Positive selection and horizontal gene transfer in the genome of a male-killing *Wolbachia*

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Abstract

Wolbachia are a genus of widespread bacterial endosymbionts in which some strains can hijack or manipulate arthropod host reproduction. Male killing is one such manipulation in which these maternally transmitted bacteria benefit surviving daughters in part by removing competition with the sons for scarce resources. Despite previous findings of interesting genome features of microbial sex ratio distomers, the population genomics of male-killers remain largely uncharacterized. Here, we uncover several unique features of the genome and population genomics of four Arizonan populations of a male-killing Wolbachia strain, wInn, that infects mushroom-feeding Drosophila innubila. We first compared the wInn genome to other closely related Wolbachia genomes of Drosophila hosts in terms of genome content and confirm that the wInn genome is largely similar in overall gene content to the wMel strain infecting D. melanogaster. However, it also contains many unique genes and repetitive genetic elements that indicate lateral gene transfers between wInn and non-Drosophila eukaryotes. We also find that, in line with literature precedent, genes in the Wolbachia prophage and Octomom regions are under positive selection. Of all the genes under positive selection, many also show evidence of recent horizontal transfer among Wolbachia symbiont genomes. These dynamics of selection and horizontal gene transfer across the genomes of several Wolbachia strains and diverse host species may be important underlying factors in Wolbachia’s success as a male-killer of divergent host species.

Introduction

Wolbachia are the most widespread endosymbionts on the planet, infecting an estimated 40-52% of all insect species (Kondo, et al. 2002; Zug and Hammerstein 2012; Weinert, et al. 2015). These obligate intracellular Gram-negative α-proteobacteria of the order Rickettsiales primarily infect the gonads of their hosts and are predominantly transmitted vertically via the cytoplasm from mother to offspring (Hertig and Wolbach 1924; Serbus and Sullivan 2007). Wolbachia of insects and other arthropods have adopted cunning techniques to facilitate their matrilineal spread by manipulating host reproduction to increase the proportion of infected, transmitting females in the population (Werren, et al. 2008; Hurst and Frost 2015; Kaur, et al. 2021). The most common form of this reproductive parasitism is cytoplasmic incompatibility (CI), where crosses between uninfected females and infected males result in death of offspring. If the mother is also infected with a compatible strain, offspring are rescued from death, giving infected females a relative fitness advantage in the population over uninfected females (Yen and Barr 1971; Turelli and Hoffman 1991; Sinkins, et al. 1995). Three other, less common forms of reproductive parasitism rely on sex ratio distortion to increase the proportion of transmitting females each generation. These phenotypes are known as parthenogenesis (asexual reproduction of females, (Russell and Stouthamer 2011)), feminization (genetic males physically develop and reproduce as females, (Bouchon, et al. 1998; Kageyama, et al. 2002)), and
male killing (infected males die early in development, (Hurst, et al. 1999; Fujii, et al. 2001; Dyson, et al. 2002)). In addition, despite being comparatively rare, there are reported cases of horizontal Wolbachia host switching. These include cases of switches either between host species where Wolbachia propagate in new taxa through mechanisms such as predation or transfer between individual hosts within a species (O’Neill, et al. 1992; Vavre, et al. 1999; Haine, et al. 2005; Riegler, et al. 2005; Werren, et al. 2008; Ilinsky 2013; Turelli, et al. 2018). These transfers likely influence the evolution of Wolbachia in relation to their hosts and play a key role in Wolbachia’s ubiquitous spread around the world (Sanaei, et al. 2020).

The incredible success of Wolbachia in becoming one of the world’s most widespread infections (Werren, et al. 2008; LePage and Bordenstein 2013) is in part due to its diverse genetic toolkit and unique genome features. Indeed, to date, 17 Wolbachia supergroups have been described, labelled supergroups A-F, H-Q, and S (Taylor, et al. 2018; Laidoudi, et al. 2020; Lefoulon, et al. 2020). Studies on strains in supergroups A and B are well represented in the literature, and include many reproductive parasite strains of hosts such as mosquitoes and a large number of Drosophila species (Gerth, et al. 2014). The first sequenced Wolbachia genome, strain wMel of Drosophila melanogaster, demonstrated that this genus deviates from the canonical model of streamlined genome content typical of other endosymbionts. It instead contains unusually high levels of repetitive DNA content and mobile elements such as prophage insertions, insertion sequence (IS) elements, and transposons along with large numbers of gene duplications and genetic rearrangements (Wu, et al. 2004). Further, there is evidence for frequent activity and transmission of these transposable elements in the genome (Cordaux, et al. 2008).

Many of the differences in content between Wolbachia genomes are contributed by prophage WO, the genome of phage WO of Wolbachia, that has inserted itself into the bacterial chromosome and replicates along with core Wolbachia genes (Ishmael, et al. 2009; Kent and Bordenstein 2010; Bordenstein and Bordenstein 2016). This prophage is found in at least five different supergroups within non-mutualist symbioses (Kaur, et al. 2021). Prophage WO sometimes retains the potential to form phage particles (Masui, et al. 2001) and sometimes degrades over time into a relic prophage, losing the potential to form new viral particles due to loss of key virus structure genes, but with other genes remaining (Metcalf, et al. 2014; Bordenstein and Bordenstein 2016). Prophages and phages are highly mobile and dynamic elements in the genome, often picking up new genes via horizontal gene transfer. Phage WO can transfer itself between Wolbachia strains coinfecting the same host, and nearby genes or gene fragments may hitchhike in the process, making the phage a vehicle of gene movement and a creator of genetic novelty (Bordenstein and Wernegreen 2004; Kent, et al. 2011). Many unique prophage WO genes have the potential to confer important functions in interactions with the eukaryotic host (Bordenstein and Bordenstein 2016). Indeed, functional and evolutionary analyses of the genetic loci that underlie CI have shown that they are in fact prophage WO associated genes that interact directly with the eukaryotic host to manipulate reproductive
and developmental processes (Beckmann, et al. 2017; LePage, et al. 2017; Lindsey, et al. 2018; Beckmann, et al. 2019). Related to phage WO is a cassette of 8 genes known as Octomom, which are associated with a greater host protective benefit against RNA viruses such as *Drosophila* C virus and Flock House virus (Chrostek, et al. 2013). This cassette contains paralogs of phage WO genes but replicates separately, and variance in copy number has functional consequences for the bacteria and host. Specifically, in a lab-derived strain of *wMel*, copy number of *Octomom* is correlated with regulation of *Wolbachia* titer making strains pathogenic to the host (Chrostek and Teixeira 2015; Duarte, et al. 2021).

Generally, specific genes and regions of genomes can horizontally transfer to and from bacteria of the same strain allowing for a recombination-like process which may facilitate adaptation. Or, these regions can transfer to different strains which allows for the acquisition of new genes, allowing for adaptation to better propagate within their hosts (Lawrence 1999; Dutta and Pan 2002). Regarding *Wolbachia* specifically, studies have demonstrated that lateral transfer with eukaryotes has likely occurred many times with *Wolbachia*, as it is common for entire *Wolbachia* genes or domains within genes to have homology with eukaryotic DNA (Wu, et al. 2004; Bordenstein and Bordenstein 2016). Indeed, the transfers occur both ways, as many arthropod and nematode genomes also contain *Wolbachia* DNA (Kondo, et al. 2002; Hotopp, et al. 2007; Funkhouser-Jones, et al. 2015; Leclercq, et al. 2016; Wang, et al. 2016). Genetic transfers may be aided by the abundant mobile elements within the genome, including phage WO and transposons. Indeed, the high and variable number of transposable elements in *Wolbachia* genomes has long been recognized as a useful method for differentiating strains due to their frequent movement (Wu, et al. 2004; Duron, et al. 2005), and they may be behind the movement of specific genes (Cooper, et al. 2019) or disruption of others (Iturbe-Ormaetxe, et al. 2005). In addition, the first plasmid of *Wolbachia* was recently described, pWCP of several populations of *Culex pipiens* mosquitoes, which itself contains a transposable element (Reveillaud, et al. 2019). Thus, the growing number of described cases of genetic transfer correlate with the unusually high number of mobile genetic elements within *Wolbachia*, as well as its ability to switch hosts.

Despite the great diversity and interest in a variety of *Wolbachia* infections, most research attention has focused on CI, largely due to its use in vector control strategies (Zabalou, et al. 2004). These programs take advantage of the natural abilities of *Wolbachia* to both block viral transmission and spread itself via reproductive parasitism (Hedges, et al. 2008; Teixeira, et al. 2008; O'Neill, et al. 2018; Mains, et al. 2019; Ross, et al. 2019). Comparatively fewer analyses have been done on *Wolbachia* genomes of strains that induce male killing (Dyer and Jaenike 2004; Ishmael, et al. 2009; Duplouy, et al. 2013; Metcalf, et al. 2014). However, male killing merits additional analysis due to its potential in vector control (Berec, et al. 2016), role in shaping arthropod evolution (Jiggins, et al. 2000), and the close relationship between CI and male killing (Dyer, et al. 2005). Indeed, the CI genetic loci are located only a few genes away from the male-
killing candidate gene, \textit{wmk} (WO-mediated killing) in the Wolbachia strain of \textit{Drosophila melanogaster} (\textit{wMel}) (Perlmutter, et al. 2019). Also, many male-killing and CI strains are closely related (Sheeley and McAllister 2009), and several strains are multipotent in that they can switch between the two phenotypes either within the same host or between different hosts (Hurst, et al. 2000; Sasaki, et al. 2002; Jaenike 2007). Also, the strength of the phenotypes can be quite variable even within strains, suggesting there is a complex relationship between symbiont genotypes and host phenotypes that requires further study (Cooper, et al. 2017; Cooper, et al. 2019). The close genetic relationship between, and likely prophage origins of, the two phenotypes indicate that studies on male killing may inform CI and vice versa. In addition, their overall similarities may help narrow down evolutionary dynamics that are unique to each phenotype or shared between them.

Several studies have set the stage for male-killing genetics and genomics. Beyond Wolbachia, genomes of \textit{Arsenophonus nasoniae} and \textit{Spiroplasma poulsonii} have been sequenced. The \textit{Arsenophonus} strain was the first full genome of a male-killer and revealed that it has a relatively large genome for an insect symbiont, with evidence of gene transfer with Wolbachia and other bacteria (Darby, et al. 2010). The \textit{Spiroplasma} genome has reduced metabolic capabilities despite its extracellular lifecycle, and also contains several lethal RIP toxins and a male-killing gene on a plasmid (Paredes, et al. 2015; Harumoto and Lemaitre 2018; Garcia-Arraez, et al. 2019). The first sequenced genome of a \textit{Wolbachia} male-killer was that of the \textit{wBoI1b} strain of \textit{Hypolimnas bolina} butterflies (Duplouy, et al. 2013). Among sequenced genomes at the time, it was most like \textit{wPip} of \textit{C. pipiens} and contains type IV secretion system genes, many ankyrin repeat domains, and several prophage regions. In addition, \textit{wBoI1b} has many genes with homology to non-Wolbachia bacteria, and a few genes homologous to genes in mosquitoes, suggesting gene transfer has occurred between this strain and both other prokaryotes and eukaryotes (Duplouy, et al. 2013). The genome of the \textit{wRec} strain that causes CI in its native host, \textit{D. recens}, and male killing in sister species \textit{D. subquinaria} shows degradation in the prophage region (relic phage), with only a few dozen phage WO genes remaining and loss of essential phage particle genes, and no genes unique to the strain (Jaenike 2007; Metcalf, et al. 2014).

Among the few known Wolbachia male-killers of flies is the strain infecting \textit{Drosophila innubila} mushroom-feeders, \textit{wInn} (Dyer and Jaenike 2004). This strain is particularly interesting as it is closely related to Wolbachia found in the main \textit{Drosophila} model species, \textit{wMel} of \textit{D. melanogaster}, which causes CI (Sheeley and McAllister 2009). In addition, the symbiosis between \textit{wInn} and its host has been maintained for thousands of years, and despite this, there is no evidence of host resistance to male killing in modern populations (Jaenike and Dyer 2008; Unckless and Jaenike 2011). A previous microarray analysis compared the content of the \textit{wInn} genome to that of several other strains, including several CI strains and non-parasitic strains. The findings were that phage and ankyrin repeat genes were amongst the most
divergent in the genome, that the ankyrins were particularly variable within wInn, and that the strain had several unique but uncharacterized hypothetical proteins (Ishmael, et al. 2009). Thus, several previous studies examined genomic content of male-killers, but little is known about their genomics on a population level. Due to the close relationship between wMel and wInn, the longstanding symbiosis of wInn with its host, and building on previous findings suggesting unique wInn genome content, we chose to conduct population genomics analysis on this strain. We aimed to identify both new genetic content and population genomic trends that may be important for Wolbachia or male-killers more specifically.

Here, we sequence the genome of the Wolbachia strain infecting D. innubila, wInn, and conduct population genomic analyses using sequences from 48 Wolbachia-infected individual wild females from four populations in Arizona. We compare the genome content to that of similar supergroup A Wolbachia of Drosophila hosts to reduce variables when narrowing down unique genomic regions or population genetic trends of this male-killer. We demonstrate overall similarity of the genome content with wMel, as previously shown, and newly identify several dozen unique genes and repetitive elements implying lateral gene transfer with divergent hosts. We determine that genes from prophage and Octomom regions show more evidence of positive selection than background genes, consistent with other strains and Wolbachia’s general ability to adapt to diverse hosts. Finally, we examine population structure and co-inheritance of Wolbachia with mitochondria to show that wInn largely exhibits patterns of strict vertical inheritance within the population and mobile elements are likely responsible for any discordant phylogenies. Variance in sequencing coverage also putatively suggest that Octomom copy number may differ considerably across individuals and that prophage WO may form active particles in this strain.

**Results**

wInn genome assembly reveals a genome similar to wMel and evidence of lateral gene transfer from multiple host genera

D. innubila is a mycophagous species in the Drosophila subgenus, found throughout the southwestern USA and northwestern Mexico on mountain-top forests known as ‘Sky Islands’, separated by large expanses of desert (Jaenike, et al. 2003; Dyer and Jaenike 2005; Dyer, et al. 2005; Jaenike and Dyer 2008). Here, we examined the genome and population genomic variation of wInn. In a previous survey we collected wild D. innubila from four isolated mountain locations and tested strains for Wolbachia using PCR to amplify the wsp locus and found 48 females infected with Wolbachia (Supplementary Table 1, 13 from the Chiricahua mountains, 27 from Prescott, 2 from the Huachucas, and 6 from the Santa Ritas) (Hill and Unckless 2020b, a).

We sequenced and assembled the genome using a combination of short and long reads for one strain. The wInn genome is a single circular chromosome 1,290,587 base pairs long, with 35.1% GC content.
(Figure 1A). We found 1341 genes, 1301 found previously in other Wolbachia: 1232 genes are shared with wMel, 1145 shared with wRec, and 1010 shared with wRi. The wInn genome had a BUSCO score of 81.9% (181 complete single copy orthologs and 2 fragmented orthologs, from a total of 221) compared to 81.3% in wMel (180 complete single copy orthologs and 2 fragmented orthologs, from a total of 221). Of the 1301 previously identified genes, 924 are conserved across all four genomes (Figure 1C, Supplementary Table 2), including 12 prophage WO-A and 54 prophage WO-B genes in all genomes (Supplementary Table 3), and 9 Octomom genes (5 orthologs to wMel and 4 paralogs of these, genes linked to Wolbachia pathogenicity) (Chrostek and Teixeira 2015). Interestingly, these Octomom genes are not found in a single cassette like in wMel but are instead spread throughout the genome (Figure 1A). The genes orthologous to WO-B genes of wMel are found in 3 groups (Figure 1A, called WOInn-B1, WOInn-B2 and WOInn-B3). Despite the fragmentation of the prophage regions, the genes are syntenic to the prophage WO-B region in wMel. We also found 10 type IV secretion system genes, found in two cassettes, as in wMel (Figure 1A). Consistent with previous results, wInn is closely related to wMel within supergroup A, clustering with other supergroup A Wolbachia genomes (Figure 1B, Supplementary Figure 1).

Three genes are shared between the male-killing wInn genome and wRec, but absent in the wMel and wRi genomes, both strains that induce CI in their native hosts (Figure 1C). Recall that wRec reportedly kills males when introgressed into a sister species, but causes CI in its native host (Jaenike 2007). All three genes are hypothetical proteins found in other Wolbachia supergroup A genomes that do not cause male killing in their native hosts (including other varieties of wMel). In addition, wInn does not appear to have a reduced, relic prophage genome like wRec, and instead shares most prophage genes with wMel despite being more diverged from wMel than wRec (Figure 1C). The 57 genes absent in wRec but present in wInn and wMel consists of 21 prophage genes, 4 transcription genes, 9 metabolism genes, and 21 genes of unknown function. We attempted to further confirm the differences in genomic content by mapping short reads from wRec, wMel, wInn and wHa to each of the genomes pairwise and find the exact same number of genes shared in each case, supporting the assembled genomes are not missing any shared genes. The wRec genome appears to be missing portions of the regions orthologous to 0.35-0.55Mb and 1.24-1.28Mb in the wInn genome, which also includes a large portion of the prophage WO-B genome.

Of the 40 wInn coding sequences not found in other Wolbachia, 23 of these have high similarity to genomic mRNA in Formica wood ants that may have an overlapping range with D. innubila (non-redundant megablast e-value < 0.00005) (Francoeur 1973; Altschul, et al. 1990). These 40 sequences largely contain predicted domains that are typically found in Wolbachia genomes, such as ankyrin repeats, PD-(D/E)XK nuclease/transposases, membrane transporters, and genes involved in amino acid and nucleotide metabolism (Bordenstein and Bordenstein 2016; Lindsey, et al. 2018; Massey and Newton 2021). Similarly, the D. innubila host also contains multiple transposable element (TE) sequences shared with Camponotus
(a genus within *Formica*) (Hill, et al. 2019). Further, we find 5 unique wInn genes have a high similarity to *Varroa destructor* mite transcriptome sequences (non-redundant megablast e-value < 0.00005, Supplementary Table 3). The 12 remaining sequences have no known orthologs. Among the 157 genes absent in wInn but present in wMel, we found no functional categories enriched (*p*-value > 0.12).

Like other *Wolbachia* (Foster, et al. 2005; Woolfit, et al. 2013) a large proportion of the wInn genome is repetitive: 12.38% of the genome consists of repetitive content (Figure 1A). Most of these sequences are short simple repeats, satellites, and insertions from 16 bacterial insertion sequences (selfish elements found in bacteria). 1.49% of the genome consists of insertions of a single hAT family element (*hobo*-like DNA transposon found in *Drosophila*, rnd-1_family-6) inserted in 14 loci across the genome and 3.74% consists of 3 LINE elements inserted in 37 loci across the genome (long interspersed nuclear elements, an RNA transposon order found in *Drosophila*), primarily in clusters (Figure 1A) (Wicker, et al. 2007). Consistent with several of the above-described unique wInn genes, one LINE element (rnd-1_family-12 with 14 insertions) is homologous to a LINE found previously in *Varroa destructor* (or a close relative), while another (rnd-1_family-165 with 12 insertions) is homologous to a LINE found in *Formica* wood ants (non-redundant megablast e-value < 0.00005) (Altschul, et al. 1990). No homologous sequence can be identified for the hAT element (non-redundant megablast e-value = 1 yielded no hits), which is also the only TE found with a complete sequence, as opposed to most of the LINE element insertions that are degraded (Supplementary Figure 2).

**Figure 1.** A. Schematic of the wInn genome. Circles correspond to the following: 1) GC content of the wInn genome in 10-kbp windows, between 30% and 40%. Darker colors have higher GC content. 2) Locations of genes thought to interact with hosts, specifically prophage orthologous to WO-A and WO-B genes in wMel (blue), Type IV secretion pumps (green), and Octomom genes (yellow). 3) Loci of non-phage genes. 4) Loci of repetitive content, with short simple repeats and interspersed satellites (SSR and IS, black), and hAT or LINE TE insertions (red). B. Phylogeny of *Wolbachia* genomes closely related to wInn for reference, as a subset of Supplementary Figure 1. Bootstrap support of each branch is shown on the nodes (of 100 bootstraps). A description of each *Wolbachia* genome and the species they infect is given in Supplementary Table 1. C. The overlap of genes between wInn, wMel, and wRi. D. Synteny between wMel and wInn, with single large inversion shown in blue, while consistent synteny groups are shown in grey.
Octomom and prophage genes are under positive selection in wInn and other Wolbachia

We next used codeML to determine genes with signatures of selection in wInn compared to the closely related Wolbachia genomes (Yang 2007). For each ortholog set, we identified the proportion of synonymous (dS) substitutions and amino acid changes to nonsynonymous substitutions (dN) (per possible synonymous or nonsynonymous substitution, respectively). We did this for substitutions occurring on each branch of the phylogeny to identify changes between the gene sequence of wInn, wHa, and wRi. We expect dN/dS to be higher when genes are under positive selection, due to more nonsynonymous fixations (Yang 2007). We chose wHa (wInn-wHa dS ~ 0.068) and wRi (wInn-wRi dS ~ 0.386) over wMel or other genomes as these genomes are diverged enough from wInn to provide some signal in synonymous divergence (unlike wMel or wRec, where dS ~ 0.001 between genomes), while not being diverged enough to have too little similarity or saturated rates of dS. Octomom and prophage genes show values consistent with positive selection on all branches (Figure 2, GLM t-value = 2.750, p-value = 0.0061). The genes with the next closest...
values include DNA metabolism genes, which showed no significant difference in rates of positive selection on the wInn branch compared to background (Figure 2, GLM t-value = 1.868, p-value = 0.0622). The DNA metabolism genes with the highest dN/dS values in wInn are the non-phage genes WD1095 (radC, a DNA repair protein), WD0065 (a DNA binding protein), WD0057 (a host integration factor) and WD0752 (xerC, a recombinase). Next, we looked at dN or dS alone to determine how selection individually differed among categories to ask if signals were driven by an increase in dN alone, or with an accompanying change in dS, which would tell us about overall genome evolution rates in wInn compared to other strains. Elevated dN alone would suggest positive selection only, while elevated dN and dS would indicate not only selection, but also rapid evolution of DNA more generally. Consistent with the elevated divergence in Octomom and phage genes being driven by positive selection, we find these two categories have elevated dN across the total phylogeny compared to genes in other categories (Octomom GLM t-value = 4.1, p-value = 6.61e-5, phage GLM t-value = 3.735, p-value = 1.1e-4), while no other categories have elevated dN compared to the background (GLM p-value > 0.05). We do not find significantly elevated dS in Octomom or phage genes on across the other branches (Octomom GLM t-value = -0.518, p-value = 0.6045, phage GLM t-value = -0.643, p-value = 0.5209). Interestingly, we find both elevated dN (Octomom GLM t-value = 3.699, p-value = 0.00028, phage GLM t-value = 3.926, p-value = 9.25e-5) and elevated dS (Octomom GLM t-value = 5.053, p-value = 5.2e-7, phage GLM t-value = 7.216, p-value = 1.1e-12) on the wInn branch in Octomom and phage genes.

Several genes have been implicated in reproductive parasitism in Wolbachia in Drosophila, so we specifically examined the evolution of these genes in wInn and the other genomes (wmk: WD0626, cifA: WD0631, cifB: WD0632). These genes show no difference from background rates of positive selection in wInn (dN/dS = 0.27-1.56 in wInn, versus dN/dS background median = 0.225, GLM t-value = 0.224, p-value = 0.642). The wmk male-killing candidate showed no significant difference on the wHa and wRi branches compared to wInn (dN/dS = 1.56, versus dN/dS background median = 0.225). Similarly, cifA & cifB (genes involved with cytoplasmic incompatibility) (Beckmann, et al. 2017; LePage, et al. 2017), had higher values (though not significantly different) in wInn than the other two strains (Figure 2, WD0631 & WD0632, dN/dS = 1.10 & 0.27 respectively, p-value = 0.845). We also examined selection of specific codons in these genes but find no specific sites that are driving positive selection in these genes (p-value > 0.05). The Type IV secretion genes also do not show significantly different rates of positive selection than the background across the total phylogeny (GLM t-value = 1.427, p-value = 0.154).

**Figure 2.** Selection on genes of the wInn branch versus evolution on the wRi/wHa branches. Functional categories of interest (DNA metabolism genes, prophage genes and Octomom genes) are highlighted by different shapes and colors. Dashed lines show dN/dS = 1 for both axes, while the dotted line shows where dN/dS is equal on the axes. Genes of interest, either due to putative involvement in Wolbachia...
pathogenicity, or due to high dN/dS in wInn exclusively are named and labeled with a black outline to distinguish them.

Some prophage genes show evidence of recent horizontal transfer in wInn and across the Wolbachia phylogeny

We suspected that many symbiont genes potentially involved in unique wInn host-microbe interactions may be more likely to experience horizontal transfer between Wolbachia strains than other genes because of their association with the prophage. We looked for evidence of horizontal gene transfer since the divergence of wInn from wHa and wRi. We used VHICA (Wallau, et al. 2016) to compare synonymous divergence (dS) for the pairwise comparisons of wInn-wHa, wInn-wRi, and wRi-wHa for genes with orthologs in all three species. We used dS as a measure of the neutral mutation rate, as we expect positive selection on synonymous mutations rarely leads to their fixation. If a gene has horizontally transferred into wInn from another Wolbachia (but not wHa or wRi), we expect the dS to be higher in these comparisons than expected based on the distribution of dS across the rest of the genome (using the mean dS plus the variance in dS as the cut off for elevated dS) (Wallau, et al. 2016). When a gene has transferred into the wInn genome, we expect there will be elevated dS in both comparisons involving wInn, but not the wHa-wRi comparison, as it would be significantly higher compared to these close relatives if it came from a more distantly related source. Overall, 13 genes have elevated dS in just the wInn comparisons (Figure 3, Supplementary Table 4). These genes are enriched for prophage WO-A & WO-B genes ($\chi^2$ test, $\chi^2 = 60.476$, df = 1, $p$-value = $7.448e-15$), and Octomom genes ($\chi^2$ test, $\chi^2 = 181.64$, df = 1, $p$-value = $2.2e-16$) that have...
elevated divergence in w_Inn. The putatively horizontally transferred genes do not significantly overlap with genes under positive selection in the w_Inn genome ($\chi^2 = 0.009$, df = 1, $p$-value = 0.9212), though genes under positive selection across the total phylogeny do significantly overlap with genes with elevated dS in all comparisons ($\chi^2 = 9.8389$, df = 1, $p$-value = 0.001709). Domain analysis of the 13 genes demonstrates that most have weakly predicted domain functions, and those that do include predicted annotations such as radC DNA repair, patatin phospholipase, and burrH-like transcription factors with ankyrin repeats, all of which are typically found within phage WO (Bordenstein and Bordenstein 2016). The 6 putatively horizontally transferred genes not associated with the prophage or Octomom regions are all genes of unknown function. Horizontal transfer of the Wolbachia organism is unlikely to play a role in the elevated divergence seen here due to the lack of a significant difference in dS for the w_Inn-w_Ri and w_Ha-w_Ri comparisons (Wilcoxon Rank Sum Test $W = 462970$, $p$-value = 0.7687).

**Figure 3.** Comparison of dS between pairs of Wolbachia suggesting horizontal transfer of genes. Point colors show the gene ontology categories (GO category) for Octomom genes and Prophage WO-B genes. Point shape indicates evidence of excessive divergence (and possible horizontal transfer) in either w_Inn, w_Ha, or both. Dashed lines show the mean + the variance of dS for each axis as a rough cutoff for elevated synonymous divergence.

To examine if these gene categories frequently experience horizontal transfer, or if these transfers are unique to w_Inn, we downloaded 54 Wolbachia genomes (all genomes available for download on NCBI genomes, described in Supplementary Table 1) and made gene alignments for all orthologs and attempted to identify gene tree/species tree discordance. We assumed that excessive gene tree/species tree discordance
would be due to large amounts of horizontal gene transfer. We attempted to find functional categories which show more tree discordance than expected and across 847 orthologous genes, and found excessive amounts of discordance for prophage WO genes (Table 1, 36 of 47 genes, degrees of freedom = 1, $\chi^2 = 111.1$, $p$-value = 5.62e-26) and Octomom genes (Table 1, 7 of 7 genes, degrees of freedom = 1, $\chi^2 = 71.27$, $p$-value = 3.395e-17) across large evolutionary distances, while no other categories have significantly more discordance than expected. We find a significant overlap in the genes which have horizontally transferred in wInn and across the whole phylogeny, when put into a 2x2 contingency table (discordant vs non-discordant in VHICA &/or Ancient HT. $\chi^2 = 49.003$, $p$-value = 2.556e-12).

**Table 1:** Summary of species tree/gene tree discordance analysis. Using 847 orthologous genes across 54 genomes (Supplementary Table 5), we identified genes which showed significant discordance from the species tree. We calculated the expected number of discordant genes in each category based on the total number of discordant genes and the proportion of total genes in this category. We then used this value and the observed number of discordant genes in each category to perform a $\chi^2$ test. The table shows the number of genes in each category showing discordance, and if this discordance is significant using a $\chi^2$ test, using an expected number of discordant genes per category based on the size of the category. Note that these $p$-values are not corrected for multiple tests.

| Functional category       | Discordant genes | Genes in category | $\chi^2$   | $p$-value  |
|---------------------------|------------------|-------------------|------------|------------|
| Biosynthesis              | 1                | 50                | 2.374      | 0.876      |
| Cell envelope             | 1                | 31                | 0.952      | 0.671      |
| Cellular processes        | 1                | 35                | 1.238      | 0.732      |
| DNA metabolism            | 1                | 42                | 1.759      | 0.815      |
| Energy metabolism         | 0                | 90                | 7.438      | 0.006      |
| Octomom                   | 7                | 7                 | 71.278     | 3.395e-17  |
| Phage WO-A                | 12               | 13                | 111.105    | 5.626e-26  |
| Phage WO-B                | 24               | 33                | 165.927    | 5.817e-38  |
| Protein synthesis         | 1                | 144               | 9.985      | 0.002      |
| Regulatory functions      | 0                | 11                | 0.909      | 0.340      |
| Transcription             | 0                | 52                | 4.298      | 0.038      |
| Transport and binding     | 0                | 44                | 3.636      | 0.057      |

proteins

Type IV                    | 0                | 10                | 0.826      | 0.363      |

Unknown function           | 22               | 285               | 0.102      | 0.749      |
wInn is highly structured between locations, and is not always co-inherited with the mitochondria

We next mapped the short-read data for 48 samples of wild D. innubila infected with wInn, collected from four locations in Arizona (Figure 4), to the repeat masked wInn genome and called polymorphism. From this, we identified 30 SNPs as coding synonymous, 69 SNPs as coding non-synonymous, and 235 SNPs as non-coding across all individuals. The wInn samples are highly structured based on both the total and synonymous variation (Figure 4). Using a principal component analysis, we find three clear clusters, separating the Chiricahua and Prescott populations, and grouping the Santa Rita and the Huachuca populations together (Figure 4), as seen with the mitochondrial genome and consistent with previous findings (Jaenike, et al. 2003; Dyer and Jaenike 2005; Jaenike and Dyer 2008; Hill and Unckless 2020a).

**Figure 4:** Principal component analysis of genetic variation in wInn A. Map of locations samples were taken from in this survey, adapted from (Hill and Unckless 2020a). Phoenix and Tucson are shown as points of reference. B. total polymorphism and C. silent polymorphism in wInn samples, colored and shaped by location of collection, Chiricahuas (CH, red circles), Huachucas (HU, orange squares), Prescott (PR, blue triangles) and Santa Ritas (SR, green diamonds).

However, when building a maximum-likelihood tree of the wInn samples using all polymorphisms in the core *Wolbachia* genes, we find some evidence of potential migration between populations (Supplementary Figure 3A). Specifically, we find two samples from PR cluster within CH and share the CH mitochondrial haplotype (Supplementary Figure 3). These two PR samples are also closer to CH than other PR samples in the principal component analysis (Figure 4B, C). This signature is not seen in the host, likely due to the recent establishment of *D. innubila* (Hill and Unckless 2020a), particularly in Prescott.

To identify if specific genomic regions are contributing to the population structure, we calculated the fixation index ($F_{ST}$), a measure of pairwise divergence between a subpopulation and the total population, between the three clustered groups. We expect $F_{ST}$ to be elevated in cases where SNPs are found at high
frequencies in a single population but not the remaining samples. As expected with the non-recombining bacterial genome, we found signatures of $F_{ST}$ are uniform genome wide, with no specific windows of elevated $F_{ST}$ compared to the rest of the genome (1kbp windows, GLM $p$-value > 0.432) and no functional categories are enriched for high or low $F_{ST}$ (Supplementary Table 6, GLM $p$-value > 0.611).

When comparing the co-inheritance of the maternally transmitted $w$Inn and $D. innubila$ mitochondria, we found little evidence of discordant inheritance, consistent with a previous study (Dyer and Jaenike 2005). We do however find evidence of 49 total SNPs spread across four clusters in the $w$Inn genome which show evidence of recombination-like events due to the presence of four allele combinations between the Wolbachia site and the mitochondria site across individuals (Supplementary Figure 4). In other words, if a mitochondrial site is polymorphic for A/C and a Wolbachia site is polymorphic for G/A, we find all pairwise combinations in the population (AA, AG, CA, CG). The two larger clusters of SNPs are contained within the mobile prophage WO-A and WO-B portions of the genome.

We also find the sequence coverage of the prophage regions differs between $w$Inn lines: the prophage region has significantly higher sequence coverage in 8 of 48 lines, varying from 1-4x the average coverage of the Wolbachia genome (Wilcoxon Rank Sum W > 443221, $p$-value < 0.04, Figure 5). In addition, phage copy number is negatively correlated with Wolbachia titer (Figure 5, Spearman’s $\rho$ = -0.584 t-value = -7.831, $p$-value = 2.35e-10). Indeed, higher phage coverage correlates with lower Wolbachia titer up to a point, consistent with potentially active phage lysing symbiont cells.

**Figure 5:** Prophage abundance genes (prophage coverage/total Wolbachia coverage) compared to the Wolbachia titer (total Wolbachia coverage/host autosomal coverage) and Octomom copies compared to Wolbachia titer (total Wolbachia coverage/host autosomal coverage). A LOESS smoothed regression was added to the data for each plot to assist in visualizing the change in gene copy number with titer, showing the fitted curve of the average Octomom copy per 2-fold Wolbachia titer.
Given the signals of selection on Octomom genes found here, and the previously identified association between increased pathogenicity and Octomom copy number in lab strains (Chrostek and Teixeira 2015), we also examined the correlation between Octomom sequencing coverage and Wolbachia titer within lines. We found all strains contain Octomom genes, some of which are in a cassette, and some not. The sequence coverage of the Octomom genes appears to also differ between lines (between 0X-16X coverage, Figure 5). We note that while Wolbachia titer is not significantly different as Octomom coverage changes (a proxy for copy number, Figure 5, Spearman’s $\rho = 0.115$, GLM t-value = 1.930, $p$-value = 0.0597), it is difficult to infer copy number when sequencing coverage is low (as it is in lower Wolbachia titer individuals) (Hill and Unckless 2020a). The lack of resolution precludes any conclusions on their relationship, as it is possible to falsely infer Octomom copy number in some samples. Variance in Octomom coverage does not significantly relate to coverage (GLM t-value = -0.311, $p$-value = 0.0637). Conservatively, we cannot confirm a relationship between Octomom copy number and Wolbachia titer in this dataset, and any potential relationships will need to be confirmed in the future. The differences in titer are not significantly associated with any SNPs or gene duplications outside of the Octomom or prophage WO regions ($p$-value > 0.186).

**Discussion**

We sequenced and assembled the genome of the Wolbachia strain infecting Drosophila innubila, wInn. wInn is one of the few strains of Wolbachia to cause male killing in Drosophila (Jaenike, et al. 2003) and so we sought to examine its population genome dynamics, with particular focus on prophage and Octomom regions that have genes putatively or empirically involved in Wolbachia pathogenicity (Metcalf, et al. 2014; Chrostek and Teixeira 2015; Bordenstein and Bordenstein 2016; Beckmann, et al. 2017; LePage, et al. 2017; Perlmutter, et al. 2019). The genome content and dynamics of wInn are largely like
other closely related strains (Figure 1), sharing most of its genome with similar supergroup A *Wolbachia* strains, as previously reported (Ishmael, et al. 2009). When comparing the genomes of *w*Inn and *w*Rec (capable of male killing) to the other closely related strains in this analysis (all CI-inducing), we identify only 3 unique genes. However, these genes are found in other *Wolbachia* strains not used in our initial analyses, based on the non-redundant BLAST database (Altschul, et al. 1990; Pruitt, et al. 2005), suggesting that these genes are more broadly found in non-male killing *Wolbachia* and were likely lost in the *w*Mel and *w*Ri strains used here. The lack of unique male-killer genes is consistent with the idea that male killing is often hidden. Indeed, many strains like *w*Rec do not cause male killing in their native hosts but do kill males when transferred to other hosts or vice versa (Fujii, et al. 2001; Sasaki, et al. 2002; Jaenike 2007; Hughes and Rasgon 2014). In addition, there is evidence of host resistance that suppresses the phenotype in many other systems, where the arms race between host and bacteria leads to loss of phenotype (Hornett, et al. 2006; Jaenike 2007; Majerus and Majerus 2010). These factors indicate that absence of phenotype does not necessarily correlate with absence of symbiont genotype, and male killing is instead also heavily dependent on host background (i.e., male killing is not a simple matter of presence/absence of a male-killing toxin gene). The fact that the *wmk* male-killing gene candidate is found in many non-male-killer genomes also supports the idea that male-killers do not necessarily have unique genetic content involved in the phenotype and a combination of host- and microbe-dependent factors are necessary for the phenotype to occur (Perlmuter, et al. 2019).

We also find that while the overall genetic content of *w*Inn is like *w*Mel, it has key differences compared to the more similar *w*Rec strain. The *w*Inn and *w*Mel strains share 57 total genes (of which 21 were prophage genes) that are unique among those strains analyzed (including *w*Rec). These differences are largely due to the loss of phage regions in *w*Rec (Metcalf, et al. 2014). It is intriguing to note that *w*Rec contains relic phage regions that have lost many genes compared to those in *w*Mel and *w*Inn, and is likely unable to produce viral particles because of the absence of key virus structural genes (Metcalf, et al. 2014). Meanwhile, *w*Inn and *w*Mel have not (Figure 1). Both *w*Inn and *w*Rec are closely related, both are capable of male killing (Jaenike, et al. 2003; Metcalf, et al. 2014), and both infect mycophagous species, yet one has an eroded prophage region while the other does not, which may potentially be due to differences in phenotypes in their native hosts (CI vs male killing, Figure 1). In addition, evidence here based on higher sequence coverage and the inverse relationship between phage titer and *Wolbachia* titer, further suggests that there may cautiously be active phage WO particle production in *w*Inn (Supplementary Figure 5). This remains to be confirmed via methods such as phage purification or EM imaging. If accurate, the variation in prophage coverage across samples may indicate that putative active phage particles are more abundant or in a different part of their replication cycle in each sample (the samples were not controlled for age or other factors that may affect the viral titer), or it could indicate variance in the number of phage insertions.
across the genomes of each sample. Similarly, the variation in Octomom sequence coverage across samples may also indicate different copy numbers across lines. It is unclear why wInn and wMel would putatively maintain viable phage particle production while wRec would not. Future research will be needed to determine any functional consequences of phage particles that may be playing a role in their retention or loss across different systems, and what role they may play in parasitic phenotypes.

We also identified 40 genes unique to the wInn genome, and most intriguingly, 28 of these are homologous to Formica wood ant genes, an additional 5 share homology with Varroa destructor mites, and there are several TEs with homology to those in Camponotus ants. Along with evidence of horizontal gene transfer and rapid evolution, this homology with divergent hosts would suggest some possibilities for the genetic transfer routes in this system. Indeed, mites are known to transfer Wolbachia infections among Drosophila populations (Brown and Lloyd 2015), and Varroa destructor and its relatives are common parasitic species found throughout the United States and the rest of the world (Rosenkranz, et al. 2010). Therefore, it is possible that this or a similar species has vectored either the entire Wolbachia symbiont or some genes among various arthropods, contributing to horizontal gene transfer in this strain. Formica wood ants and Camponotus ants are also common across the United States (Bolton, et al. 2006), indicating that there is a possibility of interaction with the mites and/or D. innubila, although the transfers may have occurred before this Wolbachia strain established itself in its current host. The homology of wInn genes with ant genes may indicate that there has been an exchange of genes or symbionts among these hosts, possibly via mites, and the ants and mites in the area may contain or have contained similar strains. In one possible model, the mites would vector either genes or symbionts among interacting hosts (Houck, et al. 1993; Brown and Lloyd 2015), and in other models, the wInn strain may have originated in these hosts. The potentially active phage particles could also play a role by primarily or excessively moving the prophage region among hosts, which would likely be much easier and more common than symbiont exchange. Given that the coverage of the phage region differs between samples, it would suggest the possibility of phage actively forming and lysing bacterial cells.

Horizontal transfer of genes between Wolbachia strains and hosts is in line with existing literature demonstrating Wolbachia’s proclivity for genetic exchange. Indeed, the horizontal transfer of individual genes or the entire phage region among Wolbachia strains that is supported here reflects previously reported cases (Wang, et al. 2016; Cooper, et al. 2019). The presence of 13 genes with elevated dS in wInn comparisons suggests horizontal acquisition from another Wolbachia, as they are divergent from recent ancestors but found in other strains. However, the genes that appear to have been horizontally acquired, are like other genes in various Wolbachia strains. The enrichment for specific phage WO genes identified here suggests they horizontally transfer between Wolbachia genomes more often than the rest of the genome, possibly due to the activity of putative phage particles (Table 1, Figure 3), or the activity of surrounding
mobile elements, as may have happened in the *D. yakuba* clade with horizontal transfer of the CI loci and nearby transposons (Cooper, et al. 2019).

Further, entire symbiont transfer may also potentially occur in this system, although it is rare if it does occur. Vertically transmitted symbionts, such as *Wolbachia*, are subject to strong selection within the host, and unlike frequently horizontally transferring microorganisms, can experience various degrees of genome reduction and other genetic adaptations that lead to essential ties with a specific host (Moran 2002; Dyer and Jaenike 2005; Jaenike and Dyer 2008; McCutcheon and Moran 2012). However, bypassing this phenomenon, there are *Wolbachia* strains that can transfer to new hosts via various modes of transmission and then sweep rapidly across a new and sometimes divergent host population (Riegler, et al. 2005; Baldo, et al. 2008; Turelli, et al. 2018; Sanaei, et al. 2020). Whole symbiont transmission could be aided by frequent horizontal transfer of genes or entire regions, such as the prophage, just as we report here in wInn.

More broadly, acquisition of new genetic variants that the eukaryotic host is unfamiliar with may confer an advantage that could allow the *Wolbachia* to be transferred to a new host or maintained in an existing host. Indeed, some known cases of horizontal phage WO gene transfer among symbionts have functional effects on the symbiont’s ability to parasitize the host (Wang, et al. 2016; Cooper, et al. 2019). Most crucially, horizontal gene transfer in *Wolbachia* is not restricted to exchange among phages or bacteria, but also with the eukaryotic host. Phage WO stands unique among other described phages with its regions of large genes containing eukaryotic-like domains that imply both lateral transfer between animal and phage WO genomes and potential interaction with the eukaryotic host (Bordenstein and Bordenstein 2016). Thus, the acquisition of genes homologous to those in ants and mites in wInn reflects *Wolbachia*’s unique genetic exchanges more broadly as well as its tripartite interactions among phages, bacteria, and animals. Elucidating the function and fitness impacts of these genes, if any, will be an interesting area of future research. Also, if there is indeed frequent horizontal exchange in this system, this may then be a driving force for maintenance of phage particles, as they may confer selective advantages over time. Further supporting the idea of recurrent genetic exchanges in the wInn ecosystem is evidence of both a complete hAT element, suggesting recent transmission of this element, and more degraded LINE elements homologous to those in *Formica* and *Varroa*, suggesting more ancient acquisition.

We find repeat content in the wInn genome that is similar other *Wolbachia* (Figure 1A, Supplementary Table 2, (Wu, et al. 2004; Ishmael, et al. 2009)). This contrasts with other obligate intracellular parasites that have relatively small, repeat free genomes (Woolfit, et al. 2013). Most of the elements found in the wInn genome are also shared with the host, *D. innubila* (Hill, et al. 2019), suggesting that inefficient selection has not allowed these TEs to be maintained for extensive periods of time, but instead these elements are recent acquisitions (Yoshiyama, et al. 2001). It is possible that eventually these TEs will be shed from the wInn genome, as similar repeat families have been acquired in the past and
eventually gone extinct and removed from the genome, in a cyclical fashion (Maruyama and Hartl 1991; Lohe, et al. 1995). Previous work in wMelPop suggests that reduced selection allowed repeats to accumulate in the Wolbachia genomes (Woolfit, et al. 2013). This could also be the case for wInn, allowing these families to be maintained in the genome for extended periods of time, as opposed to removed immediately. The lack of similarity between wInn and wMelPop repeats suggests that mobile elements have not been maintained since the common ancestor of these two Wolbachia strains, and that turnover is much more frequent.

Beyond genome content, we analyzed the population genomics of the four populations of wInn. The overall finding, similar to previous findings on phage biology and the wInn genome (Ishmael, et al. 2009; Duplouy, et al. 2013), is that some prophage and Octomom genes appear to be under positive selection in all Wolbachia branches analyzed, while others are not. Additionally, no functional categories were significantly enriched for positive selection, and those closest to the cutoff were involved in DNA repair, DNA binding, host integration, and recombination (Supplementary Table 3). Looking more specifically at the cifA/B CI genes and the wmk male-killing gene candidate, we find that they are not more rapidly evolving than the rest of the prophage region (Figure 2).

Regarding the Octomom regions, we find that these genes are under positive selection across all the Wolbachia genomes in the clade examined, as expected, not just in the male-killing Wolbachia (Figure 2). The selection on these genes may indicate they are frequently involved in host-pathogen interactions as suggested elsewhere (Chrostek and Teixeira 2015), and as is seen with immune genes and other genes involved in interspecies arms races (Dawkins and Krebs 1979; Sackton, et al. 2007; Obbard, et al. 2009; Palmer, et al. 2018). Indeed, previous studies have found an association between Octomom copy number and increased titer (Chrostek and Teixeira 2015), as we see here (Supplementary Figure 5). Additionally, while prophage may be able to excise themselves for transfer, Octomom may utilize transposable elements for horizontal transfer (Chrostek and Teixeira 2015). In line with this, the Octomom genes are fragmented across the genome rather than remaining in full cassettes, and the horizontal transfer of transposable elements is more and more frequently being found to occur between closely species with overlapping ranges (Peccoud, et al. 2017; Hill and Betancourt 2018; Wallau, et al. 2018). When looking for evidence of recent horizontal transfer to wInn more generally (after divergence from wHa and wRi), 13 genes were identified as potentially horizontally transferred to wInn. Of these, 7 were prophage or Octomom genes (Figure 3), supporting all findings so far indicating particularly frequent transfer of prophage and Octomom genes.

Finally, we tested if the samples grouped geographically and compared the inheritance of mitochondria and wInn in D. innubila populations (Figure 4) (Hill and Unckless 2020b, a). Indeed, geographically, there is structure to the samples, which mostly group by capture location. This would suggest that there little to no movement from population to population, or that at least individuals do not
reproduce after migrating. Low F\textsubscript{ST} levels across the genome indicate that the structure found among the population groupings is not driven by any region. However, we also find some phylogenetic discordance in inheritance in the \textit{Wolbachia} prophage region compared to host mitochondria, which could be due to imperfect co-inheritance, recurrent mutation, or a horizontal transfer event. Because the discordance is primarily associated with the prophage regions, the pattern is most likely due to horizontal prophage movement, rather than truly incongruous co-inheritance of symbiont and mitochondria in the population (Figure 5). This suggests that the prophages are likely transferring between symbiont genomes within their \textit{D. innubila} populations, causing recombination-like signatures and resulting in the particularly high levels of horizontal transfer of the prophage region seen here (Figure 4, Table 1, Supplementary Figure 4). Indeed, if there is active phage as suggested by the high variance in prophage coverage, the active particles would likely aid in this process of transfer between individuals in the population. When examining the phylogeny of w\textsubscript{Inn} genomes, we find some Prescott lines are grouped within Chiricahua lines (Supplementary Figure 3), potentially driven by this horizontal gene transfer. Alternatively, since the populations are recently established (Hill and Unckless 2020b, a), it may simply indicate that these lines and mitotypes differentially segregated into these populations upon establishment and that the types dominant in Chiricahuas are rarer in Prescott.

In summary, we provide an updated genome description of w\textsubscript{Inn} of \textit{D. innubila} and use various analyses to understand the population genomic trends of this symbiont. We find that the genome has many unique genes suggestive of lateral gene transfer with divergent eukaryotic hosts, along with likely recent and ancient repetitive elements that also indicate lateral transfer with diverse species has occurred on multiple occasions. It remains to be determined when these transfers occurred, if they occur in both directions, and what unique functional consequences they may have for this strain. We also find no evidence of genomic content unique to strains that can cause male killing, in line with predictions that the phenotype is not determined by microbial factors alone. Future work will need to focus on identifying host factors that may be responsible for the expression or absence of male killing in any given system. Reflecting literature precedent, parts of the prophage and Octomom regions are under positive selection, and we find that several genes have likely horizontally transferred into the symbiont from other \textit{Wolbachia}. It is unknown which strains may have been a part of any genetic exchanges with w\textsubscript{Inn}, when, or in what context they may have been in contact. We also found evidence of structured populations that suggest little to no host migration between populations, high fidelity in mitochondrion-w\textsubscript{Inn} co-inheritance, and a high likelihood of frequent prophage exchange among individuals within populations. It is unknown if most or all other male-killers exhibit such clear demographic structure among populations, and if this plays a role in the long-term maintenance of male killing. In light of the fact that not all strains that can kill males form phage particles (w\textsubscript{Rec} has a relic phage), it remains unknown if putative particles and common exchange of prophages...
among individuals plays an important role in parasitic phenotypes generally or among male-killers specifically. Finally, we provide preliminary evidence that there may be active phage particles among individuals that potentially aid in the dynamics described here. The presence or absence of phage particles and exact numbers of Octomom copies per sample will need to be confirmed in later work. Moving forward, it will be important to compare the population genomics of other male-killing strains to determine if they reflect the same principles uncovered here that may contribute to the success of Wolbachia symbionts in sex-biased populations or may reflect a broader strategy for survival and adaptation in diverse Wolbachia around the globe.

**Materials and Methods**

**Genome sequence of wInn**

For a single Wolbachia-positive strain described previously (Unckless and Jaenike 2011), we extracted DNA following the protocol described in (Chakraborty, et al. 2018). We prepared the DNA as a sequencing library using the Oxford Nanopore Technologies Rapid 48-h (SQK-RAD002) protocol, which was then sequenced using a MinION (Oxford Nanopore Technologies, Oxford, UK; NCBI SRA: TBD) (Jain, et al. 2016). The same DNA was also used to construct a fragment library with insert sizes of ~180bp, and we sequenced this library on an Illumina HiSeq 4000 (150 bp paired-end, Illumina, San Diego, CA, NCBI SRA: TBD).

Oxford Nanopore sequencing read bases were called post hoc using the built in read_fast5_basecaller.exe program with options: –f FLO-MIN106 –k SQK-RAD002 –r –t 4. We assembled the raw Oxford Nanopore sequencing reads alongside the Illumina paired-end short sequencing reads using SPAdes version 3.13.0 (Bankevich, et al. 2012), which generated an initial assembly of 83 contigs with homology to Wolbachia. We then attempted to improve this initial assembly using the 83 assembled fragments, along with Nanopore sequencing reads and Illumina paired-end short sequencing reads in MaSuRCA version 3.4.1 (Zimin, et al. 2013), defining the expected genome size as 1.5 million bp. This produced a single contig that was 1,286,799 bp long. We confirmed this contig was circular through aligning long-reads to this assembly and validating by eye. We then used Racon version 1.4.3 to polish the genome with minion fragments for 3 iterations (Walker, et al. 2014) and further polished with Pilon version 1.23 for three iterations using the short read data (Vaser, et al. 2017). We then verified the contiguity of the assembly using BUSCO version 3.0 (Simão, et al. 2015). From a search for 221 proteobacteria orthologs, we found 181 complete single copy orthologs and 2 fragmented orthologs (compared to 180 complete and 2 fragmented for the published wMel genome: NC_002978.6).

**Fly collections and Wolbachia infection confirmation**
We collected wild Drosophila at four mountainous locations across Arizona between the 22nd of August and the 11th of September 2017 (Hill and Unckless 2020b, a). Specifically, we collected at the Southwest research station in the Chiricahua mountains (31.871 latitude, -109.237 longitude), Prescott National Forest (34.540 latitude, -112.469 longitude), Madera Canyon in the Santa Rita mountains (31.729 latitude, -110.881 longitude) and Miller Peak in the Huachuca mountains (31.632 latitude, -110.340 longitude). Baits consisted of store-bought white button mushrooms (Agaricus bisporus) placed in large piles about 30cm in diameter, at least 5 baits per location. A sweep net was used to collect flies over the baits in either the early morning or late afternoon between one and three days after the bait was left. Flies were sorted by sex and species based on morphology at the University of Arizona and were flash frozen at -80°C before being shipped on dry ice to Lawrence, KS. Specifically, we separated individuals likely to be Drosophila innubila from the rest of the collections for further processing and genetic confirmation of species identification.

We further analyzed the 343 D. innubila flies which we homogenized and extracted DNA from using the Qiagen Gentra Puregene Tissue kit (USA Qiagen Inc., Germantown, MD, USA) (Hill and Unckless 2020b, a). We tested these samples for infection using Wolbachia primers specific to the Wolbachia surface protein (wsp) gene alongside a positive and negative control (Zhou, et al. 1998).

The reaction mixture for the wsp PCR consisted of 1uL DNA, 1u 10X buffer (Solis Biodyne), 1.0 µl of 20 mM MgCl2 (Solis Biodyne), 1 µl of dNTPs (20 µM each), 0.5 µl of forward (F) primer (81F 5'-TGGTCCAATAAGTGATGAAGAAAC-3', 20 µM), 0.5 µl of reverse (R) primer (691R 5'-AAAAATTAAACGCTACTCCA-3', 20 µM), 0.5 µl of Taq DNA polymerase (5 U/µl) (Solis Biodyne) and water to make up the final volume of 10 µl. The amplification reaction consisted of one cycle of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C, followed by 35 cycles of 15 s at 94°C, 1 min at 58°C and 2 min at 72°C, and one cycle of 15 s at 94°C, 1 min at 58°C and 7 min at 72°C. These conditions yielded 610 basepair (bp) PCR products, which we observed running out the product on a 1% agarose TAE gel. This survey yielded 48 Wolbachia-positive lines.

For the 48 Wolbachia-infected lines we previously extracted DNA and sequenced the host and Wolbachia genomes on two runs of an Illumina HiSeq 4000 (150 bp paired-end (Hill and Unckless 2020a, b), Illumina, San Diego, CA), producing an average of 20,618,752 reads per sample, of which an average of 436,527 (approximately 85x coverage) mapped to Wolbachia per sample, as summarized in Supplementary Table 1.

**Genome annotation**

We annotated the wInn genome using Prokka version 1.15.4 (Seemann 2014), detecting 2686 total genes, of which 1341 were retained following size and quality filtering (> 50bp, quality score > 20, Supplementary Table 2). Using this annotation of the genome, we extracted coding sequences and generated...
amino acid sequences using GFFread version 0.12.1 (Pertea 2011). We also downloaded the coding sequence and amino acid sequences for open reading frames in the *Wolbachia of Drosophila melanogaster* (Canton S strain) (*w*Mel-CS, SAMN02604000), the *Wolbachia of Drosophila simulans* (Riverside strain) (*w*Ri, SAMN02603205), the *Wolbachia of Drosophila simulans* (Hawaii strain) (*w*Ha, SAMN02604273), and the *Wolbachia of Culex pipiens* (*w*Pip, SAMN02296948). We used blastp version 2.9.0 (Altschul, et al. 1990) to identify orthologs for these genes in *w*Inn (parameters: hsp = 1, num_alignments = 1, e-value < 0.00001). For each set of orthologs we created a gene alignment using MAFFT version 7.409 (parameters: --auto) and for 100 randomly chosen genes made a visual inspection of amino acid sequences to confirm similarity of putative orthologous sequences. We then verified the completeness of the extracted amino acids sequences using BUSCO version 3.0 (Simão, et al. 2015). We then compared the orthologs to the published *w*Mel genome: NC_002978.6.

To annotate the repetitive content of the *w*Inn genome, we used RepeatModeler version 2.0.1 (Smit and Hubley 2008) and RepeatMasker version 4.0.9 (parameters: -gff -gccalc -s -norna) (Smit and Hubley 2013-2015).

**Genomic variation in *w*Inn**

For all 48 *Wolbachia*-positive lines collected in 2017, we mapped short reads to the *D. innubila* genome (Hill, et al. 2019), masked using RepeatMasker version 4.0.9 (parameters: -gff -gccalc -pa 4 -s) (Smit and Hubley 2013-2015), a custom library of *D. innubila* repeats (Hill, et al. 2019), and the masked *w*Inn genome using BWA MEM version 0.7.17-r1188 (Li and Durbin 2009) and SAMtools version 1.9 (Li, et al. 2009). We then extracted aligned reads mapping to *w*Inn and used GATK version 4.0.0 to remove optical and PCR duplicates and realign around indels (McKenna, et al. 2010; DePristo, et al. 2011). We then called variants in the *w*Inn genome of each *Wolbachia*-positive lines using GATK HaplotypeCaller version 4.0.0 (McKenna, et al. 2010; DePristo, et al. 2011), considering only variants with a quality score greater than 500. Finally, we combined VCFs using BCFtools version 1.7 (Narasimhan, et al. 2016) to create a multiple sample VCF.

Using the generated BAM files, we calculated the coverage of the whole *w*Inn genome relative to the host genome using BEDTools (Quinlan and Hall 2010). We also calculated the number of copies of prophage and Octomom per *Wolbachia* genome again using BEDTools. Following this, we fit a GLM to identify correlations between prophage & Octomom copy number and *Wolbachia* titer. We also fit a LOESS regression for visualization of these correlations in R (RCoreTeam 2020).

**Detection of selection on *Wolbachia* genes**

For each *w*Inn gene with an ortholog in *w*Ha and *w*Ri, we generated an alignment of the coding sequence of each gene using MAFFT version 7.409 (parameters: --auto). Following this alignment, we
reformatted the alignment into a PAML version 1.3.1 usable format and generated a gene tree using PRANK version 0.170427 (parameters: +F -showtree -d=paml) (Löytynoja 2014). We next used codeML (Yang 2007) to calculate the non-synonymous divergence (dN) and synonymous divergence (dS) across the entire gene tree and find the best fitting branches model (Model 7 or 8), as well as calculate dN and dS on each branch of the tree (Model 1), specifically looking at the estimates of dN/dS on the wInn branch versus all other branches. For both the total tree and specifically the wInn branch, we looked for gene functional categories with higher dN/dS than all other genes, after controlling for gene length. We fit a GLM, comparing the dN/dS on the wInn branch versus all other branches for all genes, including the genes functional group as a co-factor, Reporting the t-value and p-value when relevant or significant. We also fit a GLM comparing the dN/dS distribution for specific genes of interest (functional groups, or suspected male-killing associated genes) to the remaining genes in the genome, again reporting the t-value and p-value, which were two-tailed (RCoreTeam 2020).

Population structure across wInn populations

For synonymous sites in the VCF, we used VCFtools version 0.1.16 (Danecek, et al. 2011) to calculate the fixation index (Fst) between each population and the other populations (Brown 1970). We also performed a principal component analysis on the variation found across the samples in R version 3.5.1 (Team 2013), using the VCF input as a presence/absence matrix.

Ancient and recent horizontal transfer

We reasoned that in the absence of horizontal gene transfer, then Wolbachia variation would be perfectly linked to mitochondrial variation, while horizontal transfer would break that pattern. We wanted to test to determine if the horizontal transfer is occurring exclusively in specific genes & genomic regions, or is occurring across the whole Wolbachia genome, supporting movement of Wolbachia between organisms, instead of horizontal transfer of specific genes. To assess this, we looked at all pairwise combinations of mitochondrial and Wolbachia alleles and recorded sites with all four allele sets across the two loci across the 48 samples (e.g., GT, AT, GC and AC), giving a recombination like signature (suggesting non-vertical inheritance). We then counted the number of discordant and non-discordant SNPs in 10-kbp windows across the wInn genome to identify specific sections enriched for discordant SNPs. We used a $\chi^2$ test to identify specific functional categories enriched for discordant SNPs.

For longer term horizontal gene transfer, we used the VHICA R package version 0.2.7 to calculate synonymous divergence (dS) for all pairwise for all shared genes for pairwise combinations of wInn, wHa and wRi (Wallau, et al. 2016). We reasoned that horizontal transfer of a gene from a highly divergent Wolbachia would produce a signal of increased dS between wHa and wInn for that gene and could polarize which species had the horizontal transfer event based on the dS of that gene in the pairs wInn-wHa, wHa-
wRi and wInn-wRi. We considered dS to be excessively high in a gene if it was greater than the mean dS + the variance for all genes with a similar number of effective codons, as dS is on average higher when the effective number of codons is higher in a gene (5 codons window size, sliding 5 codons) (Wallau, et al. 2016). This cutoff was based on the publication first describing VHICA and was used in subsequent publications (Wallau, et al. 2016; Hill and Betancourt 2018; Wallau, et al. 2018). We considered a gene to be a putative horizontal acquisition in wInn (or a recent ancestor) if dS of the wInn gene compared to homologs in wHa and wRi is excessively high, but dS between the wHa and wRi homologs is not. We then performed a $\chi^2$ test to look for functional categories that are enriched for putatively horizontally acquired genes. For this test we calculated the expected number of putatively horizontally acquired genes in each gene category based on the total number of putatively horizontal genes and the proportion of the genes which make up this category. If all things being equal and genes are transferring randomly, we would expect the number of putatively horizontal genes in each category to be proportional to the number of genes in the category. We used the expected number and the observed number of putatively horizontally acquired genes in each category to calculate an enrichment and perform the $\chi^2$ test.

Finally, we assessed the extent of ancient horizontal transfer across the Wolbachia phylogeny. We downloaded all Wolbachia genomes and their annotations from the NCBI genome database (summarized in Supplementary Table 1). Then, based on the known NCBI annotations, we found groups of orthologous genes. We generated codon alignments for these orthologous genes using MAFFT (parameters: --auto) (Katoh, et al. 2002), and generated a gene tree for each gene using PhyML (model = GTR, gamma = 8, bootstraps = 100) (Guindon, et al. 2010). We also generated a whole species phylogeny for these genomes and to place wInn on the phylogeny. For all genes found in all species with high confidence (231 genes, totaling 208,911 bp of the genome), we generated a multigene phylogeny with 100 bootstraps using PhyML (model = GTR, gamma = 8, bootstraps = 100). We then attempted to assess the extent of discordance between species and gene trees using CADM.global in APE to test for consistency between phylogenies, with the null hypothesis that the phylogenies are different across 100,000 permutations per species/gene tree comparison (so a significant p-value will suggest little discordance between phylogenies). Again, we performed a $\chi^2$ test to look for functional categories that are enriched for putatively horizontally transferred genes. If horizontal transfer is random, we expect the number of horizontally transferring genes in a functional group to depend on the proportion of genes in this category. We therefore calculated the number of expected horizontal transfers per group by multiplying the number of events observed by the proportion of genes in a category. We then used this expected number of horizontal transfers and the observed number to calculate a $\chi^2$ value per group and perform a $\chi^2$ test.

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Data Availability

The wInn genome and annotation is available on the NCBI genome database under the accession SAMN18306550. All sequencing data used in this study is available on the NCBI SRA under the project accession: PRJNA524688. Additional data regarding D. innubila population genomics is available in the following FigShare folder: https://figshare.com/account/home/#/projects/87662 and the following DataDryad submission: https://datadryad.org/stash/share/wvfmDL39pdYrVUCgDFAfI33BOJu3KCJWuJyj-0M-qgA.

Author contributions

TH devised analyses, performed genome assembly, performed analyses and wrote the manuscript, JIP devised analyses and wrote the manuscript, RLU devised analyses and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest

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