Published in final edited form as:
Mol Ecol. 2014 October ; 23(19): 4871–4885. doi:10.1111/mec.12901.

Wolbachia do not live by reproductive manipulation alone: infection polymorphism in *Drosophila suzukii* and *D. subpulchrella*

Christopher A. Hamm[*,§], **David J. Begun**[*], **Alexandre Vo**[*], **Chris C. R. Smith**[*], **Perot Saelao**[*], **Amanda O. Shaver**[‡], **John Jaenike**[‡], and **Michael Turelli**[*]

[*]Department of Evolution and Ecology, University of California, One Shields Avenue, Davis, California 95616

[‡]Department of Biology, University of Rochester, Rochester, New York 14627

Abstract

Drosophila suzukii recently invaded North America and Europe. Populations in Hawaii, California, New York and Nova Scotia are polymorphic for *Wolbachia*, typically with <20% infection frequency. The *Wolbachia* in *D. suzukii*, denoted *w*Suz, is closely related to *w*Ri, the variant prevalent in continental populations of *D. simulans*. *w*Suz is also nearly identical to *Wolbachia* found in *D. subpulchrella*, plausibly *D. suzukii*'s sister species. This suggests vertical *Wolbachia* transmission through cladogenesis ("cladogenic transmission"). The widespread occurrence of 7-20% infection frequencies indicates a stable polymorphism. *w*Suz is imperfectly maternally transmitted, with wild infected females producing on average 5-10% uninfected progeny. As expected from its low frequency, *w*Suz produces no cytoplasmic incompatibility (CI), *i.e.*, no elevated embryo mortality when infected males mate with uninfected females, and no appreciable sex-ratio distortion. The persistence of *w*Suz despite imperfect maternal transmission suggests positive fitness effects. Assuming a balance between selection and imperfect transmission, we expect a fitness advantage on the order of 20%. Unexpectedly, *Wolbachia*-infected females produce fewer progeny than do uninfected females. We do not yet understand the maintenance of *w*Suz in *D. suzukii*. The absence of detectable CI in *D. suzukii* and *D. subpulchrella* makes it unlikely that CI-based mechanisms could be used to control this species without transinfection using novel *Wolbachia*. Contrary to their reputation as horizontally transmitted reproductive parasites, many *Wolbachia* infections are acquired through introgression or cladogenesis and many cause no appreciable reproductive manipulation. Such infections, likely

[§]Corresponding Author, current address: Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, Kansas 66045-7566, chamm@ku.edu, phone: (785) 864-3848.

The authors declare no conflicts of interest

Data accessibility: All chromatograms and sequences generated from the *Wolbachia* MLST protocol have been archived on the *Wolbachia* MLST database.
All data, accession numbers and statistical code have been deposited on Dryad: doi:10.5061/dryad.0pg63.

Author contributions: MT, JJ, CAH and DJB designed the research.
CAH, AV, CCRS, PS and AOS performed the research.
CAH, JJ, and MT analyzed the data.
MT and CAH wrote the paper.

to be mutualistic, may be central to understanding the pervasiveness of *Wolbachia* among arthropods.

Keywords

endosymbiont; mutualism; reproductive manipulation; fecundity; transmission

Introduction

Wolbachia are maternally transmitted, intracellular endosymbionts estimated to occur in nearly half of all insect species (Zug & Hammerstein 2012) and in many other arthropods (Bouchon *et al.* 1998) and filarial nematodes (Taylor *et al.* 2013). *Wolbachia* belong to the Rickettsiales order of α-Proteobacteria, whose members include the arthropod-vectored pathogens *Ehrlichia* and *Rickettsia* (Werren *et al.* 2008). Much *Wolbachia* research has focused on their ability to manipulate host reproduction to favor *Wolbachia* spread (Werren *et al.* 2008). Four types of reproductive manipulation are known: cytoplasmic incompatibility (CI), in which embryos produced by matings between infected males and uninfected females (or males and females with incompatible *Wolbachia*) suffer increased mortality (Hoffmann & Turelli 1997); male killing (MK), where infected females produce female-biased sex ratios (Hurst *et al.* 2000); feminization of genetic males (Rousset *et al.* 1992; Rigaud & Juchault 1993); and parthenogenesis induction (Rousset *et al.* 1992; Stouthamer *et al.* 1993). Only CI and MK are known in *Drosophila*. Transinfection experiments have established that both the nature and intensity of reproductive manipulation depend on host genetics and *Wolbachia* strain (Braig *et al.* 1994; Jaenike 2007; Zabalou *et al.* 2008; Veneti *et al.* 2012).

Despite the emphasis on reproductive effects, some *Wolbachia* infections cause little or no reproductive manipulation, including *w*Mel in *D. melanogaster* (Hoffmann 1988) and *w*Au in *D. simulans* (Hoffmann *et al.* 1996; Kriesner *et al.* 2013). Both infections exhibit imperfect maternal transmission, which should systematically reduce their frequency. Yet the *w*Mel-*D. melanogaster* association is minimally thousands of years old (Richardson *et al.* 2012; Chrostek *et al.* 2013); and *w*Au has been observed in Australia for over 20 years (approximately 200 fly generations), including a relatively rapid rise to an apparently stable equilibrium frequency near 0.6 (Kriesner *et al.* 2013). Presumably both infections persist by increasing host fitness (Hoffmann & Turelli 1997; Kriesner *et al.* 2013). Because *Wolbachia* are maternally transmitted, even when significant CI occurs, natural selection focuses on increasing fitness benefits for hosts rather than increasing reproductive manipulation (Turelli 1994; Haygood & Turelli 2009). Consistent with this expectation, *Wolbachia* have become critical to the survival and reproduction of several hosts. *Wolbachia* have been coevolving with filarial nematodes for millions of years; and removal causes various deleterious effects, including the inhibition of embryogenesis and larval development, reduced motility and adult viability, and stunted adult growth (Taylor *et al.* 2005). Within insects, *Wolbachia* are essential for female fertility in the parasitic wasp *Asobara tabida* (Dedeine *et al.* 2001). For several *Drosophila paulistorum* semispecies, *Wolbachia* have persisted through

cladogenesis and removal is lethal (Miller *et al.* 2010). In *D. mauritiana*, infected females produce four times as many eggs as uninfected females (Fast *et al.* 2011).

Wolbachia can enhance host fitness in more subtle ways, including metabolic provisioning (Brownlie *et al.* 2009) and protection from other microbes (Hedges *et al.* 2008; Teixeira *et al.* 2008). This recently discovered anti-microbial effect has revitalized efforts to use *Wolbachia* for disease control, an idea that goes back to the 1960s (Laven 1967; McGraw & O'Neill 2013). The disease-vector mosquito *Aedes aegypti* has been transinfected with two *Wolbachia* strains from *D. melanogaster* (McMeniman *et al.* 2009; Walker *et al.* 2011), and two natural Australian *Ae. aegypti* populations have been transformed with *w*Mel to suppress dengue virus transmission (Hoffmann *et al.* 2011). Recently *Anopheles stephensi* was transinfected with *Wolbachia*, making them less able to transmit the malarial parasite (Bian *et al.* 2013). These disease-suppression applications motivate additional analyses of *Wolbachia* in nature.

Although hundreds of papers concerning *Wolbachia* have appeared in the past decade, very few *Wolbachia*-host interactions have been studied intensively in nature. For fewer than 20 species do we have estimates of infection frequencies for multiple populations, analyses of transmission efficiency in nature, or analyses of reproductive manipulation or other phenotypes in the wild that might explain *Wolbachia* persistence and prevalence. Indeed, relatively complete scenarios explaining natural infection frequencies are available in very few cases, including: (1) the *w*Ri infection in *D. simulans*, which is maintained at a high level (about 93%) in most populations by a balance between fairly intense CI but imperfect maternal transmission (Turelli & Hoffmann 1991, 1995; Carrington *et al.* 2011; Kriesner *et al.* 2013); (2) the infections in the mosquitoes *Culex pipiens* (Barr 1980; Rasgon & Scott 2003) and *Ae. albopictus* (Kittayapong *et al.* 2002) that produce complete CI and exhibit essentially perfect maternal infection, so that almost all individuals in nature are infected; (3) the monomorphic infections in the *D. paulistorum* species complex, which cause CI, contribute to assortative mating and have evolved to obligate mutualism while persisting through cladogenesis (Miller *et al.* 2010); (4) the imperfectly transmitted male-killing (MK) strain in *D. innubila* that confers a selective advantage of about 5% and is maintained at ∼35% infection frequency (Dyer *et al.* 2004); and (5) the MK infection in the butterfly *Hypolimnas bolina*, which shows both high infection frequency and transmission efficiency (Charlat *et al.* 2009). In other species, such as *D. melanogaster* and *D. yakuba*, we know that *Wolbachia* persists despite imperfect maternal transmission and no appreciable reproductive manipulation (Harcombe & Hoffmann 2004; Charlat *et al.* 2004; but the fitness benefits maintaining the infection are not known with certainty, although plausible candidates exist (Teixeira *et al.* 2008; Brownlie *et al.* 2009).

Within host species, *Wolbachia* are typically maternally transmitted. In contrast, phylogenetic discordance between distantly related insect hosts and their *Wolbachia* (O'Neill *et al.* 1992; Werren *et al.* 1995) suggests that *Wolbachia* may be generally horizontally transmitted between species (Stahlhut *et al.* 2010; Jaenike 2012), unlike the co-speciation typical of some insect-endosymbiont mutualisms, such as aphids and *Buchnera* (Moran & Baumann 1994). Although coalescent analyses are consistent with some *Wolbachia* infections of *Drosophila* being relatively young, on the order of a few thousand years

(Richardson *et al.* 2012), some infections have persisted for hundreds of thousands of years (Jaenike & Dyer 2008), including through cladogenesis (Miller *et al.* 2010; Stahlhut *et al.* 2010). Because so few sister species have been examined for infection status, the frequency with which species acquire *Wolbachia* via descent (cladogenic transmission) or introgression (Rousset & Solignac 1995) versus horizontal transmission (O'Neill *et al.* 1992) is essentially unknown.

D. suzukii, the spotted wing *Drosophila*, is an invasive pest in North America and Europe that has spread rapidly, grows under a wide range of conditions (Tochen *et al.* 2014), and damages marketable fruit (Goodhue *et al.* 2011; Hauser 2011). Since its detection in coastal California in 2008, *D. suzukii* has spread through over half of the United States, Mexico and Canada (Fig. 1; Walsh 2011; Burrack *et al.* 2012; Freda & Braverman 2013). Since its discovery in Spain in 2008, it has also spread through most of continental Europe (Calabria *et al.* 2012; Cini *et al.* 2012). Given its economic importance and widespread distribution, *D. suzukii* has become a popular research organism (Hauser 2011), with an annotated genome (Chiu *et al.* 2013). *Wolbachia* was initially found in Japanese *D. suzukii* (Cordaux *et al.* 2008). Based on *wsp* sequencing, the infection was identified as *w*Ri (Bennett *et al.* 2012), the strain prevalent in most populations of *D. simulans* (Ballard 2000; Kriesner *et al.* 2013). While identical to *w*Ri at many commonly sequenced loci, a draft genome of *Wolbachia* from *D. suzukii* revealed several differences (Siozios *et al.* 2013), leading to the designation *w*Suz. Here we document *w*Suz infection in several populations of *D. suzukii* and describe initial attempts to better understand the population biology of *w*Suz and elucidate its prevalence, effects and origin, as such information might guide possible control measures. We also examined the *Wolbachia* infection in laboratory stocks of *D. subpulchrella*, plausibly *D. suzukii*'s sister species.

Materials and methods

Wolbachia detection and prevalence in natural populations

To determine *Wolbachia* prevalence in *D. suzukii*, in 2012 and/or 2013 we sampled one natural population in New York, two in California, and one in Nova Scotia (Fig. 1; Table 1). We also assayed two laboratory lines of *D. subpulchrella* and one of *D. biarmipes*. We used two concurrent PCR assays to determine the infection status of individual flies (Turelli & Hoffmann 1995). One reaction targeted the *Wolbachia*-specific 16S rDNA locus (Zhou *et al.* 1998; Werren & Windsor 2000), while the second targeted the arthropod-specific 28S rDNA (Folmer *et al.* 1994; Morse *et al.* 2009; primers used for all PCR experiments listed in Supplementary Material 1). Positive controls using single-copy nuclear genes are essential because failure to detect a *Wolbachia* PCR product could be due to: absence of *Wolbachia*, too much DNA, failure to extract DNA from the single-fly prep, or low-titer *Wolbachia* infection (*e.g.*, Miller *et al.* 2010; Schneider *et al.* 2014).

We extracted DNA following the “squish” buffer protocol (Gloor *et al.* 1993) or used a DNeasy Blood and Tissue kit (Qiagen). PCR reaction concentrations followed Hamm *et al.* (2014) and profiles were derived from Duron *et al.* (2008). PCR products were visualized on 1% agarose gels alongside a standard. We considered an individual infected when both the

Wolbachia-specific primers and nuclear controls produced fragments of the appropriate size. In population samples, *Wolbachia* infection frequency was estimated using only individuals with control-confirmed positive or negative infection status. We estimated exact 95% confidence intervals assuming a binomial distribution.

Of the California *D. suzukii* samples that failed to produce a *Wolbachia* band, 45 (15%) were randomly subjected to 1/10 and 1/100 dilutions of DNA and re-assayed. These samples were also assayed using quantitative real-time PCR (qPCR) to avoid false negative that might be produced by low-titer infections (Arthofer *et al.* 2009). These controls are important because highly variable infection titers have been reported within populations (Clark *et al.* 2005; Unckless *et al.* 2009). Using qPCR, we examined the titer of *Wolbachia* by amplifying a short segment of the *wsp* gene and comparing its abundance to that of *Rps17*, a reference nuclear gene (Osborne *et al.* 2009). All DNA utilized in qPCR experiments was extracted using a DNeasy kit. Each sample was assayed with four technical replicates per locus on an Illumina Eco™ real-time PCR machine. The concentrations and thermocycler profiles for qPCR followed Osborne *et al.* (2009) and relative *Wolbachia* density was estimated using the ΔCt method.

Wolbachia identification

To identify the *Wolbachia* strain(s) infecting *D. suzukii*, we randomly selected five infected females from Watsonville, California for multilocus sequence typing (MLST) (Baldo *et al.* 2006). We also typed the *Wolbachia* in *D. subpulchrella* line 201. Following Baldo *et al.* (2006), we sequenced five MLST protein-coding genes (*gatB, coxA, hcpA, ftsZ*, and *fbpA*) as well as *wsp*. Each gene was sequenced in both directions on an ABI 3730 DNA Analyzer (Applied Biosytems™) at the University of California, Davis DNA Sequencing Facility. The resulting chromatograms were assembled into contigs and visually inspected to ensure both reads were in agreement. These contigs were used as queries for a BLASTn search (Altschul *et al.* 1990) using the NCBI “nr” database to confirm that orthologous genes were amplified. Contigs were also used to search the *Wolbachia* MLST database (http://pubmlst.org/wolbachia/) using the “multiple locus query” feature. The allelic profiles were used to identify the *Wolbachia* strain. All chromatograms and sequences were deposited in the MLST database, and all data, accession numbers and statistical code were deposited on the DataDryad website (doi:10.5061/dryad.0pg63).

Given that new *Wolbachia* introductions should be associated with greatly reduced mitochondrial DNA variation (Turelli *et al.* 1992), we amplified ∼1300 bp of mitochondrial cytochrome oxidase I (*COI*) with PCR to estimate haplotype frequencies using 30 wild-caught individuals from California, 24 from New York, and from three lines of *D. subpulchrella* using a combination of primers (Folmer *et al.* 1994; Simon *et al.* 1994; Simon *et al.* 2006). Conditions for *COI* PCR followed the *hcpA* protocol from Baldo *et al.* (2006) or followed Haselkorn *et al.* (2013). To visualize the relationships among haplotypes, we generated a neighbor-joining tree using PAUP* (Swofford 2003) and asked if haplotypes were randomly associated with *Wolbachia* infection status, using Fisher's exact test.

Host phylogenies

Both *D. subpulchrella* (Takamori *et al.* 2006) and *D. biarmipes* have been proposed as sister to *D. suzukii* (van der Linde & Houle 2008; Yang *et al.* 2012). To reexamine these relationships, we used DNA from 13 protein-coding genes (3 mtDNA, 10 nDNA, downloaded from Genbank) for 10 members of the *melanogaster* species group (Supplementary Material 2). We conducted a partitioned Bayesian phylogenetic analysis using MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003) under the GTR substitution model with Γ-distributed rate heterogeneity. We ran this analysis using 10 chains for at least 1 million generations (with sampling every 5,000 generations) and until the standard deviation of split frequencies was below 0.05.

Maternal transmission

Wild female *D. suzukii* were collected in September 2012 from Rochester, NY. Flies were allowed five days for oviposition. Individual females were maintained in vials on 8 mL of banana medium (Drosophila Species Stock Center recipe), supplemented with pieces of strawberry as needed. Cultures were kept at 22°C, 70% RH, and a 12:12 light:dark cycle. Following the oviposition period, females were frozen for PCR *Wolbachia* screening. For *Wolbachia*-infected females, we screened all daughters, up to a maximum of 20. A second assay used wild-caught female *D. suzukii* collected in November 2013 from Watsonville, CA. Individual females were maintained as above in vials with 10 mL of “Bloomington” standard medium fly food and ½ a blueberry to stimulate oviposition. Females were allowed to oviposit for two days before being transferred to a fresh vial, and the experiment was continued for 10 days. We screened all offspring of *Wolbachia*-infected females for infection. We estimated the rate of maternal transmission and used a bias corrected and accelerated (BC_a) bootstrap to obtain 95% confidence intervals based on 10,000 pseudoreplicates (Efron & Tibshirani 1993).

Cytoplasmic incompatibility assays

Pairs of naturally infected and uninfected isofemale lines of *D. suzukii* were used for these experiments. Lines were established from wild-caught females from Rochester, NY (August 2012) and Central California (July 2009). Flies were reared as described above. Upon emergence, virgins were isolated and maintained on medium for five days. Immediately prior to mating a small piece of strawberry or ½ blueberry was provided. All four pairwise crosses between infection states were performed using single-pair matings. Flies were allowed to oviposit for 24 hours, and then transferred to fresh vials daily for four days. Following each transfer the eggs were counted, and after 48 hours the unhatched eggs were counted. We calculated the mean and standard error of hatch rate for each crossing type, averaging over the hatch rates associated with individual pairs. We did not distinguish between unfertilized eggs and dead embryos, and only crosses that generated at least 10 eggs were used in statistical analysis. Homogeneity among the four groups was examined using a Kruskal-Wallis rank-sum test. The infection status of males and females from each cross were verified by PCR. Similarly, CI assays were conducted using infected and uninfected *D. subpulchrella* laboratory strains, though here we compared the number of adult progeny produced.

Male killing (MK) assay

To investigate whether *Wolbachia* induced MK in *D. suzukii* or *D. subpulchrella*, we conducted reciprocal crosses between infected and uninfected males and females (derived from isofemale lines). For these experiments we used *D. suzukii* lines MTY3 (stock E-15003 from the Ehime, Japan Drosophila stock center), PacO (a stock previously available from Ehime), and MBW (a multi-female line established from Monterey Bay/Watsonville area by the Begun lab) and *D. subpulchrella* lines E-15201 (Ehime) and NGN5 (Ehime E-15203). Single pairs were placed in vials and maintained as above. Adults were transferred to fresh vials every two days. After the experiment, the parents were screened for *Wolbachia* to confirm infection status. Progeny from each cross were counted 14 days after the parents were removed. The total numbers of males and females for each experiment from infected lines were compared using a binomial test with $p = 0.5$.

Fecundity assays

Wild-caught *D. suzukii* were collected at an organic raspberry farm (Garroutte Farms) in Watsonville, California in October 2012 and October 2013. These flies were immediately taken to the laboratory, and individual females were reared as above with ½ a blueberry in each vial. Females were allowed to oviposit for two days before being transferred to a fresh vial, and the experiment was continued for 10 days. All ovipositing females were assayed for *Wolbachia*. The numbers of adult offspring from infected versus uninfected females were compared using a Wilcoxon signed-rank test.

We also compared the fecundity of infected versus uninfected females from laboratory stocks. Single pairs were placed in food vials and transferred to fresh vials every two days for a total of 25 days, after which the infection status of the pair was tested by PCR. After two weeks, the adult progeny were counted. We tested the infection status of five progeny from each cross. The numbers of offspring for *Wolbachia* infected and uninfected females were compared using a Wilcoxon test.

Because *Wolbachia* density can be influenced by many factors, including diet and rearing density, we conducted a fecundity experiment in which the parents were reared under more controlled conditions. We informally controlled density by placing three males and three females in food vials for three days; replicates were established for both infected and uninfected lines. From these offspring we collected virgins and held them for three days before performing reciprocal crosses (U♂ ⊥ I♀ and I♂ ⊥ U♀), placing one male and female in each vial. We transferred the pairs daily to fresh vials for five days. After two weeks the offspring from each cross were counted.

For *D. subpulchrella*, we controlled for nuclear effects on fecundity with reciprocal crosses between infected and uninfected lines. We informally controlled density by placing three males and three females in standard food vials with ½ blueberry for two days. Single pairs of virgin F_1 females and uninfected F_1 males were placed in holding vials with ½ blueberry for one day, then transferred to fresh food vials with ½ blueberry every day for five days. We counted emerged adults after 14 days, but excluded counts from females that produced fewer than 10 offspring.

Desiccation assay

To determine whether *Wolbachia* modified desiccation resistance, wild-caught *D. suzukii* males (collected from Watsonville, CA in October 2013) were placed individually in small test tubes, then transferred to a 0.04 m^3 glass aquarium with 200 gm of desiccant (Drierite). The tubes were checked every hour and dead flies removed. After 24 hours, the experiment was terminated and the flies screened for *Wolbachia*.

Starvation assay

To determine whether *Wolbachia* modified starvation resistance, we placed groups of five wild-caught *D. suzukii* females in standard *Drosophila* vials filled with 15 mL of 1% agar and placed the vials in an incubator. The vials were checked every 12 hours and dead flies were removed. After 72 hours, the experiment was terminated and the flies screened for *Wolbachia*.

Results

Wolbachia detection and prevalence

We found *D. suzukii* infected with *Wolbachia* throughout North America (Table 1). All 929 *D. suzukii* surveyed for *Wolbachia* produced a visible control band of the appropriate size for 28S rDNA. Of these, 159 were PCR-positive for *Wolbachia*. Of the 475 California flies that produced a 28S rDNA product but failed to generate a *Wolbachia* product, we assayed 45 using serial dilution, low-titer primers for the *SMArTR* and 12S rDNA loci (Schneider *et al.* 2014), and qPCR. These more sensitive assays also failed to detect *Wolbachia* infection.

In eight of our nine samples, infection frequencies ranged from 7 to 23% among the four populations surveyed. One sample from Winters, CA produced an outlier frequency of 58% (Table 1). The infection frequencies were not equal across all samples ($\chi^2 = 103.3$, df = 8, $P < 0.0001$). However, the eastern samples (New York and Nova Scotia) were statistically homogeneous ($\chi^2 = 1.2$, df = 2, $P = 0.55$), with an overall infection frequency of 0.08 and 95% confidence interval of (0.05, 0.12). Similarly, without the outlier, the California samples were homogeneous ($\chi^2 = 2.0$, df = 4, $P = 0.74$), with an overall infection frequency of 0.17 (0.14, 0.21). Even without the anomalous May 2013 sample from Winters, CA, the pooled eastern versus western samples showed significantly different frequencies ($\chi^2 = 16.5$ $P = 0.02$). The anomalous Winters sample corresponded to a statistically significant infection frequency increase between June 2012 and May 2013 ($\chi^2 = 15.3$, $P < 0.001$). Using each collection as a single observation of *Wolbachia* infection prevalence, the data suggest that the mean prevalence was greater in California than in eastern populations (Kruskal-Wallis χ^2 test; $Z = 5.4$, $P = 0.02$). Strain E-15201 of *D. subpulchrella* carried *Wolbachia*, whereas strain NGN5 of *D. subpulchrella* and the genome strain of *D. biarmipes* were uninfected.

Wolbachia identification and mtDNA diversity

Using the *Wolbachia* MLST protocol on infected *D. suzukii*, we identified the following alleles with 100% identity to previously reported alleles for each of the five protein-coding genes sequenced: *gatB* 22, *coxA* 23, *hcpA* 24, *ftsZ* 3, and *fbpA* 23. This allelic profile

corresponded with strain 17 in the MLST database, the *w*Ri strain found in *D. simulans* (Hoffmann *et al.* 1986). We observed the same allelic profile (100% identity) in *D. subpulchrella*. Similarly, *D. suzukii* and *D. subpulchrella* showed 100% sequence identity with *wsp* allele 16 found in *w*Ri of *D. simulans*.

We sequenced over 1200bp of mitochondrial *COI* for 23 female *D. suzukii* from Watsonville, CA and three *D. subpulchrella* lines. We identified five mtDNA haplotypes and found three haplotypes shared between infected and uninfected individuals. In contrast to the identity of the *Wolbachia* genotypes, we observed 11 fixed differences between the *COI* haplotypes of *D. suzukii* and *D. subpulchrella* (Fig. 2). The same five *COI* haplotypes were found in *D. suzukii* from Rochester. As expected with imperfect maternal transmission of *Wolbachia* and an infection that has approached its equilibrium frequency (Turelli *et al.* 1992), the infection was randomly associated with *D. suzukii* mtDNA haplotypes (Fisher's exact test, $P = 0.29$ based on 10,000 Monte Carlo simulations).

Host phylogenetics

To understand the evolutionary history of *Wolbachia* infection in *D. suzukii* and *D. subpulchrella*, we estimated the phylogeny of the *suzukii* subgroup, using 11,382 bp from 13 protein-coding loci. The fully resolved Bayesian tree placed the members of the *D. suzukii* subgroup in a monophyletic clade with *D. subpulchrella* sister to *D. suzukii* (a result concordant with the mtDNA tree) with high posterior support (Fig. 2).

Maternal transmission

We screened the offspring of wild-caught *D. suzukii* females infected with *Wolbachia* to estimate transmission frequency. From Rochester, NY samples we screened the female offspring of 14 *Wolbachia*-infected females that produced at least 20 offspring. We screened male and female progeny from six infected females from Watsonville, CA. Most females perfectly transmitted *Wolbachia* to both male and female offspring (Fig. 3), though six exhibited imperfect transmission with transmission rates varying from 95% to 20% (Fig. 3). The mean transmission rate was 0.86 with a 95% BC_a bootstrap confidence interval of (0.73, 0.96). Given this variation, many infected females would have to be screened to accurately assess the fraction of “low transmitters”; but maternal transmission of *Wolbachia* is clearly imperfect in natural populations of *D. suzukii*.

Cytoplasmic incompatibility (CI)

With CI, we expect reduced egg hatch when infected males mate with uninfected females. We found no evidence for CI in *D. suzukii* (Table 2). For our California analysis, hatch rates were homogeneous among all four classes of crosses (Kruskal-Wallis test, $P = 0.7$). In contrast, the hatch rates among crosses of New York *D. suzukii* were not homogeneous (Kruskal-Wallis test, $P = 0.005$). However, this was due to an unexpected low hatch rate from crosses between uninfected males and infected females, the opposite of what would be expected with CI. Similarly, we found no evidence of CI in *D. subpulchrella* (Table 3). Although the numbers of progeny were not homogeneous among groups (Kruskal-Wallis test, $P = 0.04$; Table 3) (Table 3), there was no significant difference between the potential

CI cross and its reciprocal (Wilcoxon test $P = 0.82$). In principle, CI might be observed with males of different ages (Hoffmann *et al.* 1986; Hoffmann 1988).

Male killing (MK)

With MK, we expect biased sex ratios from infected mothers. Reciprocal crosses between infected and uninfected lines of *D. suzukii* and *D. subpulchrella* revealed no evidence of female-biased sex ratio in either *D. suzukii* ($P = 0.37$; $N = 179$) or *D. subpulchrella* ($P = 0.47$; $N = 507$) (Fig. 4).

Fecundity assays

To offset imperfect maternal transmission, we expected a fecundity advantage for *Wolbachia*-infected females. We collected 40 *D. suzukii* females from Watsonville, CA in 2012 and 66 in 2013. In both 2012 and 2013, we detected a significant fecundity disadvantage for infected females [2012: I 10.3 ± 8.4 ($N = 7$); U 51.6 ± 8.3 ($N = 29$), two-tailed Wilcoxon test, $P < 0.005$; 2013: I 19.9 ± 6.5 ($N = 20$); U 54.5 ± 8.3 ($N = 9$), Wilcoxon test, $P = 0.02$]. In principle, these differences could have been caused by host genotype differences rather than infection status. However, with imperfect maternal transmission, infection status is rapidly randomized over both nuclear and mitochondrial genotypes (Turelli *et al.* 1992).

We complemented these field assays by crossing infected and uninfected laboratory lines of *D. suzukii* in both New York and California. Again we detected a significant fecundity disadvantage associated with *Wolbachia* (Table 4, Wilcoxon tests, $P < 0.01$). When repeated with density control, the infected females produced slightly fewer offspring than uninfected (I 7.6 ± 1.7 ($N = 8$); U 9.6 ± 3.4 ($N = 14$) but the difference was not statistically significant (Wilcoxon test, $P = 0.41$). Our density-controlled fecundity experiment for *D. subpulchrella* showed no statistically significant difference in the number of offspring produced by infected versus uninfected females produced from reciprocal crosses (I 30.9 ± 2.8 [$N = 15$]; U 33.7 ± 2.9 [$N = 11$]; Wilcoxon test, $P = 0.58$).

Although several tests indicated a *Wolbachia*-associated reduction in fecundity, some infected females had apparently normal numbers of offspring. We used qPCR to compare *Wolbachia* titer among females and found no significant association between the level of infection and fecundity (linear regression, $P = 0.31$, df = 1,8).

Desiccation assay

We assayed 76 male *D. suzukii* for resistance to desiccation, 12 of which were *Wolbachia*-infected. The mean survival time for infected flies was 14.25 hours [95% confidence (12.9, 15.5; $N = 12$)], while the mean survival time for uninfected flies was 13.16 hours (9.9, 16.4; $N = 64$). Our data provided no evidence that *Wolbachia* increases desiccation resistance (Wilcoxon test, $P = 0.67$). Only males were available for the desiccation assay due the requirements for females in other experiments.

Starvation assay

We assayed 70 female *D. suzukii* for resistance to starvation, 11 of which were *Wolbachia*-infected. Mortality rates were fairly high within the first 12 hours of the experiment because many flies stuck to the agar. Excluding these flies, the mean survival times for infected and uninfected flies were 26.6 (20.5, 32.8; $N = 9$) and 22.7 (18.8, 25.7; $N = 55$) hours, respectively. We detected no *Wolbachia* effect on survival (Wilcoxon test, $P = 0.205$).

Discussion

What maintains *w*Suz in *D. suzukii*?

Wolbachia are best known for reproductive manipulations that increase the representation of infected cytoplasms. Yet, we found no evidence of cytoplasmic incompatibility (CI) or male killing (MK) in *D. suzukii* or *D. subpulchrella*. Reciprocal crosses between infected and uninfected lineages do not show CI (Tables 2 and 3). Furthermore, *Wolbachia*-infected *D. suzukii* and *D. subpulchrella* females produce 1:1 offspring sex ratios, indicating neither MK nor feminization (Fig. 4). Thus, efforts to control or manipulate *D. suzukii* by *Wolbachia*-induced reproductive manipulation, if possible at all, would require transinfection with non-native *Wolbachia*. CI or MK may be observed with males and females of different ages than we have used (Hoffmann *et al.* 1986; Hoffmann 1988). This will be explored in future analyses.

With no apparent reproductive manipulation, *w*Suz also shows imperfect maternal transmission in *D. suzukii*. This should systematically reduce its frequency. Yet, the widespread geographic distribution of 7-20% infection frequencies, including our 2012-13 North American samples (Table 1) and an earlier Hawaiian sample (Bennett *et al.* 2013), strongly suggests that *w*Suz is stably maintained, as does the random association between *Wolbachia* infection and mtDNA haplotypes. The simplest explanation is that *w*Suz produces a fitness advantage that balances its imperfect transmission (Hoffmann & Turelli 1997). Under this scenario, if a fraction μ of the ova produced by infected females are uninfected, *w*Suz should persist only if it enhances the relative fitness, F, of infected females sufficiently that $F(1 - \mu) > 1$. With constant parameter values, the predicted equilibrium frequency is $p = 1 - [\mu F/(F - 1)]$. Hence, to maintain frequencies on the order of 5-20% in the face of $\mu \approx 15\%$, the selective advantage, $F - 1$, associated with *w*Suz should be appreciable, on the order of 20%.

This prediction motivated our experiments examining relative fecundity, starvation tolerance and desiccation resistance. None of our assays revealed a beneficial effect of *w*Suz. We may have assayed the wrong phenotypes, our experiments (apart from fecundity) may have been too small, or fitness benefits may be context-dependent. *Wolbachia* strains closely related to *w*Suz confer resistance to RNA viruses that are otherwise virulent to *Drosophila* (Hedges *et al.*2008; Teixeira *et al.* 2008; Osborne *et al.* 2009; Unckless & Jaenike 2012). *D. suzukii* has a relatively robust immune response, associated with increased hemocyte loads relative to *D. melanogaster* (Kacsoh & Schlenke 2012); and its largest gene-family expansions and contractions are related to immune response (Chiu *et al.* 2013). Hence, effects of *Wolbachia*

on immune response are natural candidates for the expected fitness benefits we have not yet discovered.

Temporal and spatial heterogeneity of infection frequencies

If *w*Suz confers resistance to microparasites, which often occur as epidemics, substantial fluctuations in prevalence may be expected. Thus, the significant increase in *Wolbachia* infection frequency in the Winters, CA population from 18% to 58% ($P < 0.001$) in less than one year may reflect a cryptic epidemic. Anomalous frequency changes have also been observed in *D. simulans* (Turelli & Hoffmann 1995), and Unckless *et al.* (2009) documented seasonal changes in *Wolbachia* titer in *D. innubila* that may alter fitness effects. The frequency differences observed in eastern and western samples may reflect systematic environmental differences. Alternatively, because *D. suzukii* has been detected in California longer than in northeastern US (Burrack *et al.* 2012), frequency differences may reflect increased build up in pathogen loads in the west. These conjectures will be tested in future studies, along with the effects of *w*Suz on pathogen resistance, development time, longevity and mating. For now, the apparent persistence of *w*Suz remains a mystery.

Although we have sampled only a handful of populations, we found a significantly lower frequency of *w*Suz infection in eastern (New York and Nova Scotia) than western *D. suzukii* populations. Continued sampling will reveal whether the difference persists or is a consequence of chance effects associated with recent invasion. Fig. 1 shows that *D. suzukii* had a disjunct distribution across the United States in 2013. Because this species was first discovered in California and Florida, it is likely that northeast populations were derived from the west or south, plausibly as a result of shipping strawberries or raspberries, favored *D. suzukii* breeding resources (Goodhue *et al.* 2011). If the lower eastern frequencies are founder events, infection prevalence might increase to the level seen in California. Given the inferred selective advantage of carrying *Wolbachia*, such frequency increases may be detectable within a few years.

Master manipulator or helpful guest?

The *Wolbachia* strains present in the sister taxa *D. suzukii* and *D. subpulchrella* are very closely related (identical at the five MLST loci and *wsp*), whereas their mtDNA show significant differences. Moreover, in *D. suzukii* the same mtDNA haplotypes are found among infected and uninfected individuals. These facts indicate that the association of *w*Suz with *D. suzukii* is old. The lack of differentiation between *Wolbachia* in *D. suzukii* and *D. subpulchrella*, combined with appreciable interspecific mtDNA differences, suggests that an infection in their most recent common ancestor may have persisted through cladogenesis. Cladogenic transmission has also been proposed for *Wolbachia* shared by *D. simulans* and *D. sechellia* (Rousset & Solignac 1995), for three species of the *D. testacea* group (Jaenike *et al.* 2010) and for several of the reproductively isolated *D. paulistorum* semispecies (Miller *et al.* 2010).

Although horizontal transmission of *Wolbachia* is proven by phylogenetic discordance between *Wolbachia* and distantly related hosts (O'Neill *et al.* 1992), only comparisons of sister species can reveal the relative frequencies of horizontal transmission versus

cladogenic transmission or introgression. Horizontal transmission is clearly indicated when closely related hosts harbor distantly related *Wolbachia*. In contrast, when closely related hosts harbor closely related *Wolbachia*, cladogenic transmission or introgression seems more plausible. Recent introgression can be ruled out if mtDNA and nuclear loci show concordant phylogenies. Hence, we have inferred cladogenic transmission of the closely related *Wolbachia* in *D. suzukii* and *D. subpulchrella*. Table 5 provides a preliminary summary of what is known within the genus *Drosophila* about *Wolbachia* reproductive manipulation and mode of acquisition. Except where noted, we have accepted the conclusions of the original investigators. When closely related hosts harbor very similar *Wolbachia*, we have inferred cladogenic transmission. What is most striking from Table 5 is how few infections have been characterized and how often transmission has been via cladogenesis or introgression (as evidenced by mtDNA comparisons, cf. Rousset & Solignac 1995). Table 5 proposes several new examples of cladogenic transmission among Hawaiian *Drosophila*. Additional mtDNA analyses may support introgression in some of these cases; however, our preliminary analysis suggests that fewer than half of *Wolbachia*-infected *Drosophila* received their infections via horizontal transmission (9 out of 31 cases in Table 5).

The lack of detectable reproductive manipulation by the *Wolbachia* in *D. suzukii* and *D. subpulchrella* may reflect a long history of coevolution (Hoffmann & Turelli 1997; Hornett *et al.* 2006). As Table 5 shows, lack of detectable CI or MK is not uncommon. Prior to PCR assays, there was an ascertainment bias favoring the discovery of *Wolbachia* that manipulate reproduction. With so few natural *Wolbachia* infections characterized for their effects, we know relatively little about the frequency or intensity of reproductive manipulation. For very few hosts do we know *Wolbachia* frequencies in natural populations. Finding persistent low-frequency infections rules out significant CI, which produces stable equilibrium frequencies above 0.5 (Turelli & Hoffmann 1995). Mutualistic effects may prove more common and/or more important for *Wolbachia* prevalence than reproductive manipulation (cf. Jaenike & Brekke 2011).

Combining infection studies of sister species with tests of reproductive manipulation will help us understand *Wolbachia* biology and patterns of coevolution with its hosts. In addition to providing a model for host-symbiont coevolution, *Wolbachia* hold significant promise for the control of vector-borne diseases (McGraw & O'Neill 2013). Although we do not yet understand what maintains *w*Suz in *D. suzukii*, it is clear that *Wolbachia* infections need not manipulate host reproduction to persist or spread (Hoffmann 1988; Hoffmann *et al.* 1996; Charlat *et al.* 2004; Kriesner *et al.* 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ary Hoffmann, Wolfgang Miller and an anonymous reviewer for constructive comments on an earlier versions. We thank Emma Dietrich, Wyatt Hanft, Derek Leale, and Andrew Tremain for assistance with the experiments and Artyom Kopp for providing *D. subpulchrella* stocks. This research was supported in part by NIH

grant R01 GM104325 to MT, NIH grant R01 GM084056 to DJB and NSF grant DEB-1144581 to JJ. CAH was supported by USDA-NIFA SCRI Competitive Research Grant 2010-51181-21167 to DJB.

References

Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. Journal of Molecular Biology. 1990; 215:403–410. [PubMed: 2231712]

Arthofer W, Riegler M, Schneider D, et al. Hidden *Wolbachia* diversity in field populations of the European cherry fruit fly, *Rhagoletis cerasi* (Diptera, Tephritidae). Molecular Ecology. 2009; 18:3816–3830. [PubMed: 19732336]

Bachtrog D, Thornton K, Clark A, et al. Extensive introgression of mitochondrial DNA relative to nuclear DNA in the *Drosophila yakuba* species group. Evolution. 1980; 60:292–302. [PubMed: 16610321]

Barr AR. Cytoplasmic incompatibility in natural populations of mosquito, *Culex pipiens* L. Nature. 1980; 283:71–72. [PubMed: 7350526]

Baldo L, Hotopp JCD, Jolley KA, et al. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Applied and Environmental Microbiology. 2006; 72:7098–7110. [PubMed: 16936055]

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

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

Bennett GM, Pantoja NA, O'Grady PM. Diversity and phylogenetic relationships of Wolbachia in Drosophila and other native Hawaiian insects. Fly. 2012; 6:1–11.

Bian G, Joshi D, Dong Y, et al. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. Science. 2013; 340:748–751. [PubMed: 23661760]

Bouchon D, Rigaud T, Juchault P. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. Proceedings of the Royal Society of London B. 1998; 265:1081–1090.

Bourtzis K, Nirgianaki A, Markakis G, et al. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. Genetics. 1996; 144:1063–1073. [PubMed: 8913750]

Braig HR, Guzman H, Tesh RB, et al. Replacement of the natural *Wolbachia* symbiont of Drosophila simulans with a mosquito counterpart. Nature. 1994; 367:453–455. [PubMed: 7906391]

Brownlie JC, Cass BN, Riegler M, et al. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia* pipientis, during periods of nutritional stress. PLoS Pathogens. 2009; 5:e1000368. [PubMed: 19343208]

Burrack HJ, Smith JP, Pfeiffer DG, et al. Using volunteer-based networks to track *Drosophila suzukii* (Diptera: Drosophilidae) an invasive pest of fruit crops. Journal of Integrated Pest Management. 2012; 4 2012 DOI: http://dx.doi.org/10.1603/IPM12012.

Calabria G, Máca J, Bächli G, et al. First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. Journal of Applied Entomology. 2012; 136:139–147.

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

Charlat S, Ballard JWO, Mercot H. What maintains noncytoplasmic incompatibility inducing *Wolbachia* in their hosts: a case study from a natural *Drosophila yakuba* population. J Evolutionary Biology. 2004; 17:322–330.

Charlat S, Duplouy A, Hornett EA, et al. The joint evolutionary histories of *Wolbachia* and mitochondria in *Hypolimnas bolina*. BMC Evolutionary Biology. 2009; 9:64. [PubMed: 19317891]

Chiu JC, Jiang X, Zhao L, et al. Genome of *Drosophila suzukii*, the Spotted Wing Drosophila. G3. 2013; 3:2257–2271. [PubMed: 24142924]

Chrostek E, Marialva MSP, Esteves SS, et al. *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenetic analysis. PLoS Genetics. 2013; 9:e1003896. [PubMed: 24348259]

Cini A, Ioriatti C, Anfora G. A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. Bulletin of Insectology. 2012; 65:149–160.

Clark ME, Anderson CL, Cande J, et al. Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research. Genetics. 2005; 170:1667–1675. [PubMed: 15937134]

Cordaux R, Pichon S, Ling A, et al. Intense transpositional activity on insertion sequences in an ancient obligate endosymbiont. Molecular Biology and Evolution. 2008; 25:1889–1896. [PubMed: 18562339]

Dedeine F, Vavre F, Fleury F. Removing symbiotic *Wolbachia* specifically inhibits oogenesis in a parasitic wasp. Proceedings of the National Academy of Sciences. 2001; 98:6247–6252.

Duron O, Bouchon D, Boutin S, et al. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biology. 2008; 6:27. [PubMed: 18577218]

Dyer K, Jaenike J. Evolutionarily stable infection by a male-killing endosymbiont in *Drosophila innubila*: Molecular evidence from the host and parasite genomes. Genetics. 2004; 168:1443–1455. [PubMed: 15579697]

Dyer KA, Burke C, Jaenike J. *Wolbachia*-mediated persistence of mtDNA from a potentially extinct species. Molecular Ecology. 2011; 2:2805–2817. [PubMed: 21595768]

Efron, B.; Tibshirani, RJ. An introduction to the bootstrap. New York: Chapman & Hall; 1993. p. 436

Fast EM, Toomey ME, Panaram K, et al. *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. Science. 2011; 334:990–992. [PubMed: 22021671]

Folmer O, Black M, Hoeh W, et al. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology. 1994; 3:294–299. [PubMed: 7881515]

Freda PJ, Braverman JM. *Drosophila suzukii*, or Spotted Wing Drosophila, Recorded in Southeastern Pennsylvania, U.S.A. Entomological News. 2013; 123:71–75.

Gloor GB, Preston CR, Johnson-Schlitz DM, et al. Type I repressors of P element mobility. Genetics. 1993; 135:81–95. [PubMed: 8224830]

Goodhue RE, Bolda M, Farnsworth D, et al. Spotted wing drosophila infestation of California strawberries and raspberries: economic analysis of potential revenue losses and control costs. Pest Management Science. 2011; 67:1396–1402. [PubMed: 21815244]

Haine ER, Pickup NJ, Cook JM. Horizontal transmission of *Wolbachia* in a *Drosophila* community. Ecological Entomology. 2005; 30:464–472.

Hamm CA, Handley CA, Pike A, et al. *Wolbachia* infection and Lepidoptera of conservation concern. Journal of Insect Science. 2014; 14:6.

Harcombe W, Hoffmann AA. Wolbachia effects in *Drosophila melanogaster*: in search of fitness benefits. Journal of Invertebrate Pathology. 2004; 87:45–50. [PubMed: 15491598]

Haselkorn TS, Cockburn SN, Hamilton PT, et al. Infectious adaptation: potential host range of a defensive endosymbiont in *Drosophila*. Evolution. 2013; 67:934–945. [PubMed: 23550746]

Hauser M. A historic account of the invasion of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the continental United States, with remarks on their identification. Pest Management Science. 2011; 67:1352–1357. [PubMed: 21898759]

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

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

Hoffmann AA. Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. Entomologia Experimentalis et Applicata. 1988; 48:61–67.

Hoffmann, AA.; Turelli, M. Cytoplasmic incompatibility in insects. In: O'Neill, SL.; Hoffmann, AA.; Werren, JH., editors. Influential Passengers: Inherited microorganisms and arthropod reproduction. Oxford University Press; New York, NY: 1997. p. 42-80.

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

Hoffmann AA, Turelli M, Simmons GM. Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution. 1986:692–701.

Hoffmann AA, Montgomery BL, Popovici J, et al. Successful establishment of *Wolbachia* in *Aedes populations* to suppress dengue transmission. Nature. 2011; 476:454–457. [PubMed: 21866160]

Hornett EA, Charlat S, Duplouy AMR, et al. Evolution of male killer suppression in a natural population. PLoS Biology. 2006; 4:e283. [PubMed: 16933972]

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

Jaenike J. Spontaneous emergence of a new *Wolbachia* phenotype. Evolution. 2007; 61:2244–2252. [PubMed: 17767593]

Jaenike J. Population genetics of beneficial heritable symbionts. Trends in Ecology and Evolution. 2012; 27:226–232. [PubMed: 22104387]

Jaenike J, Brekke TD. Defensive endosymbionts: a cryptic trophic level in community ecology. Ecology Letters. 2011; 14:150–155. [PubMed: 21155960]

Jaenike J, Dyer KA. No resistance to male-killing *Wolbachia* after thousands of years of infection. Journal of Evolutionary Biology. 2008; 21:1570–1577. [PubMed: 18717746]

Jaenike J, Stahlhut JK, Boello LM, et al. Association between *Wolbachia* and *Spiroplasma* within *Drosophila neotestacea*: an emerging symbiotic mutualism. Molecular Ecology. 2010; 19:414–425. [PubMed: 20002580]

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

Kacsoh BZ, Schlenke T. High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. PLoS ONE. 2012; 7:e34721. [PubMed: 22529929]

Kittayapong P, Mongkalangoon P, Baimai V, et al. Host age effect and expression of cytoplasmic incompatibility in field populations of *Wolbachia*-superinfected *Aedes albopictus*. Heredity. 2002; 88:270–274. [PubMed: 11920134]

Kriesner P, Hoffmann AA, Lee SF, et al. Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans*. PLoS Pathogens. 2013; 9:e1003607. [PubMed: 24068927]

Lachaise D, Harry M, Solignac M, et al. Evolutionary novelties in islands: *Drosophila santomea*, a new *melanogaster* sister species from São Tomé. Proceedings of the Royal Society of London B. 2000; 267:1487–1495.

Laven H. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. Nature. 1967; 216:383–384. [PubMed: 4228275]

Llopart A, Herrig D, Brud E, et al. Sequential adaptive introgression of the mitochondrial genome in *Drosophila yakuba* and *Drosophila santomea*. Molecular Ecology. 2014; 23:1124–1136. [PubMed: 24460929]

Mateos M, Castrezana SJ, Nankivell BJ, et al. Heritable endosymbionts in *Drosophila*. Genetics. 2006; 174:363–376. [PubMed: 16783009]

McGraw EA, O'Neill SL. Beyond insecticides: new thinking on an ancient problem. Nature Reviews Microbiology. 2013; 11:181–193.

McMeniman CJ, Lane RV, Cass BN, et al. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Science. 2009; 323:141–144. [PubMed: 19119237]

Merçot H, Llorente B, Jacques M, et al. Variability within the Seychelles cytoplasmic incompatibility system in *Drosophila simulans*. Genetics. 1995; 141:1015–1023. [PubMed: 8582608]

Miller WJ, Riegler M. Evolutionary dynamics of *w*Au-like *Wolbachia* variants in neotropical *Drosophila* spp. Applied and Environmental Microbiology. 2006; 72:826–835. [PubMed: 16391124]

Miller WJ, Ehrman L, Schneider D. Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. PLoS Pathogens. 2010; 6:e1001214. [PubMed: 21151959]

Montenegro H, Hatadani LM, Medeiros HF, et al. Male killing in three species of the *tripunctata* radiation of *Drosophila* (Diptera: Drosophilidae). Journal of Zoological Systematics. 2006; 44:130–135.

Moran N, Baumann P. Phylogenetics of cytoplasmically inherited microorganisms of arthropods. Trends in Ecology and Evolution. 1994; 9:15–20. [PubMed: 21236755]

Morse JG, Rugman-Jones PF, Watson GW, et al. High levels of exotic armored scales on imported avocados raise concerns regarding USDA–APHIS' phytosanitary risk assessment. Journal of Economic Entomology. 2009; 102:855–867. [PubMed: 19610397]

Müller MJ, Dörr NCD, Deprá M, et al. Reevaluating the infection status by the *Wolbachia* endosymbiont in *Drosophila* Neotropical species from the *willistoni* subgroup. Infection, Genetics, and Evolution. 2013; 19:232–239.

O'Grady PM, Lapoint RT, Bonacum J, et al. Phylogenetic and ecological relationships of the Hawaiian *Drosophila* inferred from mitochondrial DNA analysis. Molecular Phylogenetics and Evolution. 2011; 58:244–256. [PubMed: 21144904]

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

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

Osborne SE, Leong YS, O'Neill SL, et al. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. PLoS Pathogens. 2009; 5:e1000656. [PubMed: 19911047]

Rasgon JL, Scott TW. Wolbachia and cytoplasmic incompatibility in the California *Culex pipiens* mosquito species complex: parameter estimates and infection dynamics in natural populations. Genetics. 2003; 165:2029–2038. [PubMed: 14704183]

Richardson MF, Weinert LA, Welch JJ, et al. Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*. PLoS Genetics. 2012; 8:e1003129. [PubMed: 23284297]

Rigaud T, Juchault P. Conflict between feminizing sex ratio distorters and an autosomal masculinizing gene in the terrestrial isopod *Armadillidium vulgare* Latr. Genetics. 1993; 133:247–252. [PubMed: 8436273]

Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19:1572–1574. [PubMed: 12912839]

Rousset F, Bouchon D, Pintureau B, et al. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proceedings of the Royal Society of London B. 1992; 250:91–98.

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

Schneider DI, Klasson L, Lind AE, et al. More than fishing in the dark: PCR of a dispersed sequence produces simple but ultrasensitive *Wolbachia* detection. BMC Microbiology. 2014; 14:121. [PubMed: 24885505]

Simon C, Buckley T, Frati F, et al. Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. Annual Review of Ecology and Systematics. 2006; 37:545–579.

Simon C, Frati F, Beckenbach A, et al. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America. 1994; 87:651–701.

Siozios S, Cestaro A, Kaur R, et al. Draft genome sequence of the *Wolbachia* endosymbiont of *Drosophila suzukii*. Genome Announcements. 2013; 1:e00032–13. [PubMed: 23472225]

Stahlhut JK, Desjardins CA, Clark ME, et al. The mushroom habitat as an ecological arena for global exchange of *Wolbachia*. Molecular Ecology. 2010; 19:1940–1952. [PubMed: 20529071]

Sheeley SL, McAllister BF. Mobile male-killer: similar *Wolbachia* strains kill males of divergent *Drosophila* hosts. Heredity. 2009; 102:286–292. [PubMed: 19142204]

Stouthamer R, Breeuwert JA, Luck RF, et al. Molecular identification of microorganisms associated with parthenogenesis. Nature. 1993; 361:66–68. [PubMed: 7538198]

Swofford, DL. Phylogenetic analysis using parsimony (*and other methods) version 4. Sinauer Associates; Sunderland, MA, USA: 2003.

Takamori H, Watabe H, Fuyama Y, et al. *Drosophila subpulchrella*, a new species of the *Drosophila suzukii* species subgroup from Japan and China (Diptera: Drosophilidae). Entomological Science. 2006; 9:121–128.

Taylor MJ, Bandi C, Hoerauf A. *Wolbachia* bacterial endosymbionts of filarial nematodes. Advances in Parasitology. 2005; 60:245–284. [PubMed: 16230105]

Taylor MJ, Voronin D, Johnston KL, et al. *Wolbachia* filarial interactions. Cellular Microbiology. 2013; 15:520–526.

Teixeira L, Ferreira Á, Ashburner M. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biology. 2008; 6:e2. [PubMed: 19222304]

Tochen S, Dalton DT, Wiman N, et al. Temperature-related development and population parameters for *Drosophila suzukii* (Diptera: Drosophilidae) on cherry and blueberry. Environmental Entomology. 2014; 43:501–510. [PubMed: 24612968]

Turelli M. Evolution of incompatibility-inducing microbes and their hosts. Evolution. 1994; 48:1500–1513.

Turelli M, Hoffmann AA. Rapid spread of an inherited incompatibility factor in California *Drosophila*. Nature. 1991; 353:440–442. [PubMed: 1896086]

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

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

Unckless RL, Boelio LM, Herren JK, et al. *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. Proceedings of the Royal Society B. 2009; 276:2805–2811. [PubMed: 19419989]

Unckless RL, Jaenike J. Maintenance of a male-killing *Wolbachia* in *Drosophila innubila* by male-killing dependent and male-killing independent mechanisms. Evolution. 2012; 66:678–689. [PubMed: 22380432]

Van Der Linde K, Houle D. A supertree analysis and literature review of the genus *Drosophila* and closely related genera (Diptera, Drosophilidae). Insect Systematics & Evolution. 2008; 39:241–267.

Veneti Z, Zabalou S, Papafotiou G, et al. Loss of reproductive parasitism following transfer of male-killing *Wolbachia* to *Drosophila melanogaster* and *Drosophila simulans*. Heredity. 2012; 109:306–312. [PubMed: 22892635]

Walker T, Johnson PH, Moreira LA, et al. The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature. 2011; 476:450–453. [PubMed: 21866159]

Walsh DB, Bolda MP, Goodhue RE, et al. *Drosophila suzukii* (Diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. Journal of Integrated Pest Management. 2011; 2:1–7.

Watada M, Matsumoto M, Kondo M, et al. Taxonomic study of the *Drosophila auraria* species complex (Diptera: Drosophilidae) with description of a new species. Entomological Science. 2011; 14:392–398.

Werren JH, Jaenike J. *Wolbachia* and cytoplasmic incompatibility in mycophagous *Drosophila* and their relatives. Heredity. 1995; 75:320–326. [PubMed: 7558891]

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

Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nature Reviews Microbiology. 2008; 6:741–751.

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

Yang Y, Hou ZH, Qian YH, et al. Increasing the data size to accurately reconstruct the phylogenetic relationships between nine subgroups of the *Drosophila melanogaster species* group (Drosophilidae, Diptera). Molecular Phylogenetics and Evolution. 2012; 62:214–223. [PubMed: 21985965]

Zabalou S, Charlat S, Nirgianaki A, et al. Natural *Wolbachia* infections in the *Drosophila yakuba* species complex do not induce cytoplasmic incompatibility but fully rescue the *w*Ri modification. Genetics. 2004; 167:826–834.

Zabalou S, Apostolaki A, Pattas S, et al. Multiple rescue factors within a *Wolbachia* strain. Genetics. 2008; 178:2145–2160. [PubMed: 18430940]

Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proceedings of the Royal Society of London B. 1998; 265:509–515.

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

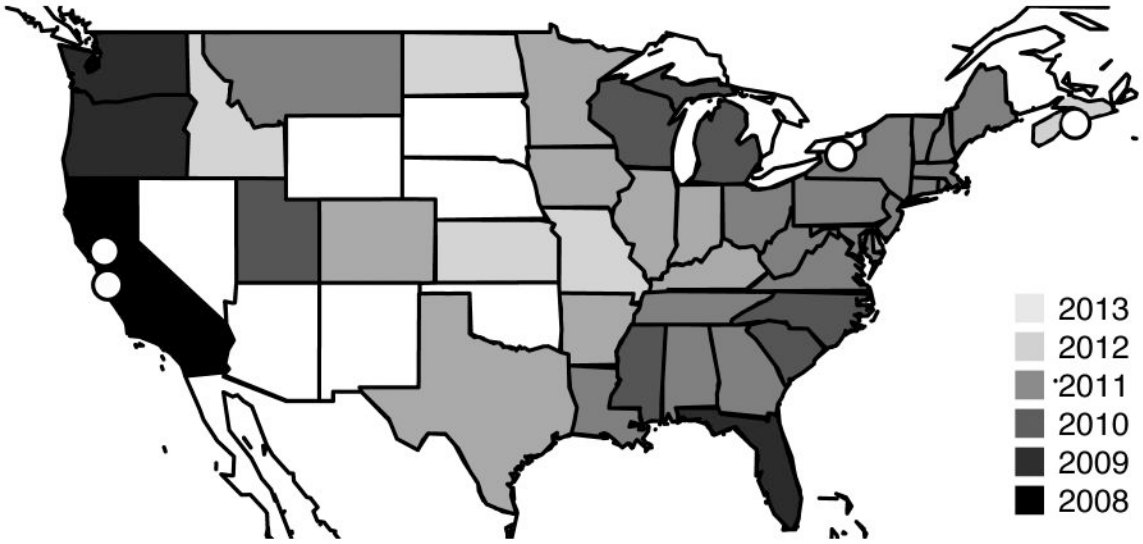

Fig. 1.

Map of *D. suzukii* samples used in this study (open circles) and timing of first detection by state (modified with permission from Burrack *et al.* 2012).

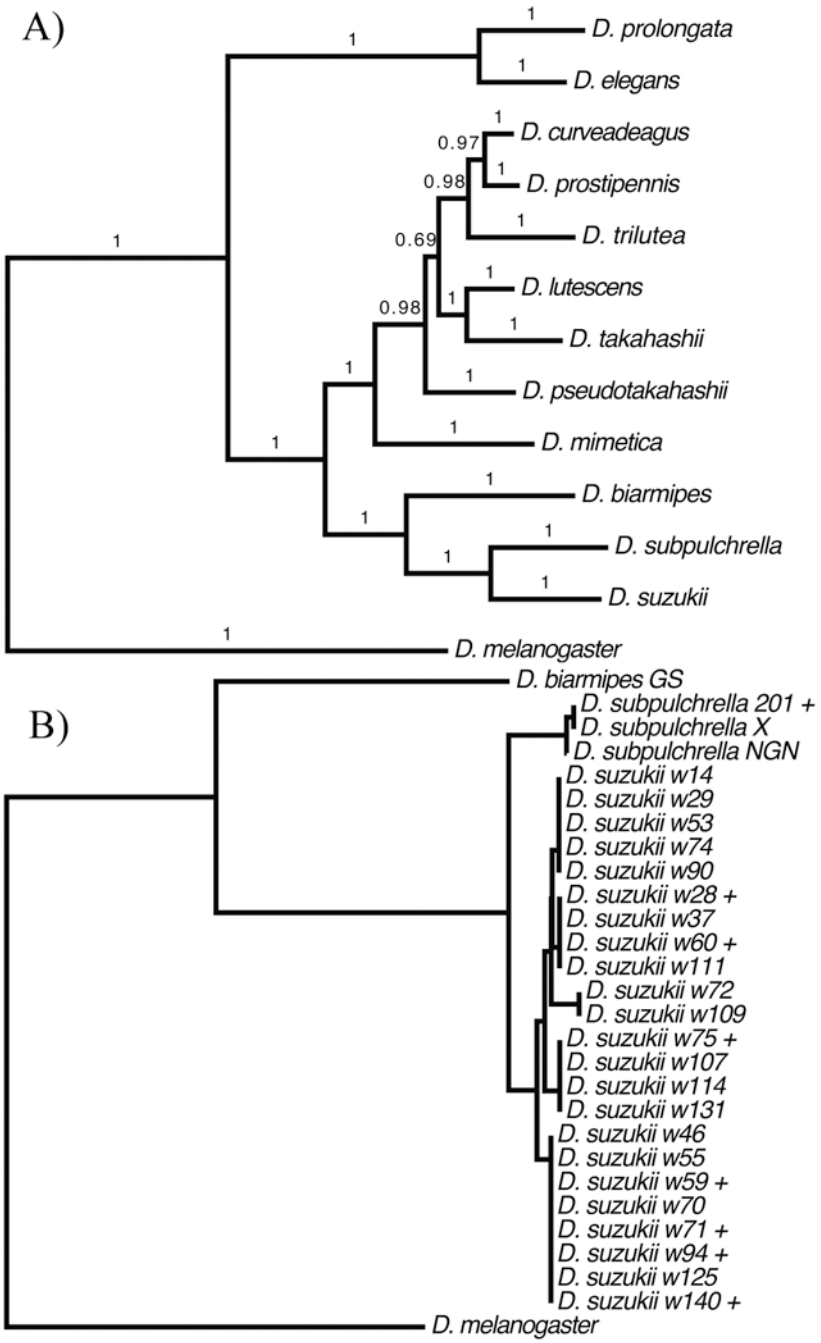

Fig. 2.

Phylogenetic trees: A) Bayesian phylogeny depicting the relationships among relatives of *D. suzukii*; B) Neighbor-joining tree of mtDNA haplotypes for *D. suzukii* and relatives. *Wolbachia*-infected individuals denoted (+).

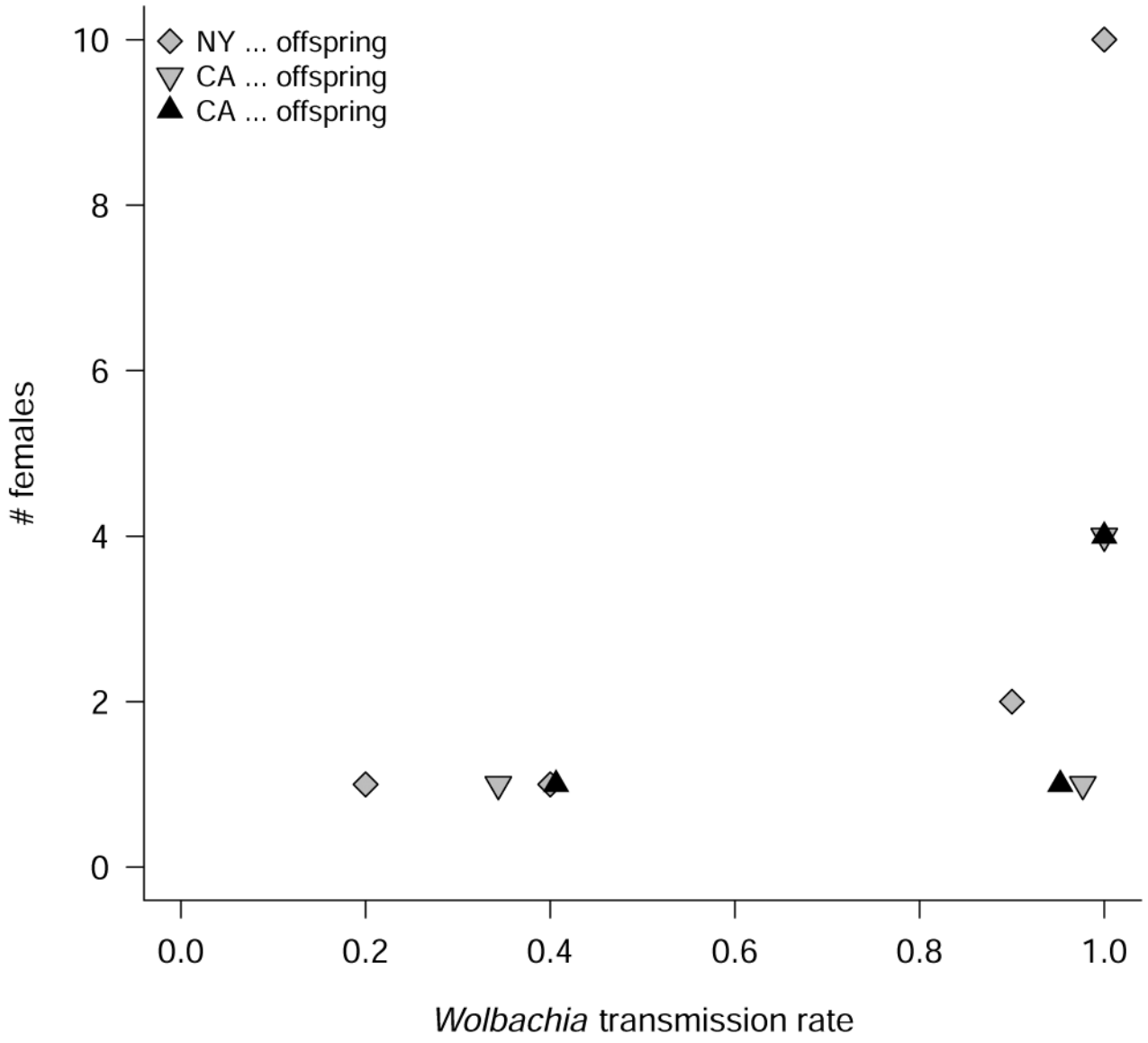

Fig. 3.
Wolbachia transmission by wild-caught *D. suzukii* females.

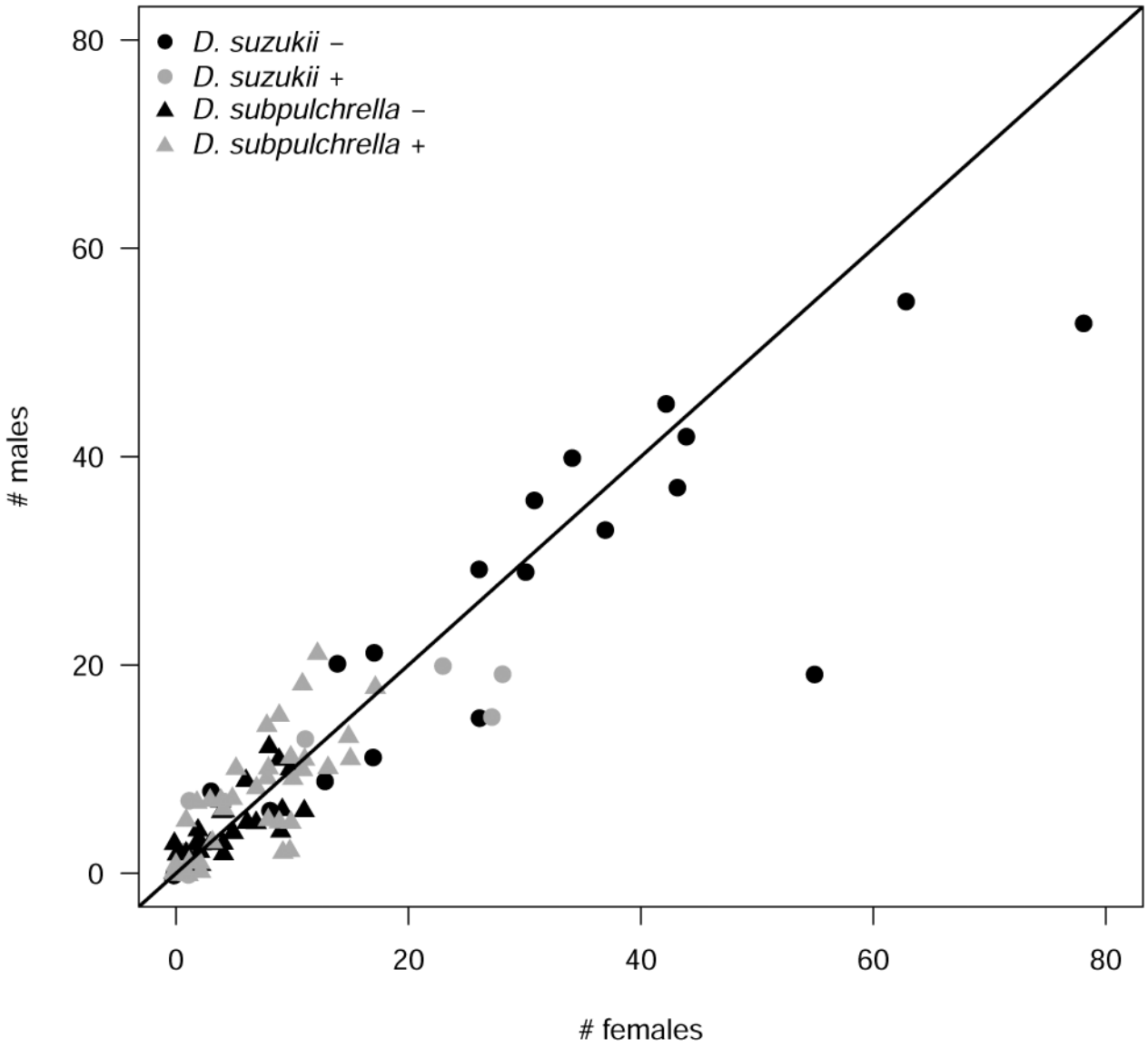

Fig. 4.

Scatterplot of the number of male and female offspring produced by *Wolbachia*-infected (+) and uninfected (–) females (mated to uninfected and infected males, respectively) of *D. suzukii* and *D. subpulchrella*. The line denotes 1:1 offspring sex ratios. Female offspring did not occur at a higher rate than males in infected *D. suzukii* + (binomial test $p = 0.5$, $P = 0.37$; $N = 179$) or *D. subpulchrella* + ($P = 0.47$; $N = 507$), indicating no male killing (or feminization).

Table 1

Sampling locations by date with sample sizes (*N*), number infected (*I*) and infection frequency, with 95% confidence intervals (CI).

Location	Date	*I*/*N*	Frequency (95% CI)
Rochester, NY	August 2012	10/109	0.092 (0.045, 0.162)
Rochester, NY	September 2012	12/178	0.067 (0.035, 0.115)
Nova Scotia, CAN	September 2013	4/34	0.117 (0.033, 0.275)
Winters, CA	June 2012	7/38	0.184 (0.077, 0.343)
Winters, CA	May 2013	41/71	0.577 (0.454, 0.694)
Watsonville, CA	September 2012	32/192	0.167 (0.117, 0.227)
Watsonville, CA	October 2012	7/40	0.175 (0.073, 0.327)
Watsonville, CA	August 2013	13/57	0.228 (0.123, 0.358)
Watsonville, CA	October 2013	33/210	0.157 (0.111, 0.214)

Table 2

Cytoplasmic incompatibility assays for *D. suzukii* from New York and California. Mean egg hatch rates (for females that laid ≥ 10 eggs) ± standard error (se), and sample sizes (*N*). U denotes *Wolbachia*-uninfected, I denotes infected. There was a difference among group hatch rates for New York flies (Kruskal-Wallis test, $P = 0.005$), but not California (Kruskal-Wallis test, $P = 0.687$).

		New York (block 1)		New York (block 2)		California	
Female	**Male**	**Mean hatch rate (± se)**	***N***	**Mean hatch rate (± se)**	***N***	**Mean hatch rate (± se)**	***N***
U	U	0.30 ± 0.06	9	0.625 ± 0.03	19	0.588 ± 0.05	23
U	I	0.51 ± 0.06	5	0.474 ± 0.05	19	0.538 ± 0.07	15
I	U	0.38 ± 0.04	11	0.307 ± 0.09	9	0.596 ± 0.03	28
I	I	0.38 ± 0.04	11	0.600 ± 0.07	15	0.547 ± 0.05	23

Table 3

Cytoplasmic incompatibility assay for *D. subpulchrella*. Mean adult numbers (for females that produced ≥ 10 progeny) ± standard error (se), and sample sizes (*N*). U denotes *Wolbachia*-uninfected, I denotes infected. There was a difference among hatch rates of all groups (Kruskal-Wallis test, $P = 0.036$); however there was no difference between CI cross and its reciprocal (two-tailed Wilcoxon test, $P = 0.65$)

Female	Male	Mean emerged adults (± se)	*N*
U	U	15.5 (± 1.56)	6
U	I	19.7 (± 2.46)	10
I	U	20.14 (± 2.25)	7
I	I	25.24 (± 2.12)	17

Table 4

Mean number of adult *D. suzukii* (± standard error, sample size) generated by *Wolbachia*-infected (I) versus uninfected (U) laboratory-reared females.

State	Block	I	U
NY	1 (October 2012)	3.84 (± 0.70, 60)	9.24 (± 1.10, 56)
	2 (March 2013)	14.25 (± 2.62, 60)	9.73 (± 2.30, 48)
CA		19.89 (± 6.49, 9)	54.45 (± 6.49, 20)

Table 5

Summary of published naturally occurring *Wolbachia* infections in *Drosophila*, indicating the *Wolbachia* strain designation, the reproductive manipulation phenotype (CI = cytoplasmic incompatibility, MK = male killing, N = very weak or none), and the mode of acquisition of the *Wolbachia* infection (C = cladogenic transmission, H = horizontal transmission, I = introgression). Empty cells indicate no information. Species in small clades in which cladogenic or introgressive inheritance of *Wolbachia* is suggested are grouped.

Species	Strain	Phenotype	Origin	References
cardini group				
arawakana	*w*Wil			*1*
arawakana	*w*Spt			*1*
arawakana				*1*
neocardini				*2*
Hawaiian species				
bristle tarsus subgroup				
nr *basimacula*	*w*Bas			*3,4*
prodita	*w*Das		C	*3,4*
redunca	*w*Das		C	*3,4*
split tarsus subgroup				
ancyla	*w*Gin		C	
fundita	*w*Fun		C	*3,4*
nr *fundita*	*w*Gin		C	*3,4*
forficata	*w*For		H[a]	*3,4*
nr *dorsigera*	*w*For			*3,4*
spoon tarsus subgroup				
dasycnemia	*w*Das			*3,4*
setiger subgroup				
eurypeza	*w*Eur			*3,4*
tetraspilota	*w*Tet			*3,4*
melanogaster group				
ananassae subgroup				
ananassae	*w*Ri	CI		*5*
ananassae	*w*Spt			*1*
pseudoananassae	*w*Pana			*1*
melanogaster subgroup				
melanogaster	*w*Mel	CI (weak)	H[a]	*6*
simulans	*w*Ri	CI	H[a]	*7*
simulans	*w*Au	N	H[a]	*8*
sechellia	*w*Ha	CI	C	*9*
sechellia	*w*No&*w*Ha	CI	C	*9*
simulans	*w*Ha	CI	C	*9, 10*

Species	Strain	Phenotype	Origin	References
simulans	*w*No&*w*Ha	CI	C	*9, 11, 12*
simulans	*w*No/*w*Ma[b]	CI	C	*9, 11, 12*
mauritiana	*w*Ma	N	I	*9*
santomea	*w*Sty	N	I or C[c]	*13, 14*
teissieri	*w*Sty	N	I or C[c]	*13, 14, 15*
yakuba	*w*Sty	N	I or C[c]	*13, 14, 15*
montium subgroup				
auraria		CI		*16*
baimaii	*w*Bai			*1*
bicornuta	*w*Bic			*1*
kikkawai	*w*Kik			*3*
nikananu	*w*Nik			*1*
triauraria/quadraria[d]	*w*Ri			*1, 17*
suzukii subgroup				
subpulchrella	*w*Suz	N	C	this study
suzukii	*w*Suz	N	C	*17, 18*
takahashii subgroup				
pseudotakahashii	*w*Pse			*1*
mitchellii group				
nigrocirrus	*w*Ela			*3*
obscura group				
ambigua			?[e]	*19*
tristis			?[e]	*19*
bifasciata	*w*Bif	MK		*20*
quinaria group				
innubila	*w*Inn	MK	H	*21*
munda	*w*Mun			*22*
quinaria			H	*23*
recens		CI	H	*24*
saltans group				
prosaltans	*w*Pro		C	*25*
septentriosaltans	*w*Spt		C	*25*
sturtevanti	*w*Stv			*1*
semieuscata group				
apicipuncta	*w*Api			*3*
testacea group				
orientacea	*w*Ori		C	*26*
neotestacea	*w*Neo		C	*26*
testacea	*w*Tes		C	*27*
virilis group				

Species	Strain	Phenotype	Origin	References
borealis		MK		*22*
willistoni group				
paulistorum semispecies	*w*Au		C	*28*
tropicalis	*w*Tro		H	*29*
willistoni	*w*Wil		H	*25*, *29*

[a] Based on the observed molecular differences between the *Wolbachia* in this species and its closest relatives, horizontal transmission seems most plausible.

[b] These infections may be identical (Ballard 2004)

[c] Lachaise *et al.* (2000) conjecture that the *Wolbachia* were transmitted by introgression. However, although strong evidence exists for mtDNA introgression (Bachtrog *et al.* 2006; Llopart *et al.* 2014), there is insufficient resolution of the *Wolbachia* differentiation to rule out cladogenic transmission.

[d] *D. quadraria* is a junior synonym of *D. triauraria* (Watada *et al.* 2011).

[e] Haine *et al.* (2005) conjecture that these *obscura* species may have experienced horizontal transmission; but given the very close relationship of the hosts and the lack of mtDNA data, cladogenic or introgressive transmission seem equally plausible.

References:

[1] Mateos *et al.* 2006,

[2] Montenegro *et al.* 2006,

[3] Bennett *et al.* 2012,

[4] O'Grady *et al.* 2011,

[5] Bourtzis *et al.* 1996,

[6] Hoffmann 1998,

[7] Hoffmann *et al.* 1986,

[8] Hoffmann *et al.* 1996,

[9] Rousset & Solignac 1995,

[10] O'Neill & Karr 1990,

[11] Merçot *et al.* 1995,

[12] James & Ballard 2000,

[13] Lachaise *et al.* 2000,

[14] Zabalou *et al.* 2004,

[15] Charlat *et al.* 2004,

[16] Bourtzis *et al.* 1996,

[17] Cordaux *et al.* 2008,

[18] Siozios *et al.* 2013,

[19] Haine *et al.* 2005,

*20*Hurst *et al.* 2000,

*21*Dyer & Jaenike 2004,

*22*Sheeley & McAlister 2009,

*23*Dyer *et al.* 2011,

*24*Werren & Jaenike 1995,

*25*Miller & Riegler 2006,

*26*Baldo *et al.* 2006,

*27*Jaenike *et al.* 2010,

*28*Miller *et al.* 2010,

*29*Müller *et al.* 2013.