Loss of cytoplasmic incompatibility and minimal fecundity effects explain relatively low *Wolbachia* frequencies in *Drosophila mauritiana*

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Abstract

Maternally transmitted *Wolbachia* bacteria infect about half of all insect species. Many *Wolbachia* cause cytoplasmic incompatibility (CI), reduced egg hatch when uninfected females mate with infected males. Although CI produces a frequency-dependent fitness advantage that leads to high equilibrium *Wolbachia* frequencies, it does not aid *Wolbachia* spread from low frequencies. Indeed, the fitness advantages that produce initial *Wolbachia* spread and maintain non-CI *Wolbachia* remain elusive. *w*Mau *Wolbachia* infecting *Drosophila mauritiana* do not cause CI, despite being very similar to CI-causing *w*No from *D. simulans* (0.068% sequence divergence over 682,494 bp), suggesting recent CI loss. Using draft *w*Mau genomes, we identify a deletion in a CI-associated gene, consistent with theory predicting that selection within host lineages does not act to increase or maintain CI. In the laboratory, *w*Mau shows near-perfect maternal transmission; but we find no significant effect on host fecundity, in contrast to published data. Intermediate *w*Mau frequencies on the island Mauritius are consistent with a balance between unidentified small, positive fitness effects and imperfect maternal transmission. Our phylogenomic analyses suggest that group-B *Wolbachia*, including *w*Mau and *w*Pip, diverged from group-A *Wolbachia*, such as *w*Mel and *w*Ri, 6–46 million years ago, more recently than previously estimated.

Keywords

host-microbe interactions; introgression; maternal transmission; mitochondria; spatial spread; WO phage

INTRODUCTION

Maternally transmitted *Wolbachia* infect about half of all species throughout all major insect orders (Werren and Windsor 2000; Zug and Hammerstein 2012; Weinert et al. 2015), as well as other arthropods (Jeyaprakash and Hoy 2000; Hilgenboecker et al. 2008) and nematodes (Taylor et al. 2013). Host species may acquire *Wolbachia* from common ancestors, from sister species via hybridization and introgression, or horizontally (O'Neill et al. 1992; Rousset and Solignac 1995; Huigens et al. 2004; Baldo et al. 2008; Raychoudhury et al. 2009; Gerth and Bleidorn 2016; Schuler et al. 2016; Turelli et al. 2018). *Wolbachia* often manipulate host reproduction, inducing cytoplasmic incompatibility (CI) and male killing in *Drosophila* (Laven 1951; Yen and Barr 1971; Hoffmann et al. 1986; Hoffmann and Turelli 1997; Hurst and Jiggins 2000). CI reduces egg hatch when *Wolbachia*-uninfected females mate with infected males. Three parameters usefully approximate the frequency dynamics and equilibria of CI-causing *Wolbachia* that do not distort sex ratios: the relative hatch rate of uninfected eggs fertilized by infected males (H), the fitness of infected females relative to uninfected females (F), and the proportion of uninfected ova produced by infected females (μ) (Caspari and Watson 1959; Hoffmann et al. 1990). To spread deterministically from low frequencies, *Wolbachia* must produce $F(1 - \mu) > 1$, irrespective of CI. Once they become sufficiently common, CI-causing infections, such as *w*Ri-like *Wolbachia* in *Drosophila simulans* and several other *Drosophila* (Turelli et al. 2018), spread to high equilibrium frequencies, dominated by a balance between CI and imperfect maternal transmission (Turelli and Hoffmann 1995; Kreisner et al. 2016). In contrast, non-CI-causing *Wolbachia*, such as *w*Au in *D. simulans* (Hoffmann et al. 1996), typically persist at lower frequencies, presumably maintained by a balance between positive *Wolbachia* effects on host fitness and imperfect maternal transmission (Hoffmann and Turelli 1997; Kreisner et al. 2013). When $H < F(1 - \mu) < 1$, "bistable" dynamics result, producing stable equilibria at 0 and at a higher frequency denoted p_s, where $0.50 < p_s \quad 1$ (Turelli and Hoffmann 1995). Bistability explains the pattern and (slow) rate of spread of *w*Mel transinfected into *Aedes aegypti* to suppress the spread of dengue, Zika and other human diseases (Hoffmann et al. 2011; Barton and Turelli 2011; Turelli and Barton 2017; Schmidt et al. 2017).

In contrast to the bistability observed with *w*Mel transinfections, natural *Wolbachia* infections seem to spread via "Fisherian" dynamics with $F(1 - \mu) > 1$ (Fisher 1937; Kriesner et al. 2013; Hamm et al. 2014). Several *Wolbachia* effects could generate $F(1 - \mu) > 1$, but we do not yet know which ones actually do. For example, *w*Ri has evolved to increase *D. simulans* fecundity in only a few decades (Weeks et al. 2007), *w*Mel seems to enhance *D. melanogaster* fitness in high and low iron environments (Brownlie et al. 2009), and several *Wolbachia* including *w*Mel protect their *Drosophila* hosts from RNA viruses (Hedges et al. 2008; Teixeira et al. 2008; Martinez et al. 2014). However, it remains unknown which if any these potential fitness benefits underlie *Wolbachia* spread in nature. For instance, *w*Mel seems to have little effect on viral abundance in wild-caught *D. melanogaster* (Webster et al. 2015; Shi et al. 2018).

D. mauritiana, D. simulans and *D. sechellia* comprise the *D. simulans* clade within the nine-species *D. melanogaster* subgroup of *Drosophila*. The *D. simulans* clade diverged from *D.*

melanogaster approximately three million years ago (mya), with the island endemics *D. sechellia* (Seychelles archipelago) and *D. mauritiana* (Mauritius) thought to originate in only the last few hundred thousand years (Lachaise et al. 1986; Ballard 2000a; Dean and Ballard 2004; McDermott and Kliman 2008; Garrigan et al. 2012; Brand et al. 2013; Garrigan et al. 2014). *D. simulans* is widely distributed around the globe, but has never been collected on Mauritius (David et al. 1989; Legrand et al. 2011). However, evidence of mitochondrial and nuclear introgression supports interisland migration and hybridization between these species (Ballard 2000a; Nunes et al. 2010; Garrigan et al. 2012), which could allow introgressive *Wolbachia* transfer (Rousset and Solignac 1995).

D. mauritiana is infected with *Wolbachia* denoted *w*Mau, likely acquired via introgression from other *D. simulans*-clade hosts (Rousset and Solignac 1995). *Wolbachia* variant *w*Mau may also infect *D. simulans* (denoted *w*Ma in *D. simulans*) in Madagascar and elsewhere in Africa and the South Pacific (Ballard 2000a; Ballard 2004). *w*Mau does not cause CI in *D. mauritiana* or when transinfected into *D. simulans* (Giordano et al. 1995). Yet it is very closely related to *w*No strains that do cause CI in *D. simulans* (Merçot et al. 1995; Rousset and Solignac 1995; James and Ballard 2000, 2002). (Also, *D. simulans* seems to be a "permissive" host for CI, as evidenced by the fact that *w*Mel, which causes little CI in its native host, *D. melanogaster*, causes intense CI in *D. simulans* [Poinsot et al. 1998].) Fast et al. (2011) reported that a *w*Mau variant increased *D. mauritiana* fecundity four-fold. This fecundity effect occurred in concert with *w*Mau-induced alternations of programmed cell death in the germarium and of germline stem cell mitosis, possibly providing insight into the mechanisms underlying increased egg production (Fast et al. 2011). However, the generality of this finding across *w*Mau variants and host genetic backgrounds remains unknown.

Here, we assess the genetic and phenotypic basis of *w*Mau frequencies in *D. mauritiana* on Mauritius by combining analysis of *w*Mau draft genomes with analysis of *w*Mau transmission in the laboratory and *w*Mau effects on host fecundity and egg hatch. We identify a single mutation that disrupts a locus associated with CI. The loss of CI in *w*Mau is consistent with theory demonstrating that selection within host species does not act to increase or maintain the level of CI (Prout 1994; Turelli 1994; Haygood and Turelli 2009), but instead acts to increase $F(1 - \mu)$, the product of *Wolbachia* effects on host fitness and maternal transmission efficiency (Turelli 1994). The loss of CI helps explain the intermediate *w*Mau frequencies on Mauritius, reported by us and Giordano et al. (1995). We find no *w*Mau effects on host fecundity, and theoretical analyses show that even a two-fold fecundity increase cannot be reconciled with the observed intermediate population frequencies, unless maternal *w*Mau transmission is exceptionally unreliable in the field. Finally, we present theoretical analyses illustrating that the persistence of two distinct classes of mtDNA haplotypes among *Wolbachia*-uninfected *D. mauritiana* is unexpected under a simple null model. Together, our results contribute to understanding the genomic and phenotypic basis of global *Wolbachia* persistence, which is relevant to improving *Wolbachia*-based biocontrol of human diseases (Ritchie 2018).

MATERIALS AND METHODS

Drosophila Husbandry and Stocks

The *D. mauritiana* isofemale lines used in this study ($N = 32$) were sampled from Mauritius in 2006 by Margarita Womack and kindly provided to us by Prof. Daniel Matute from the University of North Carolina, Chapel Hill. We also obtained four *D. simulans* stocks (lines 196, 297, 298, and 299) from the National *Drosophila* Species Stock Center that were sampled from Madagascar. Stocks were maintained on modified version of the standard Bloomington-cornmeal medium (Bloomington Stock Center, Bloomington, IN) and were kept at 25°C, 12 light:12 dark photoperiod prior to the start of our experiments.

Determining *Wolbachia* infection status and comparing infection frequencies

One to two generations prior to our experiments DNA was extracted from each isofemale line using a standard 'squish' buffer protocol (Gloor et al. 1993), and infection status was determined using a polymerase chain reaction (PCR) assay (Simpliamp ThermoCycler, Applied Biosystems, Singapore). We amplified the *Wolbachia*-specific *wsp* gene (Forward: 5'-TGGTCCAATAAGTGATGAAGAAAC-3'; Reverse: 5'-AAAAATTAAACGCTACTCCA-3'; Braig et al. 1998) and a nuclear control region of the *2L* chromosome (Forward: 5'-TGCAGCTATGGTCGTTGACA-3'; Reverse: 5'-ACGAGACAATAATATGTGGTGCTG-3'; designed here). PCR products were visualized using 1% agarose gels that included a molecular-weight ladder. Assuming a binomial distribution, we estimated exact 95% binomial confidence intervals for the infection frequencies on Mauritius. Using Fisher's Exact Test, we tested for temporal differences in *w*Mau frequencies by comparing our frequency estimate to a previous estimate (Giordano et al. 1995). All analyses were performed using R version 3.5.1 (R Team 2015).

We used quantitative PCR (qPCR) (MX3000P, Agilent Technologies, Germany) to confirm that tetracycline-treated flies were cleared of *w*Mau. DNA was extracted from *D. mauritiana* flies after four generations of tetracycline treatment (1–2 generations prior to completing our experiments), as described below. Our qPCR used a PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, California, USA) and amplified *Wolbachia*-specific *wsp* (Forward: 5'-CATTGGTGTTGGTGTTGGTG-3'; Reverse: 5'-ACCGAAATAACGAGCTCCAG-3') and *Rpl32* as a nuclear control (Forward: 5'-CCGCTTCAAGGGACAGTATC-3'; Reverse: 5'-CAATCTCCTTGCGCTTCTTG-3'; Newton and Sheehan 2014).

Wolbachia DNA extraction, library preparation, and sequencing

We sequenced *w*Mau-infected *R9*, *R29*, and *R60 D. mauritiana* genotypes. Tissue samples for genomic DNA were extracted using a modified CTAB Genomic DNA Extraction protocol. DNA quantity was tested on an Implen Nanodrop (Implen, München, Germany) and total DNA was quantified by Qubit Fluorometric Quantitation (Invitrogen, Carlsbad, California, USA). DNA was cleaned using Agencourt AMPure XP beads (Beckman Coulter, Inc., Brea, CA, U.S.A), following manufacturers' instructions, and eluted in 50 µl 1× TE Buffer for shearing. DNA was sheared using a Covaris E220 Focused Ultrasonicator (Covaris Inc., Woburn, MA) to a target size of 400 bp. We prepared libraries using NEBNext® Ultra™ II DNA Library Prep with Sample Purification Beads (New England

BioLabs, Ipswich, Massachusetts). Final fragment sizes and concentrations were confirmed using a TapeStation 2200 system (Agilent, Santa Clara, California). We indexed samples using NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 3 & Index Primers Set 4), and 10 µl of each sample was shipped to Novogene (Sacramento, CA) for sequencing using Illumina HiSeq 4000 (San Diego, CA), generating paired-end, 150 bp reads.

Wolbachia assembly

We obtained published reads (N = 6) from Garrigan et al. (2014), and assembled these genomes along with the *R9*, *R29*, and *R60* genomes that we sequenced. Reads were trimmed using Sickle v. 1.33 (Joshi and Fass 2011) and assembled using ABySS v. 2.0.2 (Jackman et al. 2017). *K* values of 41, 51, 61, and 71 were used, and scaffolds with the best nucleotide BLAST matches to known *Wolbachia* sequences with E-values less than 10^{-10} were extracted as the draft *Wolbachia* assemblies. We deemed samples infected if the largest *Wolbachia* assembly was at least 1 million bases and uninfected if the largest assembly was fewer than 100,000 bases. No samples produced *Wolbachia* assemblies between 100,000 and 1 million bases. Of the six sets of published reads we analyzed (Garrigan et al. 2014), only lines *R31* and *R41* were *w*Mau-infected. We also screened the living copies of these lines for *wsp* using PCR, and both were infected, supporting reliable *w*Mau transmission in the lab since these lines were sampled in nature.

To assess the quality of our draft assemblies, we used BUSCO v. 3.0.0 to search for homologs of the near-universal, single-copy genes in the BUSCO proteobacteria database (Simao et al. 2015). As a control, we performed the same search using the reference genomes for *w*Ri (Klasson et al. 2009), *w*Au (Sutton et al. 2014), *w*Mel (Wu et al. 2004), *w*Ha (Ellegaard et al. 2013), and *w*No (Ellegaard et al. 2013).

Wolbachia gene extraction and phylogenetics

To determine phylogenetic relationships and estimate divergence times, we obtained the public *Wolbachia* group-B genomes of: *w*AlbB that infects *Aedes albopictus* (Mavingui et al. 2012), *w*Pip_Pel that infects *Culex pipiens* (Klasson et al. 2008), *w*Pip_Mol that infects *Culex molestus* (Pinto et al. 2013), *w*No that infects *Drosophila simulans* (Ellegaard et al. 2013), and *w*VitB that infects *Nasonia vitripennis* (Kent et al. 2011); in addition to group-A genomes of: *w*Mel that infects *D. melanogaster* (Wu et al. 2004), *w*Suz that infects *D. suzukii* (Siozios et al. 2013), four *Wolbachia* that infect *Nomada* bees (*w*NFe, *w*NPa, *w*NLeu, and *w*NFa; Gerth and Bleidorn 2016), and three *Wolbachia* that infect *D. simulans* (*w*Ri, *w*Au and *w*Ha; Klasson *et al.* 2009; Sutton *et al.* 2014; Ellegaard *et al.* 2013). The previously published genomes and the five *w*Mau-infected *D. mauritiana* genomes were annotated with Prokka v. 1.11, which identifies homologs to known bacterial genes (Seemann 2014). To avoid pseudogenes and paralogs, we used only genes present in a single copy, and with no alignment gaps, in all of the genome sequences. Genes were identified as single copy if they uniquely matched a bacterial reference gene identified by Prokka v. 1.11. By requiring all homologs to have identical length in all of the draft *Wolbachia* genomes, we removed all loci with indels. 153 genes, a total of 123,720 bp, met these criteria when comparing all of these genomes. However, when our analysis was restricted to the five *w*Mau genomes, our criteria were met by 686 genes, totaling 696,312 bp. Including *w*No

with the five wMau genomes reduced our set to 651 genes with 655,380 bp. We calculated the percent differences for the three codon positions within wMau and between wMau and wNo.

We estimated a Bayesian phylogram of the *Wolbachia* sequences with RevBayes 1.0.8 under the GTR + Γ model, partitioning by codon position (Höhna et al. 2016). Four independent runs were performed, which all agreed.

We estimated a chronogram from the *Wolbachia* sequences using the absolute chronogram procedure implemented in Turelli et al. (2018). Briefly, we generated a relative relaxed-clock chronogram with the GTR + Γ model with the root age fixed to 1 and the data partitioned by codon position. The relaxed clock branch rate prior was Γ(2,2). We used substitution-rate estimates of $\Gamma(7,7) \times 6.87 \times 10^{-9}$ substitutions/3$^{\text{rd}}$ position site/year to transform the relative chronogram into an absolute chronogram. This rate estimate was chosen so that the upper and lower credible intervals matched the posterior distribution estimated by Richardson et al. (2012), assuming 10 generations/year, normalized by their median estimate of 6.87×10^{-9} substitutions/3$^{\text{rd}}$ position site/year. Although our relaxed-clock analyses allow for variation in substitution rates across branches, our conversion to absolute time depends on the unverified assumption that the median substitution rate estimated by Richardson et al. (2012) for wMel is relevant across these broadly diverged *Wolbachia*. (To assess the robustness of our conclusions to model assumptions, we also performed a strict-clock analysis and a relaxed-clock analysis with branch-rate prior Γ(7,7).) For each analysis, four independent runs were performed, which all agreed. Our analyses all support wNo as sister to wMau.

We also estimated a relative chronogram for the host species using the procedure implemented in Turelli et al. (2018). Our host phylogeny was based on the same 20 nuclear genes used in Turelli et al. (2018): *aconitase, aldolase, bicoid, ebony, enolase, esc, g6pdh, glyp, glys, ninaE, pepck, pgi, pgm, pic, ptc, tpi, transaldolase, white, wingless* and *yellow*.

Analysis of *Wolbachia* and mitochondrial genomes

We looked for copy number variation (CNV) between wMau and its closest relative, wNo across the whole wNo genome. Reads from the five infected wMau lines were aligned to the wNo reference (Ellegaard et al. 2013) with bwa 0.7.12 (Li and Durbin 2009). We calculated the normalized read depth for each alignment over sliding 1,000-bp windows by dividing the average depth in the window by the average depth over the entire wNo genome. The results were plotted and visually inspected for putative copy number variants (CNVs). The locations of CNVs were specifically identified with ControlFREEC v. 11.5 (Boeva et al. 2012), using a ploidy of one and a window size of 1,000. We calculated *P*-values for each identified CNV with the Wilcoxon Rank Sum and the Kolmogorov-Smirnov tests implemented in ControlFREEC.

We used BLAST to search for pairs of CI-factor (*cif*) homologs in wMau and wNo genomes that are associated with CI (Beckmann and Fallon 2013; Beckmann et al. 2017; LePage et al. 2017; Lindsey et al. 2018; Beckmann et al. 2019). (We adopt Beckmann et al. (2019)'s nomenclature that assigns names to loci based on their predicted enzymatic function, with superscripts denoting the focal *Wolbachia* strain.) These include predicted CI-inducing

deubiquitylase (*cid*) *w*Pip_0282-*w*Pip_0283 (*cidA-cidB*[*w*Pip]) and CI-inducing nuclease (*cin*) *w*Pip_0294-*w*Pip_0295 (*cinA-cinB*[*w*Pip]) pairs that induce toxicity and rescue when expressed/co-expressed in *Saccharomyces cerevisiae* (Beckmann et al. 2017 and Beckmann et al. 2019); WD0631-WD632 (*cidA-cidB*[*w*Mel]) that recapitulate CI when transgenically expressed in *D. melanogaster* (LePage *et al.* 2017); and *w*No_RS01055 and *w*No_RS01050 that have been identified as a Type III *cifA-cifB* pair in the *w*No genome (LePage et al. 2017; Lindsey et al. 2018). *w*No_RS01055 and *w*No_RS01050 are highly diverged from *cidA-cidB*[*w*Mel] and *cidA-cidB*[*w*Pip] homologs and from *cinA-cinB*[*w*Pip]; however, this *w*No pair is more similar to *cinA-cinB*[*w*Pip] in terms of protein domains, lacking a ubiquitin-like protease domain (Lindsey et al. 2018). We refer to these loci as *cinA-cinB*[*w*No].

We found only homologs of the *cinA-cinB*[*w*No] pair in *w*Mau genomes, which we extracted from our draft *w*Mau assemblies and aligned with MAFFT v. 7 (Katoh and Standley 2013). We compared *cinA-cinB*[*w*No] to the *w*Mau homologs to identify single nucleotide variants (SNVs) among our *w*Mau assemblies.

D. mauritiana carry either the *ma*I mitochondrial haplotype, associated with *w*Mau infections, or the *ma*II haplotype (Rousset and Solignac 1995; Ballard 2000a; James and Ballard 2000). To determine the mitochondrial haplotype of each *D. mauritiana* line, we assembled the mitochondrial genomes by down-sampling the reads by a factor of 100, then assembling with ABySS 2.0.2 using a *K* value of 71 for our data (150 bp reads) and 35 for the published data (76 bp reads) (Garrigan et al. 2014). Down-sampling reads prevents the nuclear genome from assembling but does not inhibit assembly of the mitochondrial genome, which has much higher coverage. We deemed the mitochondrial assembly complete if all 13 protein-coding genes were present on the same contig and in the same order as in *D. melanogaster*. If the first attempt did not produce a complete mitochondrial assembly, we adjusted the down-sampling fraction until a complete assembly was produced for each line.

Annotated reference mitochondrial sequences for the *D. mauritiana* mitochondrial haplotypes *ma*I and *ma*II were obtained from Ballard et al. (2000b), and the 13 protein-coding genes were extracted from our assemblies using BLAST and aligned to these references. The *ma*I and *ma*II reference sequences differ at 343 nucleotides over these protein-coding regions. We identified our lines as carrying the *ma*I haplotype if they differed by fewer than five nucleotides from the *ma*I reference and as *ma*II if they differed by fewer than five nucleotides from the *ma*II reference. None of our assemblies differed from both references at five or more nucleotides.

*w*Mau phenotypic analyses

Previous analyses have demonstrated that *w*Mau does not cause CI (Giordano et al. 1995). To check the generality of this result, we reciprocally crossed *w*Mau-infected *R31 D. mauritiana* with uninfected *R4* and measured egg hatch. Flies were reared under controlled conditions at 25°C for multiple generations leading up to the experiment. We paired 1–2-day-old virgin females with 1–2-day-old males in a vial containing spoons with cornmeal media and yeast paste. After 24 hr, pairs were transferred to new spoons, and this process was repeated for five days. Eggs on each spoon were given 24 hr at 25°C to hatch after flies were removed. To test for CI, we used nonparametric Wilcoxon tests to compare egg hatch

between reciprocal crosses that produced at least 10 eggs. All experiments were carried out at 25°C with a 12 light:12 dark photoperiod.

To determine if *w*Mau generally enhances *D. mauritiana* fecundity, we assessed the fecundity of two *w*Mau-infected isofemale lines from Mauritius (*R31* and *R41*); we also reciprocally introgressed *w*Mau from each of these lines to assess host effects. To do this we crossed *R31* females with *R41* males and backcrossed F_1 females to *R41* males—this was repeated for four generations to generate the reciprocally introgressed $R41^{R31}$ genotypes (*w*Mau variant denoted by superscripts). A similar approach was taken to generate $R31^{R41}$ genotypes. This approach has previously revealed *D. teissieri*-host effects on *w*Tei-induced CI (Cooper et al. 2017). To assay fecundity, we reciprocally crossed each genotype (*R31, R41,* $R31^{R41},$ $R31^{R41}$) to uninfected line *R4* to generate paired infected-and uninfected-F_1 females with similar genetic backgrounds. The *w*Mau-infected and uninfected F_1 females were collected as virgins and placed in holding vials. We paired 3–7-day-old females individually with an uninfected-*R4* male (to stimulate oviposition) in vials containing a small spoon filled with standard cornmeal medium and coated with a thin layer of yeast paste. We allowed females to lay eggs for 24 hours, after which pairs were transferred to new vials. This was repeated for five days. At the end of each 24-hr period, spoons were frozen until counted. All experiments were carried out at 25°C with a 12 light:12 dark photoperiod.

We also measured egg lay of *w*Mau-infected (*R31*) and tetracycline-cleared uninfected (*R31-tet*) genotypes over 24 days, on apple-agar plates, to more closely mimic the methods of Fast et al. (2011). We fed flies 0.03% tetracycline concentrated medium for four generations to generate the *R31-tet* genotype. We screened F_1 and F_2 individuals for *w*Mau using PCR, and we then fed flies tetracycline food for two additional generations. In the fourth generation, we assessed *w*Mau titer using qPCR to confirm that each genotype was cleared of *w*Mau infection. We reconstituted the gut microbiome by rearing *R31-tet* flies on food where *R31* males had fed and defecated for 48 hours. Flies were given at least three more generations to avoid detrimental effects of tetracycline treatment on mitochondrial function (Ballard and Melvin 2007). We then paired individual 6–7-day-old virgin *R31* ($N =$ 30) and *R31-tet* ($N =$ 30) females in bottles on yeasted apple-juice agar plates with an *R4* male to stimulate oviposition. Pairs were placed on new egg-lay plates every 24 hrs. After two weeks, we added one or two additional *R4* males to each bottle to replace any dead males and to ensure that females were not sperm limited as they aged.

We used nonparametric Wilcoxon tests to assess *w*Mau effects on host fecundity. We then estimated the fitness parameter *F* in the standard discrete-generation model of CI (Hoffmann et al. 1990; Turelli 1994). We used the '*pwr.t2n.test*' function in the '*pwr*' library in R to assess the power of our data to detect increases to *F*. Pairs that laid fewer than 10 eggs across each experiment were excluded from analyses, but our results are robust to this threshold.

To estimate the fidelity of maternal transmission, *R31* and *R41* females were reared at 25°C for several generations prior to our experiment. In the experimental generation, 3–5 day old inseminated females were placed individually in vials that also contained two males. These *R31* ($N =$ 17) and *R41* ($N =$ 19) sublines were allowed to lay eggs for one week. In the

following generation we screened F1 offspring for *w*Mau infection using PCR as described above.

RESULTS

Wolbachia infection status

Out of 32 *D. mauritiana* lines that we analyzed, 11 were infected with *w*Mau *Wolbachia* (infection frequency = 0.34; binomial confidence interval: 0.19, 0.53). In contrast, none of the *D. simulans* stocks ($N = 4$) sampled from Madagascar were infected, precluding our ability to directly compare *w*Mau and *w*Ma. Our new *w*Mau frequency estimate is not statistically different from a previous estimate (Giordano et al. 1995: infection frequency, 0.46; binomial confidence interval, (0.34, 0.58); Fisher's Exact Test, $P = 0.293$), based largely on assaying a heterogenous collecton of stocks in various laboratories. These relatively low infection frequencies are consistent with theoretical expectations given that *w*Mau does not cause CI (Giordano et al. 1995; our data reported below). The intermediate *w*Mau frequencies on Mauritius suggest that *w*Mau persists at a balance between positive effects on host fitness and imperfect maternal transmission. Quantitative predictions, based on the idealized model of Hoffmann and Turelli (1997), are discussed below. The maintenance of *w*Mau is potentially analogous to the persistence of other non-CI-causing *Wolbachia*, specifically *w*Au in some Australian populations of *D. simulans* (Hoffmann et al. 1996; Kriesner et al. 2013) and *w*Suz in *D. suzukii* and *w*Spc in *D. subpulchrella* (Hamm et al. 2014; Conner et al. 2017; Turelli et al. 2018; but see Cattel et al. 2018).

Draft *w*Mau genome assemblies and comparison to *w*No

The five *w*Mau draft genomes we assembled were of very similar quality (Supplemental Table 1). N50 values ranged from 60,027 to 63,676 base pairs, and our assemblies varied in total length from 1,266,004 bases to 1,303,156 bases (Supplemental Table 1). Our BUSCO search found exactly the same genes in each draft assembly, and the presence/absence of genes in our *w*Mau assemblies was comparable to those in the complete genomes used as controls (Supplemental Table 2). In comparing our five *w*Mau draft genomes over 694 single-copy, equal-length loci comprising 704,613 bp, we found only one SNP. Four sequences (*R9, R31, R41* and *R60*) are identical at all 704,613 bp. *R29* differs from them at a single nucleotide, a nonsynonymous substitution in a locus which Prokka v. 1.11 annotates as "bifunctional DNA-directed RNA polymerase subunit beta/beta."

Comparing these five *w*Mau sequences to the *w*No reference (Ellegaard et al. 2013) over 671 genes with 682,494 bp, they differ by 0.068% overall, with equivalent divergence at all three codon positions (0.067%, 0.061%, and 0.076%, respectively).

Wolbachia phylogenetics

As expected from the sequence comparisons, our group-B phylogram places *w*Mau sister to *w*No (Figure 1A). This is consistent with previous analyses using fewer loci that placed *w*Mau (or *w*Ma in *D. simulans*) sister to *w*No (James and Ballard 2000; Zabalou et al. 2008; Toomey et al. 2013). Our chronogram (Figure 1B) estimates the 95% credible interval for the split between the group-B versus group-A *Wolbachia* strains as 6 to 36 mya (point

estimate, 16 mya). Reducing the variance on the substitution-rate-variation prior by using $\Gamma(7,7)$ rather than $\Gamma(2,2)$, changes the credible interval for the A-B split to 8 to 46 mya (point estimate, 21 mya). In contrast, a strict clock analysis produces a credible interval of 12 to 64 mya (point estimate, 31 mya). These estimates are roughly comparable to an earlier result based on a general approximation for the synonymous substitution rate in bacteria (Ochman and Wilson 1987) and data from only the *ftsZ* locus (59–67 mya, Werren et al. 1995). However, our estimates are much lower than an alternative estimate based on comparative genomics (217 mya, Gerth and Bleidorn 2016). We discuss this discrepancy below.

The observed divergence between *w*No and *w*Mau is consistent across all three codon positions, similar to other recent *Wolbachia* splits like that between *w*Ri and *w*Suz (Turelli et al. 2018). Conversely, observed divergence at each codon position generally varies across the chronogram, leading to inflation of the *w*No-*w*Mau (181,476 years; credible interval = 27,711 to 701,205 years; Figure 1B) and *w*Ri-*w*Suz (16,214; credible interval = 1,254 to 70,584) divergence point estimates; the latter is about 1.6 times as large as the value in Turelli et al. (2018). (Nevertheless, the confidence intervals of our and Turelli et al. (2018)'s *w*Ri-*w*Suz divergence estimates overlap.) To obtain an alternative estimate of *w*No-*w*Mau divergence, we estimated divergence time using the observed third-position pairwise divergence (0.077%, or 0.039% from tip to MRCA) and Richardson et al. (2012)'s estimate of the "short-term evolutionary rate" of *Wolbachia* third-position divergence within *w*Mel. This approach produces a point estimate of 57,000 years, with a credible interval of 30,000 to 135,000 years for the *w*No-*w*Mau split. In Cooper et al. (2019), we address in detail how a constant substitution-rate ratio among codon positions across the tree, assumed by the model, affects these estimates.

Analysis of *Wolbachia* and mitochondrial genomes

We looked for CNVs in *w*Mau relative to sister *w*No by plotting normalized read depth along the *w*No genome. There were no differences in copy number among the *w*Mau variants, but compared to *w*No, ControlFREEC identified four regions deleted from all *w*Mau that were significant according to the Wilcoxon Rank Sum and Kolmogorov-Smirnov tests (Figure 2 and Supplemental Table 3). These deleted regions of the *w*Mau genomes include many genes, including many phage-related loci, providing interesting candidates for future work (listed in Supplementary Table 4).

To test the hypothesis that *cif* loci are disrupted, we searched for pairs of loci known to be associated with CI and found homologs to the *cinA-cinB*$^{w\mathrm{No}}$ pair in each of our draft assemblies, but we did not find homologs to the *cidA-cidB*$^{w\mathrm{Mel}}$, *cidA-cidB*$^{w\mathrm{Pip}}$, or to the *cinA-cinB*$^{w\mathrm{Pip}}$ pairs. There were no variable sites in *cinA-cinB*$^{w\mathrm{No}}$ homologs among our five *w*Mau assemblies. Relative to *w*No, all *w*Mau variants share a one base pair deletion at base 1133 out of 2091 (amino acid 378) in the *cinB*$^{w\mathrm{No}}$ homolog. This frameshift introduces over 10 stop codons, with the first at amino acid 388, potentially making this predicted CI-causing-toxin protein nonfunctional. We also identified a nonsynonymous substitution in amino acid 264 of the *cinB*$^{w\mathrm{No}}$ homolog (*w*No codon ACA, Thr; *w*Mau codon AUA, Ile) and two SNVs in the region homologous to *cinA*$^{w\mathrm{No}}$: a synonymous substitution in amino

acid 365 (*w*No codon GUC, *w*Mau codon GUU) and a nonsynonymous substitution in amino acid 397 (*w*No codon GCU, amino acid Ala; *w*Mau codon GAU, amino acid Asp). Disruption of CI is consistent with theoretical analyses showing that selection within a host species does not act directly on the level of CI (Prout 1994; Turelli 1994; Haygood and Turelli 2009). Future functional analyses will determine whether disruption of regions homologous to *cinA-cinB*$^{w\text{No}}$ underlie the lack of *w*Mau CI.

Of the *D. mauritiana* lines tested (*N* = 9), one line (uninfected-*R44*) carries the *ma*II mitochondrial haplotype, while the other eight carry *ma*I. Rousset and Solignac (1995) reported a similar *ma*II frequency, with 3 of 26 lines sampled in 1985 carrying *ma*II. The *ma*I and *ma*II references differ by 343 SNVs across the proteome, and *R44* differs from the *ma*II reference by 4 SNVs in the proteome. Four of our *ma*I lines (*R23*, *R29*, *R32*, and *R39*) are identical to the *ma*I reference, while three (*R31*, *R41*, and *R60*) have one SNV and one (*R9*) has two SNVs relative to *ma*I reference. One SNV is shared between *R9* and *R60*, but the other three SNVs are unique. Our results agree with past analyses that found *w*Mau is perfectly associated with the *ma*I mitochondrial haplotype (Rousset and Solignac 1995; Ballard 2000a; James and Ballard 2000). The presence of *ma*II among the uninfected is interesting. In contrast to *ma*I, which is associated with introgression with *D. simulans* (Ballard 2000a; James and Ballard 2000), *ma*II appears as an outgroup on the mtDNA phylogeny of the *D. simulans* clade and is not associated with *Wolbachia* (Ballard 2000b, Fig. 5; James and Ballard 2000). Whether or not *Wolbachia* cause CI, if they are maintained by selection-imperfect-transmission balance, we expect all uninfected flies to eventually carry the mtDNA associated with infected mothers (Turelli et al. 1992). We present a mathematical analysis of the persistence of *ma*II below.

Analysis of *w*Mau phenotypes

In agreement with Giordano et al. (1995), we found no difference between the egg hatch of uninfected females crossed to *w*Mau-infected males (0.34 ± 0.23 SD, *N* = 25) and the reciprocal cross (0.29 ± 0.28 SD, *N* = 24), indicating no CI. In contrast to Fast et al. (2011), we find no evidence that *w*Mau affects *D. mauritiana* fecundity (Supplemental Table 5 and Figure 3), regardless of host genetic backgrounds. Across both experiments assessing *w*Mau fecundity effects in their natural backgrounds (*R31* and *R41*), we counted 27,221 eggs and found no difference in the number of eggs laid by infected (mean = 238.20, SD = 52.35, *N* = 60) versus uninfected (mean = 226.82, SD = 67.21, *N* = 57) females over the five days of egg lay (Wilcoxon test, *W* = 1540.5, *P* = 0.357); and across both experiments that assessed *w*Mau fecundity effects in novel host backgrounds (*R31*^{R41} and *R41*^{R31}), we counted 30,358 eggs and found no difference in the number of eggs laid by infected (mean = 253.30, SD = 51.99, *N* = 60) versus uninfected (mean = 252.67, SD = 63.53, *N* = 60) females over five days (Wilcoxon test, *W* = 1869.5, *P* = 0.719). [The mean number of eggs laid over five days, standard deviation (SD), sample size (*N*), and *P*-values from Wilcoxon tests are presented in Supplemental Table 5 for all pairs.]

We sought to determine if *w*Mau fecundity effects depend on host age with a separate experiment that assessed egg lay over 24 days on apple-agar plates, similar to Fast et al. (2011). Across all ages, we counted 9,459 eggs and found no difference in the number of

eggs laid by infected (mean = 156.29, SD = 138.04, N = 28) versus uninfected (mean = 187.70, SD = 168.28, N = 27) females (Wilcoxon test, W = 409, P = 0.608) (Figure 4). While our point estimates indicate that wMau does not increase host fecundity, egg lay was generally lower and more variable on agar plates relative to our analyses of egg lay on spoons described above.

With these data, we estimated the fitness parameter F in the standard discrete-generation model of CI (Hoffmann et al. 1990; Turelli 1994). Taking the ratio of replicate mean fecundity observed for wMau-infected females to the replicate mean fecundity of uninfected females in naturally sampled $R31$ and $R41$ $D.$ $mauritiana$ backgrounds, we estimated F = 1.05 (95% BC$_a$ interval: 0.96, 1.16). Following reciprocal introgression of wMau and host backgrounds (i.e., the $R31^{R41}$ and $R41^{R31}$ genotypes), we estimated F = 1.0 (95% BC$_a$ interval: 0.93, 1.09). Finally, across all 24 days of our age-effects experiment, we estimated F = 0.83, 95% BC$_a$ interval: 0.52, 1.32) for $R31$, which overlaps with our estimate of F for $R31$ in our initial experiment (Table 1). BC$_a$ confidence intervals were calculated using the two-sample acceleration constant given by equation 15.36 of Efron and Tibshirani (1993). (Estimates of F and the associated BC$_a$ confidence intervals are reported in Table 1 for each genotype and condition.) Consistent with our other analyses, we find little evidence that wMau significantly increases fecundity. However, our data do not have much statistical power to detect values of F on the order of 1.05, which may suffice to produce $F(1 - \mu) > 1$ and deterministic spread of wMau from low frequencies. We present our power calculations in Figure 1B of the Supplementary Information.

Finally, we assessed the fidelity of wMau maternal transmission under standard laboratory conditions. We excluded sublines that produced fewer than 8 F1 offspring. In all cases, $R31$ (N = 17) sublines produced offspring that were all infected, indicating perfect maternal transmission. In contrast, one $R41$ subline produced one uninfected individual out of a total of 18 F1 offspring produced; all other $R41$ sublines meeting our criteria (N = 15) produced only infected F1 offspring, resulting in nearly perfect maternal transmission across all $R41$ sublines (μ = 0.0039).

Mathematical analyses of *Wolbachia* frequencies and mtDNA polymorphism

If *Wolbachia* do not cause CI (or any other reproductive manipulation), their dynamics can be approximated by a discrete-generation haploid selection model. Following Hoffmann and Turelli (1997), we assume that the relative fecundity of *Wolbachia*-infected females is F, but a fraction μ of their ova do not carry *Wolbachia*. Given our ignorance of the nature of *Wolbachia*'s favorable effects, the F represents an approximation for all fitness benefits. If $F(1 - \mu) > 1$, the equilibrium *Wolbachia* frequency among adults is

$$\hat{p} = 1 - \frac{\mu F}{F - 1}. \quad (1)$$

Imperfect maternal transmisson has been documented for field-collected *D. simulans* infected with wRi (Hoffmann and Turelli 1988; Turelli and Hoffmann 1995; Carrington et al. 2011), *D. melanogaster* infected with wMel (Hoffmann et al. 1998) and *D. suzukii*

infected with wSuz (Hamm et al. 2014). The estimates range from about 0.01 to 0.1. Given that we have documented imperfect maternal transmission of wMau in the laboratory, we expect (more) imperfect transmission in nature (Turelli and Hoffmann 1995; Carrington et al. 2011). In order for the equilibrium *Wolbachia* frequency to be below 0.5, approximation (1) requires that the relative fecundity of infected females satisfies

$$F < \frac{1}{1 - 2\mu}. \quad (2)$$

Thus, even for μ as large a 0.15, which greatly exceeds our laboratory estimates for wMau and essentially all estimates of maternal transmission failure from nature, *Wolbachia* can increase fitness by at most 43% and produce an equilibrium frequency below 0.5 (Supplemental Figure 1A). Conversely, (1) implies that a doubling of relative fecundity by *Wolbachia* would produce an equilibrium frequency $1 - 2\mu$. If μ 0.25, consistent with all available data, the predicted equilibrium implied by a *Wolbachia*-induced fitness doubling significantly exceeds the observed frequency of wMau. Hence, a four-fold fecundity effect, as described by Fast et al. (2011), is inconsistent with the frequency of wMau in natural populations of *D. mauritiana*. Field estimates of μ for *D. mauritiana* will provide better theory-based bounds on wMau fitness effects that would be consistent with wMau tending to increase when rare on Mauritius, i.e., conditions for $F(1 - \mu) > 1$.

Our theoretical analysis, addressing the plausibility of a four-fold fitness increase caused by wMau, assumes that the observed frequency of wMau approximates selection-transmission equilibrium, as described by (1). With only two frequency estimates (one from a heterogeneous collection of laboratory stocks), we do not know that the current low frequency is temporarlly stable. Also, we do not know that the mutations we detect in *cinA-cinB*[wNo] homologs are responsible for the lack of wMau CI. One alternative is that *D. mauritiana* has evolved to suppress CI (for host suppression of male killing, see Hornet et al. 2006 and Vanthournout and Hendrickx 2016). Host suppression of CI is expected (Turelli 1994), and it may explain the low CI caused by wMel in *D. melanogaster* (Hoffmann and Turelli 1997). However, the fact that wMau does not produce CI in *D. simulans*, a host that allows wMel and other strains to induce strong CI even though little CI is produced in their native hosts, argues against host suppression as the explanation for the lack of CI caused by wMau in *D. mauritiana*. Nevertheless, the loss of CI from wMau may be quite recent; and wMau may be on its way to elimination in *D. mauritiana*. If so, our equilibrium analysis is irrelevant – but this gradual-loss scenario is equally inconsistent with the four-fold fecundity effect proposed by Fast et al. (2011).

As noted by Turelli et al. (1992), if *Wolbachia* is introduced into a population along with a diagnostic mtDNA haplotype that has no effect on fitness, imperfect *Wolbachia* maternal transmission implies that all infected and uninfected individuals will eventually carry the *Wolbachia*-associated mtDNA, because all will have had *Wolbachia*-infected maternal ancestors. We conjectured that a stable mtDNA polymorphism might be maintained if *Wolbachia*-associated mtDNA introduced by introgression is deleterious in its new nuclear background. We refute our conjecture in Appendix 1. We show that the condition for

Wolbachia to increase when rare, $F(1 - \mu) > 1$, ensures that the native mtDNA will be completely displaced by the Wolbachia-associated mtDNA, even if it lowers host fitness once separated from Wolbachia.

How fast is the mtDNA turnover, among Wolbachia-uninfected individuals, as a new Wolbachia invades? This is easiest to analyze when the mtDNA introduced with Wolbachia has no effect on fitness, so that the relative fitness of Wolbachia-infected versus uninfected individuals is F, irrespective of the mtDNA haplotype of the uninfected individuals. As shown in Appendix 1, the frequency of the ancestral mtDNA haplotype among uninfected individuals, denoted r_t, declines as

$$r_{t + 1} = r_t/[F(1 - \mu)] . \quad (3)$$

Assuming $r_0 = 1$, recursion (3) implies that even if $F(1 - \mu)$ is only 1.01, the frequency of the ancestral mtDNA haplotype should fall below 10^{-4} after 1000 generations. A much more rapid mtDNA turnover was seen as the CI-causing wRi swept northward through California populations of D. simulans (Turelli et al. 1992; Turelli and Hoffmann 1995). Thus, it is theoretically unexpected, under this simple model, that mtDNA haplotype maII, which seems to be ancestral in D. mauritiana (Rousset and Solignac 1995; Ballard 2000a), persists among Wolbachia-uninfected D. mauritiana, given that all sampled Wolbachia-infected individuals carry maI. However, spatial variation in fitnesses is one possible explanation for this polymorphism (Gliddon and Strobeck 1975), which has persisted since at least 1985.

DISCUSSION

wMau is sister to wNo and diverged from group-A Wolbachia less than 100 mya

Our phylogenetic analyses place wMau sister to wNo, in agreement with past analyses using fewer data (James and Ballard 2000; Zabalou et al. 2008; Toomey et al. 2013). The relationships we infer agree with those from recently published phylograms (Gerth and Bleidorn 2016; Lindsey et al. 2018) (Figure 1A).

Depending on the prior used for substitution-rate variation, we estimate that wMau and other group-B Wolbachia diverged from group-A strains about 6–46 mya. This is roughly consistent with a prior estimate using only ftsZ (58–67 mya, Werren et al. 1995), but is inconsistent with a recent estimate using 179,763 bases across 252 loci (76–460 mya, Gerth and Bleidorn 2016). There are several reasons why we question the Gerth and Bleidorn (2016) calibration. First, Gerth and Bleidorn (2016)'s chronogram placed wNo sister to all other group-B Wolbachia, in disagreement with their own phylogram (Gerth and Bleidorn 2016, Figure 3). In contrast, our phylogram and that of Lindsey et al. (2018) support wAlbB splitting from all other strains at this node. Second, the Gerth and Bleidorn (2016) calibration estimated the split between wRi that infects D. simulans and wSuz that infects D. suzukii at 900,000 years. This estimate is more than an order of magnitude higher than ours (16,214 years) and nearly two orders of magnitude higher than the 11,000 year estimate of Turelli et al. (2018) who found 0.014% third position divergence between wRi and wSuz (i.e., 0.007% along each branch) over 506,307 bases. Raychoudhury et al. (2009) and

Richardson et al. (2012) both estimated a rate of about 7×10^{-9} substitutions/3rd position site/year between *Wolbachia* in *Nasonia* wasps and within *w*Mel, respectively. An estimate of 900,000 years requires a rate about 100 times slower, 7.8×10^{-11} substitutions/3rd position site/year, which seems implausible. Finally, using data kindly provided by Michael Gerth, additional analyses indicate that the third-position rates required for the *Wolbachia* divergence times estimated by Gerth and Bleidorn (2016) between *Nomada flava* and *N. leucophthalma* (1.72×10^{-10}), *N. flava* and *N. panzeri* (3.78×10^{-10}) (their calibration point), and *N. flava* and *N. ferruginata* (4.14×10^{-10}) are each more than 10 times slower than those estimated by Raychoudhury et al. (2009) and Richardson et al. (2012), which seems unlikely. Our analyses suggest that the A-B group split occurred less than 100 mya.

The lack of CI is consistent with intermediate *w*Mau infection frequencies

Across 671 genes (682,494 bases), the *w*Mau genomes were identical and differed from *w*No by only 0.068%. Across the coding regions we analyzed, we found few SNVs and no CNVs among *w*Mau variants. Our analyses did identify four large deletions shared by all *w*Mau genomes, relative to *w*No. Despite the close relationship between *w*Mau and *w*No, *w*No causes CI while *w*Mau does not (Giordano et al. 1995; Merçot et al. 1995; Rousset and Solignac 1995, our data). We searched for all pairs of loci known to cause CI and found only homologs to the *cinA-cinB*^{wNo} pair in *w*Mau genomes. All *w*Mau variants share a one-base-pair deletion in the *w*Mau region homologous to *cinB*^{wNo}. This mutation introduces a frameshift and more than ten stop codons. Future functional work will help determine if disruption of this predicted-toxin locus underlies the lack of CI in *w*Mau. Regardless, the lack of CI is consistent with the prediction that selection within host lineages does not directly act on the intensity of CI (Prout 1994; Turelli 1994). We predict that analysis of additional non-CI-causing strains will reveal additional examples of genomic remnants of CI loci. Among non-CI *Wolbachia*, the relative frequency of those with non-functional CI loci, versus no CI loci, is unknown.

Irrespective of whether CI was lost or never gained, non-CI *Wolbachia* have lower expected equilibrium infection frequencies than do CI-causing variants (Kriesner et al. 2016). The *w*Mau infection frequency of approximately 0.34 on Mauritius (Giordano et al. 1995; our data) is consistent with this prediction. Additional sampling of Mauritius, preferably over decades, will determine whether intermediate *w*Mau frequencies are temporally stable. Such temporal stability depends greatly on values of *F* and *μ* through time suggesting additional field-based estimates of these parameters will be useful.

*w*Mau co-occurs with essentially the same mitochondrial haplotype as *w*Ma that infects *D. simulans* on Madagascar and elsewhere in Africa and the South Pacific (Rousset and Solignac 1995; Merçot and Poinsot 1998; Ballard 2000a; James and Ballard 2000; James et al. 2002; Ballard 2004), suggesting that *w*Mau and *w*Ma may be the same strain infecting different host species following introgressive *Wolbachia* transfer (see below). *w*Mau and *w*Ma phenotypes are also more similar to one another than to *w*No, with only certain crosses between *w*Ma-infected *D. simulans* males and uninfected *D. simulans* females inducing CI (James and Ballard 2000). Polymorphism in the strength of CI induced by *w*Ma could result from host modification of *Wolbachia*-induced CI (Reynolds and Hoffmann 2002; Cooper et

al. 2017), or from *Wolbachia* titer variation that influences the strength of CI and/or the strength of CI rescue by infected females. Alternatively, the single-base-pair deletion in the *cinB*[wNo] homolog or other mutations that influence CI strength, could be polymorphic in *w*Ma. *w*Ma infection frequencies in *D. simulans* are intermediate on Madagascar (infection frequency = 0.25, binomial confidence intervals: 0.14, 0.40; James and Ballard 2000), consistent with no CI, suggesting replication of rarely observed *w*Ma CI is needed. Including *D. simulans* from the island of Réunion in this infection-frequency further supports the conjecture that *w*Ma causes little or no CI (infection frequency = 0.31, binomial confidence intervals: 0.20, 0.45; James and Ballard 2000). Unfortunately, no Madagascar *D. simulans* stocks available at the National *Drosophila* Species Stock Center were *w*Ma infected, precluding detailed analysis of this strain.

Our genomic data indicate that *w*Mau may maintain an ability to rescue CI, as the *cinA*[wNo] homolog is intact in *w*Mau genomes with only one nonsynonymous substitution relative to *cinA*[wNo]; *cidA* in *w*Mel was recently shown to underlie transgenic-CI rescue (Shropshire et al. 2018). *w*Ma seems to sometimes rescue CI, but conflicting patterns have been found, and additional experiments are needed to resolve this (Rousset and Solignac 1995; Bourtzis et al. 1998; Merçot and Poinsot 1998; James and Ballard 2000; Merçot and Poinsot 2003; Zabalou et al. 2008). Future work that tests for CI rescue by *w*Mau and *w*Ma-infected females crossed with males infected with *w*No or other CI-causing strains, combined with genomic analysis of CI loci in *w*Ma, will be useful.

*w*Mau does not influence *D. mauritiana* fecundity

While selection does not directly act on the level of CI (Prout 1994; Turelli 1994; Haygood and Turelli 2009), it does act to increase the product *Wolbachia*-infected host fitness and the efficiency of maternal transmission (Turelli 1994). Understanding the *Wolbachia* effects that lead to spread from low frequencies and the persistence of non-CI causing *Wolbachia* at intermediate frequencies is crucial to explaining *Wolbachia* prevalence among insects and other arthropods. The four-fold fecundity effect of *w*Mau reported by Fast et al. (2011) in *D. mauritiana* is inconsistent with our experiments and with the intermediate infection frequencies observed in nature. We find no *w*Mau effects on host fecundity, regardless of host background, with our estimates of *F* having BCa intervals that include 1. Small increases in *F* could allow the deterministic spread of *w*Mau from low frequencies, although detecting very small increases in *F* is difficult (Supplemental Figure 1B). Our results are consistent with an earlier analysis that assessed egg lay of a single genotype and found no effect of *w*Mau on host fecundity (Giordano et al. 1995). When combined with the low observed infection frequencies, our fecundity data are also consistent with our mathematical analyses indicating that *Wolbachia* can increase host fitness by at most about 50% for reasonable estimates of μ. Because fecundity is one of many fitness components, analysis of other candidate phenotypes for aiding the spread of low-frequency *Wolbachia* is needed.

Introgressive *Wolbachia* transfer likely predominates in the *D. simulans* clade

Hybridization and introgression in the *D. simulans* clade may have led to introgressive transfer of *Wolbachia* among host species (Rousset and Solignac 1995). This has been observed in other *Drosophila* (Turelli et al. 2018; Cooper et al. 2019) and *Nasonia* wasps

(Raychoudhury et al. 2009). The number of *Wolbachia* strains in the *D. simulans* clade, and the diversity of mitochondria they co-occur with, is complex. Figure 5A shows host relationships and Figure 5B shows mitochondrial relationships, with co-occurring *Wolbachia* variants in parentheses. While *D. mauritiana* is singly infected by *w*Mau, *D. simulans* is infected by several strains, including CI-causing *w*Ha and *w*No that often co-occur as double infections within individuals (O'Neill and Karr 1990; Merçot et al. 1995; Rousset and Solignac 1995). *w*Ha and *w*No are similar to *w*Sh and *w*Sn, respectively, that infect *D. sechellia* (Giordano et al. 1995; Rousset and Solignac 1995). *w*Ha and *w*Sh also occur as single infections in *D. simulans* and in *D. sechellia*, respectively (Rousset and Solignac 1995). In contrast, *w*No almost always co-occurs with *w*Ha in doubly infected *D. simulans* individuals (James et al. 2002), and *w*Sn seems to occur only with *w*Sh (Rousset and Solignac 1995). *D. simulans* has three distinct mitochondrial haplotypes (*si*I, *si*II, *si*III) associated with *w*Au/*w*Ri (*si*II), *w*Ha/*w*No (*si*I), and *w*Ma (*si*III). The *si*I haplotype is closely related to the *se* haplotype found with *w*Sh and *w*Sn in *D. sechellia* (Ballard 2000b). *w*Ma co-occurs with the *si*III haplotype, which differs over its 13 protein-coding genes by only a single-base pair from the *ma*I mitochondrial haplotype carried by *w*Mau-infected *D. mauritiana*. A second haplotype (*ma*II) is carried by only uninfected *D. mauritiana* (Ballard 2000a; James and Ballard 2000).

The lack of whole *w*Ma genome data precludes us from confidently resolving the mode of *w*Mau acquisition in *D. mauritiana*. However, mitochondrial relationships support the proposal of Ballard (2000b) that *D. mauritiana* acquired *w*Mau and the *ma*I mitochondrial haplotype via introgression from *w*Ma-infected *D. simulans* carrying *si*III. *D. mauritiana* mitochondria are paraphyletic relative to *D. sechellia* and *D. simulans* mitochondria (Solignac and Monnerot 1986; Satta and Takahata 1990; Ballard 2000a, 2000b), with *ma*I sister to *si*III and *ma*II outgroup to all other *D. simulans*-clade haplotypes (see Figure 5). Of the nine genomes we assessed, all but one (uninfected-*R44*) carry the *ma*I haplotype, and genotypes carrying *ma*I are both *w*Mau-infected ($N = 5$) and uninfected ($N = 3$). While *w*Ma-infected *D. simulans* carry *si*III, *w*No-infected *D. simulans* carry *si*I. We estimate that *w*Mau and *w*No diverged about 55,000 years ago, with only 0.068% sequence divergence over 682,494 bp. Nevertheless, it seems implausible that *w*No (versus *w*Ma) was transferred directly to *D. mauritiana* as this requires horizontal or paternal transmission of *w*No into a *D. mauritiana* background already carrying the *ma*I mitochondrial haplotype. Although our nuclear result suggests a confident phylogenetic resolution of the *D. simulans* clade (Figure 5A), this is an artifact of the bifurcation structure imposed by the phylogenetic analysis. Population genetic analyses show a complex history of introgression and probable shared ancestral polymorphisms (Kliman et al. 2000) among these three species. Consistent with this, of the 20 nuclear loci we examined, 6 (*aconitase, aldolase, bicoid, ebony, enolase, ninaE*) supported *D. mauritiana* as the outgroup within the *D. simulans* clade, 7 (*glyp, pepck, pgm, pic, ptc, transaldolase, wingless*) supported *D. sechellia* as the outgroup, and 7 (*esc, g6pdh, glys, pgi, tpi, white, yellow*) supported *D. simulans*. With successive invasions of the islands and purely allopatric speciation, we expect the outgroup to be the island endemic that diverged first. Figure 5B indicates that the *ma*II haplotype diverged from the other mtDNA haplotypes roughly when the clade diverged, with the other haplotypes subject to a complex history of introgression and *Wolbachia*-associated sweeps, as described by Ballard (2000b).

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Ballard (2000b) estimated that *si*III-*ma*I diverged about 4,500 years ago, which presumably approximates the date of the acquisition of *w*Mau (and *si*III, which became *ma*I) by *D. mauritiana*. This is surely many thousands of generations given previous estimates that consider the temperature dependence of *Drosophila* development (Cooper et al. 2014; Cooper et al. 2018). As shown by our mathematical analyses (Eq. 3), the apparent persistence of the *ma*II mtDNA among *Wolbachia*-uninfected *D. mauritiana*—without its occurrence among infected individuals—is unexpected. More extensive sampling of natural *D. mauritiana* populations is needed to see if this unexpected pattern persists. The persistence of this haplotype is inconsistent with simple null models, possibly indicating interesting fitness effects.

While paternal transmission has been observed in *D. simulans* (Hoffmann and Turelli 1988; Turelli and Hoffmann 1995), it seems to be very rare (Richardson et al. 2012; Turelli et al. 2018). *w*No almost always occurs in *D. simulans* individuals also infected with *w*Ha, complicating this scenario further. It is possible that horizontal or paternal transmission of *w*Ma or *w*No between *D. simulans* backgrounds carrying different mitochondrial haplotypes underlies the similarities of these strains within *D. simulans*, despite their co-occurrence with distinct mitochondria. Given the diversity of *Wolbachia* that infect *D. simulans*-clade hosts, and known patterns of hybridization and introgression among hosts (Garrigan et al. 2012; Brand et al. 2013; Garrigan et al. 2014; Matute and Ayroles 2014; Schrider et al. 2018), determining relationships among these *Wolbachia* and how *D. mauritiana* acquired *w*Mau will require detailed phylogenomic analysis of nuclear, mitochondrial, and *Wolbachia* genomes in the *D. simulans* clade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Appendix 1.: Mathematical analyses of mtDNA and Wolbachia dynamics

Our analysis follows the framework developed in Turelli et al. (1992), but is simplified by the lack of CI. We suppose that introgression introduces a cytoplasm carrying *Wolbachia* and a novel mtDNA haplotype, denoted B. Before *Wolbachia* introduction, we assume the population is monomorphic for mtDNA haplotype A. With imperfect maternal *Wolbachia* transmission, uninfected individuals will be produced with mtDNA haplotype B. Without horizontal or paternal transmission (which are very rare, Turelli et al. 2018), all *Wolbachia*-infected individuals will carry mtDNA haplotype B. Once *Wolbachia* is introduced, uninfected individuals can have mtDNA haplotype A or B. We assume that these three cytoplasmic types ("cytotypes") differ only in fecundity, and denote their respective

fecundities F_I, F_A and F_B. Denote the frequencies of the three cytotypes among adults in generation t by $p_{I,t}$, $p_{A,t}$ and $p_{B,t}$, with $p_{I,t} + p_{A,t} + p_{B,t} = 1$. Without CI, the frequency dynamics are

$$p_{I,t+1} = \frac{p_{I,t}F_I(1-\mu)}{\overline{F}}, p_{A,t+1} = \frac{p_{A,t}F_A}{\overline{F}}, \text{ and } p_{B,t+1} = \frac{p_{B,t}F_B + p_{I,t}\mu F_I}{\overline{F}}, \text{with} \quad (A1)$$

$$\overline{F} = F_I p_{I,t} + F_A p_{A,t} + F_B p_{B,t}. \quad (A2)$$

If the uninfected population is initially monomorphic for mtDNA haplotype A, the *Wolbachia* infection frequency will increase when rare if and only if

$$F_I(1-\mu) > F_A. \quad (A3)$$

Turelli et al. (1992) showed that if a CI-causing *Wolbachia* is introduced with a cytoplasm that contains a novel mtDNA haplotype B, which has no effect on fitness, *Wolbachia*-uninfected individuals will eventually all carry haplotype B. This follows because eventually all uninfected individuals have *Wolbachia*-infected maternal ancestors. This remains true for non-CI-causing *Wolbachia* that satisfy (A3). However, we conjectured that if the introduced B mtDNA is deleterious in the new host nuclear background, i.e., $F_A > F_B$, a stable polymorphism might be maintained for the alternative mtDNA haplotypes. The motivation was that imperfect maternal transmission seemed analogous to migration introducing a deleterious allele into an "island" of uninfected individuals. To refute this conjecture, consider the equilibria of (A1) with

$$F_I > F_A \geq F_B. \quad (A4)$$

If all three cytotypes are to be stably maintained, we expect each to increase in frequency when rare. In particular, we expect the fitness-enhancing mtDNA haplotype A to increase when the population contains only infected individuals and uninfected individuals carrying the deleterious *Wolbachia*-associated mtDNA haplotype B. From (A1), $p_{A,t}$ increases when rare if and only if

$$F_A > \overline{F} = F_I p_{I,t} + F_B(1 - p_{I,t}). \quad (A5)$$

In the absence of haplotype A, we expect p_I to be at equilibrium between selection and imperfect maternal transmission, i.e.,

$$p_I = 1 - \frac{\mu F}{F - 1}, \quad \text{(A6)}$$

with $F = F_I/F_B$ (Hoffmann and Turelli 1997). Substituting (A6) into (A5) and simplifying, the condition for $p_{A,t}$ to increase when rare is

$$F_A(F_I - F_B) > F_I(1 - \mu)(F_I - F_B). \quad \text{(A7)}$$

By assumption (A4), $F_I > F_B$; hence (A7) contradicts condition (A3), required for initial *Wolbachia* invasion. Thus, simple selection on *Wolbachia*-uninfected mtDNA haplotypes cannot stably maintain an mtDNA polymorphism. The "ancestral" mtDNA haplotype A is expected to be replaced by the less-fit *Wolbachia*-associated haplotype B.

To understand the time scale over which this replacement occurs, let r_t denote the frequency of haplotype A among *Wolbachia*-uninfected individuals, i.e., $r_t = p_{A,t}/(p_{A,t} + p_{B,t})$. From (A1),

$$r_{t+1} = \frac{r_t F_A}{r_t F_A + (1 - r_t)F_B + \mu F_I[p_{I,t}/(1 - p_{I,t})]}. \quad \text{(A8)}$$

If we assume that the mtDNA haplotypes do not affect fitness, i.e., $F_A = F_B$, and that the *Wolbachia* infection frequency has reached the equilibrium described by (A6), (A8) reduces to

$$r_{t+1} = r_t/[F(1 - \mu)], \quad \text{(A9)}$$

with $F = F_I/F_B$.

LITERATURE CITED

Baldo L, Ayoub NA, Hayashi CY, Russell JA, Stahlhut JK, and Werren JH. 2008 Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity. Molecular Ecolology 17:557–569.

Ballard JWO 2000a When one is not enough: introgression of mitochondrial DNA in *Drosophila*. Molecular Biology and Evolution 17:1126–1130. [PubMed: 10889226]

Ballard JW 2000b Comparative genomics of mitochondrial DNA in *Drosophila simulans*. Journal of Molecular Evolution 51:64–75. [PubMed: 10903373]

Ballard JWO 2004 Sequential evolution of a symbioint inferred from the host: *Wolbachia* and *Drosophila simulans*. Molecular Biology and Evolution 21:428–442. [PubMed: 14660690]

Ballard JW, and Melvin RG. 2007 Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in *Drosophila*. Insect Molecular Biology 16:799–802. [PubMed: 18093008]

Barton NH, and Turelli M. 2011 Spatial waves of advance with bistable dynamics: cytoplasmic and genetic analogues of allee effects. American Naturalist 178:E48–E75.

Beckmann JF, and Fallon AM. 2013 Detection of the *Wolbachia* protein WPIP0282 in mosquito spermathecae: implications for cytoplasmic incompatibility. Insect Biochemistry and Molecular Biology 43:867–878. [PubMed: 23856508]

Beckmann JF, Ronau JA and Hochstrasser M. 2017 A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. Nature Microbiology 2:17007.

Beckmann JF, Bonneau M, Chen H, Hochstrasser M, Poinsot D, Merçot H, Weill M, Sicard M, and Charlat S. 2019 The toxin-antidote model of cytoplasmic incompatibility: genetics and evolutionary implications. Trends in Genetics in press

Boeva V, Popova T, Bleakley K, Chiche P, Cappo J, Schleiermacher G, Janoueix-Lerosey I, Delattre O, Barillot E. 2012 Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. Bioinformatics 28:423–425. [PubMed: 22155870]

Bourtzis K, Dobson SL, Braig HR and O'Neill SL. 1998 Rescuing *Wolbachia* have been overlooked. Nature 391:852–853. [PubMed: 9495337]

Brand CL, Kingan SB, Wu L and Garrigan D. 2013 A Selective Sweep across Species Boundaries in *Drosophila*. Molecular Biology and Evolution 30:2177–2186. [PubMed: 23827876]

Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL. 2009 Evidence for Metabolic Provisioning by a Common Invertebrate Endosymbiont, *Wolbachia pipientis*, during Periods of Nutritional Stress. PLoS Pathogens 5:e1000368. [PubMed: 19343208]

Carrington LB, Lipkowitz JR, Hoffmann AA, and Turelli M. 2011 A re-examination of *Wolbachia*-induced cytoplasmic incompatibility in California *Drosophila simulans*. PLoS ONE 6:e22565 [PubMed: 21799900]

Caspari E, and Watson GS. 1959 On the evolutionary importance of cytoplasmic sterility in mosquitoes. Evolution 13:568–570.

Cattel J, Nikolouli K, Andrieux T, Martinez J, Jiggins F, Charlat S, Vavre F, Lejon D, Gibert P, and Mouton L. 2018 Back and forth *Wolbachia* transfers reveal efficient strains to control spotted wing *Drosophila* populations. Journal of Applied Ecology 55:2408–2418.

Conner WR, Blaxter ML, Anfora G, Ometto L, Rota-Stabelli O, and Turelli M. 2017 Genome comparisons indicate recent transfer of *w*Ri-like *Wolbachia* between sister species *Drosophila suzukii* and *D. subpulchrella*. Ecology and Evolution 7:9391–9404. [PubMed: 29187976]

Cooper BS, Hammad LA, and Montooth KL. 2014 Thermal adaptation of cellular membranes in natural populations of *Drosophila melanogaster*. Functional Ecology 28:886–894. [PubMed: 25382893]

Cooper BS, Ginsberg PS, Turelli M and Matute DR. 2017 *Wolbachia* in the *Drosophila yakuba* complex: pervasive frequency variation and weak cytoplasmic incompatibility, but no apparent effect on reproductive isolation. Genetics 205:333–351. [PubMed: 27821433]

Cooper BS, Sedghifar A, Nash WT, Comeault AA, Matute DR. 2018 A maladaptive combination of traits contributes to the maintenance of a *Drosophila* hybrid zone. Current Biology 28:2940–2947. [PubMed: 30174184]

Cooper BS, Vanderpool D, Conner WR, Matute DR, and Turelli M. 2019 Introgressive and horizontal acquisition of *Wolbachia* by *Drosophila yakuba*-clade hosts and horizontal transfer of incompatibility loci between distantly related *Wolbachia*. bioRxiv. 10.1101/551036.

David J, McEvey S, Solignac M and Tsacas L. 1989 *Drosophila* communities on Mauritius and the ecological niche of *D. mauritiana* (Diptera, Drosophilidae). Revue De Zoologie Africaine – Journal of African Zoology 103:107–116.

Dean MD, and Ballard JW. 2004 Linking phylogenetics with population genetics to reconstruct the geographic origin of a species. Molecular Phylogenetics and Evolution 32:998–1009. [PubMed: 15288072]

Efron B, and Tibshirani R. 1993 An Introduction to the Bootstrap Chapman & Hall, New York, NY.

Ellegaard KM, Klasson L, Näslund K, Bourtzis K and Andersson SGE. 2013 Comparative Genomics of *Wolbachia* and the Bacterial Species Concept. PLoS Genetics 9:e1003381. [PubMed: 23593012]

Fast EM, Toomey ME, Panaram K, Desjardins D, Kolaczyk ED, Frydman HM. 2011 *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. Science 334:990–992. [PubMed: 22021671]

Fisher RA 1937 The wave of advance of advantageous genes. Annals of Eugenics 7:355–369.

Garrigan D, Kingan SB, Geneva AJ, Andolfatto P, Clark AG, Thornton KR, Presgraves DC. 2012 Genome sequencing reveals complex speciation in the *Drosophila simulans* clade. Genome Research 22:1499–1511. [PubMed: 22534282]

Garrigan D, Kingan SB, Geneva AJ, Vedanayagam JP and Presgraves DC. 2014 Genome diversity and divergence in *Drosophila mauritiana*: multiple signatures of faster X evolution. Genome Biology and Evolution 6:2444–2458. [PubMed: 25193308]

Gerth M, and Bleidorn C. 2016 Comparative genomics provides a timeframe for *Wolbachia* evolution and exposes a recent biotin synthesis operon transfer. Nature Microbiology 2:16241.

Giordano R, O'Neill SL and Robertson HM. 1995 *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. Genetics 140:1307–1317. [PubMed: 7498772]

Gliddon C and Strobeck C. 1975 Necessary and sufficient conditions for multiple-niche polymorphism in haploids. American Naturalist 109:233–235.

Gloor GB, Preston CR, Johnsonschlitz DM, Nassif NA, Phillis RW, Benz WK, Robertson HM, Engels WR. 1993 Type-1 repressors of P-element mobility. Genetics 135:81–95. [PubMed: 8224830]

Gupta V, Vasanthakrishnan RB, Siva-Jothy J, Monteith KM, Brown SP, and Vale PF. 2017 The route of infection determines *Wolbachia* antibacterial protection in *Drosophila*. Proceedings of the Royal Society B: Biological Sciences 284:20170809.

Hamm CA, Begun DJ, Vo A, Smith CCR, Saelao P, Shaver AO, Jaenike J, Turelli M. 2014 *Wolbachia* do not live by reproductive manipulation alone: infection polymorphism in *Drosophila suzukii* and *D. subpulchrella*. Molecular Ecology 23:4871–4885. [PubMed: 25156506]

Haygood R, and Turelli M. 2009 Evolution of incompatibility-inducing microbes in subdivided host populations. Evolution 63:432–447 [PubMed: 19154372]

Hedges LM, Brownlie JC, O'Neill SL and Johnson KN. 2008 *Wolbachia* and virus protection in insects. Science 322:702. [PubMed: 18974344]

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A and Werren JH. 2008 How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiology Letters 281:215–220. [PubMed: 18312577]

Hoffmann AA, Turelli M, and Simmons GM. 1986 Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution 40:692–701. [PubMed: 28556160]

Hoffmann AA, and Turelli M. 1988 Unidirectional incompatibility in *Drosophila simulans*: Inheritance, geographic variation and fitness effects. Genetics 119:435–444. [PubMed: 17246430]

Hoffmann AA, and Turelli M. 1997 Cytoplasmic incompatibility in insects. In Influential passengers: inherited microorganisms and arthropod reproduction, edited by O'Neill SL, Werren JH, and Hoffmann AA. Oxford University Press, pp. 42–80.

Hoffmann AA, Clancy D and Duncan J. 1996 Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility. Heredity 76:1–8. [PubMed: 8575931]

Hoffmann AA, Hercus M and Dagher H. 1998 Population dynamics of the *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. Genetics 148:221–231. [PubMed: 9475734]

Hoffmann AA, Turelli M, and Harshman LG. 1990 Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. Genetics 126:933–948. [PubMed: 2076821]

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, and O'Neill SL. 2011 Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature 476:454–457. [PubMed: 21866160]

Höhna S, Landis MJ, Heath TA, Boussau B, Lartillot N, Moore BR, Huelsenbeck JP, Ronquist F. 2016 RevBayes: Bayesian Phylogenetic Inference Using Graphical Models and an Interactive Model-Specification Language. Systematic Biology 65:726–736. [PubMed: 27235697]

Hornett EA, Charlat S, Duplouy AMR, Davies N, Roderick GK, Wedell N, and Hurst GDD. 2006 Evolution of male-killer suppression in a natural population. PLoS Biology 4:e283. [PubMed: 16933972]

Huigens ME, de Almeida RP, Boons PA, Luck RF and Stouthamer R. 2004 Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. Proceedings of the Royal Society B: Biological Sciences 271:509–515.

Hurst GDD and Jiggins FM. 2000 Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerging Infectious Diseases 6:329–336. [PubMed: 10905965]

Jackman SD, Vandervalk BP, Mohamadi H, Chu J, Yeo S, Hammond SA, Jahesh G, Khan H, Coombe L, Warren RL, Birol I. 2017 ABySS 2.0: resource-efficient assembly of large genomes using a Bloom filter. Genome Research 27:768–777. [PubMed: 28232478]

James AC, and Ballard JWO. 2000 Expression of cytoplasmic incompatibility in *Drosophila simulans* and its impact on infection frequencies and distribution of *Wolbachia pipientis*. Evolution 54:1661–1672. [PubMed: 11108593]

James AC, Dean MD, McMahon ME and Ballard JW. 2002 Dynamics of double and single *Wolbachia* infections in *Drosophila simulans* from New Caledonia. Heredity 88:182–189. [PubMed: 11920119]

Jeyaprakash A, and Hoy MA. 2000 Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Molecular Biology 9:393–405. [PubMed: 10971717]

Joshi NA, and Fass JN. 2011 Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33) Available at https://github.com/najoshi/sickle.

Katoh K, and Standley DM. 2013 MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Molecular Biology and Evolution 30:772–780. [PubMed: 23329690]

Kent BN, Salichos L, Gibbons JG, Rokas A, Newton IL, Clark ME, Bordenstein SR. 2011 Complete bacteriophage transfer in a bacterial endosymbiont (*Wolbachia*) determined by targeted genome capture. Genome Biology and Evolution 3:209–218. [PubMed: 21292630]

Klasson L, Walker T, Sebaihia M, Sanders MJ, Quail MA, Lord A, Sanders S, Earl J, O'Neill SL, Thomson N, Sinkins SP, Parkhill J. 2008 Genome evolution of *Wolbachia* strain *w*Pip from the *Culex pipiens* group. Molecular Biology and Evolution 25:1877–1887. [PubMed: 18550617]

Klasson L, Westberg J, Sapountzis P, Näslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L, Braig HR, and Garrett R. 2009 The mosaic genome structure of the *Wolbachia* *w*Ri strain infecting *Drosophila simulans*. Proceedings of the National Academy of Sciences 106:5725–5730.

Kliman RM, Andolfatto P, Coyne JA, Depaulis F, Kreitman M, Berry AJ, McCarter J, Wakeley J, and Hey J. 2000 The population genetics of the origin and divergence of the *Drosophila simulans* species complex. Genetics 156:1913–1931. [PubMed: 11102384]

Kriesner P, and Hoffmann AA. 2018 Rapid spread of a *Wolbachia* infection that does not affect host reproduction in *Drosophila simulans* cage populations. Evolution 72:1475–1487.

Kriesner P, Conner WR, Weeks AR, Turelli M, and Hoffmann AA. 2016 Persistence of a *Wolbachia* infection frequency cline in *Drosophila melanogaster* and the possible role of reproductive dormancy. Evolution 70:979–997. [PubMed: 27076356]

Kriesner P, Hoffmann AA, Lee SF, Turelli M, and Weeks AR. 2013 Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans*. PLoS Pathogens 9:e1003607. [PubMed: 24068927]

Lachaise D, David JR, Lemeunier F, Tsacas L and Ashburner M. 1986 The reproductive relationships of *Drosophila sechellia* with *D. mauritiana*, *D. simulans*, and *D. melanogaster* from the Afrotropical region. Evolution 40:262–271. [PubMed: 28556049]

Laven H 1951 Crossing Experiments with *Culex* Strains. Evolution 5:370–375.

Legrand D, Chenel T, Campagne C, Lachaise D and Cariou ML. 2011 Inter-island divergence within *Drosophila mauritiana*, a species of the *D. simulans* complex: Past history and/or speciation in progress? Molecular Ecology 20:2787–2804. [PubMed: 21599771]

LePage DP, Metcalf JA, Bordenstein SR, On J, Perlmutter JI, Shropshire JD, Layton EM, Funkhouser-Jones LJ, Beckmann JF, Bordenstein SR. 2017 Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. Nature 543:243–247. [PubMed: 28241146]

Li H, and Durbin R. 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760. [PubMed: 19451168]

Lindsey ARI, Rice DW, Bordenstein SR, Brooks AW, Bordenstein SR, Brooks AW, Bordenstein SR, Newton IL. 2018 Evolutionary Genetics of Cytoplasmic Incompatibility Genes *cifA* and *cifB* in Prophage WO of *Wolbachia*. Genome Biology and Evolution 10:434–451. [PubMed: 29351633]

Lindsey ARI, Werren JH, Richards S and Stouthamer R. 2016 Comparative Genomics of a Parthenogenesis-Inducing *Wolbachia* Symbiont. Genes|Genomes|Genetics 6:2113–2123. [PubMed: 27194801]

Martinez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, Bourtzia K, Teixeira L, Jiggins FM. 2014 Symbionts commonly provide broad spectrum resistance to viruses in insects: A comparative analysis of *Wolbachia* strains. PLoS Pathogens 10:e1004369. [PubMed: 25233341]

Matute DR, and Ayroles JF. 2014 Hybridization occurs between *Drosophila simulans* and *D. sechellia* in the Seychelles archipelago. Journal of Evolutionary Biology 27:1057–1068. [PubMed: 24773151]

Mavingui P, Valiente Moro C, Tran-Van V, Wisniewski-Dye F, Raquin V, Minard G, Tran FH, Voronin D, Rouy Z, Bustos P, Lozano L, Barbe V, Gonzalez V. 2012 Whole-genome sequence of *Wolbachia* strain *w*AlbB, an endosymbiont of tiger mosquito vector *Aedes albopictus*. Journal of Bacteriology 194:1840. [PubMed: 22408242]

McDermott SR, and Kliman RM. 2008 Estimation of isolation times of the island species in the *Drosophila simulans* complex from multilocus DNA sequence data. PLoS One 3:e2442. [PubMed: 18560591]

Merçot H, and Poinsot D. 1998 …and discovered on Mount Kilimanjaro. Nature 391:853. [PubMed: 9495338]

Merçot H, and Poinsot D. 2003 *Wolbachia* transmission in a naturally bi-infected *Drosophila simulans* strain from New-Caledonia. Entomologia Experimentalis et Applicata 86:97–103.

Merçot H, Llorente B, Jacques M, Atlan A and Montchamp-Moreau C. 1995 Variability within the Seychelles cytoplasmic incompatibility system in *Drosophila simulans*. Genetics 141:1015–1023. [PubMed: 8582608]

Nunes MD, Wengel PO, Kreissl M and Schlotterer C. 2010 Multiple hybridization events between *Drosophila simulans* and *Drosophila mauritiana* are supported by mtDNA introgression. Molecular Ecology 19:4695–4707. [PubMed: 20958812]

Ochman H, and Wilson AC. 1987 Evolution in bacteria: evidence for a universal substitution rate in cellular organisms. Journal of Molecular Evolution 26:74–86. [PubMed: 3125340]

O'Neill SL, Giordano R, Colbert AM, Karr TL and Robertson HM. 1992 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proceedings of the National Academy of Sciences 89:2699–2702.

O'Neill SL, and Karr TL. 1990 Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348:178–180. [PubMed: 2234083]

Pinto SB, Stainton K, Harris S, Kambris Z, Sutton ER, Bonsall MB, Parkhill J, Sinkins SP. 2013 Transcriptional regulation of *Culex pipiens* mosquitoes by *Wolbachia* influences cytoplasmic incompatibility. PLoS Pathogens 9:e1003647. [PubMed: 24204251]

Poinsot D, Bourtzis K, Markakis G, Savakis C, and Merçot H. 1998 Wolbachia transfer from *Drosophila melanogaster* into *D. simulans*: host effect and cytoplasmic incompatibility relationships. Genetics 150:227–237. [PubMed: 9725842]

Prout T 1994 Some evolutionary possibilities for a microbe that causes incompatibility in its host. Evolution 48:909–911. [PubMed: 28568258]

Raychoudhury R, Baldo L, Oliveira DCSG, and Werren JH. 2009 Modes of acquisition of *Wolbachia*: horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. Evolution 63:165–183. [PubMed: 18826448]

Reynolds KT, and Hoffmann AA. 2002 Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*. Genetics Research 80:79–87.

Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM, Bergman CM. 2012 Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*. PLoS Genetics 8:e1003129. [PubMed: 23284297]

Ritchie SA 2018 *Wolbachia* and the near cessation of dengue outbreaks in Northern Australia despite continued dengue importations via travellers. Journal of Travel Medicine. 25:tay084.)

Rousset F, and Solignac M. 1995 Evolution of single and double *Wolbachia* symbioses during speciation in the *Drosophila simulans* complex. Proceedings of the National Academy of Sciences 92:6389–6393.

Satta Y and Takahata N. 1990 Evolution of *Drosophila* mitochondrial DNA and the history of the *melanogaster* subgroup. Proceedings of the National Academy of Sciences of the United States of America 87:9558–9562. [PubMed: 2124697]

Schmidt TL, Barton NH, Raši G, Turley AP, Montgomery BL, Iturbe-Ormaetxe I, Cook PE, Ryan PA, Ritchie SA, Hoffmann AA, O'Neill SL, and Turelli M. 2017 Local introduction and heterogeneous spatial spread of dengue-suppressing *Wolbachia* through an urban population of *Aedes aegypti*. PLoS Biology 15:e2001894. [PubMed: 28557993]

Schrider DR, Ayroles J, Matute DR and Kern AD. 2018 Supervised machine learning reveals introgressed loci in the genomes of *Drosophila simulans* and *D. sechellia*. PLoS Genetics 14:e1007341. [PubMed: 29684059]

Schuler H, Köppler K, Daxböck-Horvath S, Rasool B, Krumböck S, Schwarz D, Hoffmeister TS, Schlick-Steiner BC, Steiner FM, Telschow A, Stauffler C, Arthofer W, Riegler M. 2016 The hitchhiker's guide to Europe: the infection dynamics of an ongoing *Wolbachia* invasion and mitochondrial selective sweep in *Rhagoletis cerasi*. Molecular Ecology 25:1595–1609. [PubMed: 26846713]

Seemann T 2014 Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. [PubMed: 24642063]

Shi M, White VL, Schlub T, Eden J-S, Hoffmann AA, Holmes EC. 2018 No detectable effect of *Wolbachia w*Mel on the prevalence and abundance of the RNA virome of *Drosophila melanogaster*. Proceedings of the Royal Society B: Biological Sciences 285:20181165.

Shropshire JD, On J, Layton EM, Zhou H and Bordenstein SR. 2018 One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences 115:4987–4991.

Simao FA, Waterhouse RM, Ioannidis P, Kriventseva EV and Zdobnov EM. 2015 BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31:3210–3212. [PubMed: 26059717]

Siozios S, Cestaro A, Kaur R, Pertot I, Rota-Stabelli O, and Anfora G. 2013 Draft genome sequence of the *Wolbachia* endosymbiont of *Drosophila suzukii*. Genome Announcements 1, e00032–13.

Solignac M and Monnerot M. 1986 Race formation, speciation, and introgression within *Drosophila simulans*, *D. mauritiana*, and *D. schellia* inferred from mitochondrial DNA analysis. Evolution 40:531–539. [PubMed: 28556334]

Sutton ER, Harris SR, Parkhill J and Sinkins SP. 2014 Comparative genome analysis of *Wolbachia* strain *w*Au. BMC Genomics 15:928. [PubMed: 25341639]

Taylor MJ, Voronin D, Johnston KL and Ford L. 2013 *Wolbachia* filarial interactions. Cell Microbiology 15: 520–526.

Team RC 2015 R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria.

Teixeira L, Ferreira A and Ashburner M. 2008 The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biology 6:e1000002.

Toomey ME, Panaram K, Fast EM, Beatty C and Frydman HM. 2013 Evolutionarily conserved *Wolbachia*-encoded factors control pattern of stem-cell niche tropism in *Drosophila* ovaries and favor infection. Proceedings of the National Academy of Sciences 110:10788–10793.

Turelli M 1994 Evolution of incompatibility-inducing microbes and their hosts. Evolution 48:1500–1513. [PubMed: 28568404]

Turelli M and Barton NH. 2017 Deploying dengue-suppressing *Wolbachia*: robust models predict slow but effective spatial spread in *Aedes aegypti*. Theoretical Population Biology 115:45–60. [PubMed: 28411063]

Turelli M and Hoffmann AA. 1995 Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations. Genetics 140:1319–1338. [PubMed: 7498773]

Turelli M, Hoffmann AA, and McKechnie SW. 1992 Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila simulans* populations. Genetics 132:713–723. [PubMed: 1468627]

Turelli M, Cooper BS, Richardson KM, Ginsberg PS, Peckenpaugh B, Antelope CX, Kim KJ, May MR, Abrieux A, Wilson DA, Bronski MJ, Moore BR, Gao JJ, Eisen MB, Chiu JC, Conner WR, Hoffmann AA. 2018 Rapid Global Spread of *w*Ri-like *Wolbachia* across Multiple *Drosophila*. Current Biology 28:963–971. [PubMed: 29526588]

Vanthournout B and Hendrickx F. 2016 Hidden suppression of sex ratio distortion suggests Red queen dynamics between *Wolbachia* and its dwarf spider host. Journal of Evolutionaly Biology 29:1488–1494.

Webster CL, Waldron FM, Robertson S, Crowson D, Ferrari G, Quintana JF, Brouqui J-M, Bayne EH, Longdon B, Buck AH, Lazzaro BP, Akorli J, Haddrill PR, and Obbard DJ. 2015 The Discovery, Distribution, and Evolution of Viruses Associated with *Drosophila melanogaster*. PLoS Biology 13:e1002210. [PubMed: 26172158]

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, and Hoffmann AA. 2007 From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS Biology 5:e114. [PubMed: 17439303]

Weinert LA, Araujo-Jnr EV, Ahmed MZ and Welch JJ. 2015 The incidence of bacterial endosymbionts in terrestrial arthropods. Proceedings of the Royal Society of London B: Biological Sciences 282:20150249.

Werren JH, and Windsor DM. 2000 *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proceedings of the Royal Society B: Biological Sciences 267:1277–1285.

Werren JH, Zhang W and Guo LR. 1995 Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proceedings of the Royal Society B-Biological Sciences 261:55–63.

Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N. 2004 Phylogenomics of the reproductive parasite *Wolbachia pipientis w*Mel: a streamlined genome overrun by mobile genetic elements. PLoS Biology 2:e69. [PubMed: 15024419]

Yen JH and Barr AR. 1971 New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens L*. Nature 232:657–658. [PubMed: 4937405]

Zabalou S, Apostolaki A, Pattas S, Veneti Z, Paraskevopoulos C, Livadaras I, Markakis G, Brissac T, Merçot H, Bourtzis K. 2008 Multiple rescue factors within a *Wolbachia* strain. Genetics 178:2145–2160. [PubMed: 18430940]

Zug R, and Hammerstein P. 2012 Still a host of hosts for *Wolbachia*: Analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS ONE 7:e38544. [PubMed: 22685581]
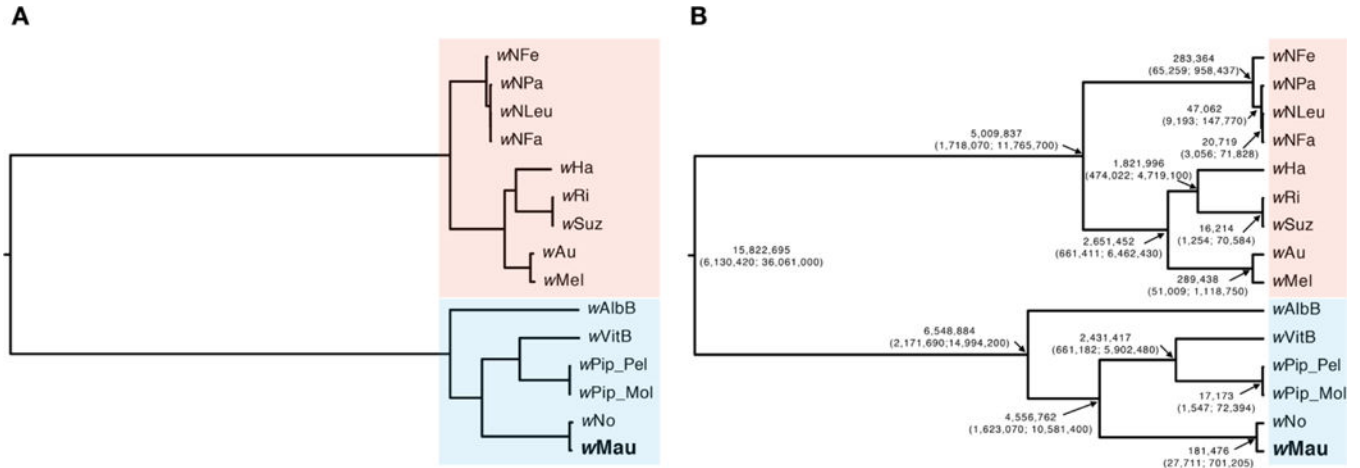
Figure 1.

A) An estimated phylogram for various group-A (red) and group-B (blue) *Wolbachia* strains. All nodes have Bayesian posterior probabilities of 1. The phylogram shows significant variation in the substitution rates across branches, with long branches separating the A and B clades. **B)** An estimated chronogram for the same strains, with estimated divergence times and their confidence intervals at each node. To obtain these estimates, we generated a relative relaxed-clock chronogram with the GTR + Γ model with the root age fixed to 1, the data partitioned by codon position, and with a $\Gamma(2,2)$ branch rate prior. We used substitution-rate estimates of $\Gamma(7,7) \times 6.87 \times 10^{-9}$ substitutions/3$^{\text{rd}}$ position site/year to transform the relative chronogram into an absolute chronogram.
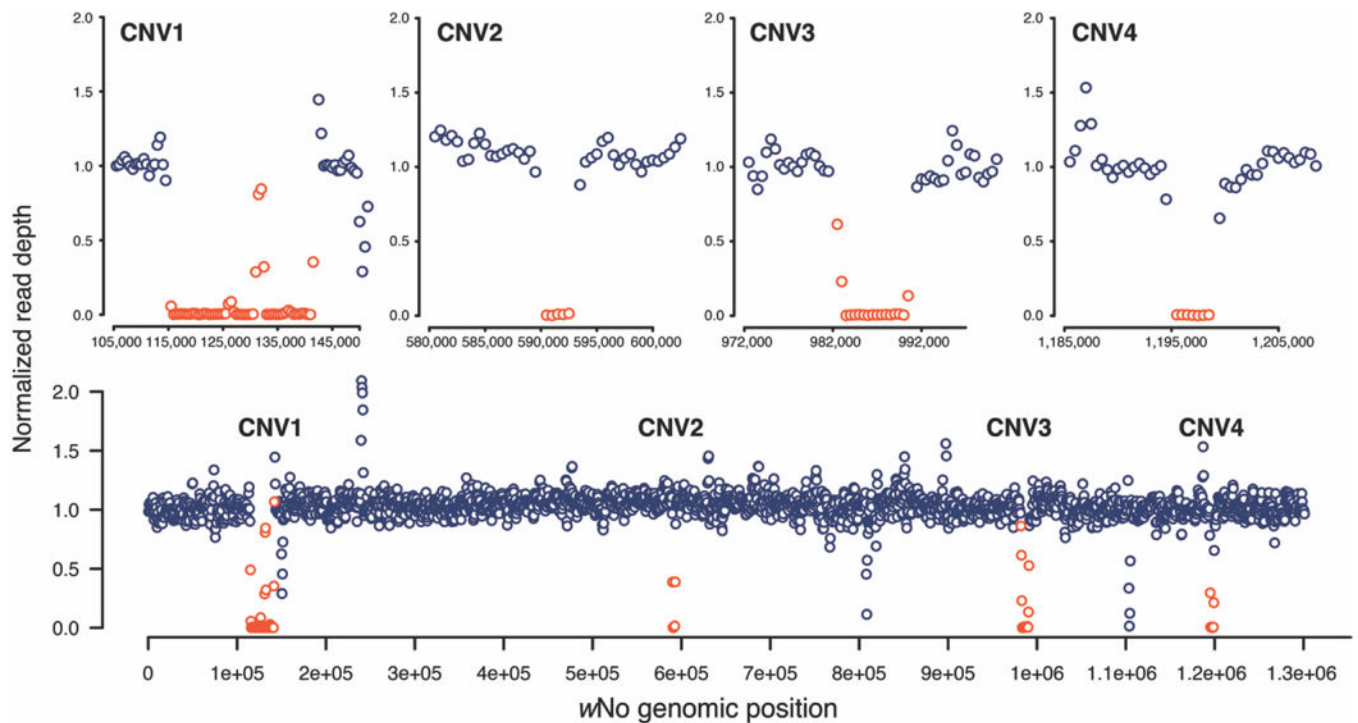
Figure 2.
All *w*Mau variants share four large deletions, relative to sister *w*No. Top panel) The normalized read depth for *w*Mau *R60* plotted across the four focal regions of the *w*No reference genome; 10 kb of sequence surrounding regions are plotted on either side of each region. Bottom panel) The normalized read depth of *w*Mau *R60* plotted across the whole *w*No reference genome. Regions that do not contain statistically significant CNVs are plotted in dark blue, and regions with significant CNVs are plotted in red. All *w*Mau variants share the same CNVs, relative to *w*No.
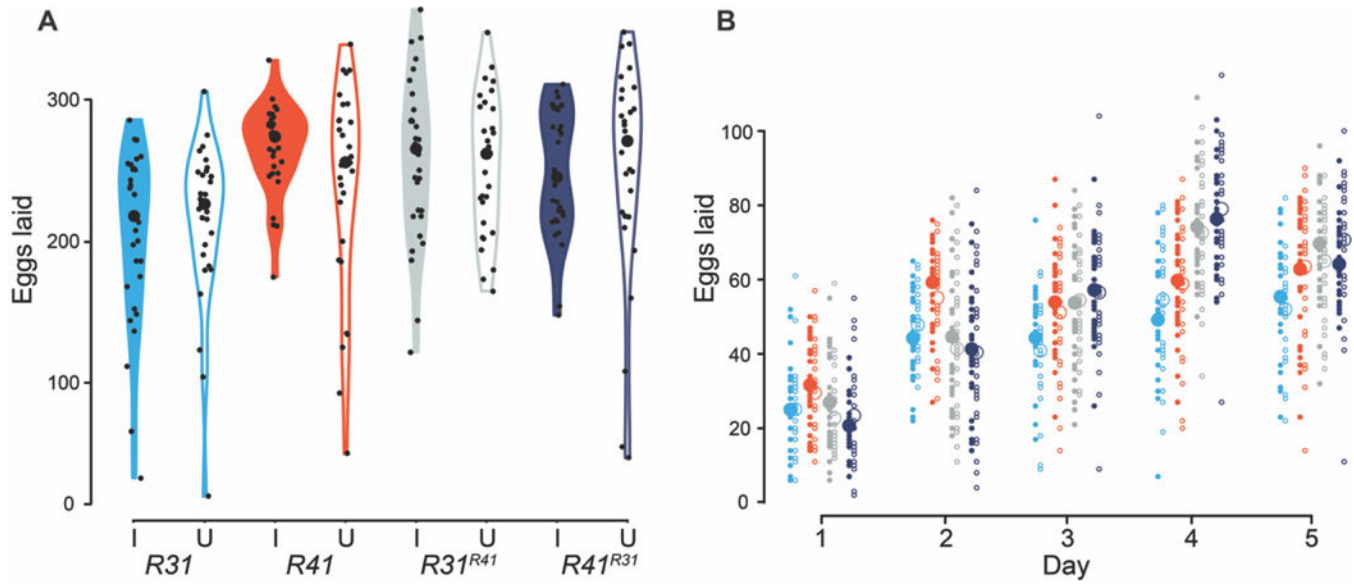
Figure 3.

*w*Mau infections do not influence *D. mauritiana* fecundity, regardless of host genomic background. **A)** Violin plots of the number of eggs laid by *D. mauritiana* females over five days when infected with their natural *w*Mau variant (*R31I* and *R41I*), when infected with a novel *w*Mau variant (*R31^{R41}I* and *R41^{R31}I*), and when uninfected (*R31U, R41U, R31^{R41}U,* and *R41^{R31}U*). Large black dots are medians, and small black dots are the eggs laid by each replicate over five days. **B)** The daily egg lay of these same infected (solid circles) and uninfected (open circles) *R31* (aqua), *R41* (red), *R31^{R41}* (gray), and *R41^{R31}* (dark blue) genotypes is reported. Large circles are means of all replicates, and small circles are the raw data. Only days where females laid at least one egg are plotted. Cytoplasm sources are denoted by superscripts for the reciprocally introgressed strains.
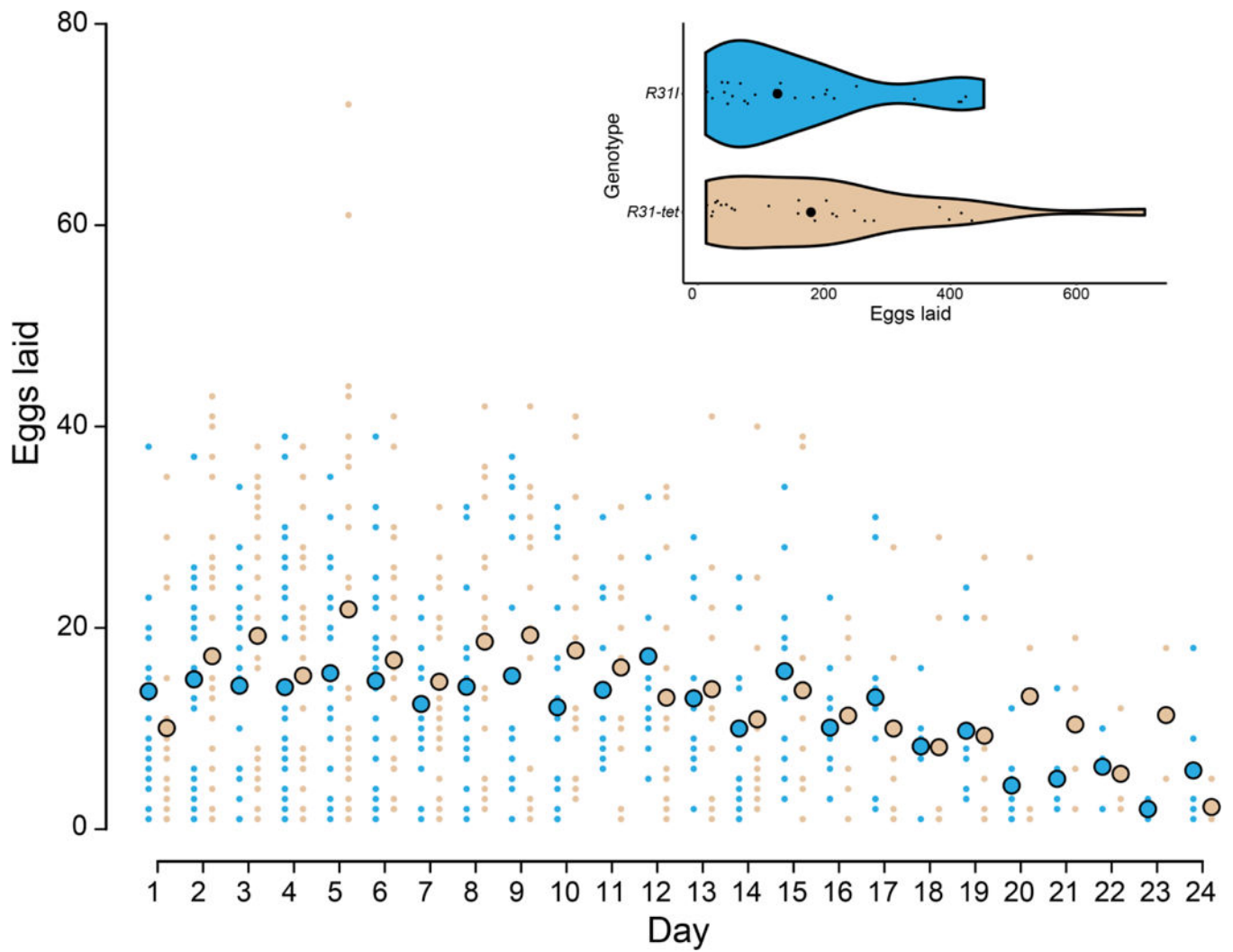
Figure 4.
The mean number of eggs laid by infected *R31* (*R31I*, large aqua dots) and uninfected *R31-tet* (large tan dots) genotypes are similar. Egg counts for each replicate are also plotted (small dots). Violin plots show egg lay across all ages for each genotype; large black circles are medians, and small black circles are the number of eggs laid by each replicate.
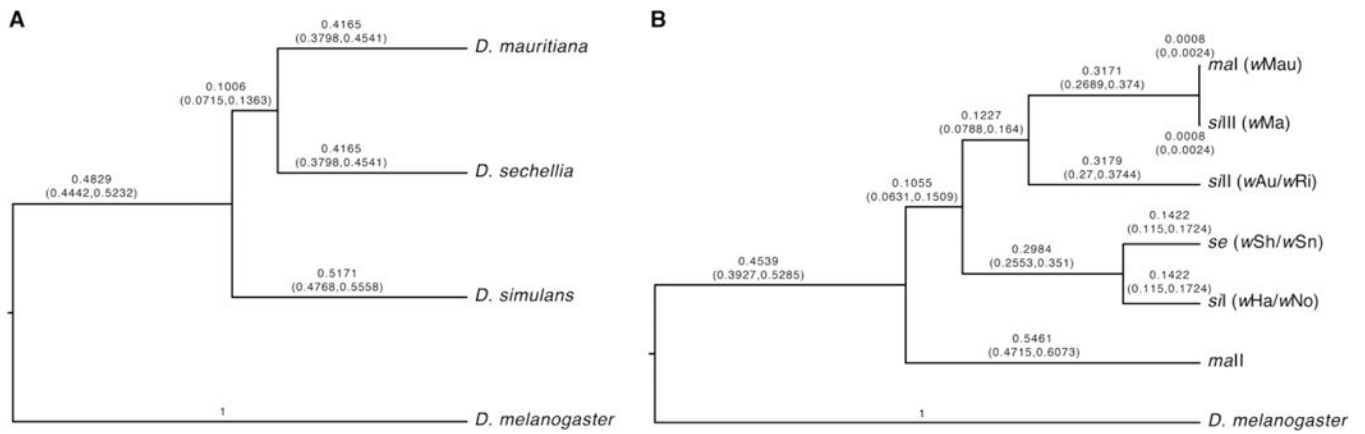
Figure 5.
A) A nuclear relative chronogram. **B)** A mitochondrial relative chronogram with co-occurring *Wolbachia* strains listed in parentheses. See the text for an interpretation of the results, including the artifactual resolution of the phylogeny of the *D. simulans* clade.

Table 1.

Estimates of the relative fitness parameter F indicate that wMau fecundity effects are likely to be minimal.

wMau variant/age class	F	95% BC$_a$ interval
$R31$	0.988	(0.862, 1.137)
$R41$	1.107	(0.995, 1.265)
$R31^{R41}$	1.012	(0.911, 1.122)
$R41^{R31}$	0.992	(0.884, 1.143)
$R31$(across age)	0.833	(0.515, 1.323)