RESEARCH ARTICLE

REVISED Diverse novel *Wolbachia* bacteria strains and genera-specific co-infections with *Asaia* bacteria in Culicine mosquitoes from ecologically diverse regions of Cameroon [version 2; peer review: 3 approved]

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Abstract

Background: The endosymbiotic bacterium *Wolbachia* infects numerous species of insects and *Wolbachia* transinfection of *Aedes* mosquito species is now being used for biocontrol programs as *Wolbachia* strains can both inhibit arboviruses and invade wild mosquito populations. The discovery of novel, resident *Wolbachia* strains in mosquito species warrants further investigation as potential candidate strains for biocontrol strategies.
Methods: We obtained mosquito specimens from diverse Culicine mosquitoes from Cameroon including ecologically diverse locations in the Central and West Regions. *Wolbachia* prevalence rates were assessed in addition to the environmentally acquired bacterial species *Asaia* in major Culicine genera. PCR-based methods were also used with phylogenetic analysis to confirm identities of host mosquito species and *Wolbachia* strains were classified using multi-locus sequence typing (MLST).

Open Peer Review

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	1	2	3
version 2 (revision) 26 Sep 2023	✓ view	✓ view	✓ view
version 1 21 Jun 2023	? view	? view	✗ view

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Results: We report high *Wolbachia* prevalence rates for Culicine species, including in a large cohort of *Aedes africanus* collected from west Cameroon in which 100% of mono-specific pools were infected. Furthermore, co-infections with *Asaia* bacteria were observed across multiple genera, demonstrating that these two bacteria can co-exist in wild mosquito populations. *Wolbachia* strain MLST and phylogenetic analysis provided evidence for diverse *Wolbachia* strains in 13 different mosquito species across seven different genera. Full or partial MLST profiles were generated from resident *Wolbachia* strains in six *Culex* species (*quinquefasciatus*, *watti*, *cinerus, nigripalpus, perexiguus* and *rima),* two *Aedes* species *(africanus* and *denderensis)* and in *Mansonia uniformis, Catageiomyia argenteopunctata, Lutzia tigripes, Eretmapodites chrysogaster* and *Uranotaenia bilineata.*
Conclusions: Our study provides further evidence that *Wolbachia* is widespread within wild mosquito populations of diverse Culicine species and provides further candidate strains that could be investigated as future options for *Wolbachia*-based biocontrol to inhibit arbovirus transmission.

Keywords
Wolbachia, bacteria, mosquitoes

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

REVISED **Amendments from Version 1**

We have taken into consideration all the very relevant reviewer comments and significantly improved all sections. Specifically, we have adjusted our title to reflect the 'genera-specific' associations seen between *Wolbachia* and *Asaia* prevalence rates, included more background information on using additional *Wolbachia* strains for dengue control in Malaysia and significantly increased the rationale for investigating Asaia-Wolbachia interactions by providing more context on previous studies looking at *Asaia* in mosquitoes. We have provided additional details on qPCR methodology and added an additional section in the methods for statistical analysis. Figure 2 now contains a more universally accessible colour scheme and we have significantly expanded our discussion to address some very valid and important reviewer queries. We have included more discussion around how our *Wolbachia* and *Asaia* prevalence rates results are more reflective of a 'genera-specific' pattern that is comparable to other previous studies and have included some limitations based on the challenging nature of mosquito morphological identification. Furthermore, we have expanded our discussion to highlight the limitations of our results based on both using whole bodies (rather than tissue-specific detection) and using monospecific pooling. We have now clearly defined what we would categorise as a 'novel' *Wolbachia* strain and acknowledged that even MLST allelic profiling may not represent the optimal loci to capture strain variation (despite being widely used to determine *Wolbachia* strain diversity).

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

Introduction

Wolbachia are endosymbiotic bacteria which reside within an estimated 40–70% of insect species[1,2]. These bacteria have been detected in numerous mosquitoes that transmit human pathogens including species within the *Aedes* (*Ae.*), *Culex* (*Cx.*) and *Anopheles* (*An.*) genera[3–9]. *Wolbachia* is now being used in applied mosquito biocontrol strategies as strains inhibit arboviruses and invade mosquito populations using the reproductive phenotype cytoplasmic incompatibility (CI). *Wolbachia*-induced CI was first used in mosquito release control trials in the 1960s in attempts to supress *Cx. quinquefasciatus* populations in Myanmar[10]. More recently, *Wolbachia*-infected *Aedes* males have been released to induce CI and the associated sterility from matings with wild-type females, resulting in inviable progeny. This method of suppressing the population, the incompatible insect technique (IIT), has seen field trials for species such as *Ae. polynesiensis* that contain natural resident strains[11,12]. Embryo microinjection has also resulted in transinfected strains in *Ae. aegypti*[13–16]. These transinfected strains, including *w*Mel, have been shown to invade wild mosquito populations and also inhibit major arbovirus transmission, such as dengue virus (DENV)[13,15,17–19]. The *w*Mel *Ae. aegypti* line, through release programmes, is now present in more than 10 countries and encouragingly, a randomised control trial in Indonesia resulted in a 77% DENV inhibition[20]. The *w*Mel strain is being continually released into additional DENV endemic countries, and based on mathematical modelling, has the capacity to reduce the DENV R0 (basic reproduction number) from 66–75%[21]. The *w*Mel strain also inhibits other medically important arboviruses such as chikungunya virus (CHIKV)[22], Yellow fever virus (YFV)[23] and Zika virus (ZIKV)[24]. There are other *Wolbachia* strains also being used in applied strategies with the *w*AlbB strain[25] now established in Malaysian *Ae. aegypti* populations and having an impact on dengue incidence[26].

Wolbachia strains that naturally reside within mosquito populations can provide important comparative data to complement biocontrol strategies. For example, whether *Wolbachia* strains in natural populations are found at a high prevalence, and whether strains are capable of inducing CI to allow rapid population invasion. This first requires the identification of strains using molecular strain typing from diverse species which would then allow more comprehensive phenotypic characterization. Intra-genera transinfection has been shown to be successful without the need for adapting *Wolbachia* strains to insect cell lines so identifying strains residing in natural mosquito populations could provide additional candidate strains for biocontrol strategies. The presence of other bacteria, such as the a-Proteobacterium *Asaia,* has been postulated to compete with *Wolbachia* to colonise the reproductive tissues of mosquitoes, including the ovaries in females[27,28]. *Asaia* has also been proposed for biocontrol strategies as this genus of bacteria forms stable associations with numerous insects that sugar feed[29] and can rapidly colonize the midgut and spread to other insect tissues after ingestion from either a sugar or blood meal[30]. *Asaia* is particularly well studied in *Anopheles* (vectors of malaria parasites) and can stably associate with multiple species and be the dominant bacteria within some species such as *An. stephensi*[31]. Unlike *Wolbachia*, *Asaia* can be cultured in cell free media and has been genetically transformed[31]. *Asaia* can also be both horizontally and vertically transmitted providing a mechanism to invade mosquito populations.

Therefore, examining the possibility of co-infections in diverse mosquito populations will investigate if any antagonistic associations between these two common bacteria are present, as has been demonstrated in lab populations[27,28]. Numerous studies which have detected natural *Wolbachia* strains in Culicines have undertaken analysis of the *16S rRNA* gene when looking at the wider microbiome[32,33]. However, a more targeted approach amplifying *Wolbachia*-specific genes is required to confirm a resident strain is present and phylogenetic analysis allows any newly discovered strains to be compared to existing strains. This then allows a more comprehensive assessment of *Wolbachia* tissue tropism (microscopy), whole genome sequencing and ultimately phenotypic characterisation. *Wolbachia* strains can be classified in Supergroups designated from A to H. A and B Supergroup strains are mostly found in arthropods, with only one strain per host, or multiple strains infecting the same host[34]. Superinfection, the infection of more than one strain of *Wolbachia* in the same host – as can be seen in *Ae. albopictus* with the *w*AlbA and *w*AlbB strains[35] – can comprise strains of differing Supergroups, such as A and B group strain co-infections.

Cameroon is a country in West Africa in which outbreaks of arboviral diseases including DENV, YFV, CHIKV and Rift Valley fever virus (RVFV)[36] have been reported. For example, DENV IgM seroprevalence among febrile children was 14%

from a cross a cross-sectional study performed in 961 children less than 15 years old attending public hospitals of Cameroon[37]. Cases of arboviruses are reported throughout the country and there is concern that rapid urbanisation seen throughout Africa could exacerbate transmission through favouring the breeding conditions of urban-adapted mosquito species[38]. Deforestation has a significant effect on the abundance and diversity of Cameroon mosquito species and could lead to spill-over transmission of additional circulating zoonotic viruses such as Semliki Forest, o'nyong'nyong and Bwamba viruses[39]. Here, we obtained specimens from entomological surveys undertaken in Central and West Cameroon to determine if diverse Culicine species harbour resident *Wolbachia* strains and if co-infections exist with *Asaia*, given this bacterium can be environmentally acquired and can compete with *Wolbachia*. We extracted RNA from preserved samples and undertook qRT-PCR analysis to make any detection of *Wolbachia* strains more likely to be from actively expressed *Wolbachia* genes. A combination of phylogenetic analysis and strain typing using multi-locus sequence typing (MLST) revealed a diversity of newly discovered *Wolbachia* strains.

Methods

Mosquito collections

A variety of adult mosquito trapping methods were used in the Central Region of Cameroon in Yaoundé (3°52'22.2"N, 11°30'38.0"E) and Olama village (3°24'45.0"N 11°17'03.0"E) from June 2019 to July 2019. Yaoundé (the capital of Cameroon) is an urban location characterised by mosquito breeding sites such as tyres, containers, and temporary water pools. In contrast, Olama village is rural, located along the Nyong River (Figure 1) and is characterised by houses built with corrugated metal roofs and mud walls. In Olama village, eight CDC miniature light traps were hung in four houses and two Stealth traps (model 14 which uses ultraviolet light for attraction) were hung in one house (one trap inside and one outside). BG Suna traps containing a carbon dioxide source from fermented yeast as an attractant were hung in trees nearby houses, approximately one metre above the ground. Traps were powered using a 12V battery over a period of 11 days from 17:00–07:00. Human landing catches were carried out in Olama Village as previously described[4]. In Yaoundé, BG Sentinel-2 traps containing a BG Sweetscent lure used as an attractant were used to collect mosquitoes for 15 days from 16:30–10:00. Traps were assembled using manufacturers guidelines and positioned nearby potential *Aedes* breeding sites under tree coverage. Larval collections using ladles and sieves were also carried out in Yaoundé in typical urban breeding sites from the following districts: Etoude, Nkolbissim and Briqueterie.

Mosquitoes were collected in the West Region from the Menoua division as previously described[40] and from two locations in the Dschang sub-division in a rural area; Fonakeukeu (05°24′73″ N, 010°04′79″ E) and a peri-urban area; Toutsang (05°25′65″ N, 010°04′05″ E). Collections were carried out in both the rainy season (from March to September 2019) and dry season (from November 2019 to February 2020) using sweep nets. Mosquitoes from Central Cameroon were morphologically identified to genera level, and from West Cameroon

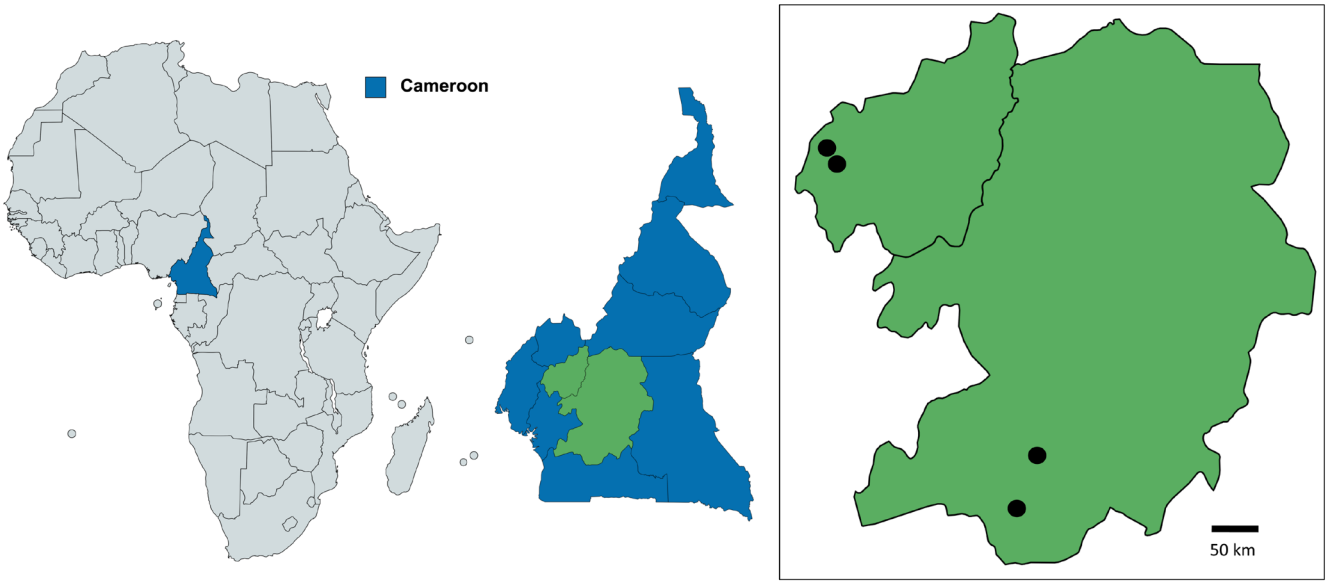

Figure 1. Mosquito collection sites in Cameroon. Collection sites in the Western Province/region were Fonakeukeu (05°24′73″ N, 010°04′79″ E) and Toutsang (05°25′65″ N, 010°04′05″ E). Collection sites in the Central Province/regions included Yaoundé (3°52′22.2″N 11°30′38.0″E) and Olama village (3°24′45.0″N 11°17′03.0″E). Maps were produced using Mapchart licensed under a Creative Commons Attribution-ShareAlike 4.0 International License.

to species level, using morphological identification keys, then preserved in RNAlater (Invitrogen) and kept at below -20°C, prior to being sent for molecular analysis at LSHTM.

RNA extraction and generation of complimentary DNA (cDNA)

From West Cameroon collections, mosquitoes of the same species, from the same location and season, were pooled prior to RNA extraction (650 mosquitoes, 192 pools, pool size range was 3–5 mosquitoes). From Central Cameroon collections, a subsample (n=576) was selected for molecular analysis based on diversity of genera and collection sites. Mosquito RNA was extracted from either pools or individuals using Qiagen 96 RNeasy Kits and a Qiagen Tissue Lyser II (Hilden, Germany) with 5 mm stainless steel beads (Qiagen) to homogenize mosquitoes. Resulting RNA was eluted in 45 µL of RNase-free water and stored at −70°C. cDNA was generated from RNA using an Applied Biosystems High-Capacity cDNA Reverse Transcription kit. Each reaction (20 µL) was made up of the following: 1 µL reverse transcriptase, 2 µL 10X RT buffer, 2 µL 10X random primers, 0.8 µL 25X dNTP (100 mM), 4.2 µL nuclease-free water and 10 µL RNA. A Bio-Rad T100 Thermal Cycler was used to generate cDNA as follows: 25°C for 10 minutes (min), 37°C for 120 min; 85°C for 5 min with all resulting cDNA stored at –20°C.

Wolbachia and *Asaia* detection

The detection of *Wolbachia* strains was initially carried out by amplification of three conserved *Wolbachia* genes; *16S rRNA* gene using primers W-Spec-16S-F: 5'-CATACCTATTCGAAGGGATA-3' and W-Spec-16s-R: 5'-AGCTTCGAGTGAAACCAATTC-3'[41], *Wolbachia* surface protein (*wsp*) gene using primers wsp81F: 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and wsp691R: 5'-AAAAATTAAACGCTACTCCA-3'[42]. A Bio-Rad T100 Thermal Cycler using standard cycling conditions was used to amplify *16S rRNA* and *wsp* gene products which were then separated and visualised using an Invitrogen E-Gel iBase Real-Time Transilluminator with 2% SYBR safe E-Gel EX agarose gels. Real-time PCR reactions for the *16S rRNA* gene were carried out with 5 µL of FastStart SYBR Green Master mix (Roche Diagnostics), primers at a final concentration of 1 µM, 1 µL of PCR grade water and 2 µL cDNA (10 µL final volume/reaction) as previously described using no template controls (NTC) and a limit of detection was previously established using a synthetic oligonucleotide standard through ten-fold serial dilutions[4]. A Roche LightCycler 96 System was used to amplify PCR products using the following cycling conditions: 15 min at 95°C, 40 cycles of 95°C for 15 seconds (sec) and 58°C for 30 sec. *Asaia* detection was also carried out using Real time PCR by amplifying the 16S *rRNA* gene[30] with the same mastermix, reagent concentrations and cycling conditions as for *Wolbachia* genes. PCR assays included a dissociation curve (95°C for 10 sec, 65°C for 60 sec and 97°C for 1 sec) to check that the correct amplicon was being amplified. Fluorescence was quantified using LightCycler 96 software (Roche Diagnostics).

Molecular mosquito species identification

For *Aedes* mosquitoes collected from Central Cameroon sites, a SYBR-green based assay that can distinguish *Ae. aegypti* from *Ae. albopictus* based on the internal transcribed spacer 1 (ITS1)[43] was used. PCR cycling conditions for the ITS1 assay were: 95°C for 15 min, 40 cycles of 95°C for 10 sec, 55°C for 30 sec, 72°C for 20 sec and a dissociation curve (see above). For *Culex* mosquitoes collected from Central Cameroon sites, a multiplex PCR assay targeting the ACE1 gene[44] that can distinguish *Cx. pipiens pipiens* from *Cx. pipiens quinquefasciatus* was also undertaken, given these sibling species are morphologically indistinguishable. *PCR* cycling conditions for the ACE1: 95°C for 10 min, 34 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and 2°C for 5 min. To determine the species for additional samples that were *Wolbachia*-positive, Sanger sequencing and phylogenetic analysis of the cytochrome c oxidase subunit 1 (*COI*) gene[45] was undertaken as this provided the most available sequences for comparison to ensure the optimal method for species identification.

Wolbachia MLST

Five conserved genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA)* were amplified to determine any newly discovered *Wolbachia* strains as previously described with the use of M13 adaptors or degenerate primers[46]. MLST PCRs consisted of 10 µL of Phire Hot Start II PCR Master Mix (Thermo Scientific), primers with a final concentration of 1 µM, 1 µL of PCR-grade water and 2 µL template cDNA (20 µL total). PCR cycling was carried out in a Bio-Rad T100 Thermal Cycler using cycling conditions that were optimised for different MLST genes tested with the Phire Hot Start II PCR Master Mix. Three genes (*gatB*, *hcpA* and *fbpA* genes) had the following cycling: 98°C for 30 sec, 34 cycles of 98°C for 5 sec, 65°C for 5 sec, 72°C for 10 sec, 72°C for 1 min. For two genes (*coxA* and *ftsZ*) cycling was: 98°C for 30 sec, 34 cycles of 98°C for 5 sec, 55°C for 5 sec and 72°C for 30 sec, 72°C for 1 min.

Sanger sequencing

PCR products were deemed worthy of sequencing when producing a strong, clear band at the correct product size when visualised using an Invitrogen E-Gel iBase Real-Time Transilluminator with 2% SYBR safe E-Gel EX agarose gels run for 10 mins. Products were sent to Source BioScience (Nottingham, UK) for cleanup prior to forward and reverse sanger sequencing. The MLST primers used were gene-specific and in the case of MLST genes just the M13 primers (M13_adaptor_F: 5'-TGTAAAACGACGGCCAGT-3' and M13_adaptor_R: 5'-CAGGAAACAGCTATGACC-3') were used if these adaptors were included in the initial PCR to generate the product. MEGAX[47] was used for all analysis of sequences with manual checking of both forward and reverse chromatograms. Editing of sequences included trimming and then alignment to produce consensus sequences was undertaken using ClustalW. Nucleotide BLAST (NCBI) database queries and searches against the *Wolbachia* MLST database were combined to determine if new alleles and strain types were

present in our collection. We also submitted our sequences to GenBank and obtained accession numbers.

Phylogenetic analysis

Alignments were constructed in MEGAX and ClustalW was also used to align our sequences alongside additional sequences obtained from NCBI BLAST and *Wolbachia* MLST database searches. Maximum Likelihood (ML) phylogenetic trees were generated after initially determining the optimal nucleotide substitution model using the "Find Best-Fit Substitution Model (ML)" option within MEGAX. The lowest Bayesian Information Criterion (BIC) score was one of the criteria used and this resulted in two models: the Jukes-Cantor model[48] and the General Time Reversible model[49]. For our phylogenetic analysis, we used the highest log likelihood and included next to the branches the percentage of trees in which the associated taxa clustered together. In all phylogenetic analyses we used a Bootstrap method with 1000 replications and Neighbor-Join and BioNJ algorithms using tMaximum Composite Likelihood (MCL). Our phylogenetic trees were then generated to scale, with branch lengths measured in the number of substitutions per site and we removed all gaps and missing data.

Statistical analysis

Fisher's exact post hoc tests in GraphPad prism version 9 ($P<0.05$ significance threshold) were used to determine any association between prevalence rates of *Wolbachia* and *Asaia* for each mosquito genus from the different regions (West and Central). Samples were categorised as *Wolbachia*-infected, *Asaia*-infected, co-infected or uninfected.

Ethical approval

We previously obtained permission and ethical approval for mosquito sampling[4,40]. Ethical approval for undertaking Human landing catches was obtained from the LSHTM ethics committee (reference no. 16684) in addition to local ethical approval (clearance no. 2016/01/685/CE/CNERSH/SP) delivered by the Cameroon National Ethics (CNE) Committee for Research on Human Health).

Results

Wolbachia and *Asaia* prevalence rates

We compared the prevalence rates of *Wolbachia* and *Asaia* rates using the *16S rRNA gene* from the three major Culicine genera collected from both the West and Central Regions, with the caveat that the West Region samples were monospecific pools from the same species at the same location (Figure 2, Table 1). In the Central Region, 97.96% (n=115) of *Aedes* genera mosquitoes were infected only with *Wolbachia*, and *Asaia* was only detected in a single mosquito as a coinfection. In contrast, the majority of *Culex* genera mosquitoes collected from the Central Region were uninfected (85.83%, n=103), with *Asaia* detected in 13.45% (n=16) of individuals and only a single individual infected with *Wolbachia*. A similar infection prevalence was observed in *Mansonia* collected from the

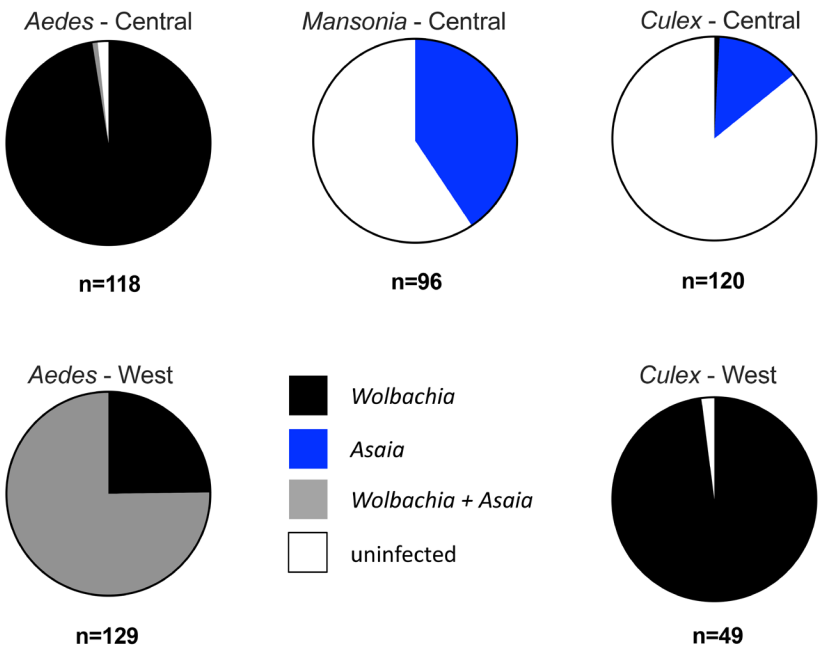

Figure 2. *Wolbachia*, *Asaia* and co-infection prevalence rates from major Culicine mosquito genera collected from the West and Central regions of Cameroon. Mosquitoes from the Central region were individually extracted and analysed. Mosquitoes from the West region were extracted from monospecific pools (same species from same collection location) and prevalence analysis represents pooled samples. No *Mansonia* mosquitoes were collected from the West region.

Table 1. Prevalence rates of *Wolbachia* (*16S rRNA* gene), *Asaia* and coinfection from mosquito genera collected from the West and Central regions of Cameroon. Mosquitoes from the Central region were individually extracted and analysed. Mosquitoes from the West region were extracted from monospecific pools (same species from same collection location) and prevalence analysis represents pooled samples.

Region	Genera	*Wolbachia* (%)	*Asaia* (%)	Co-infected (%)	Uninfected (%)	Totals	Fisher's P-value
West	*Aedes*	32 (24.81)	0 (0.00)	97 (75.19)	0 (0.00)	129	>0.99
West	*Culex*	48 (97.96)	0 (0.00)	0 (0.00)	1 (2.04)	49	>0.99
Central	*Aedes*	115 (97.46)	0 (0.00)	1 (0.85)	2 (1.69)	118	>0.99
Central	*Culex*	1 (0.83)	16 (13.45)	0 (0.00)	103 (85.83)	120	>0.99
Central	*Mansonia*	0 (0.00)	39 (40.63)	0 (0.00)	57 (59.38)	96	>0.99

Central Region, but a higher prevalence of *Asaia* was detected (40.63%, n=39) and there was no *Wolbachia* detected (59.38%, 59 individuals were uninfected for both bacterial species). In the West Region, *Aedes* mosquitoes were either co-infected (75.19%, n=97 pools) or singly infected with *Wolbachia* (24.81%, n=32 pools). For *Culex* genera mosquitoes, the vast majority (97.96%, n=48 pools) were infected with *Wolbachia* only. Results of Fisher's exact *post hoc* tests (P<0.05 significance threshold) indicated no significant associations were present in our data. As *Aedes* collections in the West Region were heavily dominated by *Ae. africanus* - a vector of YFV in forest and rainforest regions in Sub-Saharan Africa[50], we compared *Wolbachia* and *Asaia* prevalence rates for pooled mono-specific RNA pools (n=97) to look for any potential co-infections within this species. *Wolbachia* was detected in 100.00% of pools (97/97) and a high *Asaia* prevalence rate of 96.91% (94/97) pools was also seen, demonstrating a high likelihood of co-infections occurring in this species. However, as mono-specific pools consisting of an average of five female mosquitoes were used for analysis no statistical association analysis can be carried out.

Confirmation of *Wolbachia* prevalence rates through amplification of the *wsp* gene

Our preliminary assessment of *Wolbachia* prevalence rates was generated from *16S rRNA* gene amplification. However, using the *16S rRNA* gene alone is insufficient because it can also be possible that prokaryotic *16S rRNA* genes can be amplified from dead bacterial cells[51] and *Wolbachia 16S rRNA* sequences has previously been detected in water containers that contained larvae of mosquitoes[52]. We undertook further *wsp* gene amplification on a wide variety of samples collected from the West Region in which morphological identification was done to species level and individuals, of the same species were pooled into groups of up to five individuals prior to RNA extraction (monospecific pools). Screening with the *wsp* gene revealed variable estimates of *Wolbachia* prevalence rates (Table 2). Of particular interest was the high prevalence in the most abundant species *Ae. africanus* collected in both locations in West Cameroon. A total of 341 *Ae. africanus* females from Fonakeukeu were grouped into 72 pools and 65/72 pools (90.3%) were *Wolbachia*-positive based on strong amplification of the *wsp* gene. Similarly, 34/46 pools (73.9%) of the pools, representing a total of 228 *Ae. africanus* females from Toutsang amplified the *wsp* gene. Variable prevalence rates were also seen in other morphologically identified species, including *wsp* amplification in species within the *Culex*, *Aedes*, *Mansonia*, *Uranotaenia* and *Eretmapodites* genera.

Molecular species identification of selected *Wolbachia*-infected mosquito samples

After using the *16S rRNA* and *wsp* genes to provide a preliminary indication of infection status, *COI* gene barcoding[45] was done to molecularly identify the species of a sub-sample of mosquitoes, given the difficulties associated with morphological identification of less well-studied species (Table 3–Table 4, Figure 3). Our results showed that within these 13 sub-selected *Wolbachia*-infected samples, there were seven *Culex* species, three *Aedes* species and one species each of five additional genera, confirmed to species level using Sanger sequencing of the *COI* gene: *Cx. quinquefasciatus*, *Cx. watti*, *Cx. cinereus, Cx. nigripalpus, Cx. perexiguus, Cx. rima*, *Cx. cinctellus, Ae. africanus, Ae. denderensis, Ma. uniformis, Ca. argenteopunctata, Lu. tigripes, Er. chrysogaster* and *Ur. bilineata* (Table 2). To differentiate between sibling species within the *Cx. pipiens* complex, we amplified the *ACE1* gene and gel electrophoresis indicated a band size of 274 base pairs, which is diagnostic for *Cx. quinquefasciatus*. To avoid potentially mis-labelling species without sufficient sequence similarity, samples with species identity below 94% were designated '*cf*' as this would be more indicative of a species that is closely related. However, the lack of sequences available for many of these species could result in genetic variation within the same species accounting for lower-than-expected sequence similarities.

Wolbachia genetic diversity and MLST gene allelic profiling

We used *16S rRNA* phylogeny to put the strains detected in this study into context with existing strains (Figure 4). Our results showed eight strains are clustering closely together. In addition, there is sequence diversity among strains found infecting *Ae. africanus* (samples 8–10). An in-depth analysis was undertaken through MLST gene allelic profiling (Table 5) from

Table 2. *Wolbachia* infection prevalence using the *wsp* gene. Based on morphological identification to genera/species and *wsp* gene amplificaiton in mosquitoes collected from the West Region of Cameroon.

Collection site	*Genera*	Species(number)	*wsp*+/totalpools (%)
Fonakeukeu	*Aedes*	*africanus (341)*	65/72 (90.3%)
Fonakeukeu	*Aedes*	*argenteopunctatus (1)*	1/1 (100%)
Fonakeukeu	*Aedes*	*tarsalis (3)*	1/3 (33.3%)
Fonakeukeu	*Culex*	*unknown (5)*	0/5 (0.0%)
Fonakeukeu	*Culex*	*moucheti (25)*	5/8 (62.5%)
Fonakeukeu	*Culex*	*ornathotoracis (4)*	0/4 (0.0%)
Fonakeukeu	*Culex*	*tigripes (2)*	1/1 (100%)
Fonakeukeu	*Culex*	*univitattus (4)*	1/3 (33.3%)
Fonakeukeu	*Culex*	*wigglesworthi (7)*	1/3 (33.3%)
Fonakeukeu	*Mansonia*	*maculipennis (1)*	0/1 (0.0%)
Fonakeukeu	*Mansonia*	*annetii (1)*	0/1 (0.0%)
Fonakeukeu	*Eretmapodites*	*chrysogaster var (2)*	1/3 (33.3%)
Fonakeukeu	*Uranotaenia*	*billineata connali (6)*	2/4 (50%)
Toutsang	*Aedes*	*africanus (228)*	34/46 (73.9%)
Toutsang	*Aedes*	*tarsalis (2)*	0/1 (0.0%)
Toutsang	*Aedes*	*unknown (1)*	1/1 (100%)
Toutsang	*Aedes*	*circumluteolus (1)*	1/1 (100%)
Toutsang	*Aedes*	*fraseri (1)*	1/1 (100%)
Toutsang	*Aedes*	*gibbinsi (3)*	1/1 (100%)
Toutsang	*Culex*	*unknown (14)*	2/5 (40.0%)
Toutsang	*Culex*	*moucheti (28)*	2/5 (40.0%)
Toutsang	*Culex*	*tigripes (7)*	0/4 (0.0%)
Toutsang	*Culex*	*univitattus (7)*	1/2 (50.0%)
Toutsang	*Culex*	*duttoni (4)*	1/3 (33.3%)
Toutsang	*Mansonia*	*maculipennis (2)*	0/1 (0.0%)
Toutsang	*Mansonia*	*annetii (1)*	0/1 (0.0%)
Toutsang	*Eretmapodites*	*chrysogaster var (4)*	2/3 (66.7%)

representatives of each mosquito species from *wsp*-positive individuals (Central Region) or monospecific pools (West Region) after species identification was confirmed. Complete MLST sequences are present for *Cx. quinquefasciatus, Cx. watti, Ae. africanus* (Sample 8) and *Ca. argenteopunctata.* The remaining samples had sequences of sufficient quality from 2–4 genes. For example, we were only able to obtain MLST gene sequences for two genes for *Ma. uniformis (gatB* and *coxA*). All sequences of sufficient quality were submitted to Genbank to obtain accession numbers Table 6.

As expected, the MLST allelic profile for *Cx. quinquefasciatus* mostly matched with strain type (ST) 9 for the *w*Pip strain which infects *Cx. pipiens* and *Cx. quinquefasciatus,* although our sample had a match to *ftsZ* allele number 241, whereas existing profiles for ST 9 had an *ftsZ* allele number of 22. However, this represents only four nucleotide differences across the 435 base pairs for *ftsZ* alleles 241 and 22 resulting in 99.1% sequence identity. In *Cx. watti*, the *Wolbachia* strain allelic profile is most similar to Supergroup B *Wolbachia* strains found in the *Coenonympha hero* (the scarce heath

Table 3. *CO1* gene sanger sequencing for molecular confirmation of mosquito species. The NCBI BLAST percentage (%) identity and coverage are shown alongside the closest NBCI accession number (no.) and associated species. For identity 94% and under '*cf*' has been added given the uncertainty of species identification.

Sample	Collection site	identity (%)	coverage (%)	NCBI accession number	Species
S1	Yaoundé	99	99	MK300247.1	*Cx. quinquefasciatus*
S2	Olama village	98	91	KU187063.1	*Cx. watti*
S3	Toutsang	97	100	LC473616.1	*Cx. cinereus*
S4	Toutsang	94	100	MT999280.1	*Cx. cf nigripalpus*
S5	Fonakeukeu	98	99	KU380382.1	*Cx. perexiguus*
S6	Fonakeukeu	94	99	LC473614.1	*Cx. cf rima*
S7	Olama	95	89	AB738190.1	*Cx. cinctellus*
S8	Toutsang	95	100	GQ165786.1	*Ae. africanus*
S9	Toutsang	94	100	GQ165786.1	*Ae. cf. africanus*
S10	Toutsang	94	100	GQ165786.1	*Ae. cf. africanus*
S11	Fonakeukeu	99	97	GQ165787.1	*Ae. denderensis*
S12	Olama village	99	93	KU380420.1	*Ma. uniformis*
S13	Fonakeukeu	94	100	MN552301.1	*Ca. cf. argenteopunctata*
S14	Toutsang	100	99	LC507833.1	*Lu. tigripes*
S15	Toutsang	90	99	MK533645.1	*Er. cf. chrysogaster*
S16	Toutsang	99	99	LC473729.1	*Ur. bilineata*

Table 4. *CO1* and *Wolbachia 16S rRNA* GenBank accession numbers. Location, species and sample code are shown alongside Genbank accession numbers. Sample sequences without accession numbers were of insufficient quality to obtain GenBank accession numbers.

Location	Sample ID	Species	*CO1* accession number	*16S rRNA* accession number
Yaoundé	S1	*Cx. quinquefasciatus*		OP745953
Olama village	S2	*Cx. watti*	OP744462	
Toutsang	S3	*Cx. cinereus*	OP744463	
Toutsang	S4	*Cx. cf nigripalpus*	OP744465	OP746031
Fonakeukeu	S5	*Cx. perexiguus*	OP744466	OP746061
Fonakeukeu	S6	*Cx. cf rima*	OP744493	OP746056
Olama	S7	*Cx. cinctellus*		OP746069
Toutsang	S8	*Ae. africanus*	OP744519	OP746071
Toutsang	S9	*Ae. cf. africanus*	OP744523	OP747286
Toutsang	S10	*Ae. cf. africanus*		OP750996
Fonakeukeu	S11	*Ae. denderensis*	OP744531	OP747294
Olama village	S12	*Ma. uniformis*	OP744580	OP747304
Fonakeukeu	S13	*Ca. cf. argenteopunctata*	OP744988	OP747416
Toutsang	S14	*Lu. tigripes*	OP745009	OP747419
Toutsang	S15	*Er. cf. chrysogaster*	OP745018	OP747455
Toutsang	S16	*Ur. bilineata*	OP745056	OP747456

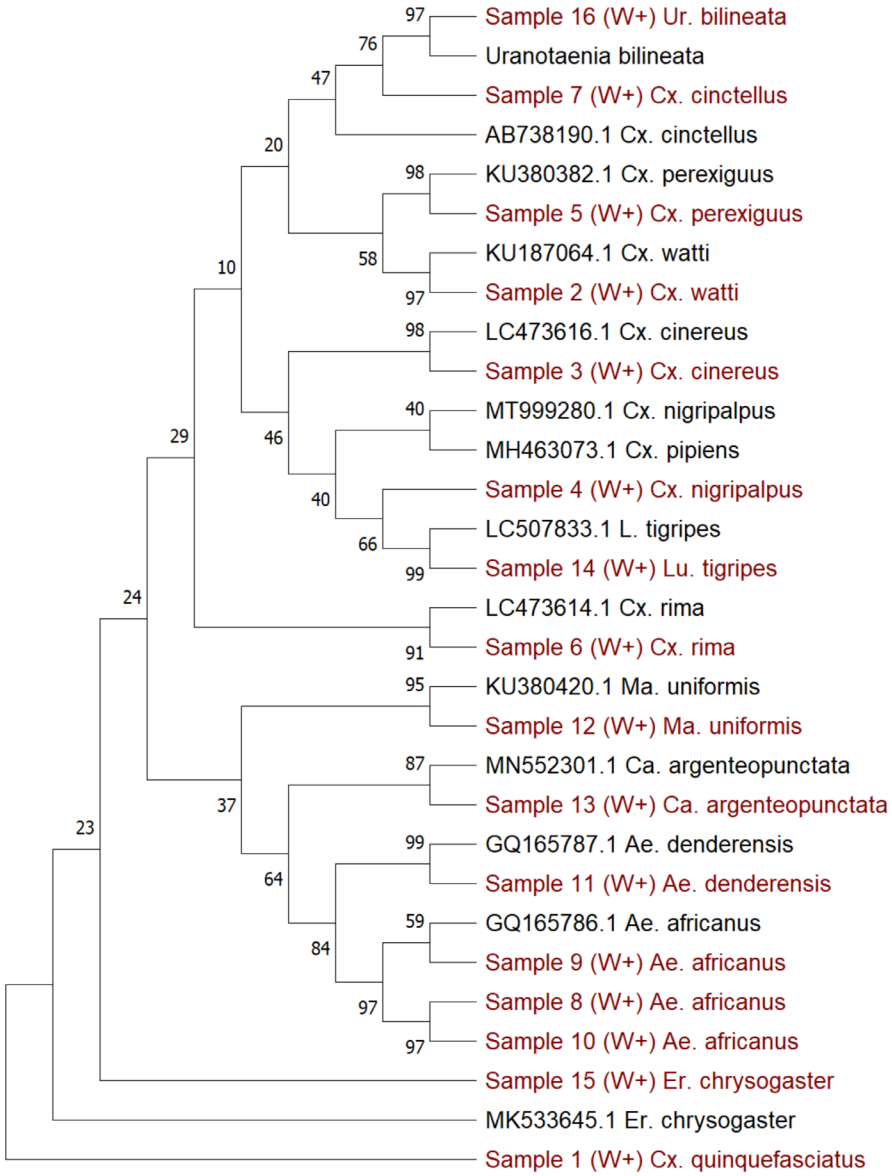

Figure 3. *CO1* gene phylogenetic analysis of mosquito species collected from Cameroon. Maximum Likelihood phylogenetic analysis using the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6443)). The tree comprises 30 nucleotide sequences with 725 positions in the dataset. Drawn to a 0.05 scale.

butterfly) – (ST 296), the moth *Amblyptilia punctidactyla*, *Coenonympha pamphilus* (the small heath butterfly), the Fischer's butterfly *Tongeia fischeri* (ST 300) and the thrip *Pezothrips kellyanus* (ST 430). However, only *gatB* (allele 9) and *ftsZ* (allele 7) show exact matches to these three strain types with *hcpA* and *fbpA* alleles being variable both for the novel strain in *Cx. watti* and STs 296, 300 and 430. The allelic profile of the *Wolbachia* strain detected in *Cx. cinerus* was most similar to 11 different strain types (3,108,151,213,366, 382,387,461,462,472,492) but appears a novel strain as none of these typed strains contain the combination of the four alleles identified in *Cx. cinerus* (we were unable to sequence the *ftsZ* gene). For example, ST 108 has the same gatB allele (71) but the remaining three genes for comparison (*coxA*, *hcpA* and *fbpA*) have variable allele numbers which are not the same as those sequenced from the *Wolbachia* strain in *Cx. cinerus.*

The *Wolbachia* strain detected in *Cx. cf. nigripalpus* produced an exact match to strain type 13 which is found in numerous insect species, including *Drosophila recens* and *Leucophenga maculosa* fruit flies *and Rhagoletis cerasi (*cherry fruit fly*)*. The widespread occurrence of this *Wolbachia* strain type across multiple insect genera requires further investigation given 19 isolates are present in the database. We were only able

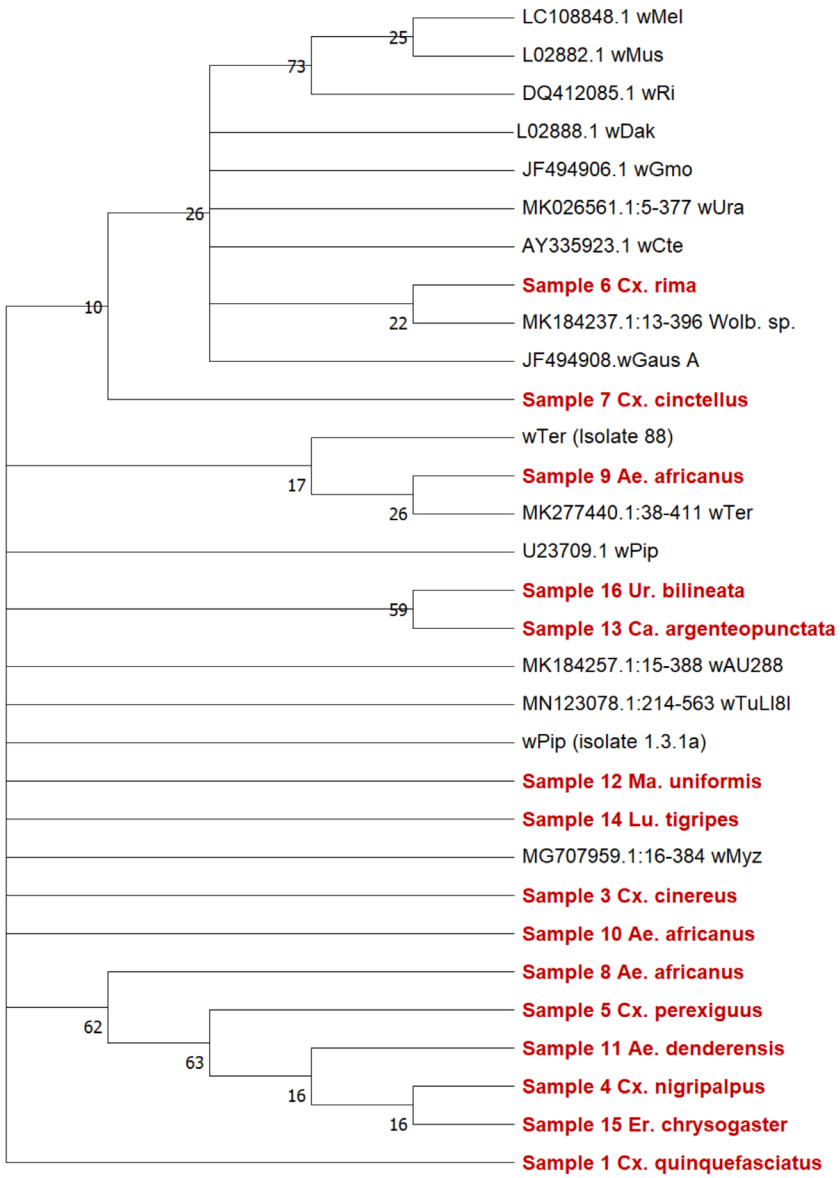

Figure 4. *16S rRNA* gene phylogenetic analysis of *Wolbachia* strains. The tree was produced using the Maximum Likelihood method and Jukes-Cantor model. The tree contains 31 nucleotide sequences and 1610 positions in the dataset. *Wolbachia* strains detected in this study are highlighted in red, sequences from additional strains obtained from Genbank with accession numbers are shown in black. Drawn to a 0.05 scale.

to sequence three genes for the *Wolbachia* strain detected in *Cx. perexiguus* (*coxA*, *fbpA* and *hcpA*) resulting in three nearest strain types (108, 187,467) of which none had allele number 11 for *coxA*. However, a complete MLST profile would help confirm what appears to be a novel strain in *Cx. perexiguus* given its unique combination of three sequenced MLST genes. Likewise, the *Wolbachia* strain detected in *Cx. rima* would appear novel given only two of four allelic loci (*gatB* and *coxA*) matched the closest ST (52) previously reported in *Anoplolepis gracillipes* (the yellow crazy ant).

Given that we collected large numbers of *Ae. africanus* from the West Region of Cameroon, we included three monospecific pools (A-C) for MLST allelic profiling (Table 5–Table 6). Our results provide evidence for multiple *Wolbachia* strains through analysis indicating allelic matches for four of five MLST genes (and divergence seen in the *16S rRNA* phylogeny between samples 8–10). Our Sanger sequencing indicated no evidence for the presence of multiple strains within the same pool, but further analysis is required to further determine if superinfections can be present within individual

Table 5. MLST gene allelic profiling. Assigned allele numbers matching those present in the *Wolbachia* MLST database (https://pubmlst.org/organisms/wolbachia-spp/) indicating nucleotide differences in brackets.

Sample ID	Mosquito species	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*
S1	*Cx. quinquefasciatus*	4	3	3 (1)	241	4
S2	*Cx. watti*	9	14 (2)	12	7	203 (1)
S3	*Cx. cinereus*	71	11	303	-	90
S4	*Cx. cf nigripalpus*	1	1	1	3	1
S5	*Cx. perexiguus*	-	11	74	-	6
S6	*Cx. cf rima*	22	2	74	258	-
S7	*Cx. cinctellus*	71	11	303	-	90
S8	*Ae. africanus*	9	8	74	106	6
S9	*Ae. cf. africanus*	9	8	74	117	203
S10	*Ae. cf. africanus*	9	8	12	117	203
S11	*Ae. denderensis*	9	11	74	106	6
S12	*Ma. uniformis*	9	14	-	-	43 (12)
S13	*Ca. cf. argenteopunctata*	9	11	303	7	446
S14	*Lu. tigripes*	71	-	74	117	6
S15	*Er. cf. chrysogaster*	-	275	-	106	6
S16	*Ur. bilineata*	9	11	74	-	90

Table 6. GenBank accession numbers for MLST gene sequences. Sample sequences without accession numbers were of insufficient quality to obtain GenBank accession numbers.

Sample ID	Mosquito species	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*
S1	*Cx. quinquefasciatus*	OQ236162	OQ236174	OQ236185	OQ236197	OQ236208
S2	*Cx. watti*	OQ236163	OQ236175	OQ236186	OQ236198	OQ236209
S3	*Cx. cinereus*	OQ236164	OQ236176	OQ236187		OQ236210
S4	*Cx. cf nigripalpus*	OQ236165		OQ236188	OQ236199	
S5	*Cx. perexiguus*		OQ236177	OQ236189		
S6	*Cx. cf rima*	OQ236166	OQ236178	OQ236190	OQ236200	
S8	*Cx. cinctellus*	OQ236167	OQ236179	OQ236191	OQ236201	
S9	*Ae. africanus*	OQ236168		OQ236192	OQ236202	OQ236211
S10	*Ae. cf. africanus*	OQ236169	OQ236180	OQ236193	OQ236203	OQ236212
S11	*Ae. cf. africanus*				OQ236204	OQ236213
S12	*Ae. denderensis*	OQ236170	OQ236181			
S13	*Ma. uniformis*	OQ236171	OQ236182	OQ236194	OQ236205	OQ236214
S14	*Ca. cf. argenteopunctata*	OQ236172		OQ236195	OQ236206	
S15	*Lu. tigripes*		OQ236183		OQ236207	OQ236215
S16	*Er. cf. chrysogaster*	OQ236173	OQ236184	OQ236196		OQ236216

Ae. africanus. A novel strain was detected in *Ae. denderensis* as only three loci *(gatB, hcpA* and *fbpA)* matched ST 467 of a *Wolbachia* strain found in *Cabera pusaria* (Common white wave moth). A complete MLST profile was generated for the *Wolbachia* strain in *Ca. argenteopunctata* which appears novel given only two loci in combination match existing strains. For *Lutzia tigripes*, we could only produce sequences for three MLST genes, but this strain also appears novel with only three of four loci matching ST 108 – a strain found in the butterfly *Brangas felderi*. Likewise, novel strains appear to be present in both *Eretmapodites chrysogaster* and *Uranotaenia bilineata* as their partial allelic profiles did not match any other strain types in the database.

Discussion

In Cameroon, we previously showed that the richness of mosquito species was dependent on both habitat type and seasonality[40]. Therefore, in this current study we analysed mosquitoes from diverse environmental settings to capture as much potential diversity in both mosquito species and corresponding resident *Wolbachia* strains. We identified what appears to be either novel strains or variants of existing characterised *Wolbachia* strains in 13 different mosquito species. A natural *Wolbachia* strain in *Cx. quinquefasciatus* mostly matching ST 9 is to be expected given the *w*Pip strain is widespread in species of the *Cx. pipiens* complex[53–55]. Our allelic profiling indicated evidence of some genetic diversity in the *ftsZ* allele although this was only four of 435 nucleotides (99.1% sequence similarity). This also highlights the requirement of using MLST allelic profiling given the *16S* analysis provided little sequence similarity to existing sequences from *Wolbachia* strains detected in the *Cx. pipiens* complex (Figure 4). Interestingly, there was no evidence of *Wolbachia* in *Cx. pipiens* collected from Madagascar[6] despite the prevailing assumption that the *w*Pip strain widely infects both *Cx. pipiens* and *Cx. quinquefasciatus* populations. Further studies across sub-Saharan Africa are needed to determine variability in both the prevalence rates and genetic strain diversity of the *w*Pip strain in members of the *Cx. pipiens* complex, given their important role as vectors of multiple human pathogens such as West Nile virus and filarial nematodes such as *Wuchereria bancrofti*.

Another vector of human pathogens analysed in our study was *Ae. africanus* – a major vector of YFV. Although *Ae. africanus* is considered a sylvatic vector in rural areas, recent studies have suggested it has the capacity to colonise peri-domestic and domestic habitats[50]. Our MLST analysis suggests there are multiple *Wolbachia* strain variants present in *Ae. africanus* with variation in gene sequences in three of the five MLST genes (Table 5). It could also be possible that resident *Wolbachia* strain superinfections occur in *Ae. africanus* as have been seen in *Ae. albopictus*[56]. *Mansonia uniformis* has been shown to transmit numerous arboviruses, such as Murray Valley encephalitis and Ross River virus, and has been shown to be a vector of Bancroftian lymphatic filariasis in Ghana[57]. Interestingly, although *Wolbachia* has been previously identified in this species, no complete allelic profile is present. Our results match *gatB* and *coxA* allelic loci from *Ma. uniformis* collected in Kenya[8] but not *fbpA*, suggesting the possibility of a different strain variant present in populations from Cameroon. The remaining novel *Wolbachia* strains that we have identified were in mosquito species that are considered either minor vectors of human disease or implicated in transmission of WNV, such as *Cx. perexiguus*, *Cx. watti*, and *Cx. rima*. With the exception of *Cx. quinquefasciatus* and *Ma. uniformis*, no *Wolbachia* MLST sequences are available (pubmlst.org/organisms/wolbachia-spp) for the remaining mosquito species.

Although we undertook molecular barcoding by sequencing the mosquito *CO1* gene to try and provide as much confidence in species identification, caution must be taken with any results as this is dependent on the availability of sequences for comparison. For example, we identified *Wolbachia* strains in multiple individuals in which sequence identity was only 94%, suggesting these may be closely related species to the closest match sequence available on GenBank. Sample 7 was identified as *Cx. cinctellus* but with only 95% identity and 89% coverage, indicating this could also be another closely-related species. The inability to accurately identify mosquito species using molecular barcoding is common for species in which few sequences have previously been made available in databases such as GenBank. However, providing the *CO1* sequences will inform future studies looking at closely related species.

Our results comparing *Wolbachia* and *Asaia* prevalence rates across major Culicine genera indicated a significant association only in *Mansonia* mosquitoes. These results for *Mansonia* are consistent with other mosquito species such as *Ae. koreicus* in which studies from field collected mosquitoes indicate a mutual exclusion between these two symbionts[58]. In contrast, high levels of co-infections (particularly within *Ae. africanus*, which dominated our collections from the West Region) add to growing evidence that *Wolbachia* and *Asaia* can co-exist in wild mosquitoes[3] despite studies clearly demonstrating an antagonistic association in lab colonies[27,28]. As *Asaia* can be acquired from the environment throughout the mosquito life cycle, the collection location becomes a significant factor that complicates this tripartite association and therefore our results are limited to both our collection locations and species collected. Another major limitation of our study is that we were unable to provide comparative data to the species level for the Central region due to the high levels of misidentification of Culicline species[59] and missing or damaged morphological features during mosquito collections. Furthermore, as tissue-specific detection was not feasible for the large number of diverse field-collected mosquitoes in our study, it could be possible that *Wolbachia* and *Asaia* co-exist within a given individual mosquito but are located in different tissues[60]. Likewise, the detection of both *Wolbachia* and *Asaia* in samples from the West region (monospecific pools) needs further investigation given the limitations of using pooled samples. The possibility of morphological misidentification resulting in the addition of an 'incorrect' species to the pool or results reflecting single infections (ie. one *Wolbachia*-infected individual, one *Asaia*-infected

individual) cannot be ruled out. However, this would seem unlikely given *Aedes* monospecific pools mosquitoes were either co-infected (75%) or singly infected with *Wolbachia* (25%) and for *Culex* 98% were infected with *Wolbachia* only. Larger cohort collections of mosquitoes from diverse environmental settings will provide further insight into how these two widespread bacteria co-exist (or do not co-exist) in different mosquito species.

Wolbachia MLST gene allelic profiling was undertaken to provide more assurances on detection of genuine endosymbiotic strains found in wild mosquito populations. We defined a 'novel' strain based on MLST to contain either new MLST gene sequences not present in https://pubmlst.org/organisms/wolbachia-spp or a combination of MLST gene sequences that does match an existing strain in the database. Despite being widely used, MLST For *Wolbachia* strains has limitations and the five genes may not represent the optimal loci to capture strain variation[61]. Furthermore, defining whether a novel strain exists based only on PCR amplification of genes is problematic given the numerous examples of environmental contamination or host genome integration[51,52]. Caution must be taken when extrapolating PCR amplification to indicate the presence of a living endosymbiotic bacterium – particularly so when only a few gene targets such as *16S rRNA* are amplified and sequenced. It has been shown that *16S rRNA* prokaryotic DNA can be amplified from dead cells[51] and *Wolbachia 16S rRNA* can be detected just from water that previously contained mosquito larvae[52]. If possible, extraction of mosquito RNA (as carried out in this study) to confirm expression of *Wolbachia* genes provides further evidence[3]. Once novel strains are detected using MLST profiling, further studies are needed to confirm a genuine stable endosymbiotic association is present with the mosquito host species. This is important when low prevalence rates are detected given this may otherwise suggest that the *Wolbachia* strain is not inducing CI. Furthermore, there are several additional experiments that can be undertaken to provide further confirmation of resident *Wolbachia* strains in mosquitoes. Methods that can visualise *Wolbachia* bacteria in mosquito tissues using microscopy, such as fluorescent *in situ* hybridization, and *Wolbachia* genome sequencing, to compare genome depth and coverage of novel strains to those of other known infections, should be carried out to fully characterise novel *Wolbachia* strains.

Conclusions

Novel *Wolbachia* strains in Culicine mosquitoes collected from ecologically diverse settings in Cameroon add to the diversity of this highly prevalent endosymbiont in insect populations. Resident *Wolbachia* strains should be further characterised to determine the tissue tropism and density of newly discovered strains. Our study also suggests that co-infection with environmentally acquired *Asaia* bacteria is widespread in wild mosquito populations (except the *Mansonia* genera) and the antagonistic relationship observed in lab colonies may not be present in some wild Culicine populations. Novel *Wolbachia* strains could be considered as candidate strains for biocontrol strategies given their ability to reside naturally within existing mosquito populations and co-exist with environmentally acquired *Asaia* bacteria.

Data availability

Underlying data

GenBanK: Wolbachia endosymbiont of Culex pipiens isolate 1 16S ribosomal RNA gene, partial sequence. Accession number OP745953; https://identifiers.org/insdc:OP745953[62]

GenBanK: Culex watti isolate 1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial. Accession number OP744462; https://identifiers.org/insdc:OP744462[63]

Additional *CO1, Wolbachia 16S* gene GenBank accession numbers are listed in Table 4;

GenBank: Wolbachia pipientis isolate S1 glutamyl-tRNA(Gln)amidotransferase subunit B (gatB) mRNA, partial cds. Accession number OQ236162; https://identifiers.org/insdc:OQ236162[64]

GenBank: Wolbachia pipientis isolate S1 cytochrome c oxidase subunit I (coxA) mRNA, partial cds. Accession number OQ236174; https://identifiers.org/insdc: OQ236174[65]

Additional *Wolbachia* multi-locus sequence typing genes GenBank accession numbers are listed in Table 6.

Extended data

Open Science Framework: Diverse Novel Wolbachia strains in Culicine mosquitoes from ecologically diverse regions of Cameroon, https://doi.org/10.17605/OSF.IO/V75DU

This project contains the raw PCR screening data.

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Open Peer Review

Current Peer Review Status: ✔ ✔ ✔

Version 2

Reviewer Report 10 October 2023

https://doi.org/10.21956/wellcomeopenres.22165.r67662

✔ **Eric P. Caragata**
Florida Medical Entomology Laboratory, Department of Entomology and Nematology, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida, USA

All of my previous comments have been addressed to my satisfaction. The only other note I want to give is that it could be beneficial to explicitly delineate methodological differences in the handling of Western and Central region mosquitoes in the methods section.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mosquito microbiology, molecular biology, immunity, and metabolism. Wolbachia. Mosquito-microbe-pathogen interactions.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 06 October 2023

https://doi.org/10.21956/wellcomeopenres.22165.r67661

✔ **Guido Favia**
School of Bioscience and Veterinary Medicine, University of Camerino, Camerino, Italy

I have read the revised version of the manuscript and in my opinion, the authors improved the its quality well addressing the concerns raised at the first evaluation. Consequently the manuscript is worthy for indexing.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 03 October 2023

https://doi.org/10.21956/wellcomeopenres.22165.r67660

Perran Ross
The University of Melbourne, Melbourne, Victoria, Australia

The authors have substantially revised the manuscript and all issues raised have been sufficiently addressed. I have no further suggestions.

Competing Interests: I am currently collaborating on a project with a lab from the Liverpool School of Tropical Medicine of which a coauthor of this paper is currently a member. The project is unrelated to the current manuscript and I have not worked with any of the other authors, so I don't believe this will affect the objectivity of my review.

Reviewer Expertise: Wolbachia infections in Aedes mosquitoes and their use in control programs

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 01 August 2023

https://doi.org/10.21956/wellcomeopenres.20603.r61513

Perran Ross
The University of Melbourne, Melbourne, Victoria, Australia

General comments

Wolbachia endosymbionts are prevalent in insect populations and can enact dramatic effects on their hosts. Understanding the prevalence and diversity of Wolbachia in natural mosquito populations has important implications for the control of mosquito-borne disease because Wolbachia infections can influence mosquito vectorial capacity and modulate the success of vector control programs. Other microbes such as Asaia can also reside in mosquito tissues where they can influence host phenotypes and interact with Wolbachia. Here, the authors performed field surveys of Culicine mosquitoes in two regions of Cameroon and screen either individuals or pools of mosquitoes for Wolbachia and Asaia using PCR detection and sequencing of 16S rRNA, wsp and MLST markers. They detect Wolbachia and Asaia at variable frequencies which depend on the mosquito genus and region. While these results provide important data for vector control programs and may lead to candidate strains for Wolbachia release programs, there are a few key limitations with their study design and their conclusions are overstated.

The authors claim to detect co-infections of Wolbachia and Asaia in field-collected mosquitoes, contradicting evidence from laboratory studies that the two microbes have an antagonistic relationship with each other. However, these two observations are not necessarily in conflict. While there may be mutual exclusion of Asaia and Wolbachia in the reproductive tissues of some mosquitoes, this does not preclude the two microbes from residing in the same mosquito but within different tissues. There are several cases where Wolbachia and Asaia have been detected in the same mosquito (e.g. Schrieke et al. 2022 Computational and Structural Biotechnology [1], Hegde et al. 2018 Frontiers in Microbiology [2], da Silva et al. 2022 genes [3], Chen et al. 2020 Frontiers in Microbiology [4]) but they may not be occupying the same tissues, especially given that Asaia can be environmentally acquired and often resides in the gut. This study also does not provide sufficient evidence for a Wolbachia-Asaia coinfection. Firstly, all samples where both Wolbachia and Asaia were detected are from pools of mosquitoes, so it is possible that these samples contain a mix of singly infected individuals. Secondly, molecular detection is not sufficient evidence of an infection as pointed out by previous studies (Chrostek and Gerth 2019 mBio [5], Ross et al. 2020 Ecol Evol [6]). This is acknowledged at the end of the discussion but the rest of the paper is written as if all these detections represent true infections.

There are also some limitations and apparent gaps/inconsistencies in their study design. Mosquitoes from the West region were pooled while mosquitoes from the Central region were tested individually, but mosquitoes were only identified to the species level in the West region. The use of pools is likely to overestimate the prevalence of Wolbachia and Asaia in a sample given that not all individuals need to test positive for the pool to be considered positive. There is also the risk that any errors with morphological species identification could lead to an incorrect assignment of Wolbachia or Asaia status of a pool if one species is positive and the other is negative. The authors also use a mix of 16S rRNA and wsp detection for Wolbachia, but the data for 16S are combined across species within a genus while the wsp data are only presented for individual species. Furthermore, the results for 16S include only Aedes, Mansonia and Culex while there are other genera included for the wsp marker. The justification for this is unclear, and it would be useful to know how concordant the two markers are with each other, especially when the authors note that the use of the 16S rRNA gene has limitations for Wolbachia detection. The authors also mention in the methods that they also used the FtsZ marker for initial Wolbachia detection but these results are not presented.

The authors then select a sample of mosquito species testing positive for Wolbachia for further

analysis of the Wolbachia strains with 16S rRNA and MLST markers. The authors place an emphasis on the fact that the Wolbachia strains that they detect are novel and diverse but these terms are not well defined. Has Wolbachia been detected in any of these species besides Cx. quinquefasciatus before, and how different do Wolbachia sequences have to be before they are considered novel and diverse? The authors compared their sequences to the MLST database but this may not represent the full diversity of Wolbachia strains that have been identified given that Wolbachia sequences have been obtained through other approaches (e.g. Scholz et al. Nature Communications). There are also some issues with the use of the MLST system for strain typing and diversity as outlined by Bleidorn and Gerth 2018 (FEMS Microbiol Ecol) [7] and these should be acknowledged.

Specific comments

Title – The title is misleading – the authors have detected Wolbachia and Asaia sequences in a range of field-collected mosquito species, but there is insufficient evidence that these represent true infections. The terms “diverse” and “novel” are not well defined and it is unclear how many of these strains have been detected for the first time.

Introduction – the authors mention that identifying Wolbachia strains can provide important comparative data but they don’t elaborate on how this information would be useful for biocontrol strategies. For instance, natural infections could interfere with releases of transinfections because they may change patterns of cytoplasmic incompatibility between the released and resident mosquitoes. The authors also note that novel strains could be considered candidates for biocontrol strategies, but what properties are you looking for in a strain for it to be considered a candidate, and what steps would need to be taken for the strain to be used (e.g. introgression or microinjection into a different mosquito line, virus blocking, cytoplasmic incompatibility and so on)?

Introduction paragraph 1 – References 11 and 12 refer to Ae. polynesiensis, not Ae. albopictus.

Introduction paragraph 1 – There is an emphasis on wMel here specifically, but also consider including the wAlbB strain which was the first transinfected strain in Ae. aegypti (Xi et al. 2005, Science) and which has also been released in wild mosquito populations to inhibit virus transmission (Nazni et al. 2019 Current Biology).

Introduction paragraph 2 – “However, a more targeted approach amplifying *Wolbachia*-specific genes is required to confirm a resident strain is present” – This really requires additional evidence such as microscopy, removal of the infection, demonstration of maternal transmission and so on.

Introduction paragraph 2 – provide an example of a superinfection comprising strains from the same supergroup

Introduction paragraph 3 – “outbreaks of arboviral diseases including DENV, YFV, CHIKV and Rift Valley fever virus (RVFV) are a possibility.” is a bit vague – do they occur and how frequently?

Methods - How many mosquitoes per pool? In the results you mention an average of 5 female mosquitoes, but how much did this vary?

Methods – sanger sequencing – which PCR products were considered to be worthy of sequencing and why?

Results paragraph 1 – When presenting West region results, do these n values represent the number of pools? Or were these actually individuals?

Results paragraph 1 - What is the hypothesis being tested with the Fisher's exact tests? Please elaborate on what associations are being tested and include these statistical tests in the methods section

Results – There appear to be some inconsistencies when presenting frequency data- sometimes samples from the West region are specifically referred to as pools while at other times there is no mention of them being individuals or pools (e.g. in the first paragraph of the results).

Table 1 – results for Wolbachia 16S are reported, but what about other genes? Were they concordant with the 16S results?

Table 1 – What is the Fisher test comparing?

Table 1 – Although mentioned as a caveat in the text, it is misleading to put "co-infection" for the West samples when these consist of pools of mosquitoes – it is plausible that pools could consist of singly infected Wolbachia and Asaia individuals

Table 2 – Only the last three rows indicate that pools were tested, but aren't all samples in this table pools?

Figure 1 – Please provide the 16S data by species to allow for a comparison with the wsp data

Results - *"Wolbachia 16S rRNA sequences has previously been detected in water containers that contained larvae of mosquitoes*" - Is this not also possible for the other genes?

Discussion – What makes a strain a candidate? Is any newly detected strain a candidate strain, or do they need to display a certain phenotype such as cytoplasmic incompatibility or virus blocking? In my opinion a candidate strain must possess some desirable traits and should be able to be maintained in the laboratory.

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: I am currently collaborating on a project with a lab from the Liverpool School of Tropical Medicine of which a coauthor of this paper is currently a member. The project is unrelated to the current manuscript and I have not worked with any of the other authors, so I don't believe this will affect the objectivity of my review.

Reviewer Expertise: Wolbachia infections in Aedes mosquitoes and their use in control programs

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

Author Response 04 Sep 2023

Thomas Walker

Dear Perran, **Thank you so much for your very comprehensive review. We have addressed your comments as follows with responses in bold:** Wolbachia endosymbionts are prevalent in insect populations and can enact dramatic effects on their hosts. Understanding the prevalence and diversity of Wolbachia in natural mosquito populations

has important implications for the control of mosquito-borne disease because Wolbachia infections can influence mosquito vectorial capacity and modulate the success of vector control programs. Other microbes such as Asaia can also reside in mosquito tissues where they can influence host phenotypes and interact with Wolbachia. Here, the authors performed field surveys of Culicine mosquitoes in two regions of Cameroon and screen either individuals or pools of mosquitoes for Wolbachia and Asaia using PCR detection and sequencing of 16S rRNA, wsp and MLST markers. They detect Wolbachia and Asaia at variable frequencies which depend on the mosquito genus and region. While these results provide important data for vector control programs and may lead to candidate strains for Wolbachia release programs, there are a few key limitations with their study design and their conclusions are overstated.

The authors claim to detect co-infections of Wolbachia and Asaia in field-collected mosquitoes, contradicting evidence from laboratory studies that the two microbes have an antagonistic relationship with each other. However, these two observations are not necessarily in conflict. While there may be mutual exclusion of Asaia and Wolbachia in the reproductive tissues of some mosquitoes, this does not preclude the two microbes from residing in the same mosquito but within different tissues. There are several cases where Wolbachia and Asaia have been detected in the same mosquito (e.g. Schrieke et al. 2022 Computational and Structural Biotechnology [1], Hegde et al. 2018 Frontiers in Microbiology [2], da Silva et al. 2022 genes [3], Chen et al. 2020 Frontiers in Microbiology [4]) but they may not be occupying the same tissues, especially given that Asaia can be environmentally acquired and often resides in the gut. **Thank you for these points. We have expanded paragraph 4 of our discussion to provide a more balanced view on the relationship between *Wolbachia* and *Asaia*.** This study also does not provide sufficient evidence for a Wolbachia-Asaia coinfection. Firstly, all samples where both Wolbachia and Asaia were detected are from pools of mosquitoes, so it is possible that these samples contain a mix of singly infected individuals. **We have added a caveat to our discussion on 'pooling' mosquitoes (paragraph 4) but given that you have pointed out that 'there are several cases where Wolbachia and Asaia have been detected in the same mosquito' it would be contradictory to those studies to assume a coinfection is not possible. Unfortunately these collections were undertaken during the covid pandemic so a pooling approach was taken to minimise time in labs under severe travel restrictions. Our data from the West region (*Aedes* monospecific pools mosquitoes were either co-infected (75%) or singly infected with *Wolbachia* (25%) and for *Culex* 98% were infected with *Wolbachia* only) would also indicate a mix of singly infected individuals is not likely to represent most of our data.** Secondly, molecular detection is not sufficient evidence of an infection as pointed out by previous studies (Chrostek and Gerth 2019 mBio [5], Ross et al. 2020 Ecol Evol [6]). This is acknowledged at the end of the discussion but the rest of the paper is written as if all these detections represent true infections. **We agree there have been many recent papers that have just presented molecular data without providing further evidence but we feel our manuscript provides sufficient caveats to justify our results as more than likely coming from genuine infections (paragraphs 4 and 5 of the discussion). We acknowledge that reliance on just one *Wolbachia* gene (eg. *16S rRNA* or wsp) would not be sufficient as has been the case for numerous recent publications. However, having multiple MLST gene sequences from *Wolbachia* housekeeping genes that match (or are very close in sequence) to existing sequencing in**

https://pubmlst.org/organisms/wolbachia-spp from RNA extracts (so active gene expression) would indicate it's much more likely to represent genuine strains than the alternatives such as environmental contamination. We support the general opinion that further experimental work is needed but methods such as whole genome sequencing and FISH are not feasible on large numbers of field collected mosquitoes (from different species) without prior knowledge of molecular evidence that strain exist (or are likely to exist based on high prevalence rates and consistent strain typing). There are also some limitations and apparent gaps/inconsistencies in their study design. Mosquitoes from the West region were pooled while mosquitoes from the Central region were tested individually, but mosquitoes were only identified to the species level in the West region. **Unfortunately we were unable to morphologically identify species in the Central region due to 1) a lack of expertise and 2) missing or damaged morphological features during mosquito collections. Morphological identification of less-well known Culicine species is particularly challenging and often results in misidentification[1]. Therefore, we firstly screened individuals for symbionts using qPCR targeting both *Wolbachia* and *Asaia 16S rRNA* genes to identify samples of interest (and limited our analysis to the genera level). MLST sequencing was then carried out on selected individuals for *Wolbachia* strain typing (our main aim of this work) followed by molecular confirmation of mosquito species using CO1 barcoding.** The use of pools is likely to overestimate the prevalence of Wolbachia and Asaia in a sample given that not all individuals need to test positive for the pool to be considered positive. There is also the risk that any errors with morphological species identification could lead to an incorrect assignment of Wolbachia or Asaia status of a pool if one species is positive and the other is negative. **We again agree that pooling is not the preferred method for detection but covid restrictions (both in the field for travel and in the lab for analysis) resulted in a pooling strategy to be able to analyse samples. We have added a caveat to our discussion on 'pooling' mosquitoes (paragraph 4) to address this point.** The authors also use a mix of 16S rRNA and wsp detection for Wolbachia, but the data for 16S are combined across species within a genus while the wsp data are only presented for individual species.Furthermore, the results for 16S include only Aedes, Mansonia and Culex while there are other genera included for the wsp marker. The justification for this is unclear, and it would be useful to know how concordant the two markers are with each other, especially when the authors note that the use of the 16S rRNA gene has limitations for Wolbachia detection. The authors also mention in the methods that they also used the FtsZ marker for initial Wolbachia detection but these results are not presented. **Please see our previous response in terms of the difficulties of morphological ID from the Central region (lack of expertise and damage from collections). We used the wsp gene on our pooled samples from the West to re-enforce what appeared to be a high prevalence rate from the monospecific *Aedes* pools. In contrast to 16S rRNA, wsp is often not amplified from low density strains (or strains that may not be in genuine endosymbiosis). For example, there are numerous studies that report the presence of Wolbachia in Anopheles species in which wsp failed to amplify (PMID: 32787974). Genbank lists 835 sequences for wsp (search wsp AND "Wolbachia pipientis"[porgn:__txid955]) compared to 2643 for 16S rRNA (search 16S rRNA AND "Wolbachia pipientis"[porgn:__txid955]) suggesting wsp amplification is more indicative of a genuine symbiotic association (and hypervariable regions can be used for strain typing in addition to MLST).** The authors then select a sample of mosquito species testing positive for Wolbachia for further

analysis of the Wolbachia strains with 16S rRNA and MLST markers. The authors place an emphasis on the fact that the Wolbachia strains that they detect are novel and diverse but these terms are not well defined. **We have added a sentence to our discussion (paragraph 5) on what criteria we used based on MLST.** Has Wolbachia been detected in any of these species besides Cx. quinquefasciatus before, and how different do Wolbachia sequences have to be before they are considered novel and diverse? **This was partly addressed in our discussion for *Mansonia* but we have now checked the most reliable source for strain typing (https://pubmlst.org/organisms/wolbachia-spp) and can confirm (based on this) that no other Culex species in our study has MLST or wsp sequences. There are also no strain profiles present for *Aedes africanus* or Aedes *denderensis or the remaining samples (Catageiomyia argenteopunctata, Lutzia tigripes, Eretmapodites chrysogaster* and *Uranotaenia bilineata).* We have added a sentence to the end of paragraph 2 of the discussion to address this.** The authors compared their sequences to the MLST database but this may not represent the full diversity of Wolbachia strains that have been identified given that Wolbachia sequences have been obtained through other approaches (e.g. Scholz et al. Nature Communications). There are also some issues with the use of the MLST system for strain typing and diversity as outlined by Bleidorn and Gerth 2018 (FEMS Microbiol Ecol) [7] and these should be acknowledged. **We have included a sentence in the discussion (paragraph 5) on the limitations of MLST despite this being widely used in the Wolbachia community for strain diversity.**

Specific comments

Title – The title is misleading – the authors have detected Wolbachia and Asaia sequences in a range of field-collected mosquito species, but there is insufficient evidence that these represent true infections. The terms “diverse” and “novel” are not well defined and it is unclear how many of these strains have been detected for the first time. **We respectively disagree that the title should be changed to ‘sequences’ given we are presenting MLST data. There are many published papers reporting Wolbachia strain detection with much less genetic data (sometimes based on sequences obtained from nested 16S rRNA) so this change seems inappropriate. We have changed our title to ‘Diverse novel *Wolbachia* bacteria strains and genera-specific co-infections with *Asaia* bacteria in Culicine mosquitoes from ecologically diverse regions of Cameroon” to reflect genera differences for Asaia co-infections.**

Introduction – the authors mention that identifying Wolbachia strains can provide important comparative data but they don’t elaborate on how this information would be useful for biocontrol strategies. For instance, natural infections could interfere with releases of transinfections because they may change patterns of cytoplasmic incompatibility between the released and resident mosquitoes. The authors also note that novel strains could be considered candidates for biocontrol strategies, but what properties are you looking for in a strain for it to be considered a candidate, and what steps would need to be taken for the strain to be used (e.g. introgression or microinjection into a different mosquito line, virus blocking, cytoplasmic incompatibility and so on)? **We have added some sentences in paragraph 2 of the introduction to address this important point.**
Introduction paragraph 1 – References 11 and 12 refer to Ae. polynesiensis, not Ae. albopictus. **Thank you for pointing this error out – we have changed this**

Introduction paragraph 1 – There is an emphasis on wMel here specifically, but also consider including the wAlbB strain which was the first transinfected strain in Ae. aegypti (Xi et al. 2005, Science) and which has also been released in wild mosquito populations to inhibit virus transmission (Nazni et al. 2019 Current Biology). **Thank you for this suggestion and we have added some information at the end of paragraph 1**
Introduction paragraph 2 – "However, a more targeted approach amplifying *Wolbachia*-specific genes is required to confirm a resident strain is present" – This really requires additional evidence such as microscopy, removal of the infection, demonstration of maternal transmission and so on. **We agree and have added a sentence to reflect this**

Introduction paragraph 2 – provide an example of a superinfection comprising strains from the same supergroup **We have not been able to find a clear example (at least within mosquitoes) so have modified this sentence to reflect this.**

Introduction paragraph 3 – "outbreaks of arboviral diseases including DENV, YFV, CHIKV and Rift
Valley fever virus (RVFV) are a possibility." is a bit vague – do they occur and how frequently? **We have modified this and added a sentence with an example for DENV in Cameroon.**

Methods - How many mosquitoes per pool? In the results you mention an average of 5 female mosquitoes, but how much did this vary? **We have added this (3-5 mosquitoes/pool) in the methods**

Methods – sanger sequencing – which PCR products were considered to be worthy of sequencing and why? **We have modified the beginning of the this section to provide further details**

Results paragraph 1 – When presenting West region results, do these n values represent the number of pools? Or were these actually individuals? **We have edited this paragraph for clarity**

Results paragraph 1 - What is the hypothesis being tested with the Fisher's exact tests? Please elaborate on what associations are being tested and include these statistical tests in the methods section **We erroneously left out a statistics section in the methods which has now been added**

Results – There appear to be some inconsistencies when presenting frequency data-sometimes samples from the West region are specifically referred to as pools while at other times there is no mention of them being individuals or pools (e.g. in the first paragraph of the results). **We have added 'pools' were appropriate to reflect the West region data**

Table 1 – results for Wolbachia 16S are reported, but what about other genes? Were they concordant with the 16S results? **We used qPCR targeting the 16S rRNA gene for both *Wolbachia* and *Asaia* to generate CTs to provide a consistent comparison and did not compare to endpoint PCR results. All raw data is available at** https://doi.org/10.17605/OSF.IO/V75DU.

Table 1 – What is the Fisher test comparing? **Please see our new 'statistical analysis' section in the methods. "Fisher's exact post hoc tests in GraphPad prism version 9 (P<0.05 significance threshold) were used to determine any association between prevalence rates of *Wolbachia* and *Asaia* for each mosquito genus from the different regions (West and Central). Samples were categorised as *Wolbachia*-infected, *Asaia*-infected, co-infected or uninfected." Table 1** – Although mentioned as a caveat in the text, it is misleading to put "co-infection" for the West samples when these consist of pools of mosquitoes – it is plausible that pools could consist of singly infected Wolbachia and Asaia individuals **We agree that we need to include more caveats in our discussion (see new paragraph 4) but disrespectfully disagree for table 1 given we have the following in the legend 'Mosquitoes from the West region were extracted from monospecific pools (same species from same collection location) and prevalence analysis represents pooled samples' to clearly show these are pooled samples. Table 2** – Only the last three rows indicate that pools were tested, but aren't all samples in this table pools? **Thank you for spotting this error – we have amended this table**

Figure 1 – Please provide the 16S data by species to allow for a comparison with the wsp data **This is a map showing the co-ordinates of the collections and has no species data**

Results - *"Wolbachia 16S rRNA sequences has previously been detected in water containers that contained larvae of mosquitoes*" - Is this not also possible for the other genes? **We agree it's theoretically possible but we feel it's only appropriate to reference published papers. Furthermore, 16S rRNA is often reported in papers in which no other *Wolbachia* gene amplification has been possible and given it's extremely conserved (V3/V4 region used for microbiome analysis) is more likely to result in environmental contamination.**

Discussion – What makes a strain a candidate? Is any newly detected strain a candidate strain, or do they need to display a certain phenotype such as cytoplasmic incompatibility or virus blocking? In my opinion a candidate strain must possess some desirable traits and should be able to be maintained in the laboratory. **Thank you for this valid point and opinion. Given that mosquito transinfection has been possible from closely related mosquito species (eg. wAlbB into Ae. aegypti and An. stephensi), strains that natural reside within mosquito species may provide an increased chance of successful transinfection (difficult to test given the technical difficulty of embryo microinjection). Cell line adaptation was seen as key reasons for the success of Drosophila Wolbachia strains wMel and wMelPop into *Ae. aegypti* so strains that can be firstly identified to be at high prevalence in mosquito field populations will allow further examination in terms of phenotypic effects through eg. cif gene analysis to indicate CI induction. We agree that there are numerous candidate strains in lab colonies but temperature has recently been shown to decrease both density and virus inhibition for some Drosophila strains (eg. wMel). Potential candidate strains that naturally exist in mosquito field populations in countries such as Cameroon with high average temperature may be less likely to see this effect. References** 1. Jourdain, F., Picard, M., Sulesco, T., Haddad, N., Harrat, Z., Sawalha, S.S., Gunay, F., Kanani, K., Shaibi, T., Akhramenko, D., et al. (2018). Identification of mosquitoes (Diptera: Culicidae): an external quality assessment of medical entomology laboratories in the MediLabSecure Network.

Parasit Vectors *11*, 553. 10.1186/s13071-018-3127-7.

Competing Interests: No competing interests were disclosed.

Reviewer Report 13 July 2023

https://doi.org/10.21956/wellcomeopenres.20603.r61511

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Guido Favia
School of Bioscience and Veterinary Medicine, University of Camerino, Camerino, Italy

The work is part of a series of works, now quite numerous, which aim to identify species of insect vectors that are infected by Wolbachia. In this sense, the research is not characterized by particular originality; nevertheless the research has some appreciable aspects as it aims to analyze several species of mosquitoes (some of which are little studied) in some areas of Cameroon, in different eco-ethological contexts. Furthermore, I appreciated that the analysis of Wolbachia distribution was carried out not exclusively through 16S amplification but also through an MLST approach resulting in phylogenetic analysis.

That said, the manuscript has some flaws which, in my opinion, should be corrected before publication.

1. For experts in the field the decision to verify the co-presence/co-absence of Wolbachia and Asaia is quite clear but for many readers it would be useful to explain why the monitoring concerned Asaia and not other symbionts (it might help in the introduction to point out that these are two of the few symbionts for which insect and vector control approaches have already been proposed and in some cases validated in semifield and field experiments).

2. Figure 2 compares the positivity between West and East mosquitoes at the genus level. I don't understand why, given that they then move on to the analysis and definition of the species, this datum is not expressed by species; it would definitely be more informative.

3. Even the logical thread expressed in the materials and methods does not seem acceptable to me. They speak first of the monitoring of Asaia and Wolbachia and then of the definition of the host species. A stringent logic that aims to monitor the distribution of two symbionts in different mosquito species, I think, would mean proceeding first with the identification of the host-species and then with monitoring the distributions of the symbionts.

4. Another aspect that should be slightly modified concerns the conclusions: the authors state that "*Our study also suggests that co-infection with environmentally acquired Asaia bacteria is widespread in wild mosquito populations and the antagonistic relationship observed in lab colonies may not be present in wild Culicine populations.*" This is not entirely true since there

are studies on some wild-species of culicine that demonstrate an almost total absence of coexistence of the two symbionts, as in the case of some invasive species of aedes. The sentence therefore should be rephrased and referred to and limited to the geographical context analyzed by the authors.

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

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

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

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

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

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: I am working in the same field. Nevertheless this hasn't in anyway affected my review

Reviewer Expertise: Molecular Entomology, Molecular Parasitology, Insect symbiosis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 Sep 2023

Thomas Walker

Dear Guido, **Thank you very much for taking the time out to review our manuscript. We have addressed your comments as follows with responses in bold:** The work is part of a series of works, now quite numerous, which aim to identify species of insect vectors that are infected by Wolbachia. In this sense, the research is not characterized by particular originality; nevertheless the research has some appreciable aspects as it aims to analyze several species of mosquitoes (some of which are little studied) in some areas of Cameroon, in different eco-ethological contexts. Furthermore, I appreciated that the analysis of Wolbachia distribution was carried out not exclusively through 16S amplification but also through an MLST approach resulting in phylogenetic analysis.

That said, the manuscript has some flaws which, in my opinion, should be corrected before

publication.

1. For experts in the field the decision to verify the co-presence/co-absence of Wolbachia and Asaia is quite clear but for many readers it would be useful to explain why the monitoring concerned Asaia and not other symbionts (it might help in the introduction to point out that these are two of the few symbionts for which insect and vector control approaches have already been proposed and in some cases validated in semifield and field experiments).

Thank you for this suggestion. We agree and have extended our introduction (paragraph 2) to incorporate a more expansive introduction to *Asaia* and its potential role for mosquito control.

1. Figure 2 compares the positivity between West and East mosquitoes at the genus level. I don't understand why, given that they then move on to the analysis and definition of the species, this datum is not expressed by species; it would definitely be more informative.

Unfortunately we were unable to morphologically identify species in the Central region due to 1) a lack of expertise and 2) missing or damaged morphological features during mosquito collections. Morphological identification of less-well known Culicine species is particularly challenging and often results in misidentification[1]. Therefore, we firstly screened individuals for symbionts using qPCR targeting both *Wolbachia* and *Asaia 16S rRNA* genes to identify samples of interest (and limited our analysis to the genera level). MLST sequencing was then carried out on selected individuals for *Wolbachia* strain typing (our main aim of this work) followed by molecular confirmation of mosquito species using CO1 barcoding.

1. Even the logical thread expressed in the materials and methods does not seem acceptable to me. They speak first of the monitoring of Asaia and Wolbachia and then of the definition of the host species. A stringent logic that aims to monitor the distribution of two symbionts in different mosquito species, I think, would mean proceeding first with the identification of the host-species and then with monitoring the distributions of the symbionts.

Please see our response to query 2. We firstly identified samples of interest from the *Wolbachia/Asaia* qPCR results and then proceeded to undertake *Wolbachia* MLST strain typing and confirm species using CO1 barcoding given the difficulties of Culicine morphological identification which in a study that used an external quality assessment resulted in only 64% accuracy to the species level across 19 participant laboratories[1].

1. Another aspect that should be slightly modified concerns the conclusions: the authors state that "*Our study also suggests that co-infection with environmentally acquired Asaia bacteria is widespread in wild mosquito populations and the antagonistic relationship observed in lab colonies may not be present in wild Culicine populations.*" This is not entirely true since there are studies on some wild-species of culicine that demonstrate an almost total absence of coexistence of the two symbionts, as in the case of some invasive species of aedes. The sentence therefore should be rephrased and referred to and limited to the geographical context analyzed by the authors.

Thank you for this suggestion and we agree. We have modified our discussion in paragraph 4 to address these very valuable points. **References** 1. Jourdain, F., Picard, M., Sulesco, T., Haddad, N., Harrat, Z., Sawalha, S.S., Gunay, F., Kanani, K., Shaibi, T., Akhramenko, D., et al. (2018). Identification of mosquitoes (Diptera: Culicidae): an external

quality assessment of medical entomology laboratories in the MediLabSecure Network. Parasit Vectors *11*, 553. 10.1186/s13071-018-3127-7.

Competing Interests: No competing interests were disclosed.

Reviewer Report 13 July 2023

https://doi.org/10.21956/wellcomeopenres.20603.r60867

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Eric P. Caragata
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In their manuscript 'Diverse novel Wolbachia bacteria strains and widespread co- infections with Asaia bacteria in Culicine mosquitoes from ecologically diverse regions of Cameroon', the authors use RT-qPCR screening of mosquito specimens collected from two areas in Cameroon to look for evidence of novel Wolbachia infections, as well as examining patterns of *Wolbachia-Asaia* co-occurrence/co-exclusion. They observe geographic and mosquito clade-specific patterns of Wolbachia and Asaia occurrence. They then use MLST sequencing data and phylogenetics to examine the similarity of their putative, new Wolbachia strains to each other and those previously characterized. Such strains, if proven to be viable and heritable, may be useful as targets to generate novel Wolbachia transinfections, but may also provide important information on the ecology, physiology, and vector competence of several mosquito species in the region.

The manuscript is well written and does a great job of mentioning caveats. The authors have been particularly conscious of the issue of false positive detection of Wolbachia in their choice of methodology, and in their acknowledgements that all of these putative strains must be further validated, for instance, by using imaging techniques. I have a few suggestions and clarifications for the revised version of the text:

1. The issue of false positive detection of Wolbachia in mosquitoes is intrinsic to this work and field of Wolbachia research and it could be briefly mentioned in the introduction. This will give your readers improved context for your choice of RNA-based quantification of Wolbachia.

2. Details on Wolbachia detection criteria (calling positives vs negatives) as well as the positive and negative controls used in the RT-qPCR assay have not been provided in the methods section. These details are vital to demonstrate efforts have been made to reduce false positive detection.

3. Please clarify how Fisher's tests were used to analyze your data. These tests were not mentioned in the methods section and are applied in pairwise comparisons, which might

explain why some tests in Table 1 have such high P values.

4. At the beginning of the results section it would be useful to include a brief summary of which mosquito species were collected, and where and when they were collected. The when is important given samples were collected across many years.

5. I do not see an equivalent of table 2 for the central region data. It looks as though Table 2 just deals with the western region.

6. Queries about phylogenetic trees:

   - In Fig. 3, is there an explanation for why Cx pipiens and Cx quinquefasciatus don't cluster together.

   - None of the phylogenetic appear to have outgroups

   - Fig. 4 - while it is useful to have a tree based on the Wolbachia 16s gene. It could be valuable to include an unrooted consensus tree based on the MLST data.

7. Is there a reason why Figure 2 and Table 2 collapse the prevalence of Wolbachia and Asaia to the genus level? This reduces the biological relevance of your findings as mosquitoes within a genus can fill distinct biological niches.

8. Figure 2 is not visually accessible (red/green colors).

9. The discussion describes the collection areas in Cameroon as containing a high degree of environment-driven variation. Reflecting on that, the decision to bin samples into two homogeneous regions (West vs Central) does not make sense. Are there any site-specific patterns that could be mentioned that better reflect site-to-site diversity in mosquito species/Wolbachia prevalence?

10. The discussion described Wolbachia/Asaia co-infection as being "widespread" but this does not appear to reflect the data given that Asaia was only highly prevalent in one mosquito genus.

11. If the introduction were to briefly discuss the impact of Asaia on mosquito vector competence and immunity it could add important context outlining the rationale for studying Asaia infections in mosquitoes.

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mosquito microbiology, molecular biology, immunity, and metabolism. Wolbachia. Mosquito-microbe-pathogen interactions.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 Sep 2023

Thomas Walker

Dear Eric, **Thank you for your very comprehensive review. We have addressed your comments as follows with responses in bold:** In their manuscript 'Diverse novel Wolbachia bacteria strains and widespread co- infections with Asaia bacteria in Culicine mosquitoes from ecologically diverse regions of Cameroon', the authors use RT-qPCR screening of mosquito specimens collected from two areas in Cameroon to look for evidence of novel Wolbachia infections, as well as examining patterns of *Wolbachia-Asaia* co-occurrence/co-exclusion. They observe geographic and mosquito clade-specific patterns of Wolbachia and Asaia occurrence. They then use MLST sequencing data and phylogenetics to examine the similarity of their putative, new Wolbachia strains to each other and those previously characterized. Such strains, if proven to be viable and heritable, may be useful as targets to generate novel Wolbachia transinfections, but may also provide important information on the ecology, physiology, and vector competence of several mosquito species in the region.

The manuscript is well written and does a great job of mentioning caveats. The authors have been particularly conscious of the issue of false positive detection of Wolbachia in their choice of methodology, and in their acknowledgements that all of these putative strains must be further validated, for instance, by using imaging techniques. I have a few suggestions and clarifications for the revised version of the text:

1. The issue of false positive detection of Wolbachia in mosquitoes is intrinsic to this work and field of Wolbachia research and it could be briefly mentioned in the introduction. This will give your readers improved context for your choice of RNA-based quantification of Wolbachia.

Thank you for your suggestion and we have added a sentence on this at the back end of the introduction.

1. Details on Wolbachia detection criteria (calling positives vs negatives) as well as the positive and negative controls used in the RT-qPCR assay have not been provided in the methods section. These details are vital to demonstrate efforts have been made to reduce false positive detection.

Apologies we have corrected this error and included details on the inclusion of NTCs and referenced the previously generated *16S rRNA* standard curve providing a limit of detection[1]. The raw qPCR data can also be found at https://doi.org/10.17605/OSF.IO/V75DU.

1. Please clarify how Fisher's tests were used to analyze your data. These tests were not mentioned in the methods section and are applied in pairwise comparisons, which might explain why some tests in Table 1 have such high P values.

Apologies we erroneously missed a 'statistics' section in the methods and have now addressed this

1. At the beginning of the results section it would be useful to include a brief summary of which mosquito species were collected, and where and when they were collected. The when is important given samples were collected across many years.

Unfortunately there was a type for the collection 'years' for the Central region (this was only in 2019) and we have modified this in the methods. This reduces any confusing in terms of 'when' they were collected and all location data is present in the methods so we don't feel a summary is now warranted.

1. I do not see an equivalent of table 2 for the central region data. It looks as though Table 2 just deals with the western region.

That is correct. Unfortunately we were unable to morphologically identify species in the Central region due to 1) a lack of expertise and 2) missing or damaged morphological features during mosquito collections. Morphological identification of less-well known Culicine species is particularly challenging and often results in misidentification[2]. Therefore, we firstly screened individuals for symbionts using qPCR targeting both *Wolbachia* and *Asaia 16S rRNA* genes to identify samples of interest (and limited our analysis to the genera level). MLST sequencing was then carried out on selected individuals for *Wolbachia* strain typing (our main aim of this work) followed by molecular confirmation of mosquito species using CO1 barcoding. Queries about phylogenetic trees:

- In Fig. 3, is there an explanation for why Cx pipiens and Cx quinquefasciatus don't cluster together. **There are multiple 16S sequences available on GenBank for *Wolbachia* strains detected in species within the *Culex pipiens*complex indicating genetic diversity in the strain group loosely labelled as 'wPip'. This would be reflective of numerous studies showing that there are multiple *Wolbachia* strains that co-existing within *Culex pipiens* populations[3-5]. We have added a sentence to our discussion to highlight this point and the value of undertaking MLST to provide greater depth of sequencing data. -** None of the phylogenetic appear to have outgroups **Out grouping for both mosquito barcoding and Wolbachia phylogenetic trees, to our knowledge, is not commonly undertaken (at least based on the vast majority of similar publications). Instead, we (and previously published studies) include mosquito barcoding gene sequences present on GenBank that closely match sequences generated in this study. For *Wolbachia* 16S, we (and previously published studies) include 16S sequences from well characterised Wolbachia strains such as wMel and wRi for context. -** Fig. 4 - while it is

useful to have a tree based on the Wolbachia 16s gene. It could be valuable to include an unrooted consensus tree based on the MLST data. **Although we agree we were not able to generate complete MLST profiles for all mosquito species (please see table 5) which is consistent with numerous other published studies. Therefore, we feel a phylogenetic tree based on MLST data would be incomplete and provide little beneficial data not present in table 5.**

1. Is there a reason why Figure 2 and Table 2 collapse the prevalence of Wolbachia and Asaia to the genus level? This reduces the biological relevance of your findings as mosquitoes within a genus can fill distinct biological niches.

I think this is referring to Figure 2 and yes we agree that it does limit our conclusions. Please see our response to point 5 but we have also included a few sentences in the discussion (paragraph 4) to highlight this.

1. Figure 2 is not visually accessible (red/green colors).

Thank you for pointing this out – we have changed the colour scheme.

1. The discussion describes the collection areas in Cameroon as containing a high degree of environment-driven variation. Reflecting on that, the decision to bin samples into two homogeneous regions (West vs Central) does not make sense. Are there any site-specific patterns that could be mentioned that better reflect site-to-site diversity in mosquito species/Wolbachia prevalence?

We agree but unfortunately the collections were affected by the covid pandemic and we had to treat samples differently (individuals vs pools, morphological identification) between West and Central collections due to restricted travel and time working in labs. In our methods we do include details on collection locations but do not feel we have sufficient 'site-specific environmental data' to justify further sub-divisions within the two Regions.

1. The discussion described Wolbachia/Asaia co-infection as being "widespread" but this does not appear to reflect the data given that Asaia was only highly prevalent in one mosquito genus.

We agree and have addressed this in our discussion (paragraph 4) and have changed the title to reflect this with a more balanced conclusion.

1. If the introduction were to briefly discuss the impact of Asaia on mosquito vector competence and immunity it could add important context outlining the rationale for studying Asaia infections in mosquitoes.

We agree and have added some information on *Asaia* and its potential use in mosquito biocontrol strategies (please see paragraph 2). References

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