereira TN, Sorgine MHF, Caragata EP, Moreira LA. 2017 Wolbachia and dengue virus infection in the mosquito Aedes flavovirus (Diptera: Culicidae). PLoS ONE 12, e0181678. (doi:10.1371/journal.pone.0181678)

5. Walker T et al. 2011 The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. Nature 476, 454–457. (doi:10.1038/nature10316)

6. Iturbe-Ormaetxe I, Walker T, Neill SLO. 2011 Wolbachia and the biological control of mosquito-borne disease. EMBO Rep. 12, 508–518. (doi:10.1038/embor.2011.84)

7. Joubert DA et al. 2016 Establishment of a Wolbachia superinfection in Aedes aegypti mosquitoes as a potential approach for future resistance management. PLoS Pathog. 12, e1005434. (doi:10.1371/journal.ppat.1005434)

8. Moreira LA et al. 2009 A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. Cell 139, 1268–1278. (doi:10.1016/j.cell.2009.11.042)

9. Bhat G, Xu L, Pau T, Wei, X, Yi Z. 2010 The endosymbiotic bacterium Wolbachia induces resistance to dengue virus in Aedes aegypti. PLoS Pathog. 6, e1000833. (doi:10.1371/journal.ppat.1000833)

10. Bilgame MS, Arias-Goeta C, Failloux AB, Sinkins SP. 2012 Wolbachia strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in Aedes aegypti. Proc. Natl. Acad. Sci. USA 109, 255–260. (doi:10.1073/pnas.1112021108)

11. Ant TH, Herd CS, Geoghegan V, Hoffmann AA, Sinkins SP. 2018 The Wolbachia strain wku provides highly efficient virus transmission blocking in Aedes aegypti. PLoS Pathog. 14, e1006815. (doi:10.1371/journal.ppat.1006815)

12. Fraser JE, De Bruyne JT, Iturbe-Ormaetxe I, Stepnell J, Burns RL, Flores HA, O’Neill SL. 2017 Novel Wolbachia-transinfected Aedes aegypti mosquitoes possess diverse fitness and vector competence phenotypes. PLoS Pathog. 13, e1006751. (doi:10.1371/journal.ppat.1006751)

13. McMeniman C, Lane RV, Cass BN, Fong MW, Sidhu M, Wang YE, O’Neill SL. 2009 Stable introduction of a life-shortening Wolbachia infection into the mosquito Aedes aegypti. Science 323, 141–144. (doi:10.1126/science.1165326)

14. Hoffmann AA et al. 2011 Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. Nature 476, 454–457. (doi:10.1038/nature10316)

15. O’Neill SL et al. 2018 Scaled deployment of Wolbachia to protect the community from dengue and other Aedes transmitted arboviruses. Gates Open Res. 2, 36. (doi:10.12688/gatesopenres.12844.3)

16. Ryan PA et al. 2019 Establishment of wMel Wolbachia in Aedes aegypti mosquitoes and reduction of local dengue transmission in Cairns and surrounding localities in northern Queensland, Australia. Gates Open Res. 3, 1547. (doi:10.12688/gatesopenres.13061.1)

17. Nazmi WR et al. 2019 Establishment of Wolbachia Strain wMelB in Malaysian populations of Aedes aegypti for dengue control. Curr. Biol. 29, 4241–4246. (doi:10.1016/j.cub.2019.11.007)

18. Walker T, Moreira LA. 2011 Can Wolbachia be used to control malaria? Mem. Inst. Oswaldo Cruz 106, 212–217. (doi:10.1590/S0073-20262010000900026)

19. Hughes GL, Koga R, Xue P, Fukatsu T, Ranson JG. 2011 Wolbachia infections are virulent and inhibit the human malaria parasite Plasmodium falciparum in Anopheles gambiae. PLoS Pathog. 7, e1002043. (doi:10.1371/journal.ppat.1002043)

20. Hughes GL, Vega-Rodriguez J, Xue P, Ranson JG. 2012 Wolbachia strain wMelB enhances infection by the rodent malaria parasite Plasmodium berghei in Anopheles gambiae mosquitoes. Appl. Environ. Microbiol. 78, 1491–1495. (doi:10.1128/AEM.06751-11)

21. Murdock CC, Blanford S, Hughes GL, Ranson JG, Thomas MB. 2014 Temperature alters Plasmid distribution by Wolbachia. Sci. Rep. 4, 3932. (doi:10.1038/srep03932)

22. Hughes GL, Rivoir A, Ranson JG. 2014 Wolbachia can enhance Plasmodium infection in mosquitoes: implications for malaria control? PLoS Pathog. 10, e1004182. (doi:10.1371/journal.ppat.1004182)

23. Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, Xu Y, Dimopoulos G, Xi Z. 2013 Wolbachia invades Anopheles stephensi populations and induces refractoriness to Plasmodium infection. Science 340, 748–751. (doi:10.1126/science.1236192)

24. Baldini F, Segata N, Potomkin J, Marcenac P, Shaw WR, Daube R, Diabate A, Levashina EA, Catteruccia F. 2014 Evidence of natural Wolbachia infections in field populations of Anopheles gambiae. Nat. Commun. 5, 5985. (doi:10.1038/ncomms4985)

25. Jeffries CL et al. 2018 Novel Wolbachia strains in Anopheles malaria vectors from Sub-Saharan Africa. Wellcome Open Res. 3, 113. (doi:10.12688/wellcomeopenres.14765.2)

26. Ngiam EHA, Basane H, Makoumdou F, Fenollar F, Weiell M, Medanikov O. 2018 First report of natural Wolbachia infection in wild Anopheles funestus population in Senegal. Malar. J. 17, 408. (doi:10.1186/s12936-018-2559-z)

27. Gomes FM et al. 2017 Effect of naturally occurring Wolbachia in Anopheles gambiae s.l. mosquitoes from Mali on Plasmodium falciparum malaria transmission. Proc. Natl. Acad. Sci. USA 114, 12566–12571. (doi:10.1073/pnas.1716111114)

28. Shaw WR, Marcenac P, Cholds LM, Buckee OC, Baldini F, Sawadogo SP, Daube RK, Diabate A, Catteruccia F. 2016 Wolbachia infections in natural Anopheles populations affect egg laying and negatively correlate with Plasmodium development. Nat. Commun. 7, 11772. (doi:10.1038/ncomms11772)

29. Baldini F, Rouge J, Keppel K, Mikandawire G, Mapusa SA, Sikulu-Lord M, Ferguson HM, Gowella N, Okumu FO. 2018 First report of natural Wolbachia infection in the malaria mosquito Anopheles arabiensis in Tanzania. Parasit. Vectors 11, 635. (doi:10.1186/s13071-018-2349-y)

30. Chrostek E, Gerth M. 2019 Is Anopheles gambiae a natural host of Wolbachia? mBio 10, 19. (doi:10.1128/mBio.00784-19)
41. Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, et al. 2014 Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. Proc. Natl Acad. Sci. USA 111, 12 498–12 503. (doi:10.1073/pnas.1408881111)

32. Carini P, Marsden PJ, Leff JW, Morgan EE, et al. 1995 Discrimination of all SINE200 retrotransposons within speciation complex by the polymerase chain reaction. Mol. Biol. Evol. 12, 2589–2601. (doi:10.1093/molbev/12.5.2589)

43. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994 DNA primers for amplification of 12S and 16S ribosomal DNA segments. J. Mol. Evol. 38, 494–502. (doi:10.1007/bf00160733)

52. Dabire RK, Diabate A, Catteruccia F, Neafsey DE, et al. 2012 Wolbachia in the reproductive organs of Anopheles gambiae in the Malaria Vector Control Research Consortium: a storage facility for core research resources. Malar. J. 11, 123. (doi:10.1186/1475-2875-11-123)

46. Kumar NP, Rajavel AR, Natarajan R, Jambulingam Y, et al. 2016 Anopheles gambiae sporozoite rate associated with low human blood index in Anopheles coluzzii. Sci. Rep. 6, 21 898–21 906. (doi:10.1038/srep21898)

50. Baldo L et al. 2006 Multilocus sequence typing system for the endosymbiont Wolbachia: pipeline. Appl. Environ. Microbiol. 72, 7107–7110. (doi:10.1128/AEM.03737-05)

54. Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, et al. 2018 Anopheles gambiae strains using wsp gene sequences. Ann. Trop. Med. Parasitol. 122, 199–210. (doi:10.1179/1749065818y.0000000001)

57. Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, et al. 2020 An assessment of Wolbachia diversity dynamics in African house mosquitoes. Parasit. Vectors 13, 273. (doi:10.1186/s12936-020-02959-2)

60. Tamura K. 1992 Estimation of the number of substitutions by the maximum-likelihood method. J. Mol. Evol. 35, 154–159. (doi:10.1007/bf01701581)

65. Vicente J, et al. 2017 Massive introgression drives species radiation at the range limit of Anopheles gambiae. Sci. Rep. 7, 46 451. (doi:10.1038/s41598-020-6245-z)

66. Vicente J, et al. 2017 Massive introgression drives species radiation at the range limit of Anopheles gambiae. Sci. Rep. 7, 46 451. (doi:10.1038/s41598-020-6245-z)

67. Deitz KC et al. 2012 Genetic isolation within the malaria mosquito Anopheles melas. Mol. Biol. Evol. 29, 4498–4513. (doi:10.1093/molbev/mss192)

68. Collins FH, Parkes W. 2007 A method for estimating Wolbachia nucleotide substitutions. J. Mol. Evol. 65, 179–185. (doi:10.1007/s00239-006-0109-8)

69. Jolley KA, Chan MS, Maiden MC. 2004 Wolbachia infection frequencies in insects: evidence of a global equilibrium? Proc. Biol. Sci. 271, 1287–1289. (doi:10.1098/rspb.2004.2585)

70. Bandibabone J, Muhigwa JB, Agramonte NM, et al. 2021 Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. Proc. Natl Acad. Sci. USA 118, 6083–6092. (doi:10.1073/pnas.2100627118)

71. Lobo NF et al. 2021 Wolbachia and Asaia bogorensis, an unusual bacterial symbiont of the hybridization of An. gambiae and An. melas Theobald. Am. J. Trop. Med. Hyg. 105, 702–704. (doi:10.4269/ajtmh.2021.1020)

72. Pombi M et al. 2017 Unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions using molecular tools. Sci. Rep. 5, 17 952. (doi:10.1038/srep17952)

73. Pombi M et al. 2018 Unexpectedly high Plasmodium sporozoite rate associated with low human blood index in Anopheles coluzzii from a LLIN-protected village in Burkina Faso. Sci. Rep. 8, 12 806. (doi:10.1038/s41598-018-31174-x)

74. Jeffreys CI, Tantely LA, Rahamahiana FN, Hurn E, Boyer S, Walker T. 2018 Diverse novel resident mosquito Wolbachia strains in Culicine mosquitoes from Madagascar. Sci. Rep. 8, 17 456. (doi:10.1038/s41598-018-35658-2)

75. McMeniman CJ, Lane AM, Fong AH, Voronin DA, Yorben-Ormaetxe I, Yamada R, et al. 2016 Anopheles gambiae symbiont complex along the Gambia River, with particular reference to the molecular forms of An. gambiae s.s. Malar. J. 15, 182. (doi:10.1186/s12936-017-2875-7–182)

5. Lobo NF et al. 2017 Unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions using molecular tools. Sci. Rep. 5, 17 952. (doi:10.1038/srep17952)

6. Overgaard HJ et al. 2012 Malaria transmission after five years of vector control on Bioko Island, Equatorial Guinea. Parasit. Vectors 5, 253. (doi:10.1186/1756-3305-7-253)

16. Hubend-Thomas DR. 1948 D.D.T. and gammexane as residual insecticides against Anopheles gambiae in African houses. Nature 159, 102. (doi:10.1038/159102a0)

19. Bruce-Chwatt LJ. 1950 Recent studies on insect vectors of yellow fever and malaria in British West Africa. J. Trop. Med. Hyg. 53, 71–79.

20. Burgess RW. 1962 Preliminary experiments on the hybridization of Anopheles gambiae s.s. and Anopheles melas Theobald. J. Trop. Med. Hyg. 55, 702–704. (doi:10.4269/ajtmh.1962.55.702)

23. Pombi M et al. 2017 Unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions using molecular tools. Sci. Rep. 5, 17 952. (doi:10.1038/srep17952)

24. Bruce-Chwatt LJ. 1950 Recent studies on insect vectors of yellow fever and malaria in British West Africa. J. Trop. Med. Hyg. 53, 71–79.

25. Burgess RW. 1962 Preliminary experiments on the hybridization of Anopheles gambiae s.s. and Anopheles melas Theobald. J. Trop. Med. Hyg. 55, 702–704. (doi:10.4269/ajtmh.1962.55.702)
75. Sinkins SP, Braig HR, O’Neill SL. 1995 Wolbachia pipientis: bacterial density and unidirectional cytoplasmic incompatibility between infected populations of Aedes albopictus. Exp. Parasitol. 81, 284–291. (doi:10.1006/expr.1995.1119)

76. Ant TH, Sinkins SP. 2018 A Wolbachia triple-strain infection generates self-incompatibility in Aedes albopictus and transmission instability in Aedes aegypti. Parasit. Vectors 11, 295. (doi:10.1186/s13071-018-2870-0)

77. Jeffries CL, Walker T. 2016 Wolbachia biocontrol strategies for arboviral diseases and the potential influence of resident Wolbachia strains in mosquitoes. Curr. Trop. Med. Rep. 3, 20–25. (doi:10.1007/s40475-016-0066-2)