Diverse novel resident Wolbachia strains in Culicine mosquitoes from Madagascar

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Wolbachia endosymbiotic bacteria are widespread throughout insect species and Wolbachia transinfected in Aedes mosquito species has formed the basis for biocontrol programs as Wolbachia strains inhibit arboviral replication and can spread through populations. Resident strains in wild Culicine mosquito populations (the vectors of most arboviruses) requires further investigation given resident strains can also affect arboviral transmission. As Madagascar has a large diversity of both Culicine species and has had recent arboviral outbreaks, an entomology survey was undertaken, in five ecologically diverse sites, to determine the Wolbachia prevalence. We detected diverse novel resident Wolbachia strains within the Aedemyia, Culex, Ficalbia, Mansonia and Uranotaenia genera. Wolbachia prevalence rates and strain characterisation through Sanger sequencing with multilocus sequence typing (MLST) and phylogenetic analysis revealed significant diversity and we detected co-infections with the environmentally acquired bacteria Asaia. Mosquitoes were screened for major arboviruses to investigate if any evidence could be provided for their potential role in transmission and we report the presence of Rift Valley fever virus in three Culex species: Culex tritaeniorhynchus, Culex antennatus and Culex decens. The implications of the presence of resident Wolbachia strains are discussed and how the discovery of novel strains can be utilized for applications in the development of biocontrol strategies.

The endosymbiotic bacterium Wolbachia naturally infects approximately 40% of insect species1, including mosquito vector species that are responsible for transmission of human diseases. Resident Wolbachia strains are present in some major arbovirus disease vectors such as Culex (Cx.) quinquefasciatus2–4 and Aedes (Ae.) albopictus5–8. Wolbachia has been considered for mosquito biocontrol strategies due to the varied phenotypic effects strains have on host mosquito species. Some Wolbachia strains can induce a reproductive phenotype termed cytoplasmic incompatibility (CI). This phenotype results in inviable offspring when an uninfected female mates with a Wolbachia-infected male. In contrast, Wolbachia-infected females produce viable progeny when they mate with both infected and uninfected male mosquitoes. This reproductive advantage over uninfected females allows Wolbachia to invade mosquito populations. The CI phenotype was utilized in trials conducted in the late 1960s to eradicate Cx. quinquefasciatus from Myanmar9. Releasing large numbers of Wolbachia-infected male mosquitoes, to compete with wild type males to induce sterility, is known as the incompatible insect technique (IIT)5,9. Field trials have been undertaken using IIT for both Ae. albopictus10 and Ae. polynesiensis, a vector of lymphatic filariasis in the South Pacific8.

Wolbachia strains have also been shown to protect their native Drosophila fruit fly hosts against infection by pathogenic RNA viruses3,11,12. This discovery led to an alternative biocontrol approach for dengue virus (DENV) transmission which utilizes Wolbachia’s ability to inhibit pathogen replication within mosquitoes13–17. Wolbachia strains were successfully established in Ae. aegypti, the principle mosquito vector of DENV, yellow fever virus (YFV) and Zika virus (ZIKV), through embryo microinjection15,16,18–21. Preliminary field releases demonstrated the ability of the transinfected wMel strain of Wolbachia to establish in wild mosquito populations15 and to provide strong inhibition of DENV in wild mosquitoes15,16. Recently it has been shown that Wolbachia frequencies have remained stable since deployment in an area of Townsville, Australia and to date no local dengue transmission has

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been confirmed after Wolbachia has established, despite local transmission events every year for the previous 13 years with a trend of increasing imported cases24. Further releases are ongoing in DENV endemic countries and mathematical models suggest the wMel strain of Wolbachia could reduce the basic reproduction number, R0, of DENV transmission by 66–75%25. Wolbachia strains also inhibit other medically important arboviruses including chikungunya virus (CHIKV)14,26, YFV27 and recently ZIKV26,28.

There is also evidence that resident Wolbachia strains in laboratory mosquito colonies can inhibit arbovirus transmission29–32. Therefore, the prevalence and diversity of natural Wolbachia strains in field populations of mosquitoes requires further investigation to fully understand how widespread this bacterium is in wild Culicine populations. For example, recent studies undertaken to determine if Ae. aegypti contains resident strains report contrasting results with no evidence seen in a large collection from 27 countries33 but detection of a resident strain in populations from the Southeastern United States34. Additional bacterial species that make up the mosquito microbiota may influence the presence of resident Wolbachia strains. In Ae. aegypti laboratory colonies, the acetic acid bacterium Asaia infects the gut and salivary glands and there is competition with transinfected Wolbachia strains for colonization of mosquito reproductive tissues35. Asaia has been detected in field populations of Cx. quinquefasciatus36 suggesting a complex association between these two bacterial species in wild mosquito populations.

The Culicinae subfamily of mosquitoes (Family Culicidae) contains a large variety of species (>3,000) particularly concentrated in tropical regions. Genera that transmit, or are implicated in transmitting, arboviruses include Aedesomyia (Ad.), Aedes, Culex, Mansonia (Ma.) and Uranotaenia (Ur.). Madagascar, a large island located off the southeast coast of mainland Africa, represents an optimal location to investigate Wolbachia prevalence in Culines given its geographic isolation and large diversity of both mosquito species and arboviral diseases. Currently there have been 237 mosquito species morphologically identified in Madagascar in 15 different genera37 with 59% of these species thought to be endemic species in Madagascar. There have also been several areas with low temperatures and altitude are described in Supplementary Table S1.

Results
Mosquito abundance and diversity at study sites. Overall, 1174 individuals belonging to seven genera were captured from five collection sites (Fig. 1; Table 1). Qualitative collection site characteristics, median humidity and temperature and altitude are described in Supplementary Table S1. Cx. antenatus was collected in large numbers from Tsaramandrosoro and Ivato Airport and Cx. univittatus was the dominant species in Ankazobe. 820 (69.85%) of the mosquitoes were caught in CDC light traps, while 354 (30.15%) were caught with Zebubaited traps. Tsaramandrosoro had the largest number of individuals caught (n = 644) and highest diversity, which may have been influenced by both a high median relative humidity (79%) and temperature (24 °C) for the collection period. In contrast, Bemokotra had the smallest number of individuals collected (n = 19) and this could have been influenced by a lower median relative humidity (50%) despite a median temperature of 26 °C. Ivato Airport had the lowest diversity of species collected in the study and this could possibly be due to a low median temperature (11 °C) despite a median relative humidity of 88%. Aedes, Aedeomyia and Ficalbia (Fi.) species were collected only at lower elevation sites (Tsaramandrosoro, Bemokotra and Culex species were collected predominantly at low and high elevation sites (Tsaramandrosoro, Ankazobe and Ivato Airport). However, it should be noted that there are many more factors that influence mosquito species abundance and diversity.

Detection of resident Wolbachia strains and mosquito species identification. We screened individual mosquitoes for the presence of resident Wolbachia strains using three conserved Wolbachia genes (16 rRNA, wsp and ftsZ) and detected resident strains in six mosquito genera (Table 2). The prevalence of Wolbachia infection within mosquito species ranged from 3% (1/32) of Cx. antenatus, to 100% for Ad. madagascariensis (9/9). PCR analysis resulted in congruency with the same individual samples amplifying fragments for all three genes, with the exception of Ma. uniformis, Fi. circumtestacea, Cx. antenatus and Cx. diotomii, in which the wsp gene was not amplified in any individuals. As Madagascar contains diverse mosquito species, we amplified a fragment of the mitochondrial cytochrome c oxidase subunit I (CO1) gene44 of Wolbachia-infected individuals to provide as much molecular confirmation of species as possible, given the lack of available CO1 sequences in certain regions of the gene for some species. We were unable to amplify any CO1 gene fragments of Fi. circumtestacea but the remaining Wolbachia-infected species were confirmed by successful CO1 sequencing (Fig. 2a). CO1 sequences were deposited in Genbank (accession numbers MK033247-MK033269, Supplementary Table S1) including the first CO1 sequences for Ad. madagascariensis, and two Uranotaenia species from Madagascar, as well as the first Cx. decens sequences covering this region of the CO1 gene.

Surprisingly, we found no evidence of resident Wolbachia strains in members of the Cx. pipientis complex indicating the absence of the wPip strain which is usually ubiquitous in Cx. pipientis pipientis and Cx. pipientis quinquefasciatus sibling species42. As species within the Cx. pipientis complex are morphologically indistinguishable, we selected a sub-sample of individuals from different collection sites to determine the species using both species-specific PCR analysis and sequencing targeting a CO1 gene fragment that discriminates species within the Cx. pipientis complex43. PCR-based species identification targeting the ace-2 gene44 revealed the presence of Cx. pipientis pipientis individuals in four collection sites (no Cx. pipientis complex individuals were collected from Bemokotra). Confirmation resulted from CO1 phylogenetic analysis and we confirmed six Wolbachia-uninfected
Figure 1. Map of Madagascar showing location of collection sites and mosquito traps used. Adult trapping was undertaken in five ecologically diverse sites using both CDC light traps (LT) and Cattle (zebu) baited traps (CT) near rice fields, poultry coups and breeding locations to maximize the diversity of species collected. Anivorano III is located within commune Anivorano Nord, Ambomihiarina is located within commune Tsaramandrosa, Antafia is located within commune Bemokotra, Ambohimarina is located within commune Ankazobe and Ivato Imerimandroso is located within commune Ivato Aeroport. Bioclimatic map of Madagascar adapted from Brunhes et al. using Microsoft Powerpoint (2010) and Paint Net (v4.0.6). Google Earth Pro (version 7.3) was used for satellite imagery as follows: Source: «Ivato Imerimandroso» 18°47′21.71″ S and 47°28′42.34″ E. Google Earth Pro. June 17, 2018. October 17, 2018, Source: «Ambomihiarina» 18°19′38.86″ S and 47°06′23.91″ E. Google Earth Pro. Novembre 10, 2016. October 17, 2018, Source: «Antafia» 17°01′26.31″ S and 46°45′41.97″ E. Google Earth Pro. April 02, 2016. October 17, 2018, Source: «Ambomihiarina» 16°21′38.92″ S and 46°59′20.90″ E. Google Earth Pro. July 24, 2016. October 17, 2018, Source: «Anivorano III» 12°45′31.11″ S and 49°14′13.08″ E. Google Earth Pro. July 31, 2016. October 17, 2018.
CO1 sequencing indicated these individuals were genetically diverse and, with the sequences currently logically identified as Cx strains and this was reflected by variation in the success of Wolbachia determination strain phylogenies. As Madagascar contains some mosquito species that are only present on the island, typing (MLST) gene fragments was undertaken with Wolbachia-infected individuals to characterise, type and adaptors and/or primers comprising increased degeneracies. Despite using a variety of primers, we were unable amplifying gene target fragments using standard primers and alternative protocols that use M13 sequencing.

Table 1. Summary of mosquito species abundance using morphological identification from five sites in Madagascar. Trapping was undertaken for two consecutive nights with CDC light traps (LT) and cattle (zebu-baited) (CT) traps. Trapping was undertaken between dusk and dawn andquitoes from CT traps were collected by mouth aspiration.

| Species                        | Anivorano Nord | Taarandomaro | Bemokostra | Ankafobe | Ivato Aeroport | Total |
|--------------------------------|----------------|--------------|------------|----------|----------------|-------|
| Aeoleomyia furfurea            | 0              | 0            | 2          | 0        | 0              | 2     |
| Aeoleomyia madagascariaca      | 0              | 0            | 9          | 0        | 0              | 9     |
| Aedes aegypti                  | 0              | 0            | 0          | 0        | 2              | 2     |
| Aedes albopictus               | 0              | 0            | 1          | 0        | 0              | 1     |
| Aedes circumluteolus           | 0              | 0            | 1          | 0        | 0              | 1     |
| Culex antennatus               | 8              | 6            | 250        | 91       | 5              | 52    |
| Culex bitaeniorhyncha         | 0              | 4            | 1          | 14       | 0              | 20    |
| Culex decens                   | 0              | 3            | 12         | 6        | 0              | 22    |
| Culex giganteus                | 4              | 0            | 1          | 0        | 0              | 1     |
| Culex pippini complex          | 1              | 1            | 9          | 0        | 32             | 44    |
| Culex potchilipes              | 0              | 2            | 17         | 0        | 2              | 26    |
| Culex tritaeniorhyncha         | 0              | 0            | 25         | 18       | 0              | 43    |
| Culex univittatus              | 2              | 6            | 17         | 95       | 0              | 250   |
| Ficalbia circumtestacea        | 0              | 0            | 3          | 0        | 1              | 4     |
| Lutzia tigripes                | 0              | 1            | 0          | 0        | 0              | 1     |
| Mansonia uniformis             | 5              | 10           | 2          | 50       | 0              | 67    |
| Uranotaenia sp.                | 0              | 5            | 0          | 23       | 0              | 31    |
| Total                          | 20             | 36           | 298        | 346      | 0              | 1174  |

Cx. pippini pippini within the Cx. pippini complex (Fig. 2b). We also analysed three females that were morphologically identified as Cx. pippini complex individuals but did not result in any species confirmation through PCR (Fig. 2b). CO1 sequencing indicated these individuals were genetically diverse and, with the sequences currently available for comparison, were most closely related to Cx. rima and sequences named Cx. sp.GP-A from Kenya – Genbank accession number KU380413.1 (94% nucleotide identities, but only 89–94% coverage to the gene fragment amplified), suggesting the possibility of unknown Culex species present in Madagascar. We also screened individuals from all mosquito species for Asaia and detected the presence of this competing bacteria in 13 of 17 species (Table 2). Variable prevalence rates were observed and we detected co-infections of Wolbachia and Asaia in Ad. madagascariaca (n = 8), Cx. antennatus (n = 1), Cx. decens (n = 2), Cx. duttoni (n = 1), Ma. uniformis (n = 5) and Uranotaenia species (n = 4). Fisher’s exact post hoc tests revealed no association between Wolbachia and Asaia prevalence rates in species that had at least one individual infected with either bacteria (Table 2).

Wolbachia strain characterisations. Sanger sequencing of 16S rRNA, wsp and multilocus sequence typing (MLST) gene fragments was undertaken with Wolbachia-infected individuals to characterise, type and determine strain phylogenies. As Madagascar contains some mosquito species that are only present on the island, we predicted the presence of divergent Wolbachia strains and this was reflected by variation in the success of amplifying gene target fragments using standard primers and alternative protocols that use M13 sequencing adaptors and/or primers comprising increased degeneracies. Despite using a variety of primers, we were unable to amplify wsp or any of the five MLST gene loci for Cx. antennatus and Cx. duttoni. For the remaining resident Wolbachia strains, we amplified wsp from all strains except wUnif-Mad (Table 3). Phylogenetic analysis based on the 16S rRNA gene indicates most of these novel Wolbachia strains are clustering with Supergroup B Wolbachia strains, such as wPip and wDei (Fig. 3). Interestingly, only wUa1 clusters with Supergroup A Wolbachia strains, such as wMel and wRi. As the wsp gene has been evolving at a faster rate and provides more informative strain phylogenies, with improved phylogenetic resolution, we compared the phylogenetic relationships for strains that amplified a wsp fragment (Fig. 4). Similar phylogenetic relationships are shown with wsp, with wUa1 sequences clustering with wMel (Supergroup A), but the remaining strains (wUa2, wDec and wMad) clustered with Supergroup B Wolbachia strains.

MLST resulted in successful sequencing of the majority of gene loci, although we were unable to amplify hcpA from wUnif-Mad despite using alternative protocols with degenerate primers (Table 3). ftsZ universal primers were also needed to obtain a sequence for wMad. Each of the wMad gene sequences produced an exact match to alleles already present in the database, with the resulting allelic profile producing an exact match with strain type 125. This allelic profile matches four isolates currently present in the MLST database, detected in three Lepidopteran species, from French Polynesia, India, Japan and Tanzania (Isolate numbers 40, 247, 270 and 322 respectively), but no isolates from mosquito or other Dipteran hosts. The remaining strains had new alleles for at least one of the MLST gene loci (sequences differed from those currently present in the database by at least one nucleotide difference per locus), resulting in new allelic profiles highlighting the diversity and novelty of these resident strains. Wolbachia gene sequences were deposited in GenBank (accession numbers MK026554-MK026563 and MK033270-MK033320, Supplementary Table S2).
Wolbachia strains in seven mosquito species: Uranotaenia spp, Aedes circumlateolus, Aedes aegypti, Culex bitaeniorhynchus, Culex antennatus, Mansonia uniformis, and Culex poicilipes. The novel resident Wolbachia strain in Cx. decens, named wDec, was only found in individuals collected from Tsaramandroso with no detectable infections in individuals from Anivorano Nord or Bemokotra. Resident Wolbachia strains in both Cx. antennatus and Aedes aegypti were detected in individuals collected from Tsaramandroso. The novel wMad Wolbachia strain was detected in all Ad. madagascarica females collected in Tsaramandroso. We sequenced the wUnif-Mad strain in Ma. uniformis from both Tsaramandroso and Anivorano Nord. The single Wolbachia-infected Fi. circumtestacea was collected from Tsaramandroso and novel Wolbachia strains in Uranotaenia species (wUra1 and wUra2) were detected in individuals from Anivorano Nord, Tsaramandroso and Bemokotra.

Table 2. Wolbachia and Asaia infection prevalence rates in adult female mosquitoes using three conserved Wolbachia genes (16S rRNA, ftsZ and wsp). An individual was considered Wolbachia-infected when any one of the three Wolbachia gene fragments were amplified. Species containing resident Wolbachia strains are in bold. Wolbachia and Asaia prevalence rates are shown with 95% confidence intervals in parentheses. Fisher’s exact post hoc P value is shown comparing Wolbachia and Asaia infections in individual mosquitoes from species that contained at least one individual of both bacterial endosymbionts. ^ denotes where CO1 sequences were obtained for phylogenetic analysis of mosquito species.

| Mosquito species          | Number | Wolbachia gene PCR amplification | Prevalence (%) | Fisher’s exact post hoc P value (Wolbachia vs. Asaia) |
|---------------------------|--------|----------------------------------|----------------|------------------------------------------------------|
| Aedes albopictus          | 1      | 16S rRNA ftsZ wsp Wolbachia Asaia| 100 (20–100)   |                                                      |
| Aedes circumlateolus      | 1      | 0 0 0                            | 100 (34–100)   |                                                      |
| Aedomyia furfuria         | 2      | 0 0 0                            | 100 (34–100)   |                                                      |
| Aedomyia madagascarica^   | 9      | 9 9 9 9                          | 100 (70–100)   | 0.99                                                 |
| Culex antennatus^         | 32     | 1 1 1                            | 3 (1–16)       | 0.99                                                 |
| Culex bitaeniorhynchus    | 12     | 0 0 0                            | 58 (32–81)     |                                                      |
| Culex decens^             | 17     | 3 3 3                            | 18 (4–43)      | 0.46                                                 |
| Culex duttoni^            | 1      | 1 1 1                            | 100 (21–100)   | 0.99                                                 |
| Culex tritaeniorhynchus^  | 19     | 0 0 0                            | 95 (74–100)    |                                                      |
| Culex giganteus           | 5      | 0 0 0                            | 80 (37–96)     |                                                      |
| Culex p. complex          | 44     | 0 0 0                            | 7 (1–30)       |                                                      |
| Culex polticipes          | 21     | 0 0 0                            | 48 (28–68)     |                                                      |
| Ficalbia circumtestacea   | 3      | 1 1 1                            | 33 (6–79)      |                                                      |
| Mansonia uniformis^       | 24     | 7 7 7                            | 29 (15–49)     | 0.65                                                 |
| Uranotaenia spp^          | 27     | 7 7 7                            | 26 (13–45)     | 0.54                                                 |

The phylogeny of these novel strains based on concatenated sequences of all five MLST gene loci (where possible) (Fig. 5), or four MLST gene loci for analysis of wUnif-Mad (htpA sequence not available) alongside the other strains (Fig. 6), also reveals significant strain divergence. As expected, due to the general incongruence between host phylogenetic relationships and their resident Wolbachia strains^40,49, the majority of the novel Wolbachia strains in our study provide no evidence for clustering with other strains that infect mosquitoes. One exception is the wMad strain which, although distinct, appears closely related to wPip (the resident strain normally present in the Cx. p. complex). The other exception is the wUnif-Mad strain (based on 4 MLST loci, without htpA), which is closely related to the Wolbachia strain previously detected in Ma. uniformis in Kenya (Isolate number 500), and where the pattern of amplification success was also matched as there was absence of amplification for both htpA and wsp. However, wUnif-Mad differs by two nucleotides within the fbpA sequence, producing a novel fbpA allele and therefore a new allelic profile (Fig. 6 and Table 3).

Site locations for Wolbachia-infected mosquitoes. The novel resident Wolbachia strain in Cx. decens, named wDec, was only found in individuals collected from Tsaramandroso with no detectable infections in individuals from Anivorano Nord or Bemokotra. Resident Wolbachia strains in both Cx. antennatus (wA) and Cx. duttoni (wD) were from individuals collected in Tsaramandroso. The novel wMad Wolbachia strain was detected in all Ad. madagascarica females collected in Tsaramandroso. We detected the wUnif-Mad strain in Ma. uniformis from both Tsaramandroso and Anivorano Nord. Our single Wolbachia-infected Fi. circumtestacea (wGir) was collected from Tsaramandroso and novel Wolbachia strains in Uranotaenia species (wUra1 and wUra2) were detected in individuals from Anivorano Nord, Tsaramandroso and Bemokotra.

Arbovirus detection in mosquitoes. In total, 704 mosquitoes of species considered potential vectors of medically important arboviruses in Madagascar were screened using arbovirus-specific PCR assays. Mosquitoes were screened as either individuals or pools of 3–5 individuals from the same location and trapping method (Table 1). RVFV RNA was detected in two individually screened female adults of Cx. decens: one collected from Tsaramandroso and one from Bemokotra. We also detected RVFV in ten individual Cx. tritaeniorhynchus females and five individual Cx. antennatus females from Tsaramandroso. We sequenced RVFV PCR products from all positive individuals to confirm correct target amplification and also used CO1 sequencing to confirm mosquito species (representative samples are shown in Fig. 2a). No evidence of infection for the remaining arboviruses was present from any mosquitoes collected from the five sites in our study.

Discussion
Madagascar contains some of the most diverse and unique flora and fauna given its geographical isolation. There are currently 237 known species of mosquitoes present on the island, but recent newly described species^47 would suggest the possibility of more species present only in Madagascar. In our study, we assessed the Wolbachia prevalence of mosquito species that have the potential to be arbovirus vectors from five diverse ecological sites. To our knowledge, we are reporting for the first time resident Wolbachia strains in seven mosquito species: Ad.
**Figure 2.** Madagascar mosquito species phylogenetic analysis using the CO1 gene. (a) Maximum Likelihood phylogenetic analysis of one fragment of the CO1 gene showing a tree with the highest log likelihood (−4419.95), comprising 26 nucleotide sequences. There were a total of 669 positions in the final dataset. (b) Maximum Likelihood phylogenetic analysis for selected *Culex* mosquito species targeting a second CO1 gene fragment showing a tree with the highest log likelihood (−2078.87), comprising 22 nucleotide sequences. There were a total of 638 positions in the final dataset. Wolbachia-infected = (W+), Wolbachia-uninfected = (W−), Rift Valley fever virus-infected = (RVFV+).

... madagascarica, *Cx. antennatus*, *Cx. decens*, *Cx. duttoni*, *Fi. circumtestacea* and two *Uranotaenia* species. A limitation of this study is that sampled mosquito species were collected at night preventing an analysis of resident Wolbachia strains present in day-active mosquitoes. Further studies should be undertaken in these sites using mosquito traps such as Biogents (BG) sentinel traps that target the collection of species more active during the day such as *Ae. aegypti* and *Ae. albopictus*.

The novel resident Wolbachia strain in *Cx. decens*, named wDec, was only found in individuals collected from one location (Tsaramandroso) and an overall prevalence of 20% indicates that prevalence rates are likely to be variable across the island. *Cx. decens* is present in all biogeographic domains of Madagascar and is particularly
abundant in the central domain. In Madagascar, this species was previously shown to be infected with WNV and Babahoyo virus and in mainland Africa has been shown to be infected with additional arboviruses including Moussa virus. The Mad strain was found in all individuals we collected from . Ad. madagascarica was previously found naturally infected with WNV and is zoophilic, with a preference for feeding on avian blood, demonstrating its likely involvement in zoonotic arbovirus transmission in Madagascar. In previous entomological surveys in north-western Madagascar where WNV is endemic, Ad. madagascarica was the most abundant species and WNV RNA was detected in one pool suggesting this species may play a role in WNV maintenance or transmission.

Novel resident Wolbachia strains were also detected in two Uranotaenia species, of which there is very limited knowledge of these species in Madagascar. The lack of available CO1 sequences prevented molecular species confirmation but is endemic to Madagascar and the Comoros archipelago and is most abundant in warmer regions of the western domain. Ur. alboabdominalis is thought to occur mainly in the eastern and western domains of Madagascar and Ur. neireti in the central and eastern domains between 900–2000 m above sea level.

There is also limited knowledge of Fi. circumtestacea in Madagascar, with reports of its presence in the eastern and western domains, but this species is not currently confirmed as being involved in arbovirus transmission. As we were only able to amplify a fragment of the 16S rRNA gene for resident strains in Cx. antennatus (wAnt)
and Cx. duttoni (wDut), further studies are needed to determine if the lack of additional Wolbachia gene amplification is due to strain variability or low-density infections preventing successful amplification. An alternative explanation could be that amplification resulted from Wolbachia genes integrated into the mosquito host genomes resulting from ancient horizontal gene transfers. Although most genes transferred between Wolbachia and their hosts are non-functional in the recipient genome, expression of genes associated with Wolbachia prophage regions was previously observed in Ae. aegypti. As we extracted RNA from field caught mosquitoes, we were able to demonstrate expression of multiple Wolbachia genes (including the ftsZ cell cycle gene that plays a central role during bacterial cytokinesis and the Wolbachia surface protein gene for most strains) which would indicate amplification is more likely of bacterial gene origin rather than integrated into the host genome.

Ma. uniformis is present in all biogeographical domains of Madagascar, is considered an anthropophilic vector of numerous arboviruses such as RVFV and WNV, and is a known vector of Wuchereria bancrofti filarial nematodes in numerous countries. Resident Wolbachia strains in Ma. uniformis have previously been shown in southeast Asia, Kenya, and more recently in Sri Lanka. Interestingly in Sri Lanka, 3/3 (100%) of Ma. uniformis individuals amplified the wsp gene but in our study all seven individuals failed to amplify wsp. Ma. uniformis was also one of the four Wolbachia-infected mosquito species from populations in Kenya and this provides the only comparative MLST and reports the requirement for nested PCR to amplify hcpA. Our Wolbachia prevalence rate of 29% (7/24) for Ma. uniformis is similar to the 29% (5/19) reported in Kenyan populations suggesting this species has variable prevalence rates in wild populations.

The absence of Wolbachia infections in the Cx. pipiens complex (particularly Cx. pipiens pipiens which were confirmed by PCR and CO1 sequencing) was surprising given high infection rates of the wPip strain are often
observed in wild populations. Furthermore, our results contrast with a study undertaken in Madagascar as part of a wider Incompatible Insect Technique (IIT) from southwestern Indian Ocean islands in which the wPip strain was detected in Cx. pipiens quinquefasciatus mosquitoes from Antananarivo. This suggests the possibility of variable infection rates between Cx. pipiens and its sibling species Cx. pipiens quinquefasciatus in Madagascar populations (which we did not collect in our study). We also undertook molecular identification of three Culex individuals (also Wolbachia-uninfected) that were phylogenetically similar to Cx. rima which is consistent with a previous entomological survey that describes an unknown species morphologically similar to Cx. rima. Further phylogenetic studies are warranted to determine the diversity of Culex species and the variability of Wolbachia prevalence within the Cx. pipiens complex.

The presence of resident Wolbachia strains in mosquito vector species which can transmit human arboviruses could be influencing arboviral transmission dynamics in field populations of Madagascar. In laboratory studies, resident Wolbachia strains have been shown to impact arboviral transmission. For example, Wolbachia was shown to reduce DENV infection of salivary glands and limit transmission in Ae. albopictus. Outbreaks of RVFV in Madagascar are thought to result from infected domestic animals imported from east Africa and Cx. antennatus has previously been identified as an important RVFV vector in Madagascar. Our results would indicate Cx. tritaeniorhynchus is also likely contributing to transmission in Tsaramandroso given we also detected RVFV RNA in multiple non-blood fed females of this species. RVFV has not previously been detected in this species in Madagascar, however, Cx. tritaeniorhynchus was implicated as a major vector during a large outbreak of RVFV in Saudi Arabia so this species contribution to transmission in Madagascar could potentially be currently underestimated.

Figure 5. Maximum Likelihood molecular phylogenetic analysis from concatenation of the five MLST loci. The tree with the highest log likelihood (−9010.45) is shown incorporating 73 nucleotide sequences. There were a total of 2063 positions in the final dataset. Novel resident strains discovered and characterised in our study are highlighted in colour and denoted with circles. Concatenated sequence data from Wolbachia strains downloaded from the MLST database for comparison are shown in teal (mosquito strains) and black (all other host strains), with isolate numbers from the MLST database shown in brackets.
The detection of RVFV in three *Culex* species at Tsaramandroso and *Cx. decens* at Bemokotra would correlate with previous studies that have shown that RVFV is one of the most abundant and widely distributed arboviruses across the island and there have been several recent RVFV outbreaks. In our study, we detected RVFV in *Wolbachia*-uninfected *Cx. decens* and *Cx. tritaeniorhynchus* individuals but our single *Wolbachia*-infected *Cx. antennatus* was co-infected with RVFV. This female was non-blood fed indicating potential dissemination of RVFV beyond the bloodmeal. We were also unable to amplify any *Wolbachia* genes other than 16S rRNA from this individual which could be explained by a very low-density resident strain present in *Cx. antennatus* potentially resulting in the low prevalence rate of 3.1% (1/32).

*Wolbachia* tissue tropism influences the potential to inhibit arboviruses as some resident *Wolbachia* strains are present predominantly in reproductive tissue and have little effects on arboviruses but transinfected strains that are present in tissues such as salivary glands have strong inhibitory effects. However, as arbovirus infection rates are normally low in mosquito populations, correlating the prevalence of *Wolbachia* and RVFV would require a significantly larger number of mosquitoes.

The co-detection of resident *Wolbachia* strains and *Asaia* in seven species also indicates the potential for complex tripartite interaction with arboviruses. In our study, *Asaia* was also detected in the single *Wolbachia*-infected *Cx. antennatus* individual so further studies should be undertaken to determine the dynamics of these microbes within wild mosquito populations. The prevalence of *Asaia* is highly dependent on the environment given this...
bacterium can be acquired through various routes including from males to females during mating and from both larval and adult mosquito sugar feeding\(^7\). For example, prevalence rates of 46% were seen in field-caught *Ae. albopictus* from Madagascar\(^8\) but the overall composition and diversity of the mosquito microbiota in field populations of *Ae. aegypti* and *Ae. albopictus* in Madagascar is also highly dependent on environmental factors\(^15,19,20\). It has also recently been shown that there is no evidence for *Wolbachia-Asaia* co-infections in a wide range of *Anopheles* species and the environment likely influences *Asaia* prevalence and density in wild mosquito populations\(^9\).

The discovery of novel resident *Wolbachia* strains in mosquito species from Madagascar may also impact future attempts to extend *Wolbachia* biocontrol strategies by using these strains for applied use through trans-infection. The naturally occurring *wAlbA* and *wAlbB* strains of *Wolbachia* that infect *Ae. albopictus* have been successfully transferred to *Ae. aegypti*\(^16,18,20\) and significantly inhibit DENV\(^16,20\). This suggests the introduction of novel resident *Wolbachia* strains from other Culicine species may generate inhibitory effects on arboviruses although this will be dependent on several factors and there is variability between *Wolbachia* strains on the strength of inhibition\(^5,16,20,21\). Further experiments should elucidate both the density and tissue tropism of these novel strains to determine candidate strains for transfer to species such as *Cx. quinquefasciatus* that are major arbovirus vectors. As *Cx. quinquefasciatus* contain a resident *Wolbachia* strain (*wPip*)\(^39\), introduction of transfected strains would require a stable association, with introduced strains growing to higher densities in specific tissues which result in inhibition of pathogen transmission\(^15\). Our discovery of novel resident strains in *Culex* species closely related to *Cx. quinquefasciatus* may improve transinfection success and ultimately lead to *Wolbachia* biocontrol strategies targeting *Culex* species.

**Methods**

**Study sites.** Five study sites were chosen according to a large variety of potential ecological factors in order to sample a wide variety of mosquito species (Fig. 1, Supplementary Table S3). Sampling was undertaken in June 2016 from the village within each of the following five study areas (located in the Northern domain), Tsaramandroso and Bemokotra (Western domain), Ankazobe and Ivato Aeroport (Central domain). The village of Anivorano III (12°45′S, 49°14′19.3″E, at 375 m above sea level (asl)) is within the district of Antsiranana rural and is located 500 m south of the city of Anivorano Nord. The village consists of 32 houses distributed on both sides of the Irodo River. This river irrigates rice fields with heterogeneous rice phenology that are restricted to a small valley floor. The village of Ambomiharina (16°21′62.2″S, 46°59′34.4″E, at 84 m asl) is located in the Ambato-Boeny district within the Tsaramandroso commune. The village consists of 35 houses and is located 5 km south east of Ankafantsika Forest, surrounded by a large rice field, irrigated by numerous large streams. The village of Antafa (17°01′37.8″S, 46°45′61.7″E, at 64 m asl) is located on the Betsiboka River, at 11 km south west of the city of Maevatanana (commune Bemokotra) and consists of 193 houses. The landscape consists of Betsiboka River in the west and large dry rice fields in the south, east and north of the village. Additionally, this site is characterized by a resident population of bats that were found both in houses and flying during dusk. The village of Ambohimarina (18°19′58.6″S, 47°06′33.3″E, at 1212 m asl) is 5 km south of the city of Ankazobe (commune Ankazobe). The village consists of 289 houses, is located on a hill and surrounded with valleys composed of rice fields. The village of Ivato Imerimandroso (18°47′31.6″S, 47°28′68.4″E, at 1261 m asl) is located 300 m from Ivato Airport in the city of Antananarivo (commune Ivato Aeroport). Rice fields are flooded and surrounded with swamp and marshes. The domestic animals and livestock consist of cattle, dogs, and poultry in all five study sites.

**Mosquito sampling.** Mosquito sampling was undertaken utilizing both CDC light traps and a net trap baited with Zebu (local species of cattle) to attract zoophilic species at each of the five study sites\(^7\). Trapping was performed over two consecutive nights per site, with CDC light traps set up at dusk to be operational during sunset and overnight, before being collected at dawn. Eight CDC light traps were placed around each site nearby dwellings, near poultry coops, inside areas of denser vegetation (typically forested areas or crop fields) and near potential breeding locations which varied depending on each site. At each site, one Zebu trap was placed in a way which simulated local animal husbandry practices (i.e. inside of a shed, nearby to a large corral, or on its own tied to a post). Zebu traps were set up following sunset and mosquitoes were collected by mouth aspiration before dawn. Temperature and relative humidity were recorded at each site using Lascar Electronics data loggers and the median was calculated over the collection period.

**Morphological identification and RNA extraction.** Mosquitoes were anesthetized with chloroform vapor and morphological identification was performed using a variety of mosquito genera keys\(^7\). Following identification, mosquitoes were placed into 96 well storage plates or Eppendorf tubes and RNAlater (Sigma, UK) was added prior to storage at 4 °C or lower to prevent viral RNA degradation. RNA was extracted from individual whole mosquitoes (or additional pools for further detection of human pathogens) using QIAGEN RNeasy 96 kits according to manufacturer’s instructions. RNA was eluted in 40 μl of RNase-free water and stored at −80 °C. A QIAGEN QuantiTect Reverse Transcription kit was used to reverse transcribe RNA, generating cDNA from all RNA extracts, according to manufacturer’s instructions.

**Molecular species identification.** Sanger sequencing targeting a fragment of the mitochondrial gene cytochrome oxidase I (CO1) gene was undertaken on mosquito cDNA extracts to confirm species identification\(^41\). Selected samples morphologically identified as part of the *Cx. pipiens* complex were further analyzed using multiplex PCR targeting the ace-2 locus\(^44\). Sanger sequencing was also undertaken targeting a different fragment of the CO1 gene specifically shown to provide the optimal discrimination of species within the *Culex pipiens* complex\(^43\).
**Wolbachia screening.** Wolbachia detection was first undertaken targeting three conserved Wolbachia genes previously shown to amplify a wide diversity of strains; 16S rDNA gene, Wolbachia surface protein (wsp) gene and FtsZ cell cycle gene. DNA extracted from a Drosophila melanogaster fruit fly (infected with the wMel strain of Wolbachia) was used as a positive control, in addition to no template controls (NTCs). 16S rDNA and wsp gene PCR reactions were carried out in a Bio-Rad T100 Thermal Cycler using standard cycling conditions and PCR products were separated and visualised using 2% E-gel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-gel iBase Real-Time Transilluminator. FtsZ gene real time PCR reactions were prepared using 5 µl of FastStart SYBR Green Master mix (Roche Diagnostics), a final concentration of 1 µM of each primer, 1 µl of PCR grade water and 2 µl template DNA, to a final reaction volume of 10 µl. Prepared reactions were run on a Roche LightCycler® 96 System for 15 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 58°C for 30 seconds. Amplification was followed by a dissociation curve (95°C for 10 seconds, 65°C for 60 seconds and 97°C for 1 second) to ensure the correct target sequence was being amplified. PCR results were analysed using the LightCycler® 96 software (Roche Diagnostics). *Asaia* detection was also undertaken targeting the 16S rRNA gene.

**Wolbachia MLST.** Multilocus sequence typing (MLST) was undertaken to characterize Wolbachia strains using the sequences of five conserved genes as molecular markers to genotype each strain. In brief, 450–500 base pair fragments of the coxA, fbpA, hcpA, gatB and ftsZ Wolbachia genes were amplified from one individual from each Wolbachia-infected mosquito species using previously optimised protocols. A Culex pipiens gDNA extraction (previously shown to be infected with the wPip strain of Wolbachia) was used a positive control for each PCR run, in addition to no template controls (NTCs). If no amplification was detected using standard primers, further PCR analysis was undertaken using alternative protocols which included utilisation of M13 sequencing adaptors and/or primers with increased degeneracies.

**Sanger sequencing.** PCR products were separated and visualised using 2% E-gel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-gel iBase Real-Time Transilluminator. PCR products were submitted to Source BioScience (Source BioScience Plc, Nottingham, UK) for PCR reaction clean-up, followed by Sanger sequencing to generate both forward and reverse reads. Sequencing analysis was carried out in MEGA7 as follows. Both chromatograms (forward and reverse traces) from each sample was manually checked, analysed, and edited as required, followed by alignment by ClustalW and checking to produce consensus sequences. Consensus sequences were used to perform nucleotide BLAST (NCBI) database queries, and Wolbachia gene loci sequences were used for searches against the Wolbachia MLST database (http://pubmlst.org/wolbachia). If a sequence produced an exact match in the MLST database, we assigned the appropriate allele number.

**Phylogenetic analysis.** Maximum Likelihood phylogenetic trees were constructed from Sanger sequences as follows. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood in each case is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. The phylogeny test was by Bootstrap method with 1000 replications. Evolutionary analyses were conducted in MEGA.

**Arbovirus screening.** Arbovirus screening using published PCR assays included the major arboviruses of public health importance, suspected or having the potential of being transmitted in Madagascar (Supplementary Table S4). PCR reactions for all assays except ZIKV were prepared using 5 µl of Qiagen SYBR Green Master mix, a final concentration of 1 µM of each primer, 1 µl of PCR grade water and 2 µl template cDNA, to a final reaction volume of 10 µl. Prepared reactions were run on a Roche LightCycler® 96 System and PCR cycling conditions are described in Supplementary Table S1. Amplification was followed by a dissociation curve (95°C for 10 seconds, 65°C for 60 seconds and 97°C for 1 second) to ensure the correct target sequence was being amplified. ZIKV screening was undertaken using a Taqman probe-based assay using 5 µl of Qiagen QuantiTect probe master mix, a final concentration of 1 µM of each primer, 1 µl of PCR grade water and 2 µl template cDNA, to a final reaction volume of 10 µl. PCR results were analysed using the LightCycler® 96 software (Roche Diagnostics). Synthetic long oligonucleotide standards (Integrated DNA Technologies) of the amplified PCR product were generated in the absence of biological virus cDNA positive controls and each assay included negative (no template) controls.

**Statistical analysis.** Fisher’s exact post hoc test in Graphpad Prism 6 was used to compare *Wolbachia* and *Asaia* prevalence rates in species that had at least one individual infected with *Wolbachia*.

**Ethics approval and consent to participate.** Ethical clearance for the use of Zebu cattle in baited traps was obtained from the ethical committee of the Livestock ministry of Madagascar which is the sole relevant authority for animal care in Madagascar. The ethical committee number is: 2012/WN/Minel/3. We followed the European guidelines (European directives EU 86/609-STE123 and 2010/63/EU) for animal handling. To minimize the risk of infection of mosquito borne diseases, an internal net was used to protect the cattle against mosquito bites.
Availability of Data And Material
All data generated or analysed during this study are included in this article.

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**Author Contributions**
All authors contributed to the design of the study. S.B. facilitated the field study that was carried out by L.M.T., F.N.R. and E.H. involving mosquito capture and morphological identification in the field. C.L.J., E.H. and T.W. performed molecular analysis of samples. C.L.J. performed Sanger sequencing analysis, MLST and phylogenetic analysis. T.W. provided overall supervision. All authors read and approved final version of the manuscript.

**Additional Information**
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