Population genomics of *Wolbachia* and mtDNA in *Drosophila simulans* from California

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ABSTRACT: *Wolbachia pipientis* is a widespread intracellular endosymbiont infecting many arthropods and filarial nematodes. Little is known about the short-term evolution of *Wolbachia* or its interaction with its host. *Wolbachia* is maternally inherited, resulting in co-inheritance of mitochondrial organelles such as mtDNA. Here I explore the short-term evolution of *Wolbachia*, and the relationship between *Wolbachia* and mtDNA, using a large inbred panel of *Drosophila simulans* infected with the *Wolbachia* strain *w*Ri. I find reduced diversity relative to expectation in both *Wolbachia* and mtDNA, but only mtDNA shows evidence of a recent selective sweep or population bottleneck. I find that all individuals in the population are infected, and we estimate *Wolbachia* and mtDNA titre in each genotype. I find considerable variation in both phenotypes, despite low genetic diversity in *Wolbachia* and mtDNA. A phylogeny of *Wolbachia* and of mtDNA show that both trees are largely unresolved, suggesting a recent origin of the infection derived from a single origin. Using Wolbachia and mtDNA titre as a phenotype, I perform an association analysis with the nuclear genome and find several regions implicated in the phenotype, including one which contains four CAAX-box protein processing genes. CAAX-box protein processing can be an important part of host-pathogen interactions in other systems, suggesting interesting directions for future research.

44 INTRODUCTION:

45 Heritable symbiotic associations such as that between *Drosophila* and *Wolbachia pipientis* have
46 widespread impact on host ecology and evolution. These types of heritable endosymbiotic relationships
47 are recognized as key drivers of evolution, but the intraspecific variation that effects their short-term
48 evolution is not well explored. *Wolbachia* are α-proteobacterial endosymbionts found in up to 40% of all
49 arthropod species[1-3]. *Wolbachia* are maternally transmitted and spread through manipulating the
50 reproductive strategies of their host, using mechanisms such as feminization, male-killing, or cytoplasmic
51 incompatibility. The most common of these is cytoplasmic incompatibility, where mating between males
52 and females of the same species results in embryonic mortality if they have different *Wolbachia* infection
53 status[4-8]. *Wolbachia* may also confer certain protections upon their host, such as increased resistance to
54 certain viruses, or increased survival when exposed to certain environmental stressors[9-15]. *Wolbachia* is
55 one of the most abundant obligate intracellular parasites, given that 85% of animal species are insects.
56 This has profound meaning for evolutionary processes such as sexual selection and speciation[16,17].

57 *Wolbachia* strain *w*Ri is known to have spread recently in the sister species to the model organism
58 *Drosophila melanogaster*, *D. simulans*[4,5,8,18]. It was at ~95-100% frequency in Southern California
59 populations at the time its original sampling in the 1980's[19,20]. It likely invaded California less than 25
60 years before it was first detected in 1984[21]. It is now thought to have been horizontally transmitted to *D.*
61 *simulans* from *D. ananassae*, though the same strain is also found in *D. suzukkii*[22]. The maternal
62 transmission of *Wolbachia* means that as the microorganism spreads all maternally inherited organelles
63 spread along with it. Most notably mtDNA will be forced through a bottleneck, lowering the diversity of
64 mtDNA in infected populations[18,23,24]. This will cause mtDNA and *Wolbachia* to be more closely
65 associated than nuclear genes, and this coupling has been demonstrated previously in *D. simulans*[18,25,26]. In
66 fact, *D. simulans* is known to have three major mitochondrial haplotypes (*si*I, *si*II, and *si*III) and two
67 subtypes (*si*IIA and *si*IIB) that harbor very little variation and that appear to be nonrandomly associated
68 with *Wolbachia* strains[27-29]. These mitochondrial haplotypes are largely allopatric, except for the presence

69 of both *si*II, and *si*III in Madagascar and La Reunion[30].

70 In *D. melanogaster* variation in *Wolbachia* has been well investigated using genomic data,
71 namely in five populations from around the world, though this has not been done in the genomic era in *D.*
72 *simulans*[31]. They found long lived associations between mitochondrial and *Wolbachia* haplotypes and
73 strong geographic structuring among cytotypes[31-33]. This study also observed that *Wolbachia* titre varied
74 among fly populations as the result of intraspecific nuclear genetic variation[31]. However, the assumption
75 that it was due to intraspecific nuclear background was based on the presence of a constant environment
76 and no polymorphisms were identified. Very little is known about how *Wolbachia* interact with their
77 hosts, though recent work has uncovered evidence that deubiquitylating enzymes produced by *Wolbachia*
78 and secreted into the host cytoplasm mediate cytoplasmic incompatibility[34]. *Wolbachia* DNA is also
79 frequently inserted into the host genome, though this has not occurred with *w*Ri in *D. simulans*[21]. Genes
80 involved in the formation of germline stem cells such as *benign gonial cell neoplasm* and *bag-of-marbles*
81 are considered candidates for interacting with *Wolbachia*, and have been found to have unusual
82 population genetic patterns in *D. melanogaster* [35,36]. *bag-of-marbles* has been suggested to interact with
83 *Wolbachia* due to fertility rescue in hypomorphs, but the interaction of this gene with *Wolbachia* in
84 natural populations is not clear[22,35-37]. Notably, *Wolbachia* localizes in tissues differently depending upon
85 the strain and species so the interactions between the host and *Wolbachia* are likely to also be
86 different[38,39].

87 *Wolbachia* infections must be maintained in host populations through transovarial transmission,
88 wherein *Wolbachia* is present in the germline at sufficient copy number to ensure transmission but not to
89 cause host pathology[40]. *Wolbachia* titre has been shown to have important phenotypic effects on the
90 host[11,41-49]. However, control of *Wolbachia* replication is not well understood, nor is the dependence of
91 this control on host background versus bacterial genotype[11,50-52]. Differences in *Wolbachia* titre when it is
92 transinfected between species suggests a role of host background in controlling copy number, population
93 genomics in *D. melanogaster* suggest an effect of host background, and there does seem to be host-

94 specific patterns of tissue colonization[53-55]. However, multiple *Wolbachia* genotypes can also behave
95 differently in the same genetic background suggesting contributions from the bacterial genome [51,56]. It is
96 also possible to select for greater *Wolbachia* densities, though the heritability of this is unclear[57,58].

97 Here I investigate the dynamics of *Wolbachia* and mtDNA in a large panel of *D. simulans* from a
98 single Californian population. I determine infection status of *Wolbachia* in the panel of *D. simulans*
99 genotypes. I look for signatures of selection in both genomes using summary statistics Tajima's *D* and π
100 and find that while *Wolbachia* patterns of variation are not unusual given its demographic history the
101 reduction in mtDNA diversity is suggestive of a recent bottleneck due either to selection or changes in
102 population size. I also measure linkage disequilibrium between mtDNA and *Wolbachia* as a proxy for co-
103 inheritance. Using whole genome sequences, I investigate the phylogeny of both *Wolbachia* and mtDNA
104 and find that in this population they are essentially unresolved. I investigate variation in the copy number
105 of both *Wolbachia* and mtDNA in this population using relative estimates derived from illumina
106 sequencing coverage compared to nuclear coverage. I find considerable copy number variation in this
107 population, and an association analysis using this as a phenotype implicates several genomic regions
108 potentially involved in mediating this phenotype. This includes a region containing multiple genes
109 involved CAAX-box protein prenylation, a process that is important for mediating the relationship
110 between host and pathogen in other systems[59-61].

111 METHODS

112 ***Drosophila* strains**

113 Strains are as described in[62]. Briefly, the 167 *D. simulans* lines were collected in the Zuma organic orchard in
114 Zuma beach, California in February of 2012 from a single pile of fermenting strawberries. Single mated females
115 were collected and inbred by 15 generations of full sib mating of their progeny. *Drosophila* were raised at a
116 constant temperature of 20º C with 12-hour light/dark cycles. They were raised on a standard glucose/yeast media,
117 and each library was constructed from adult females of similar age (less than one week).

118 **Data sources and processing**

119 The sequencing reads were downloaded from the NCBI Short Read Archive from project SRP075682.
120 Libraries were assembled using BWA mem (v. 0.7.5), and processed with samtools (v. 0.1.19) using
121 default parameters[63,64]. The *Wolbachia* reference is the *w*Ri strain previously identified in Southern
122 California (Accession number NC_012416)[21]. The mtDNA reference is from *D. simulans* w^{501}, which is
123 haplogroup *siII* as expected for *D. simulans* from California (Accession number KC244284)[65]. PCR
124 duplicates were removed using Picard MarkDuplicates (v. 2.9.4) and GATK (v. 3.7) was used for indel
125 realignment and SNP calling using default parameters (http://picard.sourceforge.net)[66]. SNPs were called
126 jointly for all genotypes using Haplotypecaller[66]. Individual consensus fasta sequences were produced
127 using SelectVariants to create individual vcf files and FastaAlternateReferenceMaker. Vcf files were
128 filtered for indels and non-biallelic SNPs using VCFtools (v. 0.1.13)[67]. The files were also filtered for
129 SNPs with more than 10% missing data and a minor allele frequency of 2%, meaning that each SNP must
130 be in the population in at least approximately 3 copies. The *Wolbachia* genome was filtered for regions of
131 unusual coverage or SNP density, for example two regions of the *Wolbachia* genome harbored ~40 SNPs
132 within two kb, far above background levels of variation (Supp. File _ for an example). These two regions
133 coincided with regions of unusually high coverage suggesting they are repeated elements.

134 **Prediction of *Wolbachia* infection status**

135 *Wolbachia* infection status was determined by calculating the mean depth of coverage of the assembly
136 and the breadth of coverage of the consensus sequence using bedtools[68]. Depth of coverage refers to the
137 average read depth across the *Wolbachia* genome, while breadth of coverage refers to the number of bases
138 covered by at least two reads. Depth of coverage at each nucleotide was estimated using the genomecov
139 function, while breadth was estimated using the coverage function. Predictions of *Wolbachia* infection
140 status using illumina data have previously been shown to have 98.8% concordance with PCR based
141 predication of infection status[32].

142 **Nucleotide diversity**

143 Levels of polymorphism for mtDNA and *Wolbachia* were estimated as π in 10 kb windows. To
144 investigate whether the frequency spectrum conformed to the standard neutral model of molecular

145 evolution I calculated Tajima's *D* in 10 kb windows using VCFtools (v0.1.14)[69]. To assess the
146 significance of deviations in Tajima's *D* and π 10,000 simulations were performed using msms
147 conditioned on the number of variable sites and with no recombination[70].

148 **Linkage disequilibrium**

149 Linkage between *Wolbachia* and mtDNA SNPs could potentially be a predictor of co-inheritance of
150 mtDNA and *Wolbachia*. Linkage was estimated using VCFtools (v0.1.14) using inter-chrom-geno-r2 to
151 estimate r^2 between each SNP in the two genomes[67].

152 **Estimation of mtDNA and *Wolbachia* copy number**

153 In insects, the phenotypic effect of *Wolbachia* will vary depending upon copy number in the host cells[9,32].
154 Given that there are two copies of autosomal DNA in a cell, I infer mtDNA and *Wolbachia* copy number
155 based on the ratio between mtDNA and autosomal DNA. This is intended to provide a relative estimate of
156 copy number rather than an absolute measure. Relative copy number estimated in this way obscures intra-
157 individual variation and variation between tissues, though the authors note that all flies used in
158 constructing the libraries were females of approximately the same age. *Wolbachia* contains several
159 regions which were excluded due to unusually high coverage across all samples (more than 3x the mean
160 coverage). Average coverage was calculated from randomly chosen and equivalently sized nuclear
161 regions for each mtDNA (Scf_3L:8000000..8014945) and *Wolbachia* (Scf_2L:11000000..11445873).
162 The average coverage of each nuclear region, respectively, was then used to normalize estimates of copy
163 number for each genotype. Previously the results of measuring *Wolbachia* copy number in the same
164 samples using both qPCR and estimates from illumina read depth had a Pearson's correlation coefficient
165 of .79, thus this is a robust approach to measuring *Wolbachia* titre[31].

166 **Phylogenomic analysis**

167 To understand the relationship between *Wolbachia* infection and mtDNA I reconstructed the genealogical
168 history of each within the sample population. Multiple alignments were generated for both mtDNA and
169 *Wolbachia* by concatenating fasta consensus sequence files for each genotype. All indels and non-biallelic
170 SNPs were excluded from the dataset prior to generating the consensus fasta for each genotype. RAxML

171 version 8.10.2 was used to reconstruct phylogenies[71]. Maximum likelihood tree searches were conducted
172 using a general time reversible (GTR) model of nucleotide substitution with CAT rate heterogeneity and
173 all model parameters estimated by RAxM[72]. Trees were inferred using the rapid bootstrap algorithm and
174 simultaneous estimation of trees and bootstrapping, with automatic estimation of the necessary number of
175 bootstrap replicates.

176 **Association Analysis**

177 The association analysis focused on a relationship between nuclear polymorphisms and *Wolbachia* and
178 mtDNA copy number. To reduce the need for correction due to multiple testing and focus on regions that
179 may have been affected by selection due to the recent invasion of *Wolbachia* I used a subset of the
180 nuclear genome identified previously as exhibiting haplotype structure suggestive of recent selection[62,73].
181 These regions are unusually long haplotype blocks, thus many of the SNPs within each block are not
182 independent, reducing the need for correction due to multiple testing. Heterozygous bases were coded as
183 missing, and all loci with more than 10% missing data were excluded from the analysis, as well as SNPs
184 with a minor allele frequency of less than 2%, meaning they were present in the population in at least 3
185 copies. mtDNA and *Wolbachia* copy number were used for a multivariate analysis of association using
186 plink.multivariate[74]. To investigate the possibility that *Wolbachia* copy number is affected by
187 polymorphisms in mtDNA, and vice versa, a single trait analysis was performed using plink v. 1.07[75].

188

189 <u>RESULTS</u>

190 **Sequencing Data**

191 The autosomal data included in this analysis was reported in[62]. There was very little variation in both
192 *Wolbachia* and mtDNA in this population. This included 78 SNPs and indels in the *Wolbachia* genome
193 and 90 in mtDNA. Reduced diversity has been reported previously in *D. simulans* mtDNA[24,25]. The
194 authors note that previous work has established that there is no unusual relatedness in the nuclear genome
195 of this population[62].

196 **Infection status**

197 In a previous study lines were scored as infected if they had a breadth of coverage greater than 90% and a
198 mean depth greater than one[32]. However, that dataset had a clearly bimodal distribution between infected
199 and uninfected lines, where uninfected lines had breadth of coverage less than 10% while infected lines
200 had a breadth of coverage of greater than 90%. As such that this demarcation was a natural interpretation
201 of the data[32]. In *D. simulans*, all lines had ~99% breadth of coverage aside from a single line with both a
202 lower overall depth of coverage and 80% breadth (Fig. 1). For this reason, all lines were scored as
203 infected. 100% infection is not unusually high for *D. simulans*.

204 **Nucleotide Diversity**

205 Estimates of π in *Wolbachia* ranged from 5.98×10^{-7} to 1×10^{-3}, with an average of 1.42×10^{-5}, within the
206 range of estimates from *Wolbachia* in *D. melanogaster* from another study ($7.9 \times 10^{-6} – 2.8 \times 10^{-5}$ [31]). The
207 mean of π in simulated populations of *Wolbachia* is 1.9×10^{-3} suggesting that variation is somewhat
208 reduced in *w*Ri. π in mtDNA is 1×10^{-4} which again is similar to estimates from *D. melanogaster* ($4.34 \times$
209 $10^{-4} – 1.51 \times 10^{-3}$)[31].

210 Overall Tajima's *D* was estimated to be -2.4 for *D. simulans* mtDNA (Fig 2). This is similar to
211 estimates in *D. melanogaster*[32]. Significance of this estimate was assessed using 10,000 simulations in
212 msms conditioned on the number of segregating sites and no recombination, and it is significant at p
213 $< .05$. Tajima's *D* in *Wolbachia* is not significantly different from expectations under neutrality based on
214 10,000 simulations. Thus, while a selective sweep seems to have strongly effected mtDNA in *D.*
215 *simulans*, the same is not true of the *Wolbachia* population (Fig 2). This is very different from *D.*
216 *melanogaster* where *Wolbachia* and mtDNA had similar patterns of nucleotide diversity[32].

217 This is also much more negative than previously reported for mtDNA in *D. simulans*[25]. It is very
218 different from the general patterns of Tajima's *D* in the nuclear genome, where average Tajima's *D* is 1
219 and the majority of the genome has a positive Tajima's *D*. Simulations in previous work suggest that the
220 pervasively positive values in the nuclear genome may be due to a population contraction, which again
221 indicates that the population dynamics affecting *D. simulans* nuclear and mtDNA genomes are very
222 different[25,62].

223 **Linkage disequilibrium**

224 There was no significant linkage disequilibrium between the genomes of *Wolbachia* and *D. simulans*
225 mtDNA. Average LD between *Wolbachia* and mtDNA SNPs was 2.06×10^{-3}. This may be because the
226 infection of *D. simulans* was too recent for variation to accumulate along particular lineages, and also
227 suggests that *D. simulans* was infected by a single invasion. As all strains were infected with *Wolbachia* it
228 is also possible that variation in both lineages is fixed rather than sorting in the population.

229

230 **Estimation of mtDNA and *Wolbachia* copy number**

231 There was considerable heterogeneity in both *Wolbachia* and mtDNA copy number (Fig. 1). Mean
232 (standard deviation) copy number of *Wolbachia* is 5.56 (2.45). This is similar to one estimate in *D.*
233 *melanogaster*, where mean copy number is 5.57 (3.95) though the standard deviation is lower in *D.*
234 *simulans* [32]. The reported mean was lower in other populations of *D. melanogaster*, though still within the
235 same range (2 - 4.5)[31]. Similarly mean mtDNA copy number is 33.85 (15.5) in *D. simulans* and 32.9
236 (44.5) in one estimate for *D. melanogaster*[32]. This is again not an absolute measure, but relative to nuclear
237 genomic coverage. The lower standard deviation could be due to more precise staging of the age of *D.*
238 *simulans*, less background variation effecting copy number (the *D. melanogaster* sample was from
239 multiple populations), or other unknown mechanisms. There was a positive relationship between mtDNA
240 and *Wolbachia* copy number (Fig. 1) ($p < 2.4 \times 10^{-7}$). While the functional reasons for or consequences of
241 this are unclear, because they are correlated they will be used in a multivariate analysis of association
242 rather than as separate analyses.

243 **Phylogenomic analysis**

244 To understand the relationship between *Wolbachia* infection status and mtDNA sequence variation I
245 reconstructed the phylogenetic history of the complete *Wolbachia* and mtDNA genome using the entire
246 set of 167 strains (Fig 3-4). What I found is consistent with the recent spread of *Wolbachia* in *D.*
247 *simulans*, as both phylogenies are essentially unresolved. This is not unexpected for mtDNA given
248 previous work in the species which found little within-haplotype variation among the three major mtDNA

249 haplotypes in *D. simulans*[25,28]. Furthermore, of the 167 sequences 88 are identical to at least one other
250 sequence in the sample. While the *Wolbachia* phylogenetic tree gives the impression of having more
251 resolution than mtDNA, this is likely due to the larger genome, as the branches have similarly low
252 support. Of the 167 strains included in the tree 18 are identical to one or more *Wolbachia* genomes. Both
253 trees are essentially star phylogenies with the majority of bootstrap support values being less than 30.
254 Bootstrap support of greater than 70, for two branches in the mtDNA tree and five in the *Wolbachia* tree,
255 is shown (Fig 3-4). If uninfected individuals had been included in the dataset perhaps it would be possible
256 to test for congruence between the two phylogenies, however the essentially unresolved trees make it
257 clear that both *Wolbachia* and mtDNA swept the population recently.
258
259 **Association Analysis**
260 Association analysis was performed using plink.multivariate by regressing the line means for mtDNA and
261 *Wolbachia* copy number on each SNP contained within the previously identified in a scan for selection[62].
262 This scan for selection focused on identifying haplotype blocks in LD. This considerably reduces the
263 number of SNPs tested for association, in addition to the fact that the SNPs are in haplotype blocks and
264 are therefore not independent tests[62,73]. This reduces the need for correction due to multiple testing. I used
265 a p-value cut-off of $p < 9 \times 10^{-6}$ and identified 16 SNPs associated with *Wolbachia* and mtDNA copy
266 number. Of these 16 SNPs 13 are located in the same region on chromosome 2R (Scf_2R: 13550916-
267 13569038). Given the concentration of significant SNPs in a single region, this is also the region I will
268 focus on the most in the following discussion. The region containing 13 SNPs contains nine genes, four of
269 which are involved in CAAX-box protein processing, *ste24a-c* and a recent duplicate of *ste24c* CG30461.
270 CAAX-box protein processing is a part of a series of posttranslational protein modifications collectively
271 called protein prenylation which are required for fully functional proteins to be targeted to cell
272 membranes or organelles. It has been shown that pathogenic bacteria can exploit the host cell's
273 prenylation machinery, though it is unclear if this occurs in *Wolbachia*[59].

274 The other five genes are *AsnRs-m*, which is largely unannotated but is thought to a mitochondrial
275 aminoacyl-tRNA synthetase[76]. *NIPP1Dm* is involved in axon guidance and negative regulation of protein
276 phosphorylation[77,78]. *CG6805* is generally unannotated but is inferred to be involved in
277 dephosporylation[76]. *Cbp53E* regulates neural development[79]. Lastly, *Ehbp1* is a developmental gene
278 implicated in regulation of the Notch pathway and membrane organization[80].
279 Of the other three SNPs identified in this association analysis two are located at Scf_2R:5814103
280 and Scf_2R:5811043, while the third is located at Scf_3L:2055556. Scf_2R:5811043and
281 Scf_2R:5814103 are located in *Su(var)2-10* and *Phax*, respectively. These are neighboring genes, though
282 there is a third gene within 10 kb, *Mys45A*. *Su(var)2-10* is involved in development and chromosome
283 organization, but it has also been implicated in the regulation of the innate immune response and defense
284 against Gram-negative bacteria[81]. *Su(var)2-10* is of particular interest given that *Wolbachia* are Gram-
285 negative bacteria, however the potential role of *Su(var)2-10* in immune response is not clear. *Phax* is not
286 well annotated but is inferred to be involved in snRNA export from the nucleus [79]. *Mys45A* is potentially
287 involved in actin cytoskeleton organization[79]. In *D. melanogaster Wolbachia* uses host actin for maternal
288 transmission, though this has not been verified in *D. simulans*[82]. The last SNP, at Scf_3L:2055556, is in
289 *Connectin*, a cell adhesion protein also involved in axon guidance[83].
290 The identification of these SNPs in association with mtDNA and *Wolbachia* copy number does
291 not imply a functional relationship. Nonetheless, I chose to investigate whether any of these substitutions
292 had an effect on the coding sequence of any of genes in the region. Of the three SNPs found outside the
293 region containing the CAAX-box proteins all were either in introns or regulatory regions. Of the 13 SNPs
294 identified between Scf_2R: 13550916-13569038 eight are in introns or untranslated regions, including
295 one in the long intron of *Cb53E*, three in the introns or noncoding transcript of *CG6805*, and two in the
296 introns of *Ephb*. Of the remaining five SNPs four are in coding regions but silent, causing no change in
297 the amino acid sequence of the protein. This includes silent mutations in the exons of *ste24c* and two
298 silent mutations in the exons of *Epbh*. One SNP located in an exon of *ste24a*, at 13558515, is an amino
299 acid substitution from a Leucine to a Valine. This is not an uncommon amino acid substitution[84,85], though

300 it can be associated with phenotypes[86,87]. Mutations in introns and untranslated regions could also be
301 having an effect on gene expression or processing, as could other linked SNPs in the region that were not
302 included in the analysis.
303
304 *Association between Wolbachia and mtDNA*
305 Association analysis was performed using plink by regressing the line means for mtDNA copy number
306 onto the *Wolbachia* genome and vice versa[75]. There was no association between *Wolbachia* SNPs and
307 mtDNA copy number, but the opposite was not true. One SNP in the *D. simulans* mtDNA affected
308 *Wolbachia* copy number at $p < 3.18 \times 10^{-6}$. It is located in the *D. simulans* homolog of *D. melanogaster*
309 *srRNA* which has been implicated in pole cell formation[88]. *Wolbachia* is incorporated into the pole cells,
310 the precursor to the germline, in order to be transmitted[89].
311
312 DISCUSSION
313 Using high through-put sequencing of a large panel of *D. simulans* I have reconstructed the complete
314 genome sequences of mtDNA and *Wolbachia*. I use these genome sequences to investigate the recent
315 history of *Wolbachia* and mtDNA in this population, as well as to estimate titre of both *Wolbachia* and
316 mtDNA. The history of *Wolbachia* in this population is reflected in the essentially star-like phylogeny of
317 both mtDNA and *Wolbachia*, indicating recent spread and co-inheritance. Lack of variation at mtDNA
318 and *Wolbachia* suggests a single spread of *w*Ri in this population as well as strict vertical transmission in
319 the maternal cytoplasm. Variation in *Wolbachia* is within the range expected under a neutral model,
320 however that was not the case for mtDNA which suggests either a selection sweep or a population
321 bottleneck. Previous studies found similar population genetic patterns at *Wolbachia* and mtDNA in *D.*
322 *melanogaster*, and thus could not distinguish whether selection on *Wolbachia* was driving similar patterns
323 in mtDNA or vice versa[31]. The much stronger pattern of negative Tajima's *D* in the mtDNA suggests that
324 in *D. simulans* selection is in fact mitochondrial. There was no linkage disequilibrium between *Wolbachia*

325 and mtDNA variants, however this is most likely due to fixation of a single mitochondrial haplotype
326 without considerable subsequent mutation.

327 Currently little is known about how *Wolbachia* interacts with its host[37-39,82,90]. Understanding these
328 interactions, including regulation of *Wolbachia* titre, will be key to understanding the evolution of
329 *Wolbachia* and its hosts. By normalizing *Wolbachia* and mtDNA copy number using coverage of the
330 nuclear genome I am able to obtain estimates of its abundance. Much as in previous work, mtDNA copy
331 number was higher than *Wolbachia* copy number, though both varied across strains[32]. As all of my data
332 was produced from adult females, at the same time, using the same techniques, there is no danger that this
333 is due to differences in methodology among samples[32]. Estimates of copy number were very similar to
334 previous work in *D. melanogaster*, performed with qPCR, and there has been shown to be a high
335 correlation between qPCR and illumina estimates of copy number[31,32]. These are not absolute measures,
336 rather they are relative to one another and to nuclear copy number, and they provide robust estimates of
337 *Wolbachia* titre within the population. As the *Wolbachia* phylogenetic tree is essentially unresolved in
338 this population but there is considerable variation in *Wolbachia* titre, it is clear that some host factors
339 must be affecting variation in *Wolbachia* titre.

340 The history of mtDNA and the nuclear genome is quite divergent in this population. The nuclear
341 genome has an average Tajima's *D* of 1 and 5 polymorphisms for every 100 bp[62]. Simulations suggest
342 that this is due to a combination of population contraction and selection, most likely from standing
343 variation, though many types of sweeps can produce similar signatures[62]. In contrast the mtDNA genome
344 contains an abundance of low frequency variation, and in fact the majority of mtDNA genomes sampled
345 in this population are identical. This is consistent with the recent spread, single origin, and maternal
346 transmission, of *w*Ri in *D. simulans*. This is consistent with previous work which found low levels of
347 mtDNA variation in *D. simulans* within a haplotype[24,91]. This is also consistent with work on *Wolbachia*
348 which documented the spread of *w*Ri in *D. simulans* in the 1980's[4,5,8,18-20].

349 While it has been proposed elsewhere, the author is not aware of another association analysis of
350 *Wolbachia* and mtDNA copy number[32]. *Wolbachia* copy number is known to be affected by host

351 background, but the genes or mechanisms involved are not known[54,55,57]. The fact that four of the nine
352 genes found in the primary region detected in the association analysis are involved in CAAX-box protein
353 processing is of particular interest, given the history of this type of gene and intracellular pathogens.
354 CAAX-box protein processing is a part of a series of posttranslational protein modifications collectively
355 called protein prenylation which are required for fully functional proteins to be targeted to cell
356 membranes or organelles. Prenylated proteins include Ras, Rac, and Rho. However, it has been shown
357 that pathogenic bacteria can exploit the host cell's prenylation machinery[59]. For example, *Salmonella-*
358 *induced filament A* is a protein from *Salmonella typhimurium*, a gram-negative facultative intracellular
359 bacterium. *Salmonella-induced filament A* has a CAAX motif required for prenylation to occur, it was
360 shown to be processed by host prenylation machinery, and it is necessary for survival of the
361 bacterium[60,92,93]. *Legionella pneumophila* Ankyrin B protein exploits the host prenylation machinery in
362 order to anchor Ankyrin B protein to the membrane of the pathogenic vacuole[61]. Proliferation of
363 *Legionella pneumophila* requires Ankyrin B, as does the manifestation of Legionnaires disease. Ankyrin
364 repeat domains are most commonly found in eukaryotes and viruses, though they are rarely found in
365 bacteria and Archaea[94]. In bacteria they are found in a few obligate or facultative intracellular
366 Proteobacteria[59]. *Wolbachia* has an unusually high number of Ankyrin repeat domains with rapid
367 evolution[94]. Ankyrin proteins play a major role in host-pathogen interactions and the evolution of
368 infections[95,96]. There is no way to know from the current analysis if the Ankyrin repeat genes are
369 exploiting the host prenylation system but it is an intriguing area for future investigation. The results of
370 this association analysis suggest that some interaction between the pathogen and its host is targeting the
371 protein prenylation machinery.
372 There was also an association between a polymorphism in *srRNA,* which has been implicated in
373 pole cell formation[88], and *Wolbachia*/mtDNA copy number. Concentration of *Wolbachia* in the posterior
374 of the embryo, where pole cells are forming, is correlated with degree of cytoplasmic incompatibility[97]. *D.*
375 *simulans* has been shown to have nearly complete cytoplasmic incompatibility, though it is possible there
376 are mutations sorting at low frequency that affect this or that mitigate negative phenotypic consequences

377 of high *Wolbachia* titre. It has also been demonstrated that *gurken* is important in for *Wolbachia* titre in
378 the germline in *D. melanogaster*, and it is involved in pole cell formation beginning at an earlier stage
379 than *sr*RNA suggesting there could be an interaction between the two factors[88,90]. *D. simulans w*Ri has a
380 different distribution in the cytoplasm from other strains of *Wolbachia*, as it tends to evenly distribute
381 throughout the embryo while other strains are either concentrated at the posterior, or at the anterior of the
382 embryo away from the pole cells[97]. Future work in related species may show that these different
383 distributions also mitigate different interactions between host and symbiont, including being effected by
384 different genes and processes within the host.

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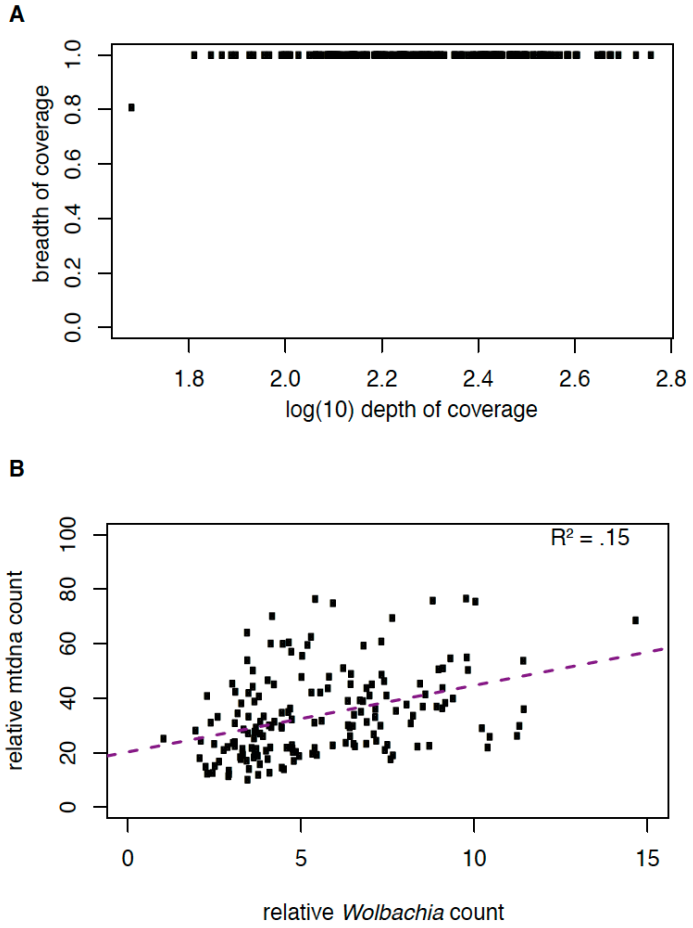

388

Figure 1. *Wolbachia* infection status and relationship to mtDNA copy number A. Relationship between depth and breadth of sequencing coverage for *Wolbachia* assemblies in the *D. simulans* panel. Depth of coverage is shown in $\log_{10}$ unites and is calculated as the number of reads present at each nucleotide in the reference averaged over every site. Breadth of coverage is the proportion of covered nucleotides in the consensus sequence relative to the reference. **B.** Relationship between relative mtDNA copy number and *Wolbachia* copy number. Both were normalized relative to nuclear coverage. Although separate regions were used to normalize mtDNA and *Wolbachia*, as they are different sizes, average values were very similar within genotypes. The relationship between mtDNA and *Wolbachia* copy number is positive ($p < 2.4 \times 10^{-7}$).

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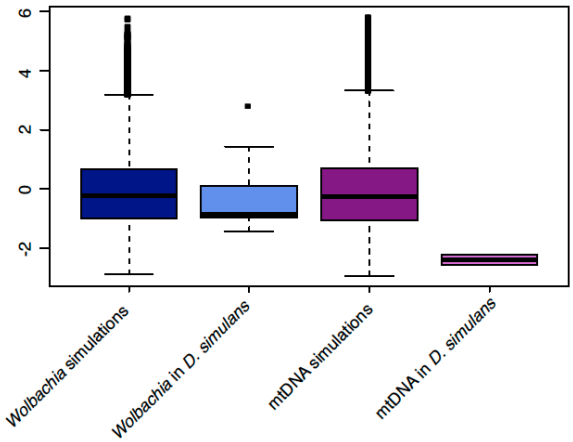
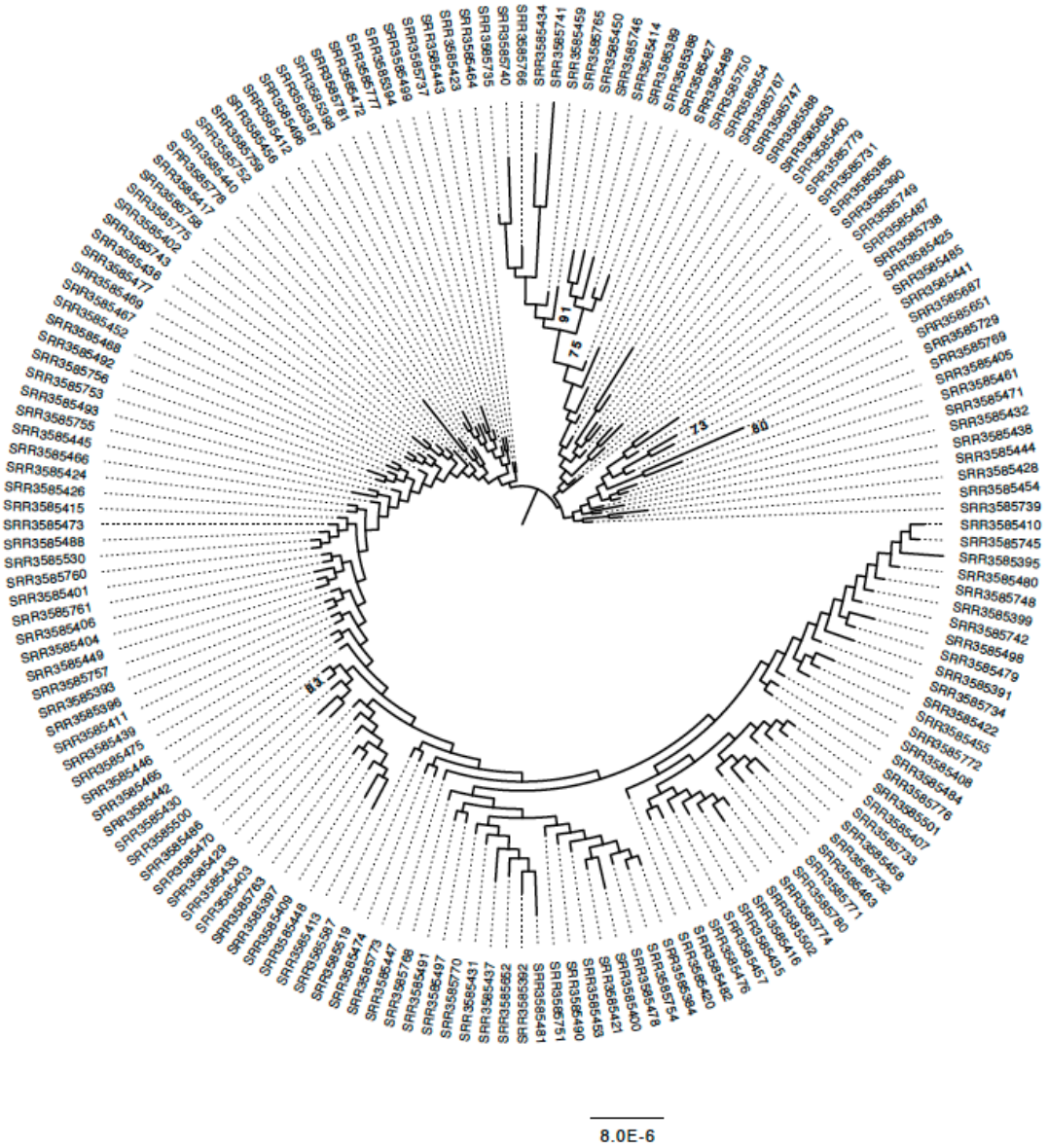


Figure 2. *Wolbachia* and mtDNA Tajima's *D*. 10,000 simulations were performed for *Wolbachia* and *D. simulans* each conditioned upon the number of polymorphisms. The actual values in *D. simulans* mtDNA are outside the 95% confidence interval of the simulations, while *Wolbachia* is not. There is considerable variation in Tajima's *D* across the *Wolbachia* genome while mtDNA is much smaller and invariant in its values of Tajima's *D*.



396

Figure 3: Maximum likelihood genealogy of the *D. simulans Wolbachia* pathogen. All strains were infected with *Wolbachia* and are included in this geneaology. The underlying data consist of an ungapped multiple alignment of 168 sequences of the entire *Wolbachia* genome. The unrooted tree was midpoint rooted for visualization and branches with > 70% RAxML bootstrap support values are shown in bold. Scale bars for branch lengths are in term of mutations per site. The majority of branches are essentially unsupported by bootstrapping.

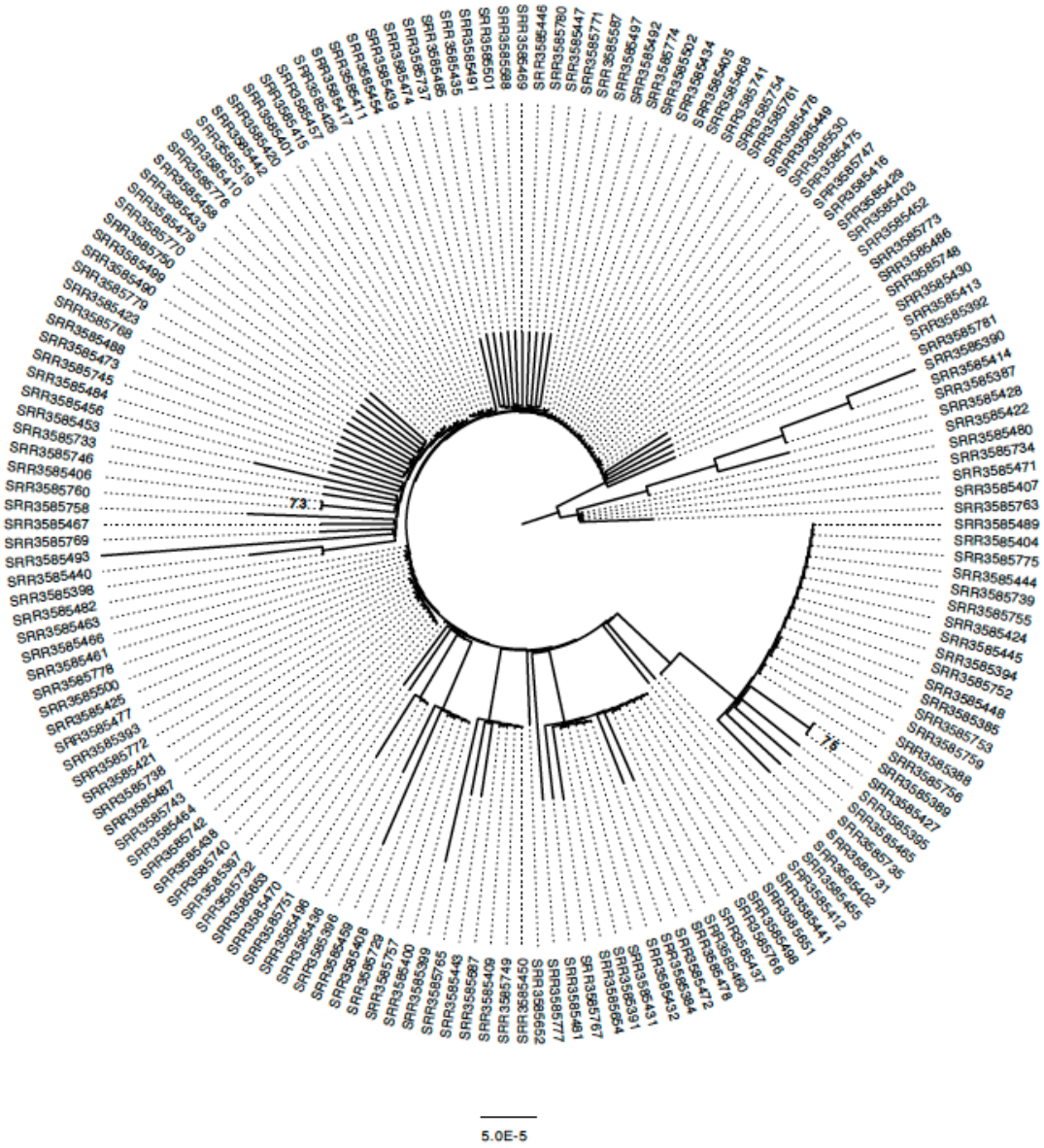


Figure 4: Maximum likelihood genealogy of the *D. simulans* mtDNA genome. The underlying data consist of an ungapped multiple alignment of 168 sequences of the entire mtDNA genome. The unrooted tree was midpoint rooted for visualization and branches with > 70% RAxML bootstrap support values are shown in bold. Scale bars for branch lengths are in term of mutations per site. The tree is largely unresolved, suggesting recent spread of this mtDNA haplotype through the population.

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