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Diverse novel resident *Wolbachia* strains in Culicine mosquitoes from Madagascar

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Wolbachia endosymbiotic bacteria are widespread throughout insect species and *Wolbachia* transfected 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 *Aedeomyia*, *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.*) *quinquefasciatus*^{2–4} and *Aedes* (*Ae.*) *albopictus*^{5,6}. *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 Myanmar⁷. 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)^{8,9}. Field trials have been undertaken using IIT for both *Ae. albopictus*¹⁰ 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 viruses^{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 mosquitoes^{13–17}. *Wolbachia* strains were successfully established in *Ae. aegypti*, the principle mosquito vector of DENV, yellow fever virus (YFV) and Zika virus (ZIKV), through embryo microinjection^{15,16,18–21}. Preliminary field releases demonstrated the ability of the transfected wMel strain of *Wolbachia* to establish in wild mosquito populations²² and to provide strong inhibition of DENV in wild mosquitoes²³. 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 ZIKV^{26,28}.

There is also evidence that resident *Wolbachia* strains in laboratory mosquito colonies can inhibit arbovirus transmission^{29–32}. 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 colonisation of mosquito reproductive tissues³⁵. *Asaia* has been detected in field populations of *Cx. quinquefasciatus*³⁶ 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 *Aedeomyia* (*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 Culicines 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 genera^{37,38}, with 59% of these species thought to be endemic species in Madagascar. There have also been several arbovirus outbreaks in recent history including Rift Valley fever virus (RVFV)³⁹, DENV (2006), CHIKV (2006, 2007, 2009 & 2010) and West Nile virus (WNV)⁴⁰. Previous studies have identified 64 species in Madagascar that are implicated in transmission of medical or veterinary pathogens³⁷. In this study, we undertook an entomology survey in five ecologically diverse sites in Madagascar to determine the prevalence of resident *Wolbachia* strains in adult female mosquitoes and discovered multiple novel *Wolbachia* strains. The characterisation, typing and phylogeny of these novel *Wolbachia* strains was analysed through Sanger sequencing, with multilocus sequence typing (MLST), to determine the diversity and evolutionary history of resident strains. We also compared *Wolbachia* and *Asaia* prevalence rates and screened mosquitoes for major medically important arboviruses to investigate if there was evidence for the involvement of any of these species as potential arbovirus vectors in these sites.

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. antennatus* was collected in large numbers from Tsaramandroso and Ivato Aeroport and *Cx. univitattus* 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. Tsaramandroso 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 Aeroport 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 (Tsaramandroso, Bemokotra) and *Culex* species were collected predominantly at low and high elevation sites (Tsaramandroso, Ankazobe and Ivato Aeroport). 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. antennatus*, 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. antennatus* and *Cx. duttoni*, 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) gene⁴¹ 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. pipiens* complex indicating the absence of the wPip strain which is usually ubiquitous in *Cx. pipiens pipiens* and *Cx. pipiens quinquefasciatus* sibling species⁴². As species within the *Cx. pipiens* 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. pipiens* complex⁴³. PCR-based species identification targeting the *ace-2* gene⁴⁴ revealed the presence of *Cx. pipiens pipiens* individuals in four collection sites (no *Cx. pipiens* complex individuals were collected from Bemokotra). Confirmation resulted from CO1 phylogenetic analysis and we confirmed six *Wolbachia*-uninfected

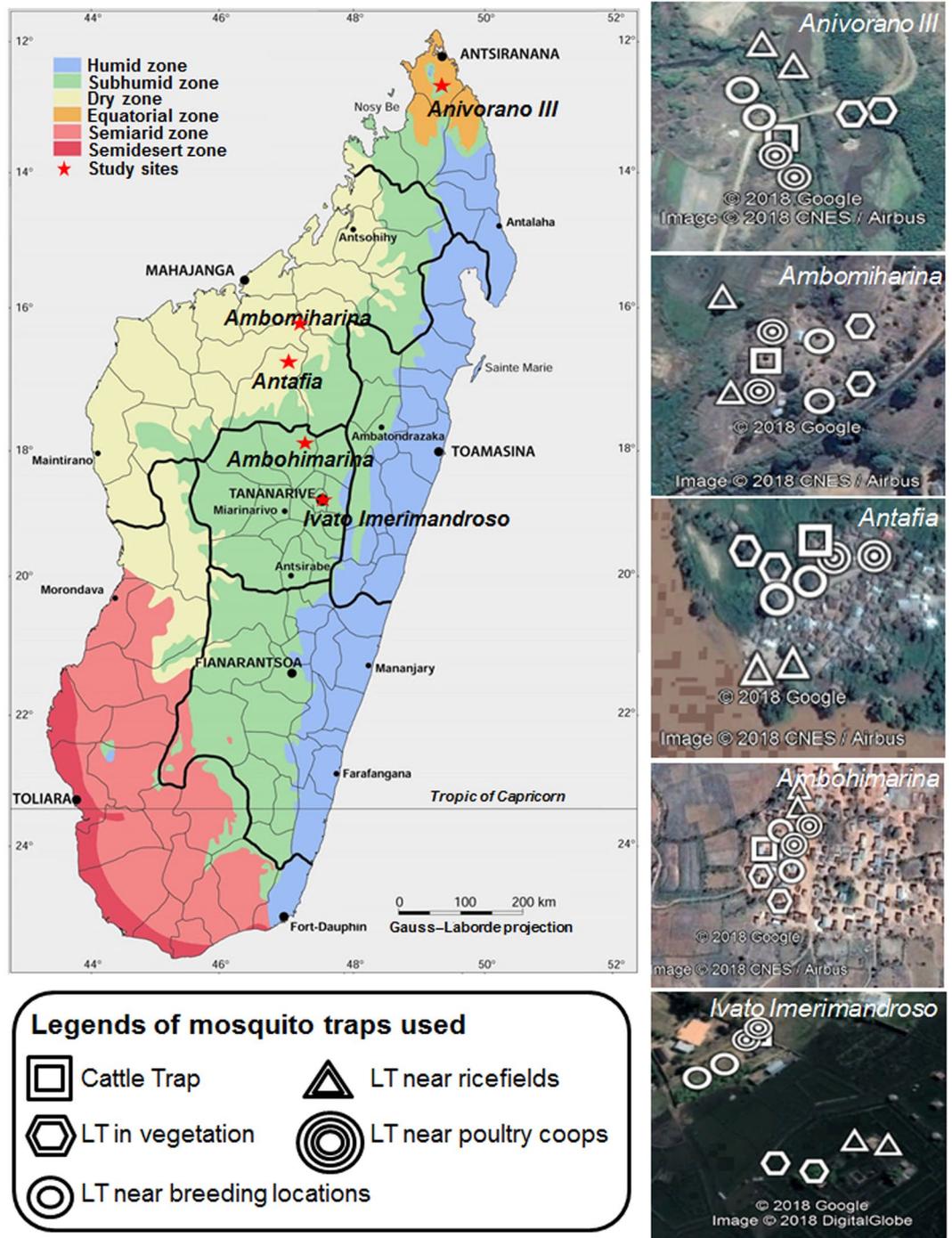


Figure 1. Map of Madagascar showing location of collection sites and mosquito traps used. Adult trapping was undertaken in five ecologically diverse sites using both CDC light traps (LT) and Cattle (zebu) baited traps (CT) near rice fields, poultry coops and breeding locations to maximize the diversity of species collected. Anivorano III is located within commune Anivorano Nord, Ambomiharina is located within commune Tsaramandroso, 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.*³⁸ 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: «Ambomiharina» 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: «Ambomiharina» 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.

	Anivorano Nord		Tsaramandroso		Bemokotra		Ankazobe	Ivato Aeroport		Total
	CT	LT	CT	LT	CT	LT	LT	CT	LT	
<i>Aedeomyia furfurea</i>	0	0	0	2	0	0	0	0	0	2
<i>Aedeomyia madagascariensis</i>	0	0	0	9	0	0	0	0	0	9
<i>Aedes aegypti</i>	0	0	0	0	0	2	0	0	0	2
<i>Aedes albocephalus</i>	0	0	0	1	0	0	0	0	0	1
<i>Aedes circumluteolus</i>	0	0	0	1	0	0	0	0	0	1
<i>Culex antennatus</i>	8	6	250	91	0	5	52	32	81	525
<i>Culex bitaeniorhynchus</i>	0	4	1	14	0	1	0	0	0	20
<i>Culex decens</i>	0	3	1	12	0	6	0	0	0	22
<i>Culex giganteus</i>	4	0	0	1	0	0	0	0	1	6
<i>Culex pipiens complex</i>	1	1	0	9	0	0	32	0	1	44
<i>Culex poicilipes</i>	0	0	2	17	0	2	1	4	0	26
<i>Culex tritaeniorhynchus</i>	0	0	25	18	0	0	0	0	0	43
<i>Culex univittatus</i>	2	6	17	95	0	5	250	0	1	376
<i>Ficalbia circumtestacea</i>	0	0	0	3	0	1	0	0	0	4
<i>Lutzia tigripes</i>	0	1	0	0	0	0	0	0	0	1
<i>Mansonia uniformis</i>	5	10	2	50	0	0	0	0	0	67
<i>Uranotaenia sp.</i>	0	5	0	23	0	3	0	0	0	31
Total	20	36	298	346	0	19	335	36	84	1174

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 and mosquitoes from CT traps were collected by mouth aspiration.

Cx. pipiens pipiens within the *Cx. pipiens* complex (Fig. 2b). We also analysed three females that were morphologically identified as *Cx. pipiens* 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. madagascariensis* (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 degeneracies⁴⁵. 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 *wUra1* 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 resolution⁴⁶, we compared the phylogenetic relationships for strains that amplified a *wsp* fragment (Fig. 4). Similar phylogenetic relationships are shown with *wsp*, with *wUra1* sequences clustering with *wMel* (Supergroup A), but the remaining strains (*wUra2*, *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 primers⁴⁷ 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).

Mosquito species	Number	Wolbachia gene PCR amplification			Prevalence (%)		Fisher's exact <i>post hoc</i> P value (Wolbachia vs. Asaia)
		16S rRNA	ftsZ	wsp	Wolbachia	Asaia	
<i>Aedes albocephalus</i>	1	0	0	0			
<i>Aedes circumlateolus</i>	1	0	0	0		100 (20–100)	
<i>Aedomyia furfuraria</i>	2	0	0	0		100 (34–100)	
<i>Aedomyia madagascariensis</i> [^]	9	9	9	9	100 (70–100)	89 (56–98)	>0.99
<i>Culex antennatus</i> [^]	32	1	1	0	3 (1–16)	56 (38–74)	>0.99
<i>Culex bitaeniorhynchus</i>	12	0	0	0		58 (32–81)	
<i>Culex decens</i> [^]	17	3	3	3	18 (4–43)	18 (4–43)	0.46
<i>Culex duttoni</i> [^]	1	1	1	0	100 (21–100)	100 (21–100)	>0.99
<i>Culex tritaeniorhynchus</i> [^]	19	0	0	0		95 (74–100)	
<i>Culex giganteus</i>	5	0	0	0		80 (37–96)	
<i>Culex pipiens complex</i>	44	0	0	0		7 (1–30)	
<i>Culex poicilipes</i>	21	0	0	0		48 (28–68)	
<i>Ficalbia circumtestacea</i>	3	1	1	0	33 (6–79)		
<i>Mansonia uniformis</i> [^]	24	7	7	0	29 (15–49)	67 (47–82)	0.65
<i>Uranotaenia spp</i> [^]	27	7	7	7	26 (13–45)	19 (8–37)	0.54

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* test 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.

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 (*hcpA* 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^{48,49}, 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. pipiens* complex). The other exception is the wUnif-Mad strain (based on 4 MLST loci, without *hcpA*), 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 *hcpA* 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* (wAnt) and *Cx. duttoni* (wDut) were from individuals collected in Tsaramandroso. The novel wMad *Wolbachia* strain was detected in all *Ad. madagascariensis* 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* (wCir) 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 species³⁷ 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.*

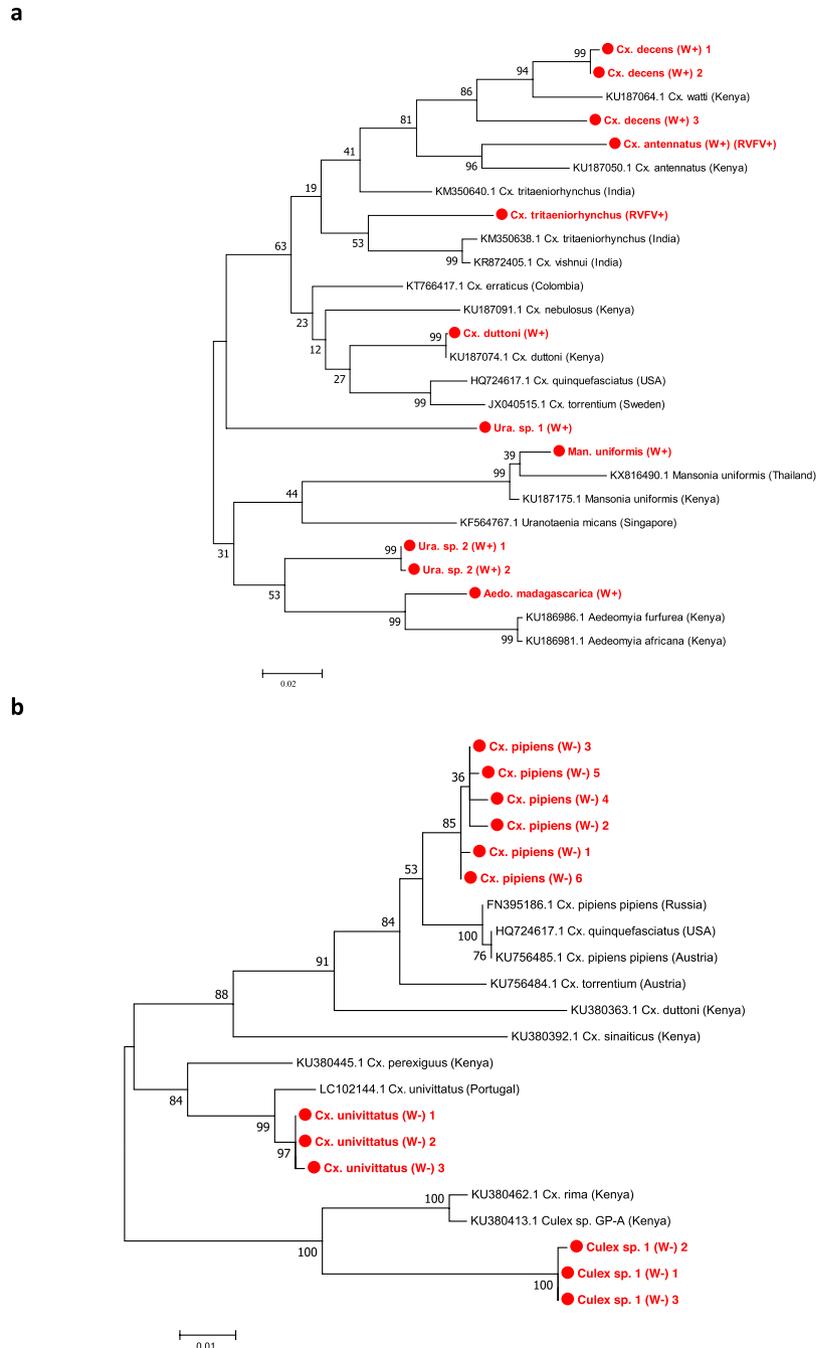


Figure 2. Madagascar mosquito species phylogenetic analysis using the CO1 gene. (a) Maximum Likelihood phylogenetic analysis of one fragment of the CO1 gene⁴¹ showing a tree with the highest log likelihood (-4419.95), comprising 26 nucleotide sequences. There were a total of 669 positions in the final dataset. (b) Maximum Likelihood phylogenetic analysis for selected *Culex* mosquito species targeting a second CO1 gene fragment⁴³ 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+).

madagascariensis, *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

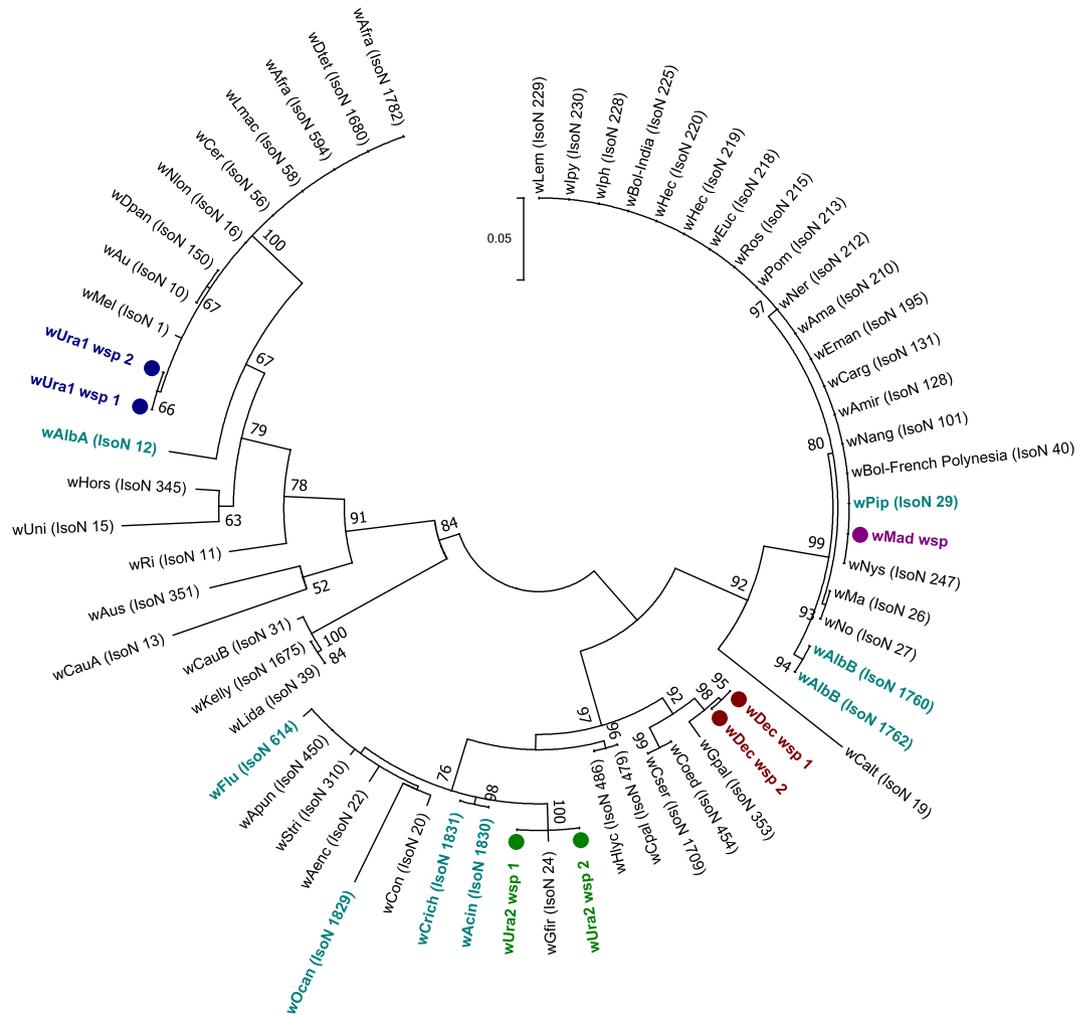


Figure 4. Maximum Likelihood phylogenetic analysis for novel *Wolbachia* strains using the *wsp* gene. The tree with the highest log likelihood (-2976.84) is shown, incorporating 62 nucleotide sequences. There were a total of 439 positions in the final dataset. Novel resident *Wolbachia* strains discovered and characterised in our study are highlighted in colour, denoted with circles and multiple *wsp* sequences from individuals of the same species are included where possible. *Wsp* 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.

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^{54,55}. Although most genes transferred between *Wolbachia* and their hosts are non-functional in the recipient genome⁵⁶, expression of genes associated with *Wolbachia* prophage regions was previously observed in *Ae. aegypti*⁵⁵. 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 RVFV⁵⁷ and WNV⁵², 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 Asia⁵⁸, Kenya⁵⁹ and more recently in Sri Lanka⁶⁰. Interestingly in Sri Lanka, 3/3 (100%) of *Ma. uniformis* individuals amplified the *wsp* gene⁶⁰ 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 Kenya⁵⁹ 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 populations⁵⁹ 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

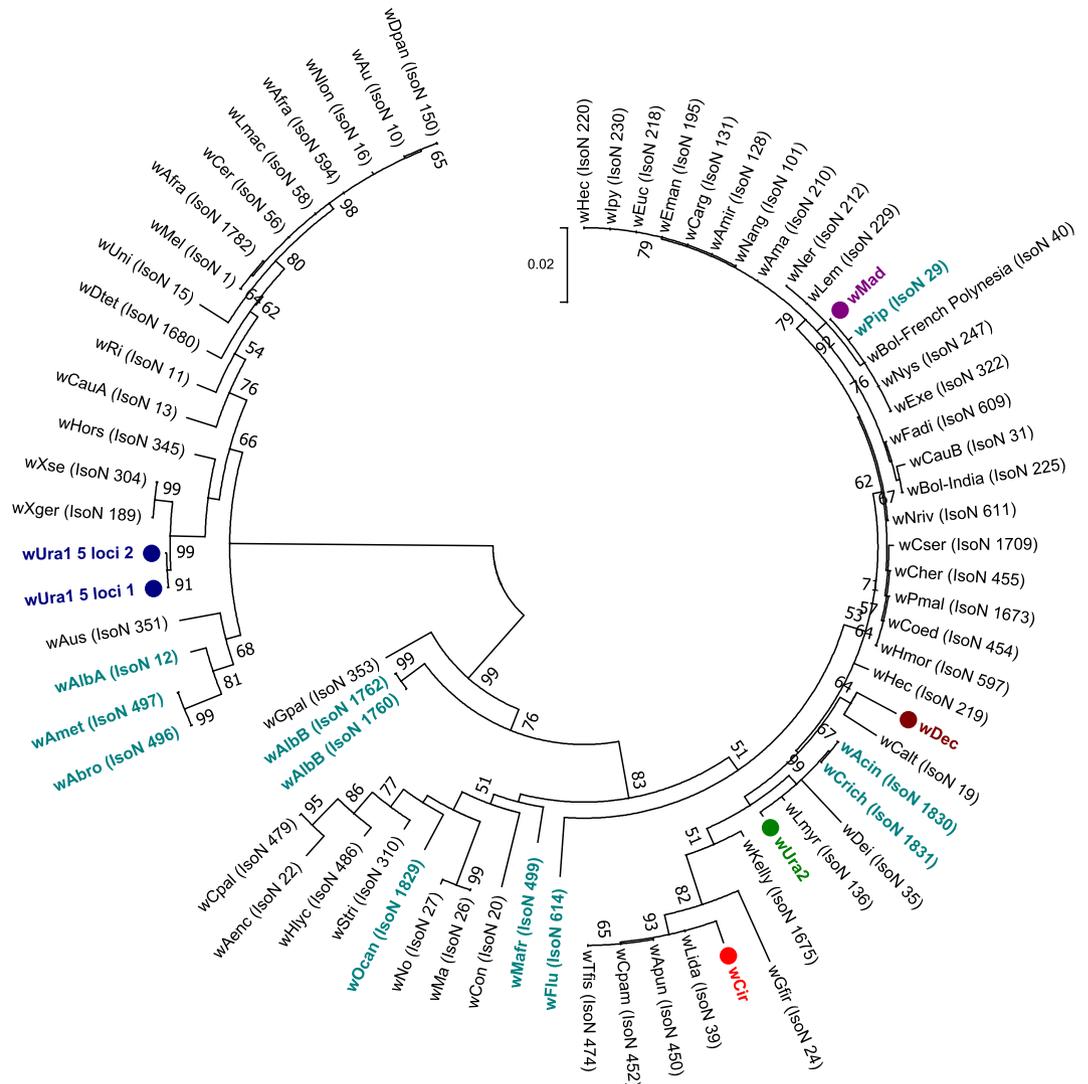


Figure 5. Maximum Likelihood molecular phylogenetic analysis from concatenation of the five MLST loci. The tree with the highest log likelihood (-9010.45) is shown incorporating 73 nucleotide sequences. There were a total of 2063 positions in the final dataset. Novel resident strains discovered and characterised 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.

observed in wild populations^{61–65}. 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 Antananarivo⁶⁶. This suggests the possibility of variable infection rates between *Cx. pipiens 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* (Fig. 2b) which is consistent with a previous entomological survey that describes an unknown species morphologically similar to *Cx. rima*⁶⁷. 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*²⁹. Outbreaks of RVFV in Madagascar are thought to result from infected domestic animals imported from east Africa⁶⁸ and *Cx. antennatus* has previously been identified as an important RVFV vector in Madagascar³⁹. 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 Arabia⁶⁹ so this species contribution to transmission in Madagascar could potentially be currently underestimated.

bacterium can be acquired through various routes including from males to females during mating and from both larval and adult mosquito sugar feeding⁷³. For example, prevalence rates of 46% were seen in field-caught *Ae. albopictus* from Madagascar⁷⁴ 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^{75,76}. 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 populations⁷⁷.

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 transinfection. 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^{15,16,20,21}. 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*)⁷⁸, introduction of transinfected strains would require a stable association, with introduced strains growing to higher densities in specific tissues which result in inhibition of pathogen transmission⁷¹. 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 one village within each of the following communes: Anivorano Nord (located in the Northern domain), Tsaramandroso and Bemokotra (Western domain), Ankazobe and Ivato Aeroport (Central domain). The village of Anivorano III (12°45'52.2"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 Ankarafantsika Forest, surrounded by a large rice field, irrigated by numerous large streams. The village of Antafia (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 sites⁷⁰. 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 keys³⁷. 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 1 (CO1) gene was undertaken on mosquito cDNA extracts to confirm species identification⁴¹. Selected samples morphologically identified as part of the *Cx. pipiens* complex were further analyzed using multiplex PCR targeting the *ace-2* locus⁴⁴. 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* complex⁴³.

Wolbachia screening. *Wolbachia* detection was first undertaken targeting three conserved *Wolbachia* genes previously shown to amplify a wide diversity of strains; 16S rDNA gene⁷⁹, *Wolbachia* surface protein (wsp) gene⁴⁶ and FtsZ cell cycle gene⁸⁰. 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^{46,79} 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^{73,81}.

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 protocols⁴⁵. A *Culex pipiens* gDNA extraction (previously shown to be infected with the wPip strain of *Wolbachia*) was used as 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 degeneracies⁴⁵.

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 MEGA7⁸² 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>)⁸³. 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 model⁸⁴. 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 MEGA7⁸².

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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