


ORIGINAL RESEARCH

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

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Abstract

Wolbachia endosymbionts may be acquired by horizontal transfer, by introgression through hybridization 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 wSuz and wSpc, very closely related to wRi, identified in California populations of *D. simulans*. However, these variants differ in their induced phenotypes: wRi causes significant cytoplasmic incompatibility (CI) in *D. simulans*, but CI has not been detected in *D. suzukii* or *D. subpulchrella*. Our draft genomes of wSuz and wSpc contain full-length copies of 703 of the 734 single-copy genes found in wRi. Over these coding sequences, wSuz and wSpc differ by only 0.004% (i.e., 28 of 704,883 bp); they are sisters relative to wRi, from which each differs by 0.014%–0.015%. Using published data from *D. melanogaster*, *Nasonia* wasps and *Nomada* bees to calibrate relative rates of *Wolbachia* versus host nuclear divergence, we conclude that wSuz and wSpc are too similar—by at least a factor of 100—to be plausible candidates for cladogenic transmission. These three wRi-like *Wolbachia*, which differ in CI phenotype in their native hosts, have different numbers of orthologs of genes postulated to contribute to CI; and the CI loci differ at several nucleotides that may account for the CI difference. We discuss the general problem of distinguishing alternative modes of *Wolbachia* acquisition, focusing on the difficulties posed by limited knowledge of variation in absolute and relative rates of molecular evolution for host nuclear genomes, mitochondria, and *Wolbachia*.

KEYWORDS

cytoplasmic incompatibility loci, horizontal transmission, introgression, molecular clocks, relative rates, spotted-wing *Drosophila*

1 | INTRODUCTION

Drosophila suzukii Matsumura (Diptera Drosophilidae) is an invasive and destructive fruit fly native to southeast Asia that has recently invaded North America, South America, and Europe (Cini, Ioriatti, & Anfora, 2012; Hauser, 2011; Rota-Stabelli, Blaxter, & Anfora, 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 (Fig. 1, Atallah et al., 2014; McEvey, 2017a, 2017b). 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 insect species 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 (Cattell et al., 2016; Hamm et al., 2014; Kriesner et al., 2013, 2016; Mazzetto, Gonella, & Alma, 2015). Indeed, it seems

increasingly plausible that even infections that cause reproductive manipulations become established 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 (Fast et al., 2011; Weeks et al., 2007), protection from viruses (Teixeira, Ferreira, & Ashburner, 2008), or metabolic provisioning (Brownlie et al., 2009).

To understand why *Wolbachia* are found in so many species, it is critical to know how *Wolbachia* infections are acquired and how long *Wolbachia*–host associations persist. As noted by Raychoudhury, Baldo, Oliveira, and Werren (2008), although *Wolbachia* are maternally transmitted, host lineages can acquire *Wolbachia* in three ways: by cladogenic transmission, in which, an infection persists through speciation; by introgression, in which, hybridization of closely related species leads to maternal 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 show that their informal methodology underestimated the relative frequencies of horizontal and introgressive transmission. Horizontal transmission of *Wolbachia* 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 the two species that have been examined most intensively, *D. simulans* (Turelli & Hoffmann, 1995) and *D. melanogaster* (Richardson et al., 2012). 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 multilocus sequence typing (MLST) loci used to classify *Wolbachia* (Baldo et al., 2006), cladogenic *Wolbachia* acquisition was likely. Here, we use comparative analyses of draft *Wolbachia* genomes, and extensive nuclear data from *Drosophila* and other insect hosts, to refute this hypothesis.

The three alternative modes of *Wolbachia* acquisition would be trivial to distinguish if reliable chronograms (dated phylogenies) were available for the nuclear, mitochondrial, and *Wolbachia* genomes. Under cladogenic transmission, without subsequent introgression or horizontal transmission, roughly concordant chronograms for all three genomes are expected. From the arguments of Gillespie and Langley (1979), we expect a slightly longer divergence time for

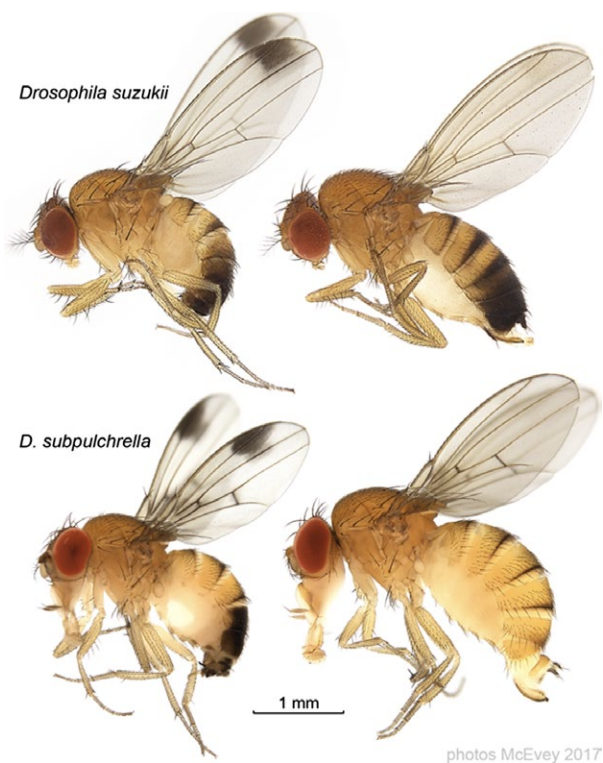


FIGURE 1 *Drosophila suzukii* and *D. subpulchrella*, with males on the left. The photographs are from McEvey (2017a, 2017b); the composite image is courtesy of Shane McEvey

nuclear than mitochondrial or *Wolbachia* given the greater intraspecific variation observed in nuclear DNA. However, for typical pairs of *Drosophila* species that diverged on the order of 10^6 years ago (Coyne & Orr, 1989, 1997), this discordance under cladogenic acquisition is unlikely to be as large as a factor of two. Under introgression without subsequent horizontal transmission, the mitochondrial and *Wolbachia* chronograms should be concordant (because they are simultaneously maternally transmitted) and show more recent divergence than the bulk of the nuclear genome. Finally, under horizontal transmission, more recent divergence is expected between infecting *Wolbachia* than between either the host 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 all available 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. Our conclusion does not exclude the possibility that *D. suzukii* and *D. subpulchrella* retained a *Wolbachia* infection from their common ancestor. Our data indicate only that their current *Wolbachia* are too similar to have been diverging since the speciation of their hosts. In principle, one could establish cladogenic transmission followed by introgression or horizontal transmission if traces of historical infections could be found in host genomes (Hotopp et al., 2007). Unfortunately, as shown below, no such traces were found in our *D. suzukii* or *D. subpulchrella* genomes.

In addition to assessing *Wolbachia* acquisition, we examine patterns of molecular evolution by comparing the draft genomes for wSuz (Siozos et al., 2013) and wSpc (this paper) to the wRi reference genome (Klasson et al., 2009). We consider both a general pattern, namely the relative frequencies of nonsynonymous and synonymous substitutions, and sequence divergence for candidate loci associated with two *Wolbachia*-induced phenotypes, life shortening and CI. The "Octomom" duplication, which distinguishes wMelPop (Min & Benzer, 1997) from wMel (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; but see Rohrscheib et al., 2016 for a critique and LePage et al., 2017 and Chrostek & Teixeira, 2017 for support of the hypothesis connecting these loci to life shortening or *Wolbachia* titer). Beckmann and Fallon (2013) used proteomics to identify the locus wPip_0282 in wPip, the *Wolbachia* found in *Culex pipiens*, as a candidate for producing CI. They found at least one homolog of this WO prophage locus in several CI-causing *Wolbachia*, including wMel and wRi. Within wPip 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 wMel, and there are three homologous/paralogous pairs in wRi. Beckmann, Ronau, and Hochstrasser (2017) and LePage et al. (2017) provide experimental and bioinformatic evidence that *WD0631* and *WD0632* contribute to CI (but LePage et al. (2017) argue against the operon hypothesis). We examine differences in homologs and paralogs of these loci among wSuz, wSpc, and wRi.

2 | MATERIALS AND METHODS

2.1 | 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*, wSuz, presented in Siozos et al. (2013). Illumina HiSeq2000 120-base, paired-end sequence data were generated from two libraries of 180 and 300 base pair (bp) inserts. The *D. subpulchrella* genome data were generated from a stock maintained at the Fondazione Edmund Mach laboratory that was established from San Diego *Drosophila* species stock center strain 14023-0401.00, originally from Japan. Illumina HiSeq2000 125-base, paired-end sequence data were generated from two libraries of 350 and 550 bp inserts.

2.2 | Assembly of *Wolbachia* in *D. subpulchrella*

To assemble wSpc, 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, ..., 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 (Mbp). 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 wSpc. We also attempted filtering the reads by alignment to wRi and assembling with SPAdes 3.0 (Bankevich et al. 2012). The assembly of wSpc is available from GenBank under accession number NTHL00000000 (project number PRJNA401169, Biosample SAMN07599555).

To assess the quality of our draft wSpc and wSuz assemblies, we used BUSCO v. 3.0.0 (Simão et al., 2015) to search for orthologs of the near-universal, single-copy genes in the BUSCO proteobacteria database. As a control, we performed the same search using the complete reference genomes for wRi (Klasson et al., 2009), wAu (Sutton, Harris, Parkhill, & Sinkins, 2014), wMel (Wu et al., 2004), wHa, and wNo (Ellegaard et al., 2013).

2.3 | Phylogeny and estimates of divergence of wSpc and wSuz

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 wSpc and wSuz were identical both to each other and to those of the wRi 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 wSuz and wSpc with Prokka v 1.11 (Seemann, 2014), which identifies orthologs to reference bacterial genes. To normalize our comparisons, we also annotated the genomes of wRi (Klasson et al., 2009), wAu (Sutton et al., 2014), and wMel (Richardson et al., 2012; Wu et al., 2004). We selected 512 genes present in full length and single copy in all five genomes, avoiding incomplete or pseudogenes and loci with paralogs. Genes were treated as single copy if no other gene in the genome was matched to the same reference bacterial gene by Prokka, and as full length if the orthologs in the other *Wolbachia* genomes all had the same length. The nucleotide sequences of the genes were aligned with MAFFT v. 7 (Kato, 2013) and concatenated, giving an alignment of 480,831 bp. The strain phylogeny was estimated with a phylogram constructed with MrBayes v. 3.2 (Ronquist & Huelsenbeck, 2003) using the GTR+ Γ model, partitioned by codon position. All model parameters for each partition were allowed to vary independently, except topology and branch length. 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, Suchard, & Drummond 2013) to ensure convergence of all continuous parameters. The first 25% of the generations were discarded as burn-in. Only one topology had posterior probability >.001.

To estimate the divergence between wSuz and wSpc, 703 genes present in full length and single copy in wSuz, wSpc, and wRi (spanning a total of 704,883 bp) were extracted and aligned with MAFFT v. 7. As an additional assessment of the completeness of the wSuz and wSpc assemblies, we calculated the number of single-copy genes in the wRi reference and found 734. The resulting alignments were concatenated. To estimate a chronogram, we assumed for simplicity that each partition evolved at a constant rate across the tree (allowing the rates to differ among codon positions). The constant-rate chronogram was estimated with MrBayes v. 3.2, using the same procedure as our five-sequence *Wolbachia* phylogram (which included wMel and wAu). The age of the wSuz–wSpc node was set at 1, as an arbitrary scaling of relative ages.

2.4 | Nuclear divergence between *D. subpulchrella* and *D. sukuzii*

Hamm et al. (2014) used *Drosophila* nuclear data extracted from Yang et al. (2012) to assess the relationships of *D. sukuzii*, *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. sukuzii* and *D. subpulchrella*. We identified in FlyBase 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*), plus ten additional nuclear loci (*aconitase*, *enolase*, *glyp*, *glys*, *pepck*, *pgi*, *pgm*, *tpi*, *white*, and *wg*). We used BLAST to identify orthologs in the *D. sukuzii* 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, Eisen, Thornton, & Andolfatto, 2013). Data for *H2A* and *amylase* were eliminated because *H2A* had multiple nonidentical paralogs in each species and homologs of *D. melanogaster amylase* could not be found in the assemblies. The coding sequences for the remaining 18 loci were aligned with MAFFT v. 7 and concatenated. (Our nuclear data from *D. subpulchrella* are available from GenBank under accession numbers MF908506–MF909523.) The alignment was analyzed with MrBayes v. 3.2 using the same model and procedures used for our *Wolbachia* analyses, except that we partitioned the data by both gene and codon position. We estimated both a phylogram and a constant-rate chronogram. The latter assumed that each partition evolved at a constant rate over the tree. The age of the most recent common ancestor (MRCA) of *D. sukuzii* and *D. subpulchrella* was set at 1, as an arbitrary scaling of relative ages. To test the robustness of our relative divergence-time estimates for the host species, we also estimated the chronogram using a relaxed-clock model in RevBayes (Hoehna et al., 2016). This analysis also partitioned the data by gene and codon position and used the GTR+ Γ model, but it assumed uncorrelated lognormal rate variation across branches. Following the RevBayes tutorial (https://github.com/revbayes/revbayes_tutorial/blob/master/RB_BayesFactor_Tutorial/scripts/marginal_likelihood_GTR_Gamma_inv.Rev), we used a lognormal prior with mean and standard deviation parameters ($-X^2/2$, X) and a lognormal hyperprior on X with parameters ($\ln(2)/4$, $\text{Sqrt}[\ln(2)/2]$). To estimate k_s and k_a between *D. sukuzii* and *D. subpulchrella*, we used DNAsp v. 5.10 (Rozas, 2009).

Following Hotopp et al. (2007), we looked for evidence of genetic transfer from wSuz and wSpc (or other *Wolbachia*) to these hosts. The *D. sukuzii* 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.

2.5 | Analysis of divergence between wSpc, wSuz, and wRi

The trimmed Illumina reads from *D. sukuzii* and *D. subpulchrella* were aligned to the wRi reference (Klasson et al., 2009) with bwa v. 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 wRi reference. Normalized read depth for each alignment was calculated over sliding 1,000-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 ControlFREENC v. 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 wRi, we used a ploidy of two. We calculated *p*-values for each putative CNV using the Kolmogorov–Smirnov test implemented in ControlFREENC.

Sequences for the “Octomom” genes *WD0507–WD0514* (Chrostek & Teixeira, 2015; cf. Rohrscheib et al., 2016; Chrostek & Teixeira, 2017) were extracted from the *wMel* reference (Richardson et al., 2012; Wu et al., 2004). Using BLAST, we identified orthologs in the *wRi* reference (Klasson et al., 2009) and the draft assemblies for *wSuz* and *wSpc*.

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

To identify a specific insertion of the transposable element ISWpi7, which occurs in 21 identical copies in *wRi*, and whose position differentiates *wSpc* and *wSuz* from *wRi*, an additional assembly step was required. The novel insertion occurs in the *wSpc* and *wSuz* orthologs of *WRI_006720*, one of the CI-associated loci discussed below. The *D. suzukii* and *D. subpulchrella* reads were aligned to the *wSpc*

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. To test this bioinformatic result, we designed two pairs of PCR primers that spanned the hypothesized junctions between the ortholog of *WRI_006720* and ISWpi7. For the first set of primers (forward: ATGGTCACATTGAACAGAGGAT, reverse: GTTGGTGCTGCAATGCGTAA), the forward primer attaches at 728945–728966, part of *WRI_006720*. For the second set of primers (forward: AGCGTTGTGGAGGAACTCAG, reverse: CGTCATGCTGCAGTGCTTAG), the reverse primer attaches at 729570–729589, part of *WRI_006720*. No detectable product is expected with either primer set in *wRi*, which does not contain the insert in *WRI_006720*, whereas each primer set is expected to produce a unique band with *wSpc* and *wSuz*.

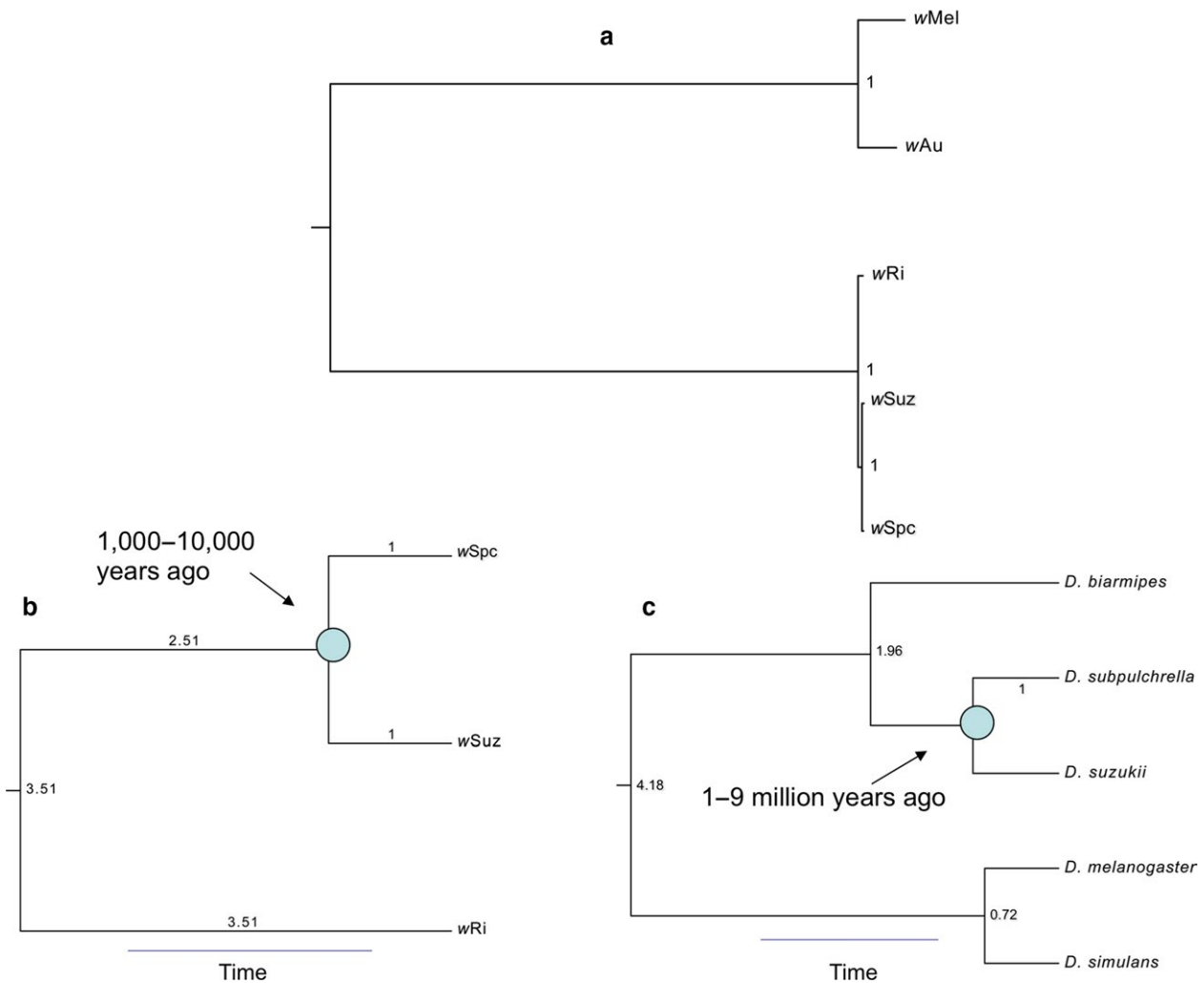


FIGURE 2 Phylogram and chronograms for the *Wolbachia* and hosts discussed. Clade posterior probabilities are shown. (a) *Wolbachia* phylogram. (b) *Wolbachia* chronogram with an estimate of the divergence time for *wSuz* and *wSpc*. Branch lengths relative to the *wSpc*–*wSuz* divergence are shown. All clade posterior probabilities are 1.0. (c) Host chronogram with an estimate of divergence time for *Drosophila suzukii* and *D. subpulchrella*. Branch lengths relative to the *D. suzukii*–*D. subpulchrella* divergence are shown. All clade posterior probabilities are 1.0

3 | RESULTS

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

We generated a draft assembly of wSpc by filtering contigs from a joint *Wolbachia*-*D. subpulchrella* assembly. The draft wSpc assembly was in 100 contigs with N50 length of 31,871 bp and total length of 1.42 Mbp. This length is close to the 1.45 Mbp wRi reference (Klasson et al., 2009), suggesting that it may represent a nearly complete genome. In contrast, the assembly produced by SPAdes 3.0 had N50 of 8,360 bp and total length of 1.20 Mbp.

Out of 221 near-universal, single-copy orthologs in proteobacteria, BUSCO 3.0.0 (Simão et al., 2015) found effectively the same number in all of the tested genomes (wRi, wAu, wMel, wHa, wNo, and the drafts of wSuz and wSpc). Our draft assemblies for wSpc and wSuz contain two BUSCO-annotated genes not found in wRi and wMel. See Table S1 for detailed information.

3.2 | *Wolbachia* divergence

We aligned and compared wSpc and wSuz at 703 protein-coding loci (704,883 bp) and identified only 28 single-nucleotide variants (SNV), an overall divergence of 0.004%. wSuz had 103 SNV compared to wRi (0.015% divergence), and wSpc had 99 SNV (0.014% divergence) (Table S2). 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 placed wSuz and wSpc as sisters relative to wRi (Fig. 2a). For wSuz and wSpc, 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). 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. 2b) shows that the divergence time of wRi from its MRCA with wSpc and wSuz is 3.51 times the divergence time of wSpc and wSuz, 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 synonymous, k_s , and nonsynonymous, k_a , substitution rates were very similar (Table 1).

3.3 | Host divergence

The host phylogram (data not shown) and chronogram (Fig. 2c) demonstrate 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 1.96 times the divergence time for *D. subpulchrella*

TABLE 1 Estimated number of substitutions per site by codon position between wSuz and wSpc, plus estimates of synonymous (k_s) and nonsynonymous (k_a) substitution rates, see the text for details

Position	Point estimates	95% confidence interval
1st	5.0×10^{-5}	$(3.0 \times 10^{-5}, 7.0 \times 10^{-5})$
2nd	3.2×10^{-5}	$(1.6 \times 10^{-5}, 4.6 \times 10^{-5})$
3rd	4.0×10^{-5}	$(2.4 \times 10^{-5}, 5.6 \times 10^{-5})$
Overall (k_s, k_a)	$(3 \times 10^{-5}, 4 \times 10^{-5})$	

and *D. suzukii*, with 95% confidence interval (1.84, 2.08). The *D. melanogaster* and *D. simulans* divergence-time estimate is 0.72 times as large as the estimated divergence time for *D. subpulchrella*-*D. suzukii*, with a 95% confidence interval of (0.65, 0.78). 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). Our estimate of the third-codon position substitutions per site, which we use to date *D. subpulchrella*-*D. suzukii* divergence, is 9.20×10^{-2} and a 95% confidence interval of $(8.6 \times 10^{-2}, 9.80 \times 10^{-2})$.

Our RevBayes (Hoehna et al. 2016) relaxed-clock chronogram (data not shown) was very similar to Fig. 2c. The divergence time of *D. biarmipes* from its MRCA with *D. subpulchrella* and *D. suzukii* was estimated to be 1.84 times the divergence time for *D. subpulchrella* and *D. suzukii* (instead of 1.96). Similarly, the *D. melanogaster* and *D. simulans* divergence-time estimate is 0.76 times as large as the estimated divergence time for *D. subpulchrella*-*D. suzukii* (instead of 0.72). We note that the model underlying this analysis assumes for computational convenience that each partition undergoes proportional rate variation across each branch, that is, each partition speeds up or slows down by the same amount along each branch (but see Langley & Fitch, 1974).

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

3.4 | 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 wSpc and wSuz. 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; wNlonB1 versus wNgrIB), 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 supports relatively recent *Wolbachia* transfer between *D. suzukii* and *D. subpulchrella*, being inconsistent with ratios observed under cladogenic *Wolbachia* acquisition. Given that we are looking at only single wSpc and wSuz sequences, their divergence time provides an upper bound for the

time of interspecific transfer (Gillespie & Langley, 1979). Additional support for noncladogenic transmission comes from the analyses of Richardson et al. (2012), who inferred that *Wolbachia* substitution rates were roughly 10-fold lower than the noncoding nuclear mutation rate for *D. melanogaster*, which is often considered a reasonable approximation for the rate of third-position substitutions (at least for fourfold degenerate sites, Obbard et al. 2012). This is clearly inconsistent with the three-order-of-magnitude difference we estimate (Table 3).

Comparing wSuz and wSpc, we found no difference in k_s and k_a (Table 1). This is also true for wMel variation in *D. melanogaster* (Richardson et al., 2012). M. Gerth and C. Bleidorn (2016, personal communication) find essentially identical estimates of k_s and k_a for all pairwise comparisons of the *Wolbachia* in the clade ([*Nomada leucophthalma*, *Nomada flava*], *Nomada panzeri*). In contrast, comparing wRi and wAu using the 429,765-bp data set of single-copy, full-length genes (Table S3), we estimate k_s of 4.34%, whereas the estimated k_a is only 0.65% (or $k_s/k_a = 6.7$). Similarly, when comparing the *Wolbachia* of the outgroup host, *Nomada ferruginata*, to the *Wolbachia* of the three ingroup species, M. Gerth and C. Bleidorn (2016, personal communication) observed k_s/k_a values of 2.8, 2.8, and 2.5. In their comparisons of wNlonB1 and wNgirB from *Nasonia longicornis* and *Nasonia 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 nonsynonymous substitutions.

Estimating absolute divergence times (i.e., times to the MRCA) for wSuz and wSpc and their hosts is more difficult. Assuming 10 generations per year in *Drosophila* and using the wMel-derived estimate of $(2.88 \times 10^{-10}, 1.29 \times 10^{-9})$ changes/site/host-generation as the 95% confidence interval for the third-position substitution rate of *Wolbachia* (Richardson et al., 2012), wSuz and wSpc diverged about 1,600–7,000 years ago. Using the 95% confidence interval for first- and second-position substitution rates from Richardson et al. (2012) yields wSuz–wSpc divergence dates of 1,200–9,100 years. Given that *D. sukuzii* and *D. subpulchrella* seem to be temperate species (Ometto et al., 2013; Takamori et al., 2006), 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* k_s of 4.7×10^{-9} changes/synonymous site/year in *Nasonia*. Using our k_s from Table 1 with the *Nasonia* calibration, the estimated divergence for wSuz and wSpc is 6,400 years, which is consistent with our *Drosophila* calibration. These analyses suggest that wSuz and wSpc 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. sukuzii* (Fig. 2c), we infer divergence times for *D. subpulchrella* and *D. sukuzii* ranging from about 1 to 9 million years, two orders of

TABLE 2 Estimated number of substitutions per site by codon position between *Drosophila sukuzii* and *D. subpulchrella* for 18 nuclear loci, plus estimates of synonymous (k_s) and nonsynonymous (k_a) substitution rates, see the text for details

Position	Point estimates	95% confidence interval
1st	1.10×10^{-2}	$(9.91 \times 10^{-3}, 1.21 \times 10^{-2})$
2nd	4.73×10^{-3}	$(4.12 \times 10^{-3}, 5.28 \times 10^{-3})$
3rd	9.20×10^{-2}	$(8.64 \times 10^{-2}, 9.80 \times 10^{-2})$
Overall (k_s, k_a)	$(1.2 \times 10^{-1}, 5.3 \times 10^{-3})$	

TABLE 3 Estimated frequencies of synonymous (k_s) versus nonsynonymous (k_a) substitutions per site for *Wolbachia* in various hosts

Data source	Species 1	Species 2	Host		<i>Wolbachia</i>		k_s ratio
			k_s	k_a	k_s	k_a	
This work	<i>Drosophila sukuzii</i>	<i>Drosophila subpulchrella</i>	1.2×10^{-1}	6.8×10^{-3}	3×10^{-5}	4×10^{-5}	0.00025
Raychoudhury et al. (2008)	<i>Nasonia giraulti</i>	<i>Nasonia longicornis</i>	1.22×10^{-2}	5.4×10^{-3}	3.7×10^{-3}	2.2×10^{-3}	0.30
Gerth and Bleidorn (2016)	<i>Nomada ferruginata</i>	<i>Nomada leucophthalma</i>	1.95×10^{-2}	2.6×10^{-3}	2.5×10^{-3}	9×10^{-4}	0.13
Gerth and Bleidorn (2016)	<i>N. ferruginata</i>	<i>N. flava</i>	1.92×10^{-2}	2.7×10^{-3}	2.5×10^{-3}	9×10^{-4}	0.13
Gerth and Bleidorn (2016)	<i>N. ferruginata</i>	<i>N. panzeri</i>	1.84×10^{-2}	3.1×10^{-3}	2.7×10^{-3}	1.1×10^{-3}	0.15
Gerth and Bleidorn (2016)	<i>N. leucophthalma</i>	<i>N. flava</i>	6.8×10^{-3}	4×10^{-4}	1×10^{-4}	1×10^{-4}	0.015
Gerth and Bleidorn (2016)	<i>N. leucophthalma</i>	<i>N. panzeri</i>	5.8×10^{-3}	8×10^{-4}	3×10^{-4}	2×10^{-4}	0.052
Gerth and Bleidorn (2016)	<i>N. flava</i>	<i>N. panzeri</i>	5.5×10^{-3}	9×10^{-4}	3×10^{-4}	3×10^{-4}	0.055

magnitude larger than our estimates for *wSuz* versus *wSpc*. Hence, despite great uncertainties, our data clearly preclude cladogenic transmission of *wSuz* and *wSpc*. This conclusion is further supported in the Discussion by a review of variation in rates of bacterial molecular evolution.

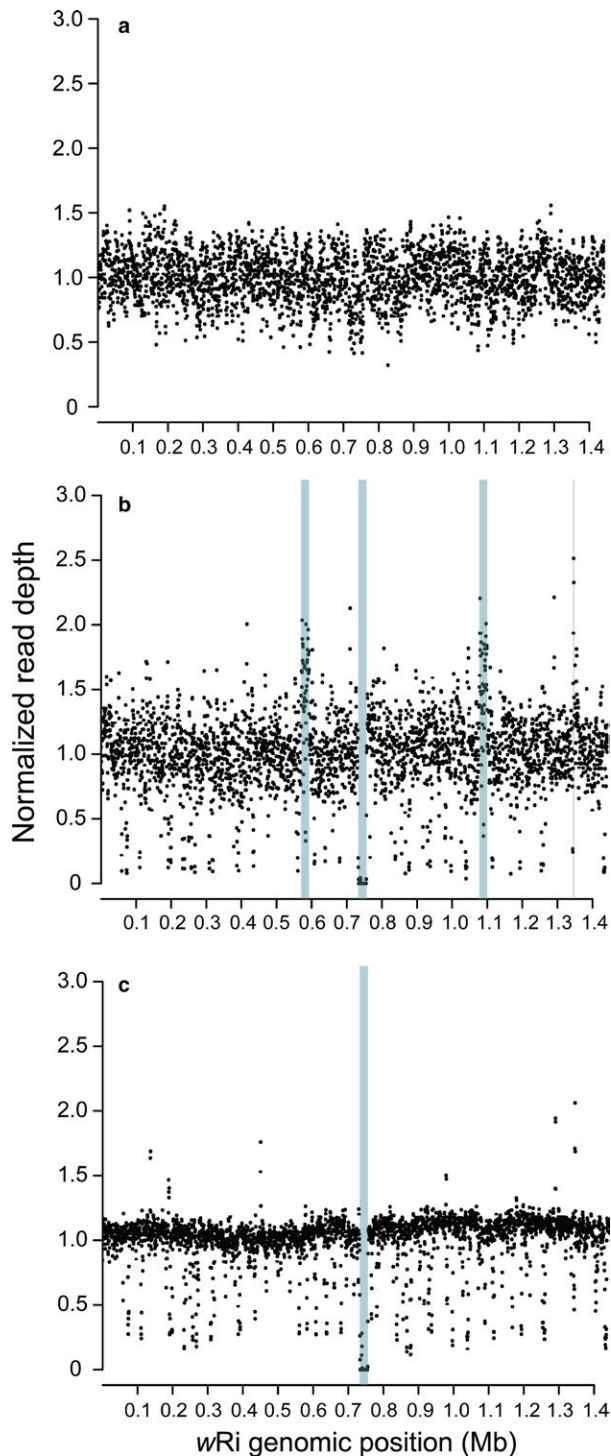


FIGURE 3 We compare normalized read density relative to the *wRi* reference sequence of Klasson et al. (2009) for: (a) the Illumina reads from the Riv84 version of *wRi* reported by Iturbe-Ormaetxe et al. (2010), (b) the *wSuz* reads from Ometto et al. (2013), and (c) the *wSpc* reads from this study

3.5 | Genome differences between *wSpc*, *wSuz*, and *wRi*: Structural variation and candidate genes

We identified CNV in *wSuz* and *wSpc* relative to the *wRi* reference sequence by plotting read depth along each genome (Fig. 3; Table 4). *wSpc* and *wSuz* share a deletion relative to *wRi* of 23,000 bp, between positions 733,000 and 756,000. *wSuz* 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 *wRi*, 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). *wSuz* 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 *wMel* (shortened life, high *Wolbachia* titer) has been associated with eight loci (*WD0507*–*WD0514*, Chrostek & Teixeira, 2015; but see also Chrostek & Teixeira, 2017; Rohrscheib et al., 2016). In the *wRi* 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 *wRi* WO-B prophages. A single *WD0509*-like gene was present, from 1,419,589 to 1,421,396, not associated with WO-B prophage. These two genes are not neighbors in *wRi*, *wSpc*, or *wSuz*, and they are not within regions that differentiate *wSpc* and *wSuz* from *wRi*.

Table 5 lists the orthologs and paralogs in *wMel*, *wRi*, *wSuz*, and *wSpc* of *wPip_0282* and *wPip_0283*, the loci originally identified as CI-causing by Beckmann and Fallon (2013) in *wPip*, 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 with LePage et al., 2017). The orthologs in *wMel* are *WD0631* and *WD0632*. As shown in Table 5, there are two copies of the type I pair in *wRi*, one copy in each of the two complete copies of the WO-B prophage. As noted by Beckmann and Fallon (2013), in *wRi*, 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 S5 lists genes included in the CNV regions of *wSuz* and *wSpc* relative to *wRi*. Notably, the orthologs of *WD0631* and *WD0632*, implicated in causing CI (Beckmann & Fallon, 2013; Beckmann et al., 2017; LePage et al., 2017), are in a partial third copy of prophage WO-B found in *wSuz*. Hence, *wSuz* contains three copies of these two loci, whereas *wSpc* has only two (see Table 5). The CNVs in *wSuz* or *wSpc* do not affect the type II loci.

Table 6 reports differences among *wRi*, *wSuz*, and *wSpc* at orthologs of the CI-associated loci *WD0631*, *WD0632*, *wRi_006710*, and *wRi_006720*. The duplicate orthologs of *WD0631* in *wRi* are *wRi_005370* and *wRi_010030*. As noted by Beckmann and Fallon (2013), the (duplicate) orthologs of *WD0632* in *wRi* have been annotated as pseudogenes, *wRi_p005380* and *wRi_p010040*, because of premature stop codons, but they retain large, intact coding regions and may be functional (LePage et al., 2017 and Beckmann et al., 2017 provide evidence supporting function). Even with multiple orthologs of *WD0631* and *WD0632* in each genome (two in *wRi*, two in *wSpc*, and three in *wSuz*), all copies within each genome are identical and

TABLE 4 Copy-number variants in *wSuz* and *wSpc* relative to *wRi*. All positions are given relative to the *wRi* reference of Klasson et al. (2009)

Start position	End position	Copy-number change	Kolmogorov–Smirnov <i>p</i> -value	Affected genomes
570,000	592,500	2 → 3 ^a	<.0001	<i>wSuz</i>
733,000	756,000	1 → 0	<.0001	<i>wSuz</i> , <i>wSpc</i>
1,077,500	1,100,000	2 → 3 ^a	<.0001	<i>wSuz</i>
1,345,000	1,347,500	1 → 2	.016	<i>wSuz</i>

^aThis sequence is duplicated in the *wRi* genome, so it was treated as diploid in our ControlFREEC v. 8.0 analysis.

TABLE 5 Homologs of CI-associated loci in *wMel*, *wRi*, *wSuz*, and *wSpc*. The gene designations in *wSpc* and *wSuz* reflect homology to loci identified in *wMel* and *wRi*

<i>Wolbachia</i>	Gene pair ^a	Gene 1	Gene 2	WO prophage association ^b
<i>wMel</i>	I	WD0631 (<i>cifA/cidA</i>) ^c (antidote?)	WD0632 (<i>cifB/cidB</i>) ^c (toxin?)	Yes
<i>wRi</i>	I.1	<i>wRi_005370</i>	<i>wRi_p005380</i> ^d	Yes
	I.2	<i>wRi_010030</i>	<i>wRi_p010040</i> ^d	Yes
	II	<i>wRi_006720</i>	<i>wRi_006710</i>	No
<i>wSpc</i>	I.1	<i>wSpc_0631.I.1</i>	<i>wSpc_0632.I.1</i>	Yes
	I.2	<i>wSpc_0631.I.2</i>	<i>wSpc_0632.II.2</i>	Yes
	II	<i>wSpc_6720</i> (disrupted)	<i>wSpc_6710</i>	No
<i>wSuz</i>	I.1	<i>wSuz_0631.I.1</i>	<i>wSuz_0632.I.1</i>	Yes
	I.2	<i>wSuz_0631.I.2</i>	<i>wSuz_0632.II.2</i>	Yes
	I.3	<i>wSuz_0631.I.3</i>	<i>wSuz_0632.II.3</i>	Partial ^e
	II	<i>wSuz_6720</i> (disrupted)	<i>wSuz_6710</i>	No

^aRoman numerals follow the “type” designations in LePage et al. (2017).

^bThis refers to location within an intact WO prophage, as opposed to a “WO-like island” (cf. LePage et al., 2017).

^cAlternative designations (*cif* vs. *cin*) from LePage et al. (2017) and Beckmann et al. (2017), respectively. Beckmann et al. (2017) propose that WD0631 produces an antidote to the toxin produced by WD0632.

^dAnnotated as pseudogenes, but see text.

^eThis third copy in *wSuz* exists in the 1077500–1100000 CNV, noted in Table 4, which is a partial copy of the WO-B prophage.

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

Our bioinformatic and PCR data show that in both *wSpc* and *wSuz* (but not *wRi*), an IS element, identical to ISWpi7 of *wRi* (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 *wRi*, each 1480 bp long with the transposase gene flanked on each

side by about 200 bp. Clearly, this ISWpi7 insertion predates the divergence of *wSpc* and *wSuz*.

4 | DISCUSSION

4.1 | Genomic data indicate noncladogenic acquisition of *wSuz* and *wSpc*

Despite considerable uncertainty in divergence-time estimates for both *wSuz* and *wSpc* and their hosts, *D. sukukii* 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

Location (gene, amino acid)	wRi codon (codon, translation)	wSpc codon (codon, translation)	wSuz codon (codon, translation)
WD0631 ^a (antidote?)			
363	AAA, Lys	GAA, Glu	GAA, Glu
473	AAA, Lys	AGA, Arg	AGA, Arg
WD0632 ^b (toxin?)			
91	GGA, Gly	GGG, Gly	GGG, Gly
176	TAT, Tyr	GAT, Asp	GAT, Asp
213	TAT, Tyr	TAC, Tyr	TAC, Tyr
1,118	TTA, Leu	TGA, STOP	TTA, Leu
WRi_006710			
663	TAT, Tyr	CAT, His	CAT, His
WRi_006720			
1–108	Present	Disrupted, see text	Disrupted, see text

^aThe duplicate orthologs in wRi are WRi_005370 and WRi_010030.

^bThe duplicate orthologs in wRi are WRi_p005380 and WRi_p010040.

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, Lipkowitz, & Brandvain, 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 (Lachaise et al., 2000; Rousset & Solignac, 1995). 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 preparation).

Understanding the frequency of alternative modes of *Wolbachia* transmission is clearly related to determining how long *Wolbachia* infections typically persist in host lineages. Bailly-Bechet et al. (2017) provide a meta-analysis of more than 1,000 arthropod species from Tahiti that suggests average durations on the order of 7 million years. However, their molecular data, which involve only two *Wolbachia* loci and the CO1 mtDNA locus, do not have sufficient power to resolve the issue. Moreover, as they note, their analysis conflates imperfect maternal transmission with the gain and loss of *Wolbachia* infections within lineages. As our analyses indicate, nearly complete *Wolbachia* and mitochondrial genomes will often be needed to unravel the acquisition and retention of closely related *Wolbachia* within host clades.

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

Gerth and Bleidorn (2016) proposed a time scale for *Wolbachia* evolution based on the apparent codivergence of *Wolbachia* and nuclear

TABLE 6 Comparisons between wRi, wSpc, and wSuz at the CI-associated loci (type I, possible antidote, toxin), WD0631 and WD0632, from wMel, and the paralogous loci (type II), WRi_006710 and WRi_006720 from wRi. All reads from wSpc and wSuz are consistent with the differences shown

genomes in a clade of four *Nomada* bee species. Our discussion of their data emphasized comparisons between the outgroup host *N. ferruginata* and the three ingroup hosts, noting that the codivergence 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 and Bleidorn (2016), we would infer much slower divergence of their *Wolbachia* (which recently acquired a biotin synthesis operon). For *N. leucophthalma* and *N. flava*, M. Gerth and C. Bleidorn (2016, personal communication) estimated synonymous nuclear substitution divergence of 6.8×10^{-3} , with a corresponding *Wolbachia* synonymous substitution divergence of only 1.0×10^{-4} (Table 3). 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 1/68th 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 1,000-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.

Although there are relatively few taxa for which we can quantify the relative rates of nuclear versus *Wolbachia* molecular evolution, there are extensive data assessing the relative constancy of bacterial molecular evolution. Kuo and Ochman (2009) provide an overview, emphasizing that variation across taxa is too great for any locus or group of loci to provide a broadly applicable “molecular clock” for

bacteria. Nevertheless, their analyses indicate that variation across lineages is typically much less than 10-fold. Yet, if *wSuz* and *wSpc* were cladogenically inherited and we assume the implausibly short host divergence time of 500,000 years (half of our lowest plausible estimate, see Fig. 2), the inferred upper bound on the rate of *Wolbachia* silent-site substitutions is about 1.0×10^{-11} per site per year. In contrast, the inferred rates of silent-site substitutions from the *Nasonia* and *Nomada* data (Table 3) are at least two orders of magnitude faster. Such variation in *Wolbachia* substitution rates over many loci would be unprecedented among bacteria.

4.3 | Comparative genomics and CI

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

The published crossing studies in *D. sukuzii* and *D. subpulchrella*, which found no statistically significant CI caused by *wSuz* or *wSpc*, are relatively small (Cattell et al., 2016; Hamm et al., 2014). 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, Ballard, & Mercot, 2004; Zabalou et al., 2004). However, larger experiments by Cooper, Ginsberg, Turelli, and Matute (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. sukuzii* and *D. subpulchrella*, as well as investigation of whether CI is produced when *wSpc* and *wSuz*, are transfected 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).

5 | CONCLUSIONS AND OPEN QUESTIONS

Understanding how host species acquire *Wolbachia* requires comparing divergence-time estimates for closely related *Wolbachia* in host

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, Anderson, Genchi, & Blaxter, 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. sukuzii* and *D. subpulchrella*, distinguishing between introgression and horizontal transmission requires mtDNA sequences, which will be analyzed in our forthcoming *D. subpulchrella* genome paper.

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 nonsynonymous substitutions. This is consistent with the pattern of variation seen for *wMel* 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 *wAu* and *wRi* infecting *D. simulans*. Does *Wolbachia* "invasion" of a new host represent a relaxation of selective constraint or an opportunity for adaptation? The reigning paradigm for molecular evolution of endosymbionts involves the fixation of slightly deleterious mutations (Kuo & Ochman, 2009; Moran, 1996), consistent with relaxed constraints and reduced effective population size. However, we can test for rapid adaptation of *Wolbachia* to hosts by moving near-identical *Wolbachia* between closely related hosts and comparing fitness (and reproductive) effects in native versus novel hosts.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

The genomic data for *D. subpulchrella* and *wSpc* 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

Our nuclear data from *D. subpulchrella* are available from GenBank under accession numbers MF908506–MF909523. For wSpc, the Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NTHL00000000. The version described in this paper is version NTHL01000000.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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