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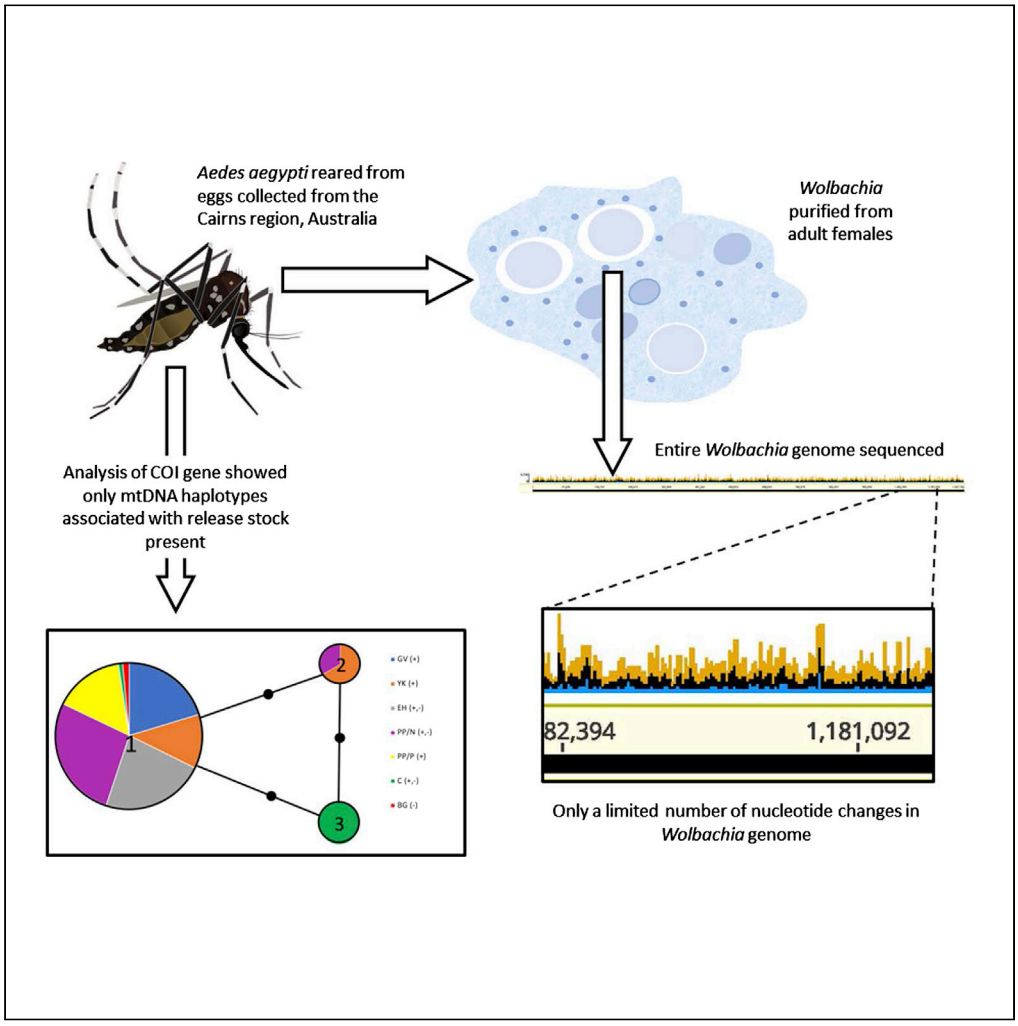
Wolbachia Genome Stability and mtDNA Variants in *Aedes aegypti* Field Populations Eight Years after Release



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HIGHLIGHTS

Long-term effect of *Wolbachia* on dengue virus blocking in *Aedes aegypti* is unknown

There were minimal changes in *Wolbachia* genomes up to 8 years post-release

Mitochondrial DNA indicated rare loss of *Wolbachia* by maternal leakage

These results suggest the stability of the *Wolbachia* genome in field populations

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Wolbachia Genome Stability and mtDNA Variants in *Aedes aegypti* Field Populations Eight Years after Release

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SUMMARY

A dengue suppression strategy based on release of *Aedes aegypti* mosquitoes infected with the bacterium *Wolbachia pipientis* is being trialed in many countries. *Wolbachia* inhibits replication and transmission of dengue viruses. Questions remain regarding the long-term stability of virus-suppressive effects. We sequenced the *Wolbachia* genome and analyzed *Ae. aegypti* mitochondrial DNA markers isolated from mosquitoes sampled 2–8 years after releases in the greater Cairns region, Australia. Few changes were detected when *Wolbachia* genomes of field mosquitoes were compared with *Wolbachia* genomes of mosquitoes obtained soon after initial releases. Mitochondrial variants associated with the initial *Wolbachia* release stock are now the only variants found in release sites, highlighting maternal leakage as a possible explanation for rare *Wolbachia*-negative mosquitoes and not migration from non-release areas. There is no evidence of changes in the *Wolbachia* genome that indicate selection against its viral-suppressive effects or other phenotypes attributable to infection with the bacterium.

INTRODUCTION

Record levels of dengue virus transmission, coupled with the recent global Zika virus emergency, reflect the difficulties confronting current insecticide-based control programs targeting *Aedes aegypti*, the primary urban vector of these viruses (Anonymous, 2019; Musso et al., 2019). An alternative strategy involving the release of mosquitoes transinfected with the *Wolbachia* bacterium is being trialed in a number of countries (Flores and O'Neill, 2018). This approach exploits two phenotypes that the maternally transmitted *Wolbachia* confers on *Ae. aegypti*: (1) inhibition of virus transmission and (2) cytoplasmic incompatibility (CI) to drive the bacterium into field populations of mosquitoes. Evidence from field releases with the *w*Mel strain in north Queensland, Australia, and the *w*AlbB strain in Kuala Lumpur, Malaysia, suggest that *Wolbachia* can be established in wild populations of *A. Aegypti*, and this has coincided with a decrease in the incidence of local dengue virus transmission (Nazni et al., 2019; Ryan et al., 2020).

The continued success of *Wolbachia*-based approaches will depend on the stability of the mosquito-bacteria-virus association, and ongoing monitoring will be essential to identify changes in any of these components or their interaction (Ritchie et al., 2018). Selection pressure may assist to maintain this complex association (Ford et al., 2019). However, evolution of the *Ae. aegypti* or *Wolbachia* genome may impact the expression of the virus inhibition or CI phenotypes, effects of *Wolbachia* on host fitness and thus maintenance of the transinfection, and rates of maternal transfer. Changes to the virus may also potentially lead to escape from blocking effects (Bull and Turelli, 2013). There is evidence for the evolution of such components in natural *Wolbachia* infections, as in the case of *Drosophila simulans* flies evolving to overcome negative effects on fecundity due to changes in *Wolbachia* or other maternally inherited components (Weeks et al., 2007) and the loss of male killing in the butterfly *Hypolimnas bolina* due to the evolution of suppressors encoded in the host DNA (Hornett et al., 2009).

Any changes in the dynamics of *Wolbachia* following introduction can also be tracked through changes in mitochondrial (mt) DNA variants associated with the *Wolbachia* at the time of release compared with variation present in background populations (Yeap et al., 2016). This includes maternal leakage, which results in loss of *Wolbachia* infection but retention of mtDNA variants, associated with releases being detected in

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Base	Genome Site	Change	Type of Change	CDS Position	ORF Effect
T	1,006,083	+T	Insertion	950	Frameshift
T	1,020,480	(T)4 -> (T)5	Insertion (tandem repeat)		
T	1,094,461	(T)2 -> (T)3	Insertion (tandem repeat)	29	Frameshift
A	1,097,797	T - > A	SNP (transversion)		
-	1,103,472	(T)4 -> (T)3	Deletion (tandem repeat)	286	Frameshift
T	1,161,856	(T)5 -> (T)6	Insertion (tandem repeat)	51	Frameshift
–	1,163,171	-C	Deletion	1146	Frameshift
–	1,177,855	-C	Deletion	370	Frameshift

Table 1. Nucleotide Differences between *Wolbachia* from the Colony Mosquitoes and the *w*Mel Reference Sequence (19)

the remaining uninfected individuals in a population, versus ongoing migration, which would introduce new mtDNA variants into this remaining uninfected component. Paternal transmission or horizontal transmission of *Wolbachia* can also be detected by the presence of new mtDNA variants in the infected component of the population (Yeap et al., 2016).

Here we apply whole-genome sequencing and analysis to determine whether there have been changes in the *Wolbachia* genome in *Ae. aegypti* populations in Cairns (Queensland, Australia) and the surrounding suburbs, the locations of the first releases in 2011 until 2017 (Hoffmann et al., 2011). We also examine mtDNA variants associated with the infected and uninfected components of the *Ae. aegypti* population in locations with different invasion histories, taking advantage of the facts that the original mtDNA variants in the release stock were relatively uncommon (frequency of 8%–13%) in the natural mosquito population where they were released (Yeap et al., 2016) and that uninfected mosquitoes continue to appear in the *Ae. aegypti* population 10 years after the releases (Hoffmann et al., 2014; Ryan et al., 2020; Schmidt et al., 2018). Taken together, these data indicate stability of the *Wolbachia* genome after establishment in the local mosquito population and an ongoing low level of maternal leakage in the *Wolbachia* population rather than migration or paternal/horizontal transmission.

RESULTS AND DISCUSSION

Genome Sequencing Reveals Minimal Change in *Wolbachia* Post-release

As a baseline sample, sequencing of the *Wolbachia* genome was performed on mosquitoes from a *w*Mel-infected *Ae. aegypti* colony that originated from mosquitoes collected in 2013 soon after the initial releases. We ascertained that there were eight sites in the genome where there were nucleotide differences between these colony mosquitoes and the *w*Mel reference sequence, which was derived from a *Drosophila melanogaster* laboratory culture (Table 1) and used in creating the original *w*Mel *Ae. aegypti* line (Walker et al., 2011). These differences could be a result of changes occurring since the *Wolbachia* was transinfected from *D. melanogaster* into the *Ae. aegypti* line as *Wolbachia* can evolve in cell lines used before microinjection (Woolfit et al., 2013). Alternatively, as many of these changes disrupted potential or known reading frames and often occurred in homopolymeric runs, they were more likely sequencing errors in the reference.

Genome sequencing was then performed on field samples. *Ae. aegypti* were reared from eggs collected in April 2019 from 23 sites in the Cairns region encompassing six suburbs where *Wolbachia*-infected mosquitoes were released between 2011 and 2017 (Figure 1). Multiple oviposition trap samples were taken at most sites (Table 2). Mosquitoes were reared from collected eggs, and pools of two to five adult female *Ae. aegypti* were formed from progeny from each trap collection. As a control location, traps were also deployed at Caravonica, a suburb where *Wolbachia*-infected mosquitoes had not been deployed. However, a pool reared from one of the Caravonica traps was positive for *Wolbachia* infection, suggesting migration or importation from a release location, and this sample was also included in the analysis. As expected, the eight sites where there were nucleotide differences in the genome of the colony mosquitoes compared with the reference were also present in the genomes of the field-collected mosquitoes (two to five adults per sample).

				Majority Nucleotide at 229,585 (%)		Majority Nucleotide at 1,174,712 (%)[a]	
Release site[b] (or Colony)	Release Date	No. Mosquitoes	No. Pools	C	A	T	C
Colony (~400 mosquitoes)	NA	400	1	100	–	100	-
Caravonica	NA	2	1	100	-	100	-
Gordonvale	January 2011	30	6	100	-	67	33
Yorkeys Knob	January 2011	5	2	100	-	100	-
Edge Hill	January 2013	25	5	80	20	80	20
Parramatta Park	January 2013	8	2	100	-	100	-
Bungalow	July 2014	5	1	100		100	-
Cairns North	August 2014	5	1	100	-	-	100
Cairns North	March 2017	15	3	67	33	-	100
Parramatta Park	March 2017	7	2	100	-	100	-

Table 2. Genome Polymorphisms Identified in *Wolbachia* Isolated from *Aedes aegypti* Collected from the Cairns Region, Northern Australia, in 2019
NA, not applicable.
[a]Silent mutation to hypothetical ORF.
[b]There were two release dates for different areas of Parramatta Park and North Cairns.

The main observation of importance was the limited number of changes in the *Wolbachia* genomes of the population sampled up to 8 years post-release. Owing to the nature of samples (i.e., pooled material), a polymorphism was defined as the detection of a nucleotide at a genome site that differed from the colony mosquitoes, and that was present in the majority of reads. There were two such polymorphisms detected. The site showing the most nucleotide change was located at genome position 1,174,712 and was a change from T to C relative to the reference sequence (Table 2). This change was silent (i.e., no amino acid change) for the predicted open reading frame (ORF) for a hypothetical gene (GeneID: 29554797). This polymorphism was present in all samples from Cairns North and was also present in Edge Hill (20% of samples) and Gordonvale (33% of samples). The former two release sites are near each other, so this polymorphism could have arisen at one of these locations and been transferred to the other through dispersal of mosquitoes. Alternatively, it could have arisen in *Wolbachia* from a geographically disparate release site and been incidentally introduced by human transport (Schmidt et al., 2018), or even arisen independently at both sites. When individual pooled samples were inspected for the presence of the T to C change, this change was detected in most samples, often at a lower frequency (Data S1. *Wolbachia* genome nucleotide variation, related to Table 2.). For all samples, the overall frequency for this change was 0–100% of reads (average 35%). Interestingly, the T to C change was also detected in the pooled colony mosquito reads at a frequency of 6%, suggesting that this polymorphism may be due to a founder effect resulting from the bottleneck produced by the mosquito release.

A second polymorphism at nucleotide site 229,585 was a C to A change relative to the reference sequence. This change would also result in a leucine to methionine change to a hypothetical 16-amino acid ORF (GeneID: 41335129). However, this is a predicted ORF whose status as a functional gene is currently unclear and biological significance is unknown. This polymorphism was present in 20% of Edge Hill samples and 33% of samples from the Cairns North sites where releases were conducted in 2017. The C to A change was present in a total of three samples with a frequency of 0–99% of reads (average 10%). The colony mosquito pooled sample had a mix of C (94%) and A (6%) at this site, similar to the other polymorphism in that the nucleotide differencing from the reference at that site (i.e., A) occurred at a low frequency. Hence, this polymorphism might also be a result of a founder effect post-release from the original mosquito release.

Nucleotide variation at two other sites on the genome was present but at lower frequencies. At genome position 184,222–184,225 (i.e., 4 nucleotides), there was a change from ACTG to TATA relative to the reference sequence (Data S1; *Wolbachia* genome nucleotide variation, Related to Table 2.). This change was in the 23S ribosomal

Figure 1. Sampling Locations in the Greater Cairns Area, North Queensland

Eggs were collected in ovitraps deployed in the suburbs of Gordonvale, Yorkeys Knob, Caravonica, Edge Hill, Cairns North, Paramatta Park, Portsmith, and Bungalow. Note that there were two releases of *Wolbachia*-infected mosquitoes in two geographically separated zones in Paramatta Park (January 2013 and March 2017) and Cairns North (August 2014 and March 2017). Samples collected from Portsmith were only included in the mitochondrial DNA analysis.

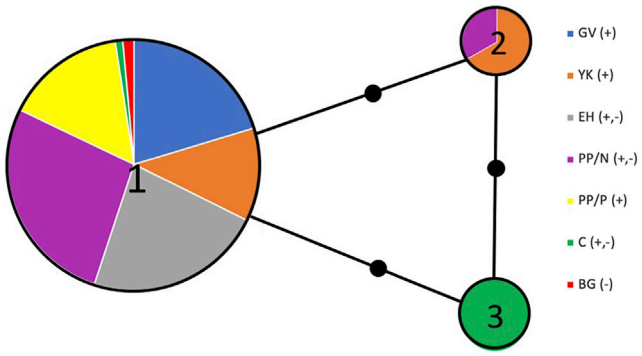

Figure 2. Haplotype Network for *COI* and Table of Frequencies

Each colored node represents an observed haplotype with circle size indicating the number of individuals with each numbered haplotype. Solid black nodes represent a single base change. Abbreviations: G, Gordonvale; YK, Yorkeys Knob; C, Caravonica; EH, Edge Hill; PP/N, Parramatta Park/Cairns North; PP/P, Parramatta Park/Portsmith; BG, Bungalow. (+), *w*Mel infected; (–), uninfected. Note that Haplotype 1 is equivalent to Haplotype 8 in Yeap et al. (2016), which was found in the *w*Mel release strain and Haplotype 2 is equivalent to Haplotype 10 in Yeap et al. (2016), which was at a low frequency in the *w*Mel release strain.

RNA sequence of the *Wolbachia* genome. The TATA sequence was present in most samples in the minority of the reads, and it varied among individual samples with a frequency of 0–24% of reads (average 6%). The significance of this low-frequency change is unclear. A fourth change was a single-nucleotide deletion present in a minority of reads (31%) in only one of the mosquito samples and, hence, was present in at least one of the five mosquitoes in that sample (Data S1; *Wolbachia* genome nucleotide variation, Related to Table 2.). This would have disrupted an ORF with predicted similarity to the "RDD family" of proteins, which have recently been functionally characterized as an $Na^+(Li^+)/H^+$ antiporter (Shao et al., 2018). The significance, if any, of the function of this protein to virus suppression is unclear. However, as this change was only observed at a low frequency in one sample, this is potentially a spontaneous mutation unrelated to selection pressure.

Analysis of Mosquito Mitochondrial DNA Reveals Only Rare Maternal Loss of *Wolbachia*

We analyzed the cytochrome oxidase I (*COI*) region in the mitochondria from both *w*Mel-infected and uninfected *Ae. aegypti* field samples from Queensland, including the same samples as tested for *Wolbachia* (Table S1). A 750-bp region (trimmed from 970 bp) on the reverse strand of the *COI* gene was compared among locations and to the original samples described in Yeap et al. (2016). Three haplotypes were identified (Figure 2, Table S2). Mosquitoes from all locations, except for the pooled negative sample from Caravonica, had haplotype 1. This includes both *Wolbachia*-positive and *Wolbachia*-negative samples from all areas at different time periods post-release (Ryan et al., 2020). An infected mosquito from Yorkeys Knob from 2018 and one uninfected mosquito from Parramatta Park/Cairns North also from 2018 had haplotype 2 (Figure 2). This haplotype had previously been collected from one infected mosquito from Yorkeys Knob in 2011 (Yeap et al., 2016) and is likely to have been present in the original release stock. We therefore did not find many other common haplotypes originally present in the population before release (Yeap et al., 2016) in the release areas, reflecting complete replacement of the mtDNA of *Ae. aegypti* by the variants present in the release stock. This effectively reflects an mtDNA sweep akin to that originally observed in natural populations of *Drosophila simulans* following a sweep by the *w*Riv *Wolbachia* infection (Hale and Hoffmann, 1990; Turelli et al., 1992). Note that we also used the genomic data to examine other variations in the mtDNA across samples, but only found one change in the ND5 gene region in one sample (210323S4) from Cairns North at genome position 6,900, when there was a change from G to A. However, this was not detected in the other Cairns North samples and the other 22 field samples. This lack of variability in the rest of the mtDNA genome is consistent with ddRAD-derived SNP data (9).

The pool of five uninfected mosquitoes from Caravonica was haplotype 3, which was also present in the original Cairns population before releases (Yeap et al., 2016). As Caravonica had not been fully invaded by *Wolbachia* at the time of collection because no releases had been carried out there, with invasion instead relying on natural *Wolbachia* spread also observed in the rest of Cairns (Schmidt et al., 2017), we expected uninfected individuals in this area to retain the original mtDNA constitution. Haplotype 3 was previously common in the original population (Yeap et al., 2016). We interpret the lack of this haplotype

in the release areas as evidence for limited migration of *Ae. aegypti* into the release locations from other uninfected areas and an ongoing low level of maternal loss of *Wolbachia* in invaded areas. This leakage is not expected to have much influence on *Wolbachia* infection levels, which are high because of ongoing cytoplasmic incompatibility, but likely reflects the effects of heat stress in some larval development containers, which can clear *Wolbachia* infections (Ross et al., 2020). The results also highlight the absence of any detectable paternal transmission or horizontal transmission in the population.

Overall, there appears to be minimal change in the *Wolbachia* genomes in mosquitoes sampled at the release sites over time. All the polymorphisms identified were rare and resulted in minor changes or occur at a low frequency. There was no clear correlation between the nucleotide frequency and release dates and locations. Thus, there seems to be little evidence for any selection process in the time since release. Importantly, the lack of change in the *Wolbachia* genome and associated mtDNA variants suggests that the virus-inhibiting and CI-inducing phenotypes are stable and that maternal transmission is imperfect, although leakage rates are relatively low, highlighting the ongoing viability of *Wolbachia*-based population replacement strategies.

Limitations of the Study

We note three limitations of the study. First, our data indicated minimal changes in the *Wolbachia* genome. Changes may also occur in the genome of the mosquito host, which could potentially reduce virus blocking. However, previous studies have indicated the persistence of cytoplasmic incompatibility post-releases, with symbiont-induced fitness cost and associated virus inhibition, which suggest a lack of evolutionary changes in host mosquitoes (Frentiu et al., 2014; Hoffmann et al., 2014). Future RNA sequencing studies will attempt to determine any changes in mosquito gene expression. Second, the focus in this study was on SNPs, but we cannot entirely rule out the possibility of large-scale genome rearrangements that may have been missed with our use of pooled mosquito samples. Finally, although the genetic diversity in our mosquito study population is comparable to that of Asia (Rasic et al., 2014), our observations are not necessarily applicable outside Australia. Hence, additional studies are required to determine whether the experience in dengue hyper-endemic regions in Asia or the Americas parallels that in Australia.

Resource Availability

Lead Contact

For additional information please contact David Warrilow (David.Warrilow@health.qld.gov.au).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Raw sequence data are available at NCBI SRA: PRJNA641232.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101572.

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AUTHOR CONTRIBUTIONS

B.H., A.A.H, S.A.R., A.F.v.d.H., and D.W. designed research; S.A.R. performed field work; B.H., Q.Y., A.F.v.d.H., and D.W. performed research; A.A.H. contributed reagents/analytic tools; B.H., A.A.H., Q.Y., and D.W. analyzed data; A.F.v.d.H. and D.W. wrote the first draft of the manuscript, and all authors approved the final version.

B.H. and Q.Y. contributed equally to this work.

DECLARATION OF INTERESTS

Although Scott Ritchie is currently employed with the World Mosquito Program, the study reported herein was conducted before commencing in that position. His current employers had no input into the design, outcomes, and interpretation of the work presented in the current study.

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Supplemental Information

Wolbachia Genome Stability and mtDNA Variants in *Aedes aegypti* Field Populations Eight Years after Release

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Supplementary Information for

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Table S1. Number of ***Aedes aegypti*** samples used for mtDNA analysis. Related to Figure 2.

Location (Suburb name)	Year *Wolbachia* infected mosquitoes released	Infection status	Year of collection		
			2011	2018	2019*
			Amplicon sequencing		Mosquito sequencing
Gordonvale	2011	+		12	30
Yorkeys Knob	2011	+	10†	12	5
Edge Hill	2013	+		20	25
		-		2	
Parramatta Park and Cairns North	2013, 2014 and 2017	+		17	28
		-		12	
Parramatta Park and Portsmith	2014 and 2017	+		20	12
Bungalow	2014	-		3	
Caravonica	No releases	+			2
		-			5

*The samples used for the 2019 analysis are the same as those used for the genome sequencing of *Wolbachia*

†Original 2011 samples from Yeap et al (Yeap et al., 2016) were included to represent the field release stock.

Table S2. Haplotype combinations for *CO*I region. Haplotypes are numbered in the first column on the left. Positions of SNPs in the 750 bp fragment, 1: **282**, 2: **531**, 3: **744;** Position underlined is the single nucleotide polymorphism that is associated with the *Wolbachia* infection. Related to Figure 2.

	1	2	3
	C	G	T
1	-	A	G
2	-	-	-
3	T	-	G

Transparent methods

Sample collection. A *w*Mel-infected colony of *Ae. aegypti* was initially started from mosquitoes collected from Cairns, Queensland, Australia in 2013, in areas where the *w*Mel infection had established. To maintain this colony, *Wolbachia*-infected females have been crossed 1-2 times a year to uninfected males sourced from recently collected field material.

Eggs of *Ae. aegypti* were collected using ovitraps deployed on 2-4 April 2019 in 6 release suburbs in Cairns and one isolated suburb where *Wolbachia* had not been released (Fig 1). Eggs from each ovitrap were hatched and larvae reared at 26 ^{o}C and 12:12 L:D. On days 5-7 post emergence, adults from each site were immobilised on wet ice, sorted into pools of up to 5 females and frozen at -80 ^{o}C. The mtDNA variation was analysed in *Ae. aegypti* individuals from the above source as well as field collections of uninfected and infected mosquitoes (Table S1) originating from eggs from ovitraps collected in 2018, with emerging adults then screened for *Wolbachia* (as in Ross et al. (Ross et al., 2020)). Note that original samples from Yeap et al (Yeap et al., 2016) were also included to represent the field release stock.

Sample processing and analysis: *w*Mel. *Wolbachia* DNA was purified from the pooled *Ae. aegypti* field material (Iturbe-Ormaetxe et al., 2011). There was a total of 23 field samples with mosquito numbers per pool of 2-5, with a mean of 4.4. A subcellular fraction was then prepared from the lysed mosquitoes which was enriched for *Wolbachia* (Iturbe-Ormaetxe et al., 2011), and DNA was extracted using the QIAamp Viral RNA extraction kit (Qiagen). Nextera XT libraries were prepared from the DNA and these were sequenced on an Illumina NextSeq500 (paired end 2 x 150 nt), obtaining approximately 5-15 million total reads per sample. The genomes were assembled from the Illumina data using *w*Mel (NC_002978.6) of total size 1,267,782 nt as a reference (Wu et al., 2004). For field samples, genome coverage varied from 99.7 – 100%. Pools from 22 of the sites in release suburbs, the positive Caravonica pool and a colony control sample produced a minimum genome coverage of 99.97% for at least one sample for each suburb. A single nucleotide polymorphism (SNP) analysis tool from the Geneious package (Kearse et al., 2012) was used to find differences between the sampled genomes and the reference. This tool was set at a minimum depth of 30 reads and a minimum frequency of 0.25 (default). Where a polymorphism was detected, this site was further analyzed in all samples to determine the within-sample nucleotide frequency.

Sample processing and analysis: mtDNA. The COI amplicon sequencing is based on Yeap et al. (Yeap et al., 2016) who identified SNP variation associated with the *Wolbachia* infection in this region (9). Samples were analysed for a 750bp region within the COI region (positions 1994-2743 on GenBank: EU352212.1) using forward primers 5'AGTTTTAGCAGGAGCAATTACTAT3' and reverse primers 5'TCCAATGCACTAATCTGCCATATTA3'. PCR amplicons from individuals were sequenced in both forward and reverse directions using Sanger Sequencing (Macrogen, Inc., Geumcheongu, Seoul, South Korea). The sequence 970bp region was analysed using Geneious package (Kearse et al., 2012) to investigate SNP variation among samples. We also lined up the rest of the mtDNA genome for the 2019 field samples where full sequence data was available. The mtDNA genomes were assembled from the Illumina data using the *A. aegypti* LVP_AGWG mitochondrial genome (NC_035159), of total size 16,790 nt, as a reference. Low-coverage positions (coverage < 3) were masked. Sequence regions within the COX2 (174bp), ATP6 (582

bp), ND1 (683 bp), ND3 (240 bp), ND4 (719 bp), ND5 (902 bp) and CYTB (968 bp) genes were analysed for SNP variation among samples.

Supplemental references

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