Genome comparisons indicate recent transfer of *w*Ri-like *Wolbachia* between sister species *Drosophila suzukii* and *D. subpulchrella*

William R. Conner*, Mark L. Blaxter†, Gianfranco Anfora‡§, Lino Ometto‡[1], Omar Rota-Stabelli‡, and Michael Turelli*[2]

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

‡Institute of Evolutionary Biology and GenePool Genomics Facility, University of Edinburgh, Edinburgh EH9 3JT, UK

†Chemical Ecology Lab, Department of Sustainable Agro-ecosystems and Bio-resources, Fondazione Edmund Mach, San Michele all'Adige (TN), Italy

§Centre Agriculture Food Environment, University of Trento, San Michele all'Adige (TN), Italy

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[1]Current address: Independent scientist (lino.ometto@gmail.com), Mezzocorona (TN), Italy

[2]**Correspondence:** Michael Turelli, mturelli@ucdavis.edu, Fax: 1-530-752-1449

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Abstract

Wolbachia endosymbionts may be acquired by horizontal transfer, by introgression between closely related species, or by cladogenic retention during speciation. All three modes of acquisition have been demonstrated, but their relative frequency is largely unknown. *Drosophila suzukii* and its sister species *D. subpulchrella* harbor *Wolbachia*, denoted *w*Suz and *w*Spc. These *Wolbachia* are very closely related to *w*Ri, identified in California populations of *D. simulans*. Nevertheless, these variants differ in the phenotypes they induce: *w*Ri causes significant cytoplasmic incompatibility (CI) in *D. simulans*, but CI has not been detected in *D. suzukii* or *D. subpulchrella*. Draft genomes of *w*Suz and *w*Spc show that they differ by only 0.004% in their coding sequences; they are sisters relative to *w*Ri, from which they differ by 0.015%. Despite uncertainties about molecular divergence rates for *Drosophila* and *Wolbachia*, *w*Suz and *w*Spc are not plausible candidates for cladogenic transmission, as their divergence is too recent compared to their hosts' – by at least a factor of 100. These three *w*Ri-like *Wolbachia* have different copy numbers of orthologs of genes postulated to contribute to CI, and also display several single nucleotide differences in the CI loci. These differences may account for the different levels of CI they produce. We discuss the general problem of distinguishing alternative modes of *Wolbachia* acquisition, focusing on the difficulties posed by limited knowledge of variation in rates of molecular evolution for host nuclear genomes, mitochondria and *Wolbachia*.

Introduction

Drosophila suzukii Matsumura (Diptera Drosophilidae) is an invasive and destructive fruit fly native to South East Asia that has recently invaded North America, South America and Europe (Hauser 2011; Cini *et al.* 2012; Rota-Stabelli *et al.* 2013). While most *Drosophila* species oviposit in fermenting fruits, *D. suzukii* and its close relative *D. subpulchrella* Takamori and Watabe use their atypical serrated ovipositors to pierce the skin of ripening soft fruits and lay eggs in them. Leveraging the genetic resources of *D. melanogaster*, *D. suzukii* and *D. subpulchrella* (both members of the *D. melanogaster* species group) are becoming model species for fundamental and applied studies.

Wolbachia are obligately intracellular, maternally inherited alpha-proteobacteria found in about half of all insects and many other terrestrial arthropods and nematodes (Weinert *et al*. 2015). *Wolbachia* are often associated with reproductive manipulations, including cytoplasmic incompatibility (CI) (Hoffmann & Turelli 1997), male killing (Hurst & Jiggins 2000), feminization (Rousset *et al*. 1992) and parthenogenesis induction (Stouthamer *et al*. 1993), all of which enhance the relative fitness of infected females. But many *Wolbachia* infections, including those in *D. suzukii* and its sister species *D. subpulchrella*, cause no detectable reproductive manipulation and presumably persist by enhancing host fitness (Kriesner *et al*. 2013; Hamm *et al*. 2014; Mazzetto *et al*. 2015; Kriesner *et al*. 2016; Cattel *et al*. 2016). Indeed, it seems increasingly plausible that even infections that cause reproductive manipulations establish in new hosts because they enhance fitness, and hence tend to increase in frequency even when very rare (Kriesner *et al*. 2013). For example, the most common *Wolbachia* reproductive manipulation is CI, in which embryos produced by uninfected females mated with infected males suffer increased mortality. Because CI is essentially irrelevant to the frequency dynamics of rare infections, initial spread of both CI-causing infections and infections that do not manipulate reproduction is likely to be driven by mutualistic effects such as fecundity enhancement (Weeks

et al. 2007), protection from viruses (Teixeira *et al*. 2008) and metabolic provisioning (Brownlie *et al*. 2009).

To understand why *Wolbachia* are found in so many species, it is critical to understand how *Wolbachia* infections are acquired and how long *Wolbachia*-host associations persist. As noted by Raychoudhury *et al*. (2008), although *Wolbachia* are maternally transmitted, lineages can acquire *Wolbachia* in three ways: by cladogenic transmission, in which an infection persists through speciation; by introgression, in which hybridization between closely related species leads to cytoplasm transfer; or by horizontal transmission, in ways that remain indeterminate, in which *Wolbachia* are transferred between closely or distantly related species through non-sexual mechanisms (such as predation or parasitism).

To complement an analysis of *Wolbachia* population biology and effects in *Drosophila suzukii* and its sister species *D. subpulchrella*, Hamm *et al*. (2014) presented a meta-analysis of *Wolbachia* infections in *Drosophila* species that addressed the frequency of both reproductive manipulation and alternative modes of acquisition. However, we suggest they may have underestimated the relative frequencies of horizontal and introgressive transmission compared to cladogenic retention. Horizontal transmission of *Wolbachia* between species was first demonstrated by extreme discordance of the phylogenies of distantly related hosts and their infecting *Wolbachia* (O'Neill *et al*. 1992). In contrast, horizontal transmission seems negligible within *D. simulans* (Turelli & Hoffmann 1995) and *D. melanogaster* (Richardson *et al*. 2012) populations. Hamm *et al*. (2014) implicitly assumed that if two closely related host species share closely related *Wolbachia*, the infections are likely to have been acquired by either cladogenic transmission or introgression. In particular, Hamm *et al*. (2014) postulated that because *D. suzukii* and its sister *D. subpulchrella* have concordant mitochondrial and nuclear phylogenies and harbor very similar *Wolbachia*, as indicated by identity at the Multi Locus Sequence Typing (MLST) loci used to classify *Wolbachia* (Baldo *et al*. 2006), cladogenic transmission of

Wolbachia was likely. Here we use comparative analyses of draft *Wolbachia* genomes, and extensive nuclear data from their *Drosophila* hosts (and relatives), to refute this hypothesis.

The three alternative modes of *Wolbachia* acquisition would be trivial to distinguish if reliable chronograms (dated phylogenies) for the nuclear, mitochondrial and *Wolbachia* genomes were available. Under cladogenic transmission, without subsequent introgression or horizontal transmission, concordant chronograms for all three genomes are expected. Under introgression without subsequent horizontal transmission, the mitochondrial and *Wolbachia* chronograms should be concordant and show more recent divergence than the bulk of the nuclear genome. Finally, under horizontal transmission, more recent divergence is expected between the *Wolbachia* than either the mitochondrial or nuclear genomes. These simple criteria are difficult to apply because of uncertainty concerning the relative rates of nuclear, mitochondrial and *Wolbachia* divergence. Here, using comparative data for *Wolbachia* and host divergence, we conclude that the *Wolbachia* in *D. suzukii* and *D. subpulchrella* are far too similar to make cladogenic transmission plausible.

In addition to assessing *Wolbachia* acquisition, we examine patterns of molecular evolution by comparing the relatively complete draft genomes for *w*Suz (Siozos *et al*. 2013) and *w*Spc (this paper) to the *w*Ri reference genome (Klasson *et al.* 2009). We consider both a general pattern, namely, the relative frequencies of non-synonymous and synonymous substitutions, and sequence divergence for candidate loci associated with two *Wolbachia*-induced phenotypes, life shortening and CI. The "Octomom" duplication, which distinguishes *w*MelPop (Min & Benzer 1997) from *w*Mel (Wu *et al*. 2004), contains the genes *WD0507-WD0514* and is associated with extremely high *Wolbachia* titer and life shortening in *D. melanogaster* (Chrostek & Teixeira 2015). Beckmann & Fallon (2013) used proteomics to identify the locus wPip_0282 in *w*Pip, the *Wolbachia* found in *Culex pipiens,* as a candidate for producing CI. They found at least one homolog of this locus in several CI-causing *Wolbachia*, including *w*Mel and *w*Ri. Within *w*Pip

and other *Wolbachia* genomes, *wPip_0282* and each homolog seemed to be part of two-gene operons, with *wPip_0282* adjacent to *wPip_0283*. This pair is orthologous to *WD0631* and *WD0632* in *w*Mel, and there are three homologous/paralogous pairs in *w*Ri. Beckmann *et al*. (2017) and LePage *et al*. (2017) provide experimental and bioinformatic evidence that *WD0631* and *WD0632*, within the WO prophage, contribute to CI. We examine differences in homologs and paralogs of these loci among *w*Suz, *w*Spc and *w*Ri.

Materials and methods

Sequence data

Genome data for *D. suzukii* and *D. subpulchrella* were generated by Edinburgh Genomics. The *D. suzukii* genome data were generated from an inbred Italian line (the Trento strain) as presented in Ometto *et al*. (2013), with the *Wolbachia*, *w*Suz, presented in Siozos *et al*. (2013). Two libraries of 180 and 300 base pairs were sequenced using 100-base, paired-end Illumina HiSeq 2000 sequencing. The *D. subpulchrella* genome data were generated from a stock maintained at the Fondazione Edmund Mach lab that was established from San Diego Stock center strain 14023-0401.00, originally from Japan. Two libraries of 350 and 550 base pairs were sequenced using 125-base, paired-end Illumina HiSeq 2500 sequencing.

Assembly of Wolbachia *in* D. subpulchrella

To assemble *w*Spc, we initially cleaned, trimmed and assembled reads for the *Wolbachia*-infected *D. subpulchrella* using Sickle (https://github.com/najoshi/sickle) and SOAPdenovo v. 2.04 (Luo *et al*. 2012). For the assembly, *K* values of 31, 41, ... and 101 were tried; and the best assembly (fewest contigs and largest N50) was kept. This preliminary assembly had over 100,000 contigs with a total length of 243 megabases. Details of the *D. subpulchrella* assembly will be published elsewhere, together with a comparison to the *D. suzukii* genome. Most of the contigs were identified through BLAST search as deriving from *Drosophila*. Minor

contamination from microbiota (such as *Acetobacter* spp.) was identified. Contigs with best nucleotide BLAST matches (with e-values less than 10^{-10}) to known *Wolbachia* sequences were extracted as the draft assembly for *w*Spc. The assembly and annotation of *w*Sub are available from Genbank under accession XXXXX [to be advised].

Phylogeny and estimates of divergence of w*Spc and* w*Suz*

The *Wolbachia* MLST loci *gatB, hcpA, coxA, fbpA, and ftsZ* (Baldo *et al*. 2006) were identified in the assemblies using BLAST. As reported in Hamm *et al*. (2014), the MLST sequences from *w*Spc and *w*Suz were identical both to each other and to those of the *w*Ri reference genome from *D. simulans* (Klasson *et al.* 2009).

To distinguish these *Wolbachia* and determine their relationships, we extracted additional orthologous loci from the draft genomes. We annotated the genomes of *w*Suz and *w*Spc with Prokka v 1.11 (Seemann 2014). To normalize our comparisons, we also annotated the genomes of *w*Ri (Klasson *et al.* 2009), *w*Au (Sutton *et al.* 2014) and *w*Mel (Wu *et al*. 2004; Richardson *et al.* 2012). We selected 512 genes present in full length and single copy in all five genomes, avoiding incomplete or pseudogenes and loci with paralogs. The nucleotide sequences of the genes were aligned with MAFFT v. 7 (Katoh 2013) and concatenated, giving an alignment of 480,831 bases. The strain phylogeny was estimated with MrBayes v. 3.2 (Ronquist & Huelsenbeck 2003) using the GTR+Γ model, partitioned by codon positions. We ran two independent chains, each with four incrementally heated subchains, for 1,000,000 generations. Trace files for each analysis were visualized in Tracer v. 1.6 (Rambaut *et al.* 2014) to ensure convergence of all continuous parameters. The first 25% of the generations were discarded as burn-in. Only one topology had posterior probability > 0.001.

To estimate the divergence between *w*Suz and *w*Spc, 703 genes present in full length and single copy in *w*Suz, *w*Spc, and *w*Ri (spanning a total of 704,883 base pairs) were extracted and

aligned with MAFFT v. 7. The resulting alignments were concatenated and used to estimate an ultrametric tree under the GTR+Γ model with rate multipliers partitioned by codon using MrBayes v. 3.2. All model parameters for each codon position were allowed to vary independently, except topology and branch length. The age of the *w*Suz-*w*Spc node was set at 1. Each analysis was run as with the *Wolbachia* sequences.

Nuclear divergence between D. subpulchrella *and* D. suzukii

Hamm *et al*. (2014) used *Drosophila* nuclear data extracted from Yang *et al*. (2012) to assess the relationships of *D. suzukii*, *D. subpulchrella* and *D. biarmipes*, but these data have subsequently been shown to be unreliable (Catullo & Oakeshott 2014). We reassessed these relationships and compared the *Wolbachia* and nuclear chronograms for *D. suzukii* and *D. subpulchrella*. We identified complete coding regions for *D. melanogaster* for the ten nuclear loci used by Hamm *et al*. (2014) (*H2A*, *Adh*, *amylase*, *amyrel*, *cdc6*, *ddc*, *esc*, *hb*, *nucl*, and *ptc*) in FlyBase. Orthologs were then identified using BLAST in the *D. suzukii* assembly of Ometto *et al*. (2013), the unpublished draft *D. subpulchrella* assembly described above, a *D. biarmipes* assembly (Chen *et al*. 2014), and a second-generation *D. simulans* assembly (Hu *et al*. 2012). Data for *H2A* and *amylase* were eliminated because *H2A* had multiple non-identical paralogs in each species and homologs of *D. melanogaster amylase* could not be found in the assemblies. The coding sequence for the remaining eight loci were aligned with MAFFT v. 7 and concatenated. The alignment was used to estimate an ultrametric tree with MrBayes v. 3.2 under the GTR+Γ model with rate multipliers partitioned by codon. All model parameters for each codon position were allowed to vary independently, except topology and branch length. The age of the most recent common ancestor (MRCA) of *D. suzukii* and *D. subpulchrella* was set at 1, as an arbitrary scaling of relative ages. Each analysis was run as two independent chains, each with four incrementally heated subchains, for 1,000,000 generations. The first 25% of the generations were

discarded as burn-in. Trace files for each analysis were visualized in Tracer v. 1.6. To estimate k_s and k_a between *D. suzukii* and *D. subpulchrella*, we used DNAsp v. 5.10 (Rozas 2009).

Following Hotopp *et al.* (2007), we looked for evidence of genetic transfer from *w*Suz and *w*Spc (or other *Wolbachia*) to these hosts. The *D. suzukii* and *D. subpulchrella* assemblies (including the *Wolbachia* contigs) were BLASTed against both all known *melanogaster* group nuclear sequences and all known *Wolbachia* sequences. We sought contigs for which part mapped to a *Drosophila* nuclear sequence and not to any *Wolbachia* sequence while another part mapped to a *Wolbachia* sequence and not to any *Drosophila* nuclear sequence.

Analysis of divergence between w*Spc,* w*Suz and* w*Ri*

The trimmed Illumina reads from *D. suzukii* and *D. subpulchrella* were aligned to the *w*Ri reference (Klasson *et al.* 2009) with bwa 0.7.12 (Li & Durbin 2009). As a control, we also aligned Illumina reads from Riv84 (Iturbe-Ormaetxe *et al*. 2010), the *D. simulans* line used to make the *w*Ri reference. Normalized read depth for each alignment was calculated over sliding 1000 bp windows by dividing the average depth in the window by the average depth over the entire genome. Putative copy number variant (CNV) locations were identified with ControlFREEC 8.0 (Boeva *et al.* 2012), using 500 bp windows and the Riv84 alignment as a control. For the bulk of the genomes, we used an expected ploidy of one, but for variants involving sequences duplicated in *w*Ri, we used a ploidy of two. We calculated *P*-values for each putative CNV using the Kolmogorov-Smirnov test implemented in ControlFREEC 8.0.

Sequences for the "Octomom" genes *WD0507-WD0514* (Chrostek & Teixeira 2015) were extracted from the *w*Mel reference (Wu *et al*. 2004; Richardson *et al.* 2012) and orthologs identified in the *w*Ri reference (Klasson *et al*. 2009) and the draft assemblies for *w*Suz and *w*Spc using BLAST.

Sequences homologous to loci putatively involved in CI in other *Wolbachia* strains (Beckmann & Fallon 2013; LePage *et al*. 2017; Beckmann *et al*. 2017) were extracted from *w*Ri (Klasson *et al*. 2009) and the draft assemblies for *w*Suz and *w*Spc. Differences among these three genomes at these loci were assessed by aligning the *w*Suz and *w*Spc reads to the *w*Ri reference and calculating the percentage of reads with the non-*w*Ri base.

To unravel an insertion of the transposable element ISWpi7, which occurs in 21 identical copies in *w*Ri, and differentiates *w*Spc and *w*Suz from *w*Ri, an additional assembly step was required. The novel insertion occurs in the *w*Spc and *w*Suz orthologs of *WRi_006720*, one of the CI-associated loci discussed below. The *D. suzukii* and *D. subpulchrella* reads were aligned to the *w*Spc assembly with bwa 0.7.12 (Li & Durbin 2009). For both contigs that contain part of the *WRi_006720* gene, reads mapping to the ISWpi7 transposable element plus the neighboring 500 bp were extracted and assembled with SOAPdenovo v. 2.04 (Luo *et al*. 2012), using a K value of 55. Both the *D. suzukii* and *D. subpulchrella* reads assembled into a single contig containing the two pieces of *WRi_006720* interrupted by a single copy of ISWpi7.

Results

Draft genome assembly for wSpc, *the* Wolbachia *from* D. subpulchrella

We generated a draft assembly of *w*Spc by filtering contigs from a joint *Wolbachia-D. subpulchrella* assembly. The draft *w*Spc assembly was in 100 contigs with N50 length of 31,871 bp and total length 1.42 Mb. This length is close to the 1.45 Mb *w*Ri reference (Klasson *et al*. 2009), suggesting that it may represent a nearly complete genome.

Wolbachia divergence

We aligned and compared *w*Spc and *w*Suz at 703 protein-coding loci (704,883 bp) and identified only 28 single-nucleotide variants (SNV), an overall divergence of 0.004%. *w*Suz had 103 SNV

compared to *w*Ri (0.015% divergence) and *w*Spc 99 SNV (0.014% divergence) (Table S1). Most (87) of these SNV are shared. There were too few differences to definitively determine whether these genomes are recombinant (Ellegaard *et al*. 2013), but the data were fully consistent with no recombination (i.e., with so few differences, we have no power to detect recombination). Bayesian phylogenetic analysis differentiated the three *w*Ri-like variants, with *w*Suz and *w*Spc sisters relative to *w*Ri (Fig. 1A). For *w*Suz and *w*Spc, we derived point estimates and 95% confidence intervals for divergence at each codon position, calculated as the rate multiplier for that position times the branch length (fixed to 1) (Table 1). (Note: The model underlying this analysis assumes for computational convenience that all three codon positions undergo proportional rate variation across each branch, *i.e*., each position speeds up or slows down by the same amount along each branch [cf. Langley & Fitch 1974]. The rate multipliers express the relative rate of evolution for each codon position. Hence, the expected number of substitutions for each codon position along each branch of the phylogram is the branch length times the rate multiplier for that position.) The estimated chronogram (Fig. 1B) shows that the divergence time of *w*Ri from its MRCA with *w*Spc and *w*Suz is 3.51 times the divergence time of *w*Spc and *w*Suz, with a 95% confidence interval of (2.41, 4.87). We found no difference in the rates of divergence for first, second and third codon positions, as also observed in the codivergence of *Wolbachia* and mtDNA haplotypes in *D. melanogaster* (Richardson *et al*. 2012). Following from this, estimates of k_s and k_a were very similar (Table 1).

Host divergence

The host chronogram (Fig. 1C) shows that *D. subpulchrella* and *D. suzukii* are sisters relative to *D. biarmipes*, as reported by Hamm *et al*. (2014). The divergence time of *D. biarmipes* from its MRCA with *D. subpulchrella* and *D. suzukii* was estimated to be 2.19 times the divergence time for *D. subpulchrella* and *D. suzukii*, with 95% confidence interval (2.00, 2.40). The *D.*

subpulchrella-D. suzukii divergence time estimate is 1.15 times as large as the estimated divergence time for *D. melanogaster* and *D. simulans*, with a 95% confidence interval of (1.03, 1.31). Point estimates and 95% confidence intervals for divergence at each codon position between *D. subpulchrella* and *D. suzukii* were calculated as the rate multiplier for that position times the branch length (fixed to 1) (Table 2).

We found no evidence for partial integration of any *Wolbachia* sequence into the nuclear genomes of either *D. subpulchrella* or *D. suzukii*.

Calibrations for Wolbachia versus host genome divergence and interpretation

We used estimates of relative divergence of the *Wolbachia* and *Drosophila* genomes to assess cladogenic versus lateral transmission of *w*Spc and *w*Suz. Our strategy was to compare our estimates of relative *Wolbachia*/host divergence to ratios obtained from published examples of cladogenic *Wolbachia* transmission. Table 3 summarizes our data and the data from two *Nasonia* wasp species (Raychoudhury *et al.* 2008, *w*NlonB1 versus *w*NgirB) and four *Nomada* bee species (Gerth & Bleidorn 2016, plus unpublished data kindly provided by the authors). Our ratio of *Wolbachia* to host silent-site divergence estimates is two or three orders of magnitude lower than found for *Nasonia* or *Nomada*. This strongly indicates relatively recent *Wolbachia* transfer between *D. suzukii* and *D. subpulchrella* rather than cladogenic *Wolbachia* acquisition. Given that we are looking at only single exemplars of *w*Spc and *w*Suz, the divergence times for these sequences provides an upper bound for the time of interspecific transfer (Gillespie & Langley 1979). Additional support for non-cladogenic transmission comes from the analyses of Richardson *et al*. (2012), who inferred that *Wolbachia* substitution rates were roughly ten-fold lower than the non-coding nuclear mutation rate for *D. melanogaster*, which is often considered a reasonable approximation for the rate of third-position substitutions (at least for four-fold

degenerate sites, Obbard *et al*. (2012)). This is clearly inconsistent with the three-order-of-magnitude difference we estimate (Table 3).

Comparing *w*Suz and *w*Spc, we found no difference in synonymous versus nonsynonymous substitution rates (Table 1). This is also true for *w*Mel variation in *D*. *melanogaster* (Richardson *et al*. 2012). Gerth & Bleidorn (2016, pers. comm.) find essentially identical estimates of k_s and k_a for all pairwise comparisons of the *Wolbachia* in the clade ((*N. leucophthalma*, *N. flava*), *N. panzeri*). In contrast, comparing *w*Ri and *w*Au using the 429,765 bp dataset of single-copy, full-length genes (Table S1), we estimate a synonymous substitution frequency of 4.34%; whereas the estimated nonsynonymous frequency is only 0.65% (or $k_s/k_a = 6.7$). Similarly, when comparing the *Wolbachia* of the outgroup host, *N. ferruginata*, to the *Wolbachia* of the three ingroup species, Gerth & Bleidorn (2016, pers. comm.) observed $k_s/k_a = 2.8$, 2.8 and 2.5. In their comparisons of *w*NlonB1 and *w*NgirB from *N. longicornis* and *N. giraulti*, Raychoudhury *et al.* (2008) estimated $k_s/k_a = 0.0037/0.0022 = 1.7$. Our data and those from other very recently diverged *Wolbachia* are consistent with either accelerated adaptive *Wolbachia* evolution in a new host or a relaxation of constraints on non-synonymous substitutions.

Estimation of absolute divergence times (i.e., times to the MRCA) for *w*Suz and *w*Spc and their hosts is more difficult. Assuming 10 generations per year and the *w*Mel-derived estimate of the 95% confidence interval for the third-position substitution rate of *Wolbachia* (2.88×10^{-10}, 1.29×10^{-9}) changes/site/generation; Richardson *et al*. 2012), *w*Suz and *w*Spc diverged about 1,600 to 7,000 years ago. Using the 95% confidence interval for first- and second-position substitution rates from Richardson *et al*. (2012) yields *w*Suz-*w*Spc divergence dates of 1,200 to 9,100 years. (Given that *D. suzukii* and *D. subpulchrella* seem to be temperate species [Takamori *et al*. 2006; Ometto *et al*. 2013], the number of generations per year may be overestimated by a factor of two, which would inflate the *Wolbachia* divergence time by a factor of two. This does not affect our conclusions.) Raychoudhury *et al.* (2008) estimated a *Wolbachia* synonymous rate

of 4.7×10^{-9} changes/synonymous site/year in *Nasonia*. Using our synonymous rate from Table 1 with the Nasonia calibration, the estimated divergence for *w*Suz and *w*Spc is 6,400 years, which is consistent with our *Drosophila* calibration. These analyses suggest that *w*Suz and *w*Spc diverged on the order of 1,000-10,000 years ago, orders of magnitude shorter than typical time scales for *Drosophila* speciation (10^5-10^6 years, Coyne & Orr 2004, p. 75; Obbard *et al*. 2012). Molecular estimates of *Drosophila* divergence times generally depend on speculative inferences from the phylogeography of the Hawaiian *Drosophila* radiation (Obbard *et al*. 2012). Using the Obbard *et al.* (2012) summary of available estimates for *D. melanogaster* and *D. simulans* divergence and our relative chronogram for *D. subpulchrella* and *D. suzukii* (Fig. 1C), we infer divergence times for *D. subpulchrella* and *D. suzukii* ranging from about one to nine million years, two orders of magnitude larger than our estimates for *w*Suz versus *w*Spc. Hence, despite great uncertainties, our data clearly preclude cladogenic transmission of *w*Suz and *w*Spc.

Genome differences between w*Spc,* w*Suz and* w*Ri: structural variation and candidate genes*

We identified copy-number variants (CNV) in *w*Suz and *w*Spc relative to the *w*Ri reference sequence by plotting read depth along each genome (Fig. 2; Table 4). *w*Spc and *w*Suz share a deletion relative to *w*Ri of 23,000 bp, between positions 733,000-756,000. *w*Suz has duplications 22,500 bp long from about 570,000 to 592,500 and 1,077,500 to 1,100,000. Both regions are part of the WO-B prophage. In *w*Ri, there are two nearly identical copies (99.4%) of WO-B, from about 565,000 to 636,000 and from about 1,071,000 to 1,142,000 (Klasson *et al*. 2009). *w*Suz had an additional duplication between 1,345,000 and 1,347,500, outside of the WO prophage regions (Table 4).

We identified homologs in our target *Wolbachia* genomes of loci implicated in producing phenotypic effects. The Octomom phenotype of *w*Mel (shortened life, high *Wolbachia* titer) is associated with eight loci (*WD0507-WD0514*, Chrostek & Teixeira 2015). In the *w*Ri reference,

we found homologs of only *WD0508* and *WD0509*. There were two *WD0508*-like genes, at 632,500-633,385 and 1,138,959-1,139,844, within the *w*Ri WO-B prophages. A single *WD0509*-like gene was present, from 1,419,589-1,421,396, not associated with WO-B prophage. These two genes are not neighbors in *w*Ri and are not within regions that differentiate *w*Spc and *w*Suz from *w*Ri.

Table 5 lists the orthologs and paralogs in *w*Mel, *w*Ri, *w*Suz and *w*Spc of *wPip_0282* and *wPip_0283*, the loci originally identified as CI-causing by Beckmann & Fallon (2013) in *w*Pip, the *Wolbachia* in *Culex pipiens*. These loci occur in pairs; and the "type I" pairs, orthologs of *wPip_0282* and *wPip_0283*, may be a toxin-antidote operon (cf. Beckmann *et al.* 2017; LePage *et al*. 2017). The orthologs in *w*Mel are *WD0631* and *WD0632*. As shown in Table 5, there are two copies of the type I pair in *w*Ri, one copy in each of the two complete copies of the WO-B prophage. As noted by Beckmann & Fallon (2013), in *w*Ri, there is also a paralogous pair (*wRi_006720* and *wRi_006710*), termed "type II" by LePage et al. (2017), that exists within what they term a "WO-like island."

Table S4 lists genes included in the CNV regions of *w*Suz and *w*Spc relative to *w*Ri. Notably, the orthologs of *WD0631* and *WD0632*, implicated in causing CI (Beckmann & Fallon 2013; LePage *et al.* 2017; Beckmann *et al.* 2017), are in a partial third copy of prophage WO-B found in *w*Suz. Hence, *w*Suz contains three copies of these two loci, whereas *w*Spc has two (see Table 5). The copy-number variants in *w*Suz or *w*Spc do not affect the type II loci.

Table 6 reports differences among *w*Ri, *w*Suz and *w*Spc at orthologs of the CI-associated loci *WD0631*, *WD0632*, *WRi_006710*, and *WRi_006720*. The duplicate orthologs of *WD0631* in *w*Ri are *WRi_005370* and *WRi_010030*. As noted by Beckmann & Fallon (2013), the (duplicate) orthologs of *WD0632* in *w*Ri have been annotated as pseudogenes, *WRi_p005380* and *WRi_p010040*, because of premature stop codons; but they retain large, intact coding regions intact and may be functional. Even with multiple orthologs of *WD0631* and *WD0632* in each

genome (two in *w*Ri, two in *w*Spc, three in *w*Suz), all copies within each genome are identical and all interspecific comparisons consistently show the single nucleotide differences reported in Table 6. *w*Suz and *w*Spc share two missense substitutions in *WD0631* and one in *WD0632*. As shown in Table 6, *w*Suz and *w*Spc also share one missense substitution in *wRi_006710*. This indicates that the duplications unique to *w*Suz occurred after the split of (*w*Suz, *w*Spc) from *w*Ri. *w*Spc has a nonsense mutation at position 3,353 of *WD0632*, which results in a protein lacking the last 56 amino acids produced in *w*Ri. These differences may account for the fact that while *w*Ri causes appreciable CI in *D. simulans* and detectable CI in *D. melanogaster*, neither *w*Suz nor *w*Spc causes detectable CI in its native host (Hamm *et al*. 2014).

In both *w*Spc and *w*Suz, an IS element, identical to ISWpi7 of *w*Ri (Klasson *et al*. 2009, Table S5), has inserted before base 323 of the ortholog to *WRi_006720*. There are 21 identical copies of the ISWpi7 transposon in *w*Ri, each 1480 bp long with the transposase gene flanked on each side by about 200 bp. Clearly, this insertion predates the divergence of *w*Spc and *w*Suz.

Discussion

Genomic data indicate non-cladogenic acquisition of w*Suz and* w*Spc*

Despite considerable uncertainly in divergence-time estimates for both *w*Suz and *w*Spc and their hosts, *D. suzukii* and *D. subpulchrella*, genomic data on relative rates of *Wolbachia* and host divergence contradict the conjecture by Hamm *et al*. (2014) that these species share similar *Wolbachia* because of cladogenic transmission. Based on this result, we must also revisit the Hamm *et al*. (2014) conclusion that cladogenic transmission of *Wolbachia* may be relatively common among *Drosophila*. That conclusion was based on the erroneous assumption that cladogenic transmission was the most plausible explanation for sister species sharing very similar *Wolbachia*. Given that on the order of half of *Drosophila* speciation events show evidence for reinforcement (i.e., accelerated rates of evolution for premating isolation associated with overlapping ranges) (Coyne & Orr 1989, 1997; Turelli *et al*. 2014), hybridization is

apparently common among sister species of *Drosophila*. Introgression has been invoked to explain the closely related *Wolbachia* found within the *simulans* and *yakuba* clades in the *D. melanogaster* subgroup (Rousset and Solignac 1995; Lachaise *et al*. 2000). In both cases, the introgression hypothesis is favored over horizontal transmission because the hosts also share essentially identical mitochondrial DNA. *Wolbachia* transmission within the *yakuba* clade is currently being reanalyzed using complete *Wolbachia*, mitochondrial and nuclear genomes (Turelli, Conner, Turissini, Matute and Cooper, in prep.).

Extremely variable rates of Wolbachia *molecular evolution seem an implausible alternative*

Gerth & Bleidorn (2016) have proposed a time scale for *Wolbachia* evolution based on the apparent co-divergence of *Wolbachia* and nuclear genomes in a clade of four *Nomada* bee species. In our discussion of their data above, we emphasized comparisons between the outgroup host *N. ferruginata* and the three ingroup hosts, noting that the co-divergence of these hosts and their *Wolbachia* produced relative rates of molecular divergence comparable to those inferred for a pair of *Nasonia* (Raychoudhury *et al*. 2008) and for *D. melanogaster* (Richardson *et al*. 2012). However, if we consider instead the sister species *N. leucophthalma* and *N. flava* from Gerth & Bleidorn (2016), we would infer much slower divergence of their *Wolbachia* (which recently acquired a biotin synthesis operon). For *N. leucophthalma* and *N. flava*, Gerth & Bleidorn (2016, pers. comm.) estimated synonymous nuclear substitution rates of 6.8×10^{-3}, with a corresponding *Wolbachia* synonymous substitution rates of only 1.0×10^{-4}. Under cladogenic transmission, this implies *Wolbachia* divergence that is roughly an order of magnitude slower than inferred from the three outgroup comparisons, with *Wolbachia* divergence at $1/68^{th}$ the rate of the host nuclear genomes rather than 1/8. This indicates either 8.5-fold rate variation for *Wolbachia* molecular evolution or that cladogenic transmission does not apply to this sister pair.

To explain our *D. suzukii* and *D. subpulchrella* data with cladogenic transmission and relative rate heterogeneity, we require that *Wolbachia* divergence is more than 1000-fold slower than third-position nuclear divergence. This relative rate is 100-fold slower than inferred for *D. melanogaster* and 30-fold slower than the slow rate implied by cladogenic transmission between *N. leucophthalma* and *N. flava*. Such extreme heterogeneity seems implausible, but more examples of cladogenic *Wolbachia* transmission are needed to definitively rule this out.

Comparative genomics and cytoplasmic incompatibility

Recent experiments strongly suggest that the *w*Mel loci *WD0631* and *WD0632*, contained within the WO-B prophage, cause CI (Beckmann & Fallon 2013, LePage et al. 2017; Beckmann *et al.* 2017). Despite having orthologs of both loci that are fairly similar to those in *w*Ri, *D. suzukii* and *D. subpulchrella* show no apparent CI. There are two copies of these CI-associated loci in *w*Ri, two in *w*Spc, and three in *w*Suz. As argued above, the additional copy in *w*Suz was acquired after *w*Suz and *w*Spc diverged. The differences we document in Table 6 between *w*Ri, *w*Suz and *w*Spc at the CI-associated loci may be informative about the portions of those loci essential to CI. Unpublished data (L. Mouton, pers. comm.) show that *w*Ri causes detectable, but slight, CI when introduced into *D. suzukii.* Given the high level of CI that *w*Ri causes in *D. simulans*, these data suggest that *D. suzukii* may suppress CI, perhaps indicating a relatively old association with CI-causing *Wolbachia* (Turelli 1994; Hoffmann & Turelli 1997). We may be able to determine whether *D. suzukii* or *D. subpulchrella* was the donor of their closely related *Wolbachia* from population genomic analyses of their mtDNA and *Wolbachia*. Genomes from a geographically diverse sample of *D. suzukii* are currently being analyzed and may resolve the direction of *Wolbachia* transfer (J. C. Chiu, pers. comm.).

The published crossing studies in *D. suzukii* and *D. subpulchrella*, finding no statistically significant CI caused by *w*Suz or *w*Spc, are relatively small (Hamm *et al*. 2014; Cattel *et al*.

2016). They are comparable to the experiments that inferred no CI associated with the native *Wolbachia* infections in *D. yakuba*, *D. teissieri* and *D. santomea* (Charlat *et al.* 2004; Zabalou *et al*. 2004). However, larger experiments by Cooper *et al*. (2017) revealed consistent, albeit weak, CI in all three *yakuba* clade species and interspecific CI between these species. More replicated assays for CI in *D. suzukii* and *D. subpulchrella*, as well as investigation of whether CI is produced when *w*Spc and *w*Suz are transinfected into CI-expressing hosts such as *D. simulans*, will indicate whether the differences described in Table 6 are candidates for disrupting the molecular processes underlying CI (Beckmann *et al*. 2017, LePage *et al*. 2017).

Conclusions and open questions

Understanding how host species acquire *Wolbachia* requires comparing divergence-time estimates for closely related *Wolbachia* in sister species to divergence-time estimates for both their hosts' nuclear genes and mtDNA. To make confident inferences, we need better estimates of both the mean and variance of relative divergence rates for these three genomes. The variance for mtDNA divergence can be obtained from extant data, such as the many available *Drosophila* genomes. Estimates for nuclear, mitochondrial and *Wolbachia* genomes can be obtained from groups like the filarial nematodes for which co-divergence of the hosts and their obligate *Wolbachia* is well established (Bandi *et al*. 1998). Our ability to infer processes of *Wolbachia* acquisition will be greatly enhanced by additional examples of cladogenic transmission among insects, besides *Nasonia* wasps (Raychoudhury *et al*. 2008) and *Nomada* bees (Gerth & Bleidorn 2016). For *D. suzukii* and *D. subpulchrella*, distinguishing between introgression and horizontal transmission requires mtDNA sequences.

It is a challenge to understand the pattern of molecular evolution between closely related *Wolbachia* whereby all three nucleotide positions evolve at similar rates, producing comparable rates of synonymous versus non-synonymous substitutions. This is consistent with the pattern of

variation seen for *w*Mel within *D. melanogaster* (Richardson et al. 2012). In contrast, k_s/k_a increases to 2-3 for the cladogenically transmitted *Wolbachia* in *Nasonia* and *Nomada*; then increases to about 7 for the more distantly related *w*Au and *w*Ri infecting *D. simulans*. Does *Wolbachia* "invasion" of a new host represent a relaxation of selective constraint or an opportunity for adaptation?

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Author Contributions

The genomic data for *D. subpulchrella* and *w*Spc were generated by O. R.-S., L. O., M. B. and G. A. The bioinformatic analyses were performed by W. R. C. with input from M. T., M. B. and O. R.-S. The first draft of the manuscript was produced by M. T. , W. R. C. and M. B. with subsequent improvements by all authors.

Data Accessibility

The assembly of *w*Spc will be made available on Genbank once the manuscript is accepted for publication.

Supporting information

Additional information may be found in the online version of this article.

Table S1.Observed pairwise genomic differences between *Wolbachia* strains, given as percentage of polymorphic sites in single-copy, full-length genes present in all three strains.

Table S2 Matrix of k_a (below diagonal) and k_s (above diagonal) estimates for *w*Suz, *w*Spc, *w*Ri, *w*Au and *w*Mel (using the 429,765 bp data set from Table S1).

Table S3 The 28 substitutions differentiating *w*Spc and *w*Suz.

Table S4 Genes present in CNV regions of *w*Suz or *w*Spc relative to *w*Ri. All locations are relative to the *w*Ri reference sequence of Klasson *et al*. (2009).

Figure Legends

Fig. 1 Phylogram and chronograms for the *Wolbachia* and hosts discussed. Clade posterior probabilities are shown. A) *Wolbachia* phylogram. B) *Wolbachia* chronogram with an estimate of the divergence time for *w*Suz and *w*Spc. Branch lengths relative to the *w*Spc-*w*Suz divergence are shown. All clade posterior probabilities are 1.0. C) Host chronogram with an estimate of divergence time for *D. suzukii* and *D. subpulchrella*. Branch lengths relative to the *D. suzukii-D. subpulchrella* divergence are shown. All clade posterior probabilities are 1.0.

Fig. 2 We compare normalized read-density relative to the *w*Ri reference sequence of Klasson *et al*. (2009) for: A) the Illumina reads from Riv84 version of *w*Ri were reported by Iturbe-Ormaetxe *et al*. (2010), B) the *w*Suz reads are from Ometto *et al*. (2014), and C) the *w*Spc reads are from this study.

Table 1 Estimated number of substitutions per site by codon position between *w*Suz and *w*Spc, plus estimates of synonymous (k_s) and non-synonymous (k_a) substitution rates, see the text for details.

Position	Point Estimates	95% Confidence Interval
1st	5.0×10^{-5}	$(3.0\times10^{-5}, 7.0\times10^{-5})$
2nd	3.2×10^{-5}	$(1.6\times10^{-5}, 4.6\times10^{-5})$
3rd	4.0×10^{-5}	$(2.4\times10^{-5}, 5.6\times10^{-5})$
Overall ($\boldsymbol{k_s}$, $\boldsymbol{k_a}$)	$(3\times10^{-5}, 4\times10^{-5})$	

Table 2 Estimated number of substitutions per site by codon position between *D. suzukii* and *D. subpulchrella* for nuclear coding regions, plus estimates of synonymous (k_s) and non-synonymous (k_a) substitution rates, see the text for details.

Position	Point Estimates	95% Confidence Interval
1st	1.20×10^{-2}	$(1.03\times10^{-2}, 1.36\times10^{-2})$
2nd	5.65×10^{-3}	$(4.68\times10^{-3}, 6.48\times10^{-3})$
3rd	9.19×10^{-2}	$(8.41\times10^{-2}, 1.00\times10^{-1})$
Overall ($\boldsymbol{k_s}$, $\boldsymbol{k_a}$)	$(1.2\times10^{-1}, 5.3\times10^{-3})$	

1

Table 3 Estimated frequencies of synonymous (k_s) versus non-synonymous (k_a) substitutions per site for *Wolbachia* in various hosts.

Data source	Species 1	Species 2	Host		*Wolbachia*		k_s ratio
			k_s	k_a	k_s	k_a	
this work	*Drosophila suzukii*	*Drosophila subpulchrella*	1.2×10^{-1}	5.3×10^{-3}	3×10^{-5}	4×10^{-5}	0.00025
Raychoudhury et al.	*Nasonia giraulti*	*Nasonia longicornis*	1.22×10^{-2}	5.4×10^{-3}	3.7×10^{-3}	2.2×10^{-3}	0.30
Gerth & Bleidorn	*Nomada ferruginata*	*Nomada leucophthalma*	1.95×10^{-2}	2.6×10^{-3}	2.5×10^{-3}	9×10^{-4}	0.13
ibid.	*N. ferrug.*	*N. flava*	1.92×10^{-2}	2.7×10^{-3}	2.5×10^{-3}	9×10^{-4}	0.13
ibid.	*N. ferrug.*	*N. panzeri*	1.84×10^{-2}	3.1×10^{-3}	2.7×10^{-3}	1.1×10^{-3}	0.15
ibid.	*N. leuco.*	*N. flava*	6.8×10^{-3}	4×10^{-4}	1×10^{-4}	1×10^{-4}	0.015
ibid.	*N. leuco.*	*N. panzeri*	5.8×10^{-3}	8×10^{-4}	3×10^{-4}	2×10^{-4}	0.052
ibid.	*N. flava*	*N. panzeri*	5.5×10^{-3}	9×10^{-4}	3×10^{-4}	3×10^{-4}	0.055

2

3

Table 4 Copy-number variants in *w*Suz and *w*Spc relative to *w*Ri. All positions are given relative to the *w*Ri reference of Klasson *et al*. (2009).

Start position	End position	Copy number change	Kolmogorov-Smirnov *P*-value	Affected genomes
570000	592500	2 → 3*	<0.0001	*w*Suz
733000	756000	1 → 0	<0.0001	*w*Suz, *w*Spc
1077500	1100000	2 → 3*	<0.0001	*w*Suz
1345000	1347500	1 → 2	0.016	*w*Suz

*****This sequence is duplicated in the *w*Ri genome, so it was treated as diploid in our ControlFREEC 8.0 analysis.

Table 5 Homologs of CI-associated loci in *w*Mel, *w*Ri, *w*Suz and *w*Spc. The gene designations in *w*Spc and *w*Suz reflect homology to loci identified in *w*Mel and *w*Ri.

Wolbachia	**gene pair***	**Gene 1**	**Gene 2**	**WO prophage association†**
*w*Mel	I	*WD0631* (*cifA*/*cidA*)‡ (antidote?)	*WD0632* (*cifB*/*cidB*)‡ (toxin?)	yes
*w*Ri	I.1	*wRi_005370*	*wRi-p005380*§	yes
	I.2	*wRi_010030*	*wRi_p010040*§	yes
	II	*wRi_006720*	*wRi_006710*	no
*w*Spc	I.1	*wSpc_0631.I.1*	*wSpc_0632.I.1*	yes
	I.2	*wSpc_0631.I.2*	*wSpc_0632.II.2*	yes
	II	wSpc_6720 (disrupted)	wSpc_6710	no
*w*Suz	I.1	*wSuz_0631.I.1*	*wSuz_0632.I.1*	yes
	I.2	*wSuz_0631.I.2*	*wSuz_0632.II.2*	yes
	I.3	*wSuz_0631.I.3*	*wSuz_0632.II.3*	partial¶
	II	*wSuz_6720* (disrupted)	*wSuz_6710*	no

*Roman numerals follow the "type" designations in LePage *et al*. (2017).

†This refers to location within an intact WO prophage, as opposed to a "WO-like island" (*cf.* LePage *et al*. 2017).

‡Alternative designations (*cif* versus *cin*) from LePage *et al*. (2017) and Beckmann *et al*. (2017), respectively. Beckmann *et al*. (2017) proposes that *WD0631* produces an antidote to the toxin produced by *WD0632*.

§Annotated as pseudogenes, but see text.

¶This third copy in *w*Suz exists in the 1077500-1100000 CNV, noted in Table 4, which is a partial copy of the WO-B prophage.

Table 6 Comparisons between *w*Ri, *w*Spc and *w*Suz at the CI-associated (type I, possible antidote, toxin) loci, *WD0631* and *WD0632*, from *w*Mel, and the paralogous loci (type II), *WRi_006710* and *WRi_006720* from *w*Ri. All reads from *w*Spc and *w*Suz are consistent with the differences shown.

Location (gene, amino acid)	*w*Ri codon (codon, translation)	*w*Spc codon (codon, translation)	*w*Suz codon (codon, translation)
WD0631[*] (antidote?)			
363	AAA, Lys	GAA, Glu	GAA, Glu
473	AAA, Lys	AGA, Arg	AGA, Arg
WD0632[†] (toxin?)			
91	GGA, Gly	GGG, Gly	GGG, Gly
176	TAT, Tyr	GAT, Asp	GAT, Asp
213	TAT, Tyr	TAC, Tyr	TAC, Tyr
1118	TTA, Leu	TGA, STOP	TTA, Leu
WRi_006710			
663	TAT, Tyr	CAT, His	CAT, His
WRi_006720			
1 to 108	Present	Disrupted, see text	Disrupted, see text

[*]The duplicate orthologs in *w*Ri are *WRi_005370* and *WRi_010030*.

[†]The duplicate orthologs in *w*Ri are *WRi_p005380* and *WRi_p010040*.

1

Table S1 Observed pairwise genomic differences between *Wolbachia* strains, given as percentage of polymorphic sites in single-copy, full-length genes present in all three strains.

*w*Spc-*w*Suz-*w*Ri dataset (704,883 base pairs, 703 genes)		
Genomes	**Number of differences**	**Percent difference**
*w*Spc v. *w*Suz	28	0.004%
*w*Ri v. *w*Suz	103	0.014%
*w*Ri v. *w*Spc	99	0.015%
*w*Spc-*w*Suz-*w*Ri-*w*Mel-*w*Au dataset (480,831 base pairs, 512 genes)		
Genomes	**Number of differences**	**Percent difference**
*w*Spc v. *w*Suz	21	0.005%
*w*Suz v. *w*Ri	62	0.014%
*w*Spc v. *w*Ri	59	0.014%

2

Table S2 Matrix of k_a (below diagonal) and k_s (above diagonal) estimates for *w*Suz, *w*Spc, *w*Ri, *w*Au and *w*Mel (using the 429,765 bp data set from Table S1).

	***w*Suz**	***w*Spc**	***w*Ri**	***w*Au**	***w*Mel**
***w*Suz**		0.002%	0.017%	4.58%	4.58%
***w*Spc**	0.005%		0.019%	4.57%	4.58%
***w*Ri**	0.013%	0.014%		4.57%	4.58%
***w*Au**	0.78%	0.77%	0.77%		0.20%
***w*Mel**	0.78%	0.78%	0.78%	0.10%	

3

4

Table S3 The 28 substitutions differentiating *w*Spc and *w*Suz.

Gene	Amino Acid	*w*Spc codon	*w*Suz codon	Gene Description
WRi_000230	228	GTT(Val)	ATT(Ile)	DNA-directed RNA polymerase beta subunit
WRi_000410	67	GCT(Ala)	GCC(Ala)	hypothetical protein
WRi_000410	118	TCG(Ser)	TTG(Leu)	hypothetical protein
WRi_000780	310	TGC(Cys)	TGT(Cys)	GTP/ATP binding protein putative
WRi_001670	103	GAC(Asp)	GAT(Asp)	enoyl-(acyl-carrier-protein) reductase
WRi_002520	228	GAT(Asp)	CAT(His)	GTP-binding protein
WRi_002650	6	GAA(Glu)	AAA(Lys)	hypothetical protein
WRi_003080	374	ACA(Thr)	ACG(Thr)	succinate dehydrogenase flavoprotein subunit
WRi_003240	59	TCT(Ser)	CCT(Pro)	hypothetical protein
WRi_003580	722	TTA(Leu)	CTA(Leu)	hypothetical protein
WRi_004080	303	CGG(Arg)	TGG(Trp)	bicyclomycin resistance protein
WRi_004790	793	CCT(Pro)	TCT(Ser)	hypothetical protein
WRi_004810	31	AAT(Asn)	ACT(Thr)	protoheme IX farnesyltransferase
WRi_006490	372	AAC(Asn)	GAC(Asp)	deoxyguanosinetriphosphate triphosphohydrolase
WRi_006610	26	CAA(Gly)	CCA(Pro)	polysaccharide deacetylase putative
WRi_007380	335	AAT(Asn)	AGT(Ser)	peptidase M16 family
WRi_007510	47	GGG(Gly)	GCG(Ala)	hypothetical protein
WRi_008460	296	TAT(Tyr)	CAT(His)	iron compound ABC transporter periplasmic iron compound-binding protein
WRi_008830	45	ACT(Thr)	GCT(Ala)	hypothetical protein
WRi_008830	29	GCA(Ala)	GTA(Val)	hypothetical protein
WRi_010700	179	ATT(Ile)	ATG(Met)	permease putative
WRi_010800	415	TTG(Leu)	TTT(Phe)	sodium/alanine symporter family protein
WRi_010800	226	ATG(Met)	ATT(Ile)	sodium/alanine symporter family protein
WRi_011150	29	GAA(Glu)	GCA(Ala)	putative monovalent cation/H+ antiporter subunit D
WRi_011880	231	GAT(Asp)	AAT(Asn)	Succinyl-CoA synthetase beta subunit
WRi_012790	260	GGG(Gly)	GAG(Glu)	Type IV secretion system protein VirB9 putative
WRi_012830	128	ATT(Ile)	ATG(Met)	rod shape-determining protein RodA
WRi_012980	22	TGT(Cys)	TTT(Phe)	HIT family protein

1

Table S4 Genes present in CNV regions of *w*Suz or *w*Spc relative to *w*Ri. All locations are relative to the *w*Ri reference sequence of Klasson *et al*. (2009).

CNV Location	Copy number change	Affected genomes	Kolmogorov-Smirnov p-value
570000-592500, 1077500-1100000	2 → 3	*w*Suz only	<0.0001

Gene start	Gene end	Gene ID	Gene Description
571723	573147	*WRi_005370*	Hypothetical protein: ortholog to WD0631, one of the tandem putative CI loci in wMel.
573202	576723	*WRi_p005380*	Putative pseudogene, a truncated ortholog of *WD0632* (and *wPip_0283*)
577843	580743	*WRi_005390*	Ankyrin repeat domain protein
581150	582643	*WRi_005400*	site-specific recombinase resolvase family
582831	583663	*WRi_005420*	transposase
584009	584467	*WRi_005440*	ankyrin repeat domain protein
584493	585227	*WRi_005450*	ankyrin repeat domain protein
585395	586555	*WRi_005460*	hypothetical protein
586555	587346	*WRi_005470*	baseplate assembly protein J putative
587349	587684	*WRi_005480*	baseplate assembly protein W putative
587687	587941	*WRi_005490*	hypothetical protein
587949	588413	*WRi_005500*	baseplate assembly protein V
588400	588876	*WRi_005510*	hypothetical protein
588873	589394	*WRi_005520*	minor tail protein Z putative
589396	589701	*WRi_005530*	hypothetical protein
589799	590803	*WRi_005540*	hypothetical protein
590841	591212	*WRi_005550*	hypothetical protein
591287	592348	*WRi_005560*	minor capsid protein C putative
1078182	1079606	*WRi_010030*	Hypothetical protein: ortholog to *WD0631*, one of the tandem putative CI loci in wMel.
1079661	1083182	*WRi_p010040*	Putative pseudogene, a truncated ortholog of *WD0632* (and *wPip_0283*)
1084302	1087202	*WRi_010050*	Ankyrin repeat domain protein
1087609	1089102	*WRi_010060*	site-specific recombinase resolvase family
1089290	1090122	*WRi_010080*	transposase
1090468	1090926	*WRi_010100*	ankyrin repeat domain protein
1090952	1091686	*WRi_010110*	ankyrin repeat domain protein
1091854	1093014	*WRi_010120*	hypothetical protein
1093014	1093805	*WRi_010130*	baseplate assembly protein J putative
1093808	1094143	*WRi_010140*	baseplate assembly protein W putative
1094146	1094400	*WRi_010150*	hypothetical protein
1094408	1094872	*WRi_010160*	baseplate assembly protein V

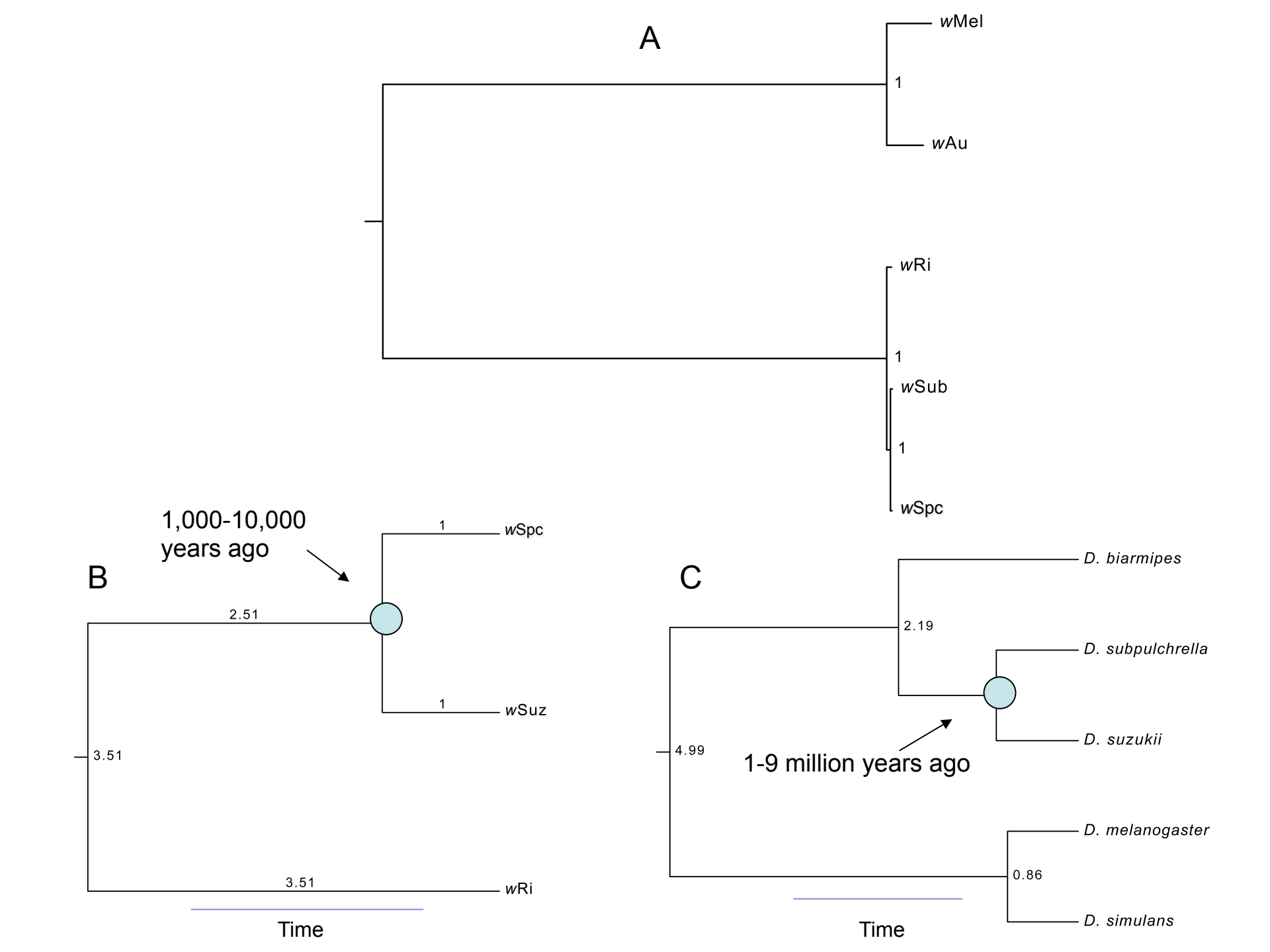
1094859	1095335	*WRi_010170*	hypothetical protein
1095332	1095853	*WRi_010180*	minor tail protein Z putative
1095855	1096160	*WRi_010190*	hypothetical protein
1096258	1097262	*WRi_010200*	hypothetical protein
1097300	1097671	*WRi_010210*	hypothetical protein
1097746	1098807	*WRi_010220*	minor capsid protein C putative

CNV Location	**Copy number change**	**Affected genomes**	**Kolmogorov-Smirnov *P*-value**
733000-756000	1 → 0	*w*Suz and *w*Spc	<0.0001

Gene start	**Gene end**	**Gene ID**	**Gene Description**
733007	734389	*WRi_006770*	transposase
735447	736526	*WRi_006790*	hypothetical protein
736739	737194	*WRi_006800*	Small heat shock protein
737637	738647	*WRi_006810*	ankyrin repeat domain protein
738683	739515	*WRi_006820*	transposase IS5 family
741777	749201	*WRi_006850*	ankyrin repeat domain protein
749574	750653	*WRi_006860*	ankyrin repeat domain protein
750749	753349	*WRi_006870*	ankyrin repeat domain protein
754223	755143	*WRi_006880*	patatin family protein
755153	755371	*WRi_006890*	hypothetical protein
755496	756032	*WRi_006900*	ankyrin repeat domain protein
755998	756978	*WRi_006910*	tail protein D putative

CNV Location	**Copy number change**	**Affected genomes**	**Kolmogorov-Smirnov *P*-value**
1345000-1347500	1 → 2	*w*Suz only	0.016

Gene start	**Gene end**	**Gene ID**	**Gene Description**
1345028	1345492	*WRi_012540*	baseplate assembly protein V
1345769	1347103	*WRi_012560*	transposase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wAu
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wSub
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wSpc
1,000-10,000
years ago
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1
wSpc
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1
wSuz
3.51
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wRi
Time
C
D. biarmipes
2.19
D. subpulchrella
D. suzukii
4.99
1-9 million years ago
D. melanogaster
0.86
D. simulans
Time