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 we 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. We find reduced diversity relative to expectation in both *Wolbachia* and mtDNA, but only mtDNA shows evidence of a recent selective sweep or population bottleneck. We find that all individuals in the population are infected, and we estimate *Wolbachia* and mtDNA titre in each genotype. We 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 and a single origin. Using Wolbachia and mtDNA titre as a phenotype, we 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 invovled 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 *D. simulans* lines were collected in the Zuma organic orchard in Zuma
114 beach, California in February of 2012 from a single pile of fermenting strawberries. Single mated females were
115 collected and inbred by 15 generations of full sib mating of their progeny. *Drosophila* were raised at a constant
116 temperature of 20º C with 12-hour light/dark cycles. They were raised on a standard glucose/yeast media, and
117 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 at each
137 nucleotide was estimated using the genomecov function, while breadth was estimated using the coverage
138 function. Predictions of *Wolbachia* infection status using illumina data have previously been shown to
139 have 98.8% concordance with PCR based predication of infection status[32].

140 **Nucleotide diversity**

141 Levels of polymorphism for mtDNA and *Wolbachia* were estimated as π in 10 kb windows. To
142 investigate whether the frequency spectrum conformed to the standard neutral model of molecular
143 evolution we calculated Tajima's *D* in 10 kb windows using VCFtools (v0.1.14)[69]. To assess the

144 significance of deviations in Tajima's D and π 10,000 simulations were performed using msms
145 conditioned on the number of variable sites and with no recombination[70].

146 **Linkage disequilibrium**

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

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

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

164 **Phylogenomic analysis**

165 To understand the relationship between *Wolbachia* infection and mtDNA we reconstructed the
166 genealogical history of each within the sample population. Multiple alignments were generated for both
167 mtDNA and *Wolbachia* by concatenating fasta consensus sequence files for each genotype. All indels and
168 non-biallelic SNPs were excluded from the dataset prior to generating the consensus fasta for each
169 genotype. RAxML version 8.10.2 was used to reconstruct phylogenies[71]. Maximum likelihood tree

170 searches were conducted using a general time reversible (GTR) model of nucleotide substitution with
171 CAT rate heterogeneity and all model parameters estimated by RAxM[72]. Trees were inferred using the
172 rapid bootstrap algorithm and simultaneous estimation of trees and bootstrapping, with automatic
173 estimation of the necessary number of bootstrap replicates.

174 **Association Analysis**

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

185

186 RESULTS

187 **Sequencing Data**

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

193 **Infection status**

194 In a previous study lines were scored as infected if they had a breadth of coverage greater than 90% and a
195 mean depth greater than one[32]. However, that dataset had a clearly bimodal distribution between infected

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

201 **Nucleotide Diversity**

202 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
203 range of estimates from *D. melanogaster* in another study ($7.9 \times 10^{-6} – 2.8 \times 10^{-5}$ [31]). The mean of π in
204 simulated populations of *Wolbachia* is 1.9×10^{-3} suggesting that variation is somewhat reduced in *w*Ri. π
205 in mtDNA is 1×10^{-4} which again is similar to estimates from *D. melanogaster* ($4.34 \times 10^{-4} – 1.51 \times 10^{-3}$
206 [31]. However, compared to simulated populations this is reduced, as the mean of 10,000 simulations is $1 \times$
207 10^{-3}.

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

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

221 **Linkage disequilibrium**

222 There was no significant linkage disequilibrium between the genomes of *Wolbachia* and *D. simulans*
223 mtDNA. Average LD between *Wolbachia* and mtDNA SNPs was 2.06 x 10^{-3}. This may be because the
224 infection of *D. simulans* was too recent for variation to accumulate along particular lineages, and also
225 suggests that *D. simulans* was infected by a single invasion. As all strains were infected with *Wolbachia* it
226 is also possible that variation in both lineages is fixed rather than sorting in the population.
227
228 **Estimation of mtDNA and *Wolbachia* copy number**
229 There was considerable heterogeneity in both *Wolbachia* and mtDNA copy number (Fig. 1). Mean
230 (standard deviation) copy number of *Wolbachia* is 5.56 (2.45). This is similar to one estimate in *D.*
231 *melanogaster*, where mean copy number is 5.57 (3.95) though the standard deviation is lower in *D.*
232 *simulans* [32]. The reported mean was lower in other populations of *D. melanogaster*, though still within the
233 same range (2 - 4.5)[31]. Similarly mean mtDNA copy number is 33.85 (15.5) in *D. simulans* and 32.9
234 (44.5) in one estimate for *D. melanogaster*[32]. This is again not an absolute measure, but relative to nuclear
235 genomic coverage. The lower standard deviation could be due to more precise staging of the age of *D.*
236 *simulans*, less background variation effecting copy number (the *D. melanogaster* sample was from
237 multiple populations), or other unknown mechanisms. There was a positive relationship between mtDNA
238 and *Wolbachia* copy number (Fig. 1) ($p < 2.4 \times 10^{-7}$).
239 **Phylogenomic analysis**
240 To understand the relationship between *Wolbachia* infection status and mtDNA sequence variation we
241 reconstructed the phylogenetic history of the complete *Wolbachia* and mtDNA genome using the entire
242 set of 167 strains (Fig 3-4). What we found is consistent with the recent spread of *Wolbachia* in *D.*
243 *simulans*, as both phylogenies are essentially unresolved. This is not unexpected for mtDNA given
244 previous work in the species which found little within-haplotype variation among the three major mtDNA
245 haplotypes in *D. simulans*[25,28]. Furthermore, of the 167 sequences 88 are identical to at least one other
246 sequence in the sample. While the *Wolbachia* phylogenetic tree gives the impression of having more
247 resolution than mtDNA, this is likely due to the larger genome, as the branches have similarly low

248 support. Of the 167 strains included in the tree 18 are identical to one or more *Wolbachia* genomes. Both
249 trees are essentially star phylogenies with the majority of bootstrap support values being less than 30.
250 Bootstrap support of greater than 70, for two branches in the mtDNA tree and five in the *Wolbachia* tree,
251 is shown (Fig 3-4). If uninfected individuals had been included in the dataset perhaps it would be possible
252 to test for congruence between the two phylogenies, however the essentially unresolved trees make it
253 clear that both *Wolbachia* and mtDNA swept the population recently.
254
255 **Association Analysis**
256 Association analysis was performed using plink.multivariate by regressing the line means for mtDNA and
257 *Wolbachia* copy number on each SNP contained within the previously identified in a scan for selection[62].
258 This scan for selection focused on identifying haplotype blocks in LD. This considerably reduces the
259 number of SNPs tested for association, in addition to the fact that the SNPs are in haplotype blocks and
260 are therefore not independent tests[62,73]. This reduces the need for correction due to multiple testing. We
261 used a p-value cut-off of $p < 9 \times 10^{-6}$ and identified 16 SNPs associated with *Wolbachia* and mtDNA copy
262 number. Of these 16 SNPs 13 are located in the same region on chromosome 2R (Scf_2R: 13550916-
263 13569038). Given the concentration of significant SNPs in a single region, this is also the region we will
264 focus on the most in the following discussion. The region containing 13 SNPs contains nine genes, four of
265 which are involved in CAAX-box protein processing, *ste24a-c* and a recent duplicate of *ste24c* CG30461.
266 CAAX-box protein processing is a part of a series of posttranslational protein modifications collectively
267 called protein prenylation which are required for fully functional proteins to be targeted to cell
268 membranes or organelles. It has been shown that pathogenic bacteria can exploit the host cell's
269 prenylation machinery, though it is unclear if this occurs in *Wolbachia*[59].
270 The other five genes are *AsnRs-m*, which is largely unannotated but is thought to a mitochondrial
271 aminoacyl-tRNA synthetase[76]. *NIPP1Dm* is involved in axon guidance and negative regulation of protein
272 phosphorylation[77,78]. *CG6805* is generally unannotated but is inferred to be involved in

273 dephosporylation[76]. *Cbp53E* regulates neural development[79]. Lastly, *Ehbp1* is a developmental gene
274 implicated in regulation of the Notch pathway and membrane organization[80].
275 Of the other three SNPs identified in this association analysis two are located at Scf_2R:5814103
276 and Scf_2R:5811043, while the third is located at Scf_3L:2055556. Scf_2R:5811043and
277 Scf_2R:5814103 are located in *Su(var)2-10* and *Phax*, respectively. These are neighboring genes, though
278 there is a third gene within 10 kb, *Mys45A*. *Su(var)2-10* is involved in development and chromosome
279 organization, but it has also been implicated in the regulation of the innate immune response and defense
280 against Gram-negative bacteria[81]. *Su(var)2-10* is of particular interest given that *Wolbachia* are Gram-
281 negative bacteria, however the potential role of *Su(var)2-10* in immune response is not clear. *Phax* is not
282 well annotated but is inferred to be involved in snRNA export from the nucleus [79]. *Mys45A* is potentially
283 involved in actin cytoskeleton organization[79]. In *D. melanogaster Wolbachia* uses host actin for maternal
284 transmission, though this has not been verified in *D. simulans*[82]. The last SNP, at Scf_3L:2055556, is in
285 *Connectin*, a cell adhesion protein also involved in axon guidance[83].
286 The identification of these SNPs in association with mtDNA and *Wolbachia* copy number does
287 not imply a functional relationship. Nonetheless, we chose to investigate whether any of these
288 substitutions had an effect on the coding sequence of any of genes in the region. Of the three SNPs found
289 outside the region containing the CAAX-box proteins all were either in introns or regulatory regions. Of
290 the 13 SNPs identified between Scf_2R: 13550916-13569038 eight are in introns or untranslated regions,
291 including one in the long intron of *Cb53E*, three in the introns or noncoding transcript of *CG6805*, and
292 two in the introns of *Ephb*. Of the remaining five SNPs four are in coding regions but silent, causing no
293 change in the amino acid sequence of the protein. This includes silent mutations in the exons of *ste24c*
294 and two silent mutations in the exons of *Epbh*. One SNP located in an exon of *ste24a*, at 13558515, is an
295 amino acid substitution from a Leucine to a Valine. This is not an uncommon amino acid substitution[84,85],
296 though it can be associated with phenotypes[86,87]. Mutations in introns and untranslated regions could also
297 be having an effect on gene expression or processing, as could other linked SNPs in the region that were
298 not included in the analysis.

299

300 *Association between Wolbachia and mtDNA*

301 Association analysis was performed using plink by regressing the line means for mtDNA copy number
302 onto the *Wolbachia* genome and vice versa[75]. There was no association between *Wolbachia* SNPs and
303 mtDNA copy number, but the opposite was not true. One SNP in the *D. simulans* mtDNA affected
304 *Wolbachia* copy number at $p < 3.18 \times 10^{-6}$. It is located in the *D. simulans* homolog of *D. melanogaster*
305 *srRNA* which has been implicated in pole cell formation[88]. *Wolbachia* is incorporated into the pole cells,
306 the precursor to the germline, in order to be transmitted[89].

307

308 DISCUSSION

309 Using high through-put sequencing of a large panel of *D. simulans* we have reconstructed the complete
310 genome sequences of mtDNA and *Wolbachia*. We use these genome sequences to investigate the recent
311 history of *Wolbachia* and mtDNA in this population, as well as to estimate titre of both *Wolbachia* and
312 mtDNA. The history of *Wolbachia* in this population is reflected in the essentially star-like phylogeny of
313 both mtDNA and *Wolbachia*, indicating recent spread and co-inheritance. Lack of variation at mtDNA
314 and *Wolbachia* suggests a single spread of *w*Ri in this population as well as strict vertical transmission in
315 the maternal cytoplasm. Variation in *Wolbachia* is within the range expected under a neutral model,
316 however that was not the case for mtDNA which suggests either a selection sweep or a population
317 bottleneck. Previous studies found similar population genetic patterns at *Wolbachia* and mtDNA in *D.*
318 *melanogaster*, and thus could not distinguish whether selection on *Wolbachia* was driving similar patterns
319 in mtDNA or vice versa[31]. The much stronger pattern of negative Tajima's *D* in the mtDNA suggests that
320 in *D. simulans* selection is in fact mitochondrial. There was no linkage disequilibrium between *Wolbachia*
321 and mtDNA variants, however this is most likely due to fixation of a single mitochondrial haplotype
322 without considerable subsequent mutation.

323 Currently little is known about how *Wolbachia* interacts with its host[37-39,82,90]. Understanding these
324 interactions, including regulation of *Wolbachia* titre, will be key to understanding the evolution of

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

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

345 While it has been proposed elsewhere, the author is not aware of another association analysis of
346 *Wolbachia* and mtDNA copy number[32]. *Wolbachia* copy number is known to be affected by host
347 background, but the genes or mechanisms involved are not known[54,55,57]. The fact that four of the nine
348 genes found in the primary region detected in the association analysis are involved in CAAX-box protein
349 processing is of particular interest, given the history of this type of gene and intracellular pathogens.
350 CAAX-box protein processing is a part of a series of posttranslational protein modifications collectively

351 called protein prenylation which are required for fully functional proteins to be targeted to cell
352 membranes or organelles. Prenylated proteins include Ras, Rac, and Rho. However, it has been shown
353 that pathogenic bacteria can exploit the host cell's prenylation machinery[59]. For example, *Salmonella-*
354 *induced filament A* is a protein from *Salmonella typhimurium*, a gram-negative facultative intracellular
355 bacterium. *Salmonella-induced filament A* has a CAAX motif required for prenylation to occur, it was
356 shown to be processed by host prenylation machinery, and it is necessary for survival of the
357 bacterium[60,92,93]. *Legionella pneumophila* Ankyrin B protein exploits the host prenylation machinery in
358 order to anchor Ankyrin B protein to the membrane of the pathogenic vacuole[61]. Proliferation of
359 *Legionella pneumophila* requires Ankyrin B, as does the manifestation of Legionnaires disease. Ankyrin
360 repeat domains are most commonly found in eukaryotes and viruses, though they are rarely found in
361 bacteria and Archaea[94]. In bacteria they are found in a few obligate or facultative intracellular
362 Proteobacteria[59]. *Wolbachia* has an unusually high number of Ankyrin repeat domains with rapid
363 evolution[94]. Ankyrin proteins play a major role in host-pathogen interactions and the evolution of
364 infections[95,96]. There is no way to know from the current analysis if the Ankyrin repeat genes are
365 exploiting the host prenylation system but it is an intriguing area for future investigation. The results of
366 this association analysis suggest that some interaction between the pathogen and its host is targeting the
367 protein prenylation machinery.

368 There was also an association between a polymorphism in *srRNA,* which has been implicated in
369 pole cell formation[88], and *Wolbachia*/mtDNA copy number. Concentration of *Wolbachia* in the posterior
370 of the embryo, where pole cells are forming, is correlated with degree of cytoplasmic incompatibility[97]. *D.*
371 *simulans* has been shown to have nearly complete cytoplasmic incompatibility, though it is possible there
372 are mutations sorting at low frequency that affect this or that mitigate negative phenotypic consequences
373 of high *Wolbachia* titre. It has also been demonstrated that *gurken* is important in for *Wolbachia* titre in
374 the germline in *D. melanogaster*, and it is involved in pole cell formation beginning at an earlier stage
375 than *sr*RNA suggesting there could be an interaction between the two factors[88,90]. *D. simulans w*Ri has a
376 different distribution in the cytoplasm from other strains of *Wolbachia*, as it tends to evenly distribute

377 throughout the embryo while other strains are either concentrated at the posterior, or at the anterior of the

378 embryo away from the pole cells[97]. Future work in related species may show that these different

379 distributions also mitigate different interactions between host and symbiont, including being effected by

380 different genes and processes within the host.

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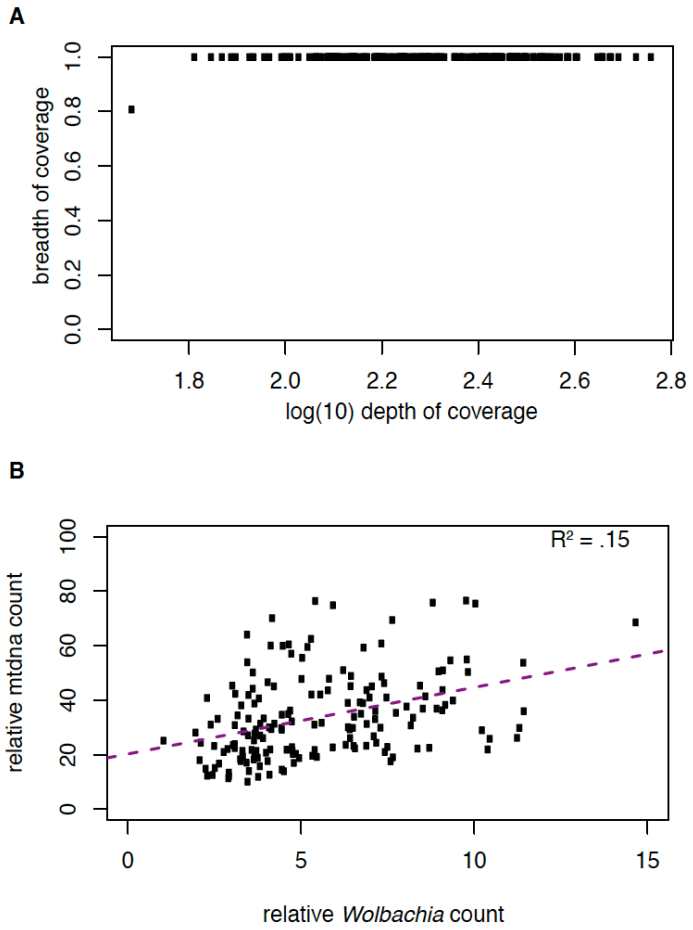

384

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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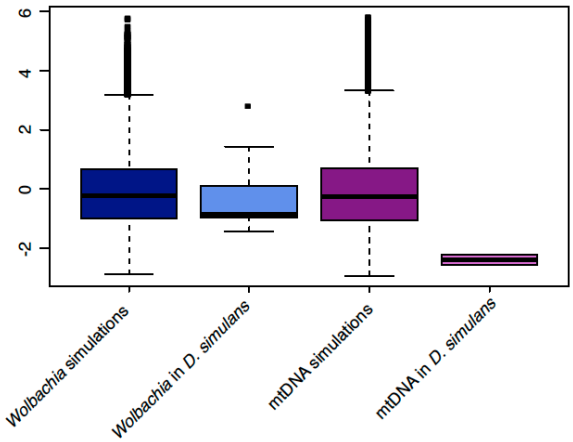
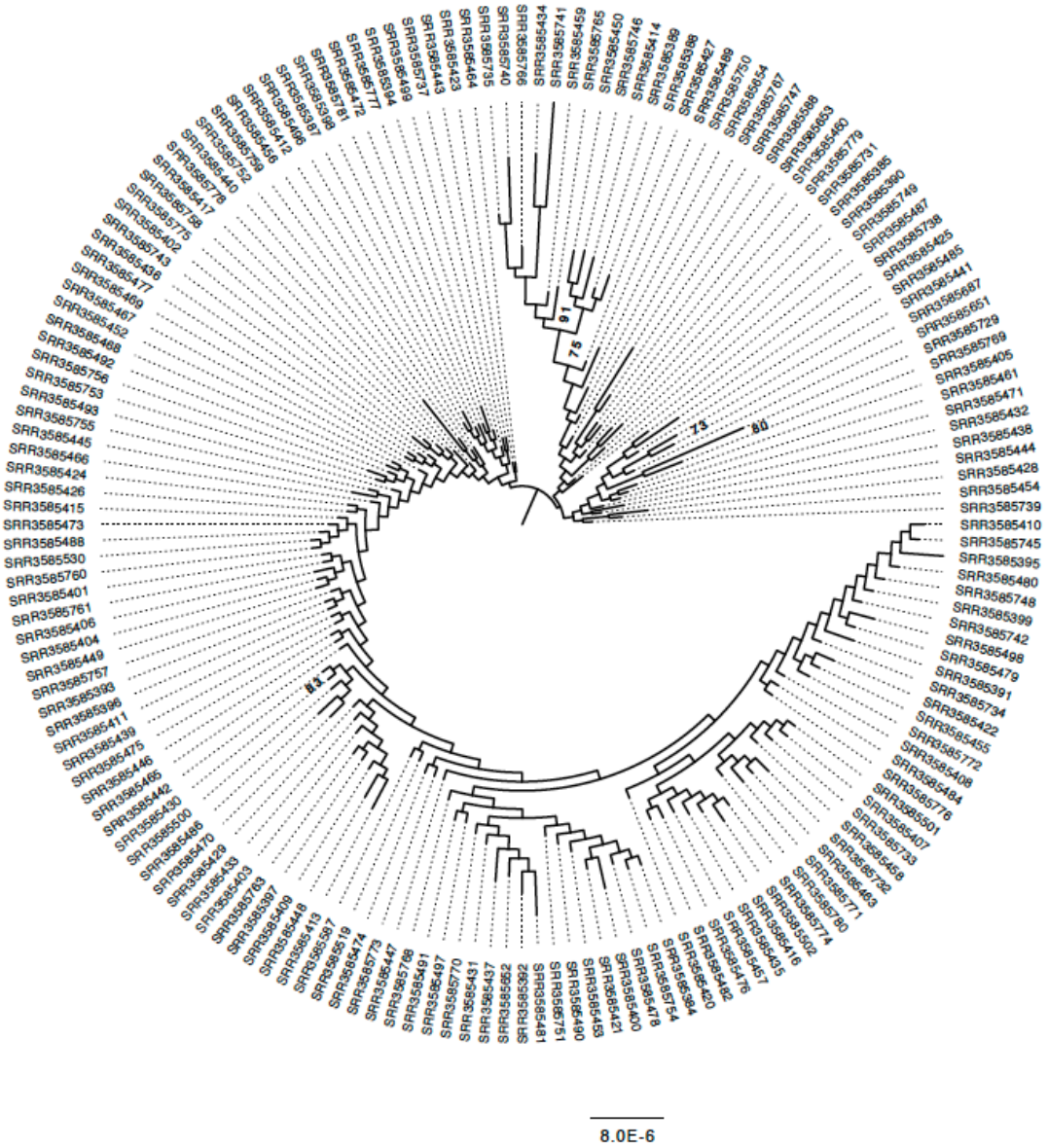


Figure 2. *Wolbachia* and mtDNA Tajima's *D*. 10,000 simulations were performed for *Wolbachia* and *D. simulans* each conditioned upon the number of polymorphisms. The actual values in *D. simulans* mtDNA are outside the 95% confidence interval of the simulations, while *Wolbachia* is not. There is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



392

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.

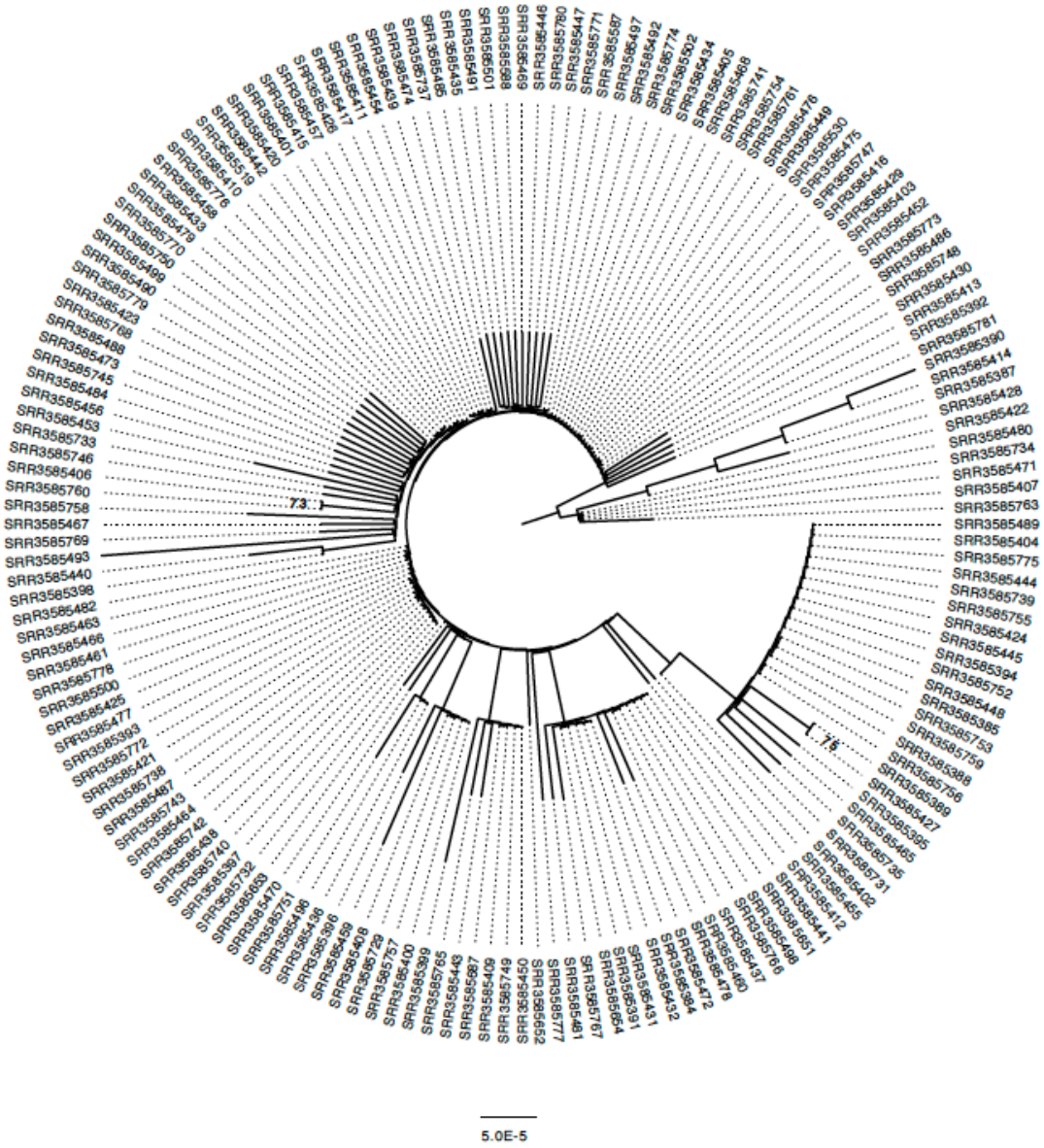


Figure 4: Maximum likelihood genealogy of the *D. simulans* mtDNA genome. The underlying data consist of an ungapped multiple alignment of 168 sequences of the entire mtDNA genome. The unrooted tree was midpoint rooted for visualization and branches with > 70% RAxML bootstrap support values are shown 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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