Fine-scale landscape genomics helps explain the slow spread of *Wolbachia* through the *Aedes aegypti* population in Cairns, Australia

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Abstract

The endosymbiotic bacterium *Wolbachia* is a promising tool for controlling arboviral diseases transmitted by the mosquito *Aedes aegypti*, and can spread unassisted through wild mosquito populations following local introductions. Recent *Wolbachia* introductions into *Ae. aegypti* populations in Cairns, Australia have produced a slower than anticipated spread that could be due to: i) barriers to *Ae. aegypti* dispersal; ii) dispersal being leptokurtically biased towards long distances in adults; and iii) non-perfect transmission of *Wolbachia* from mother to offspring. We investigated these three potential causes using double-digest RAD-sequencing and *Wolbachia* screening in 161 *Ae. aegypti* collected from Cairns in 2015. We detected a significant barrier effect of Cairns highways on *Ae. aegypti* dispersal using distance-based redundancy analysis (dbRDA) and patch-based simulation analysis. We detected putative full-siblings in ovitraps 1312 m apart, indicating the potential for long-distance movement in *Ae. aegypti* females that are generally considered weak dispersers. We also detected a pair of full-siblings, only one of which was *Wolbachia*-infected, indicating transmission failure of *Wolbachia* in the field. While the long-distance movement and *Wolbachia* loss currently represent single observations, these findings together with the identified dispersal barriers help explain the slow spread of *Wolbachia* through the *Ae. aegypti* population in Cairns. Our landscape genomics approach can be extended to other host/symbiont systems that are increasingly being considered for the biocontrol of disease vectors and pests.

Introduction

The mosquito *Aedes aegypti* (Diptera, Culicinae) is the primary vector of arboviral diseases such as Dengue, Zika and Chikungunya fever that pose an increasing burden to human health worldwide (Weaver and Lecuit, 2015; Bogoch *et al.,* 2016). The conventional approaches to combatting these diseases have involved the suppression of *Ae. aegypti* populations through source reduction or insecticide-based programs, but these have demonstrated a limited efficacy in combatting worldwide escalation of disease risk (Focks *et* al., 2000; Gubler, 2002). One of several alternative strategies involves the release of the virus-inhibiting, endosymbiotic bacteria *Wolbachia* into *Ae. aegypti* populations (McGraw and O'Neill, 2013). Once they are widespread in host populations, *Wolbachia* are expected to diminish the disease transmission rate enough to prevent outbreaks (Ferguson *et al.*, 2015). *Aedes aegypti* does not naturally carry *Wolbachia,* but several *Wolbachia* strains have been successfully transferred into *Ae. aegypti* from other hosts like *Drosophila melanogaster* (Walker *et al.*, 2011) and *Ae. albopictus* (Xi *et al.* 2005).

The *Wolbachia* strain *w*Mel, originating from *D. melanogaster*, has proven suitable for field deployments in *Ae. aegypti* given its substantial viral blockage (Ferguson *et al.*, 2015), relatively small fitness cost (Walker *et al.*, 2011), high transmission fidelity from mother to offspring (Hoffmann *et al.*, 2014) and complete cytoplasmic incompatibility (Blagrove *et al.*, 2013). Cytoplasmic incompatibility (CI) is a phenomenon where- offspring of uninfected females mated with *Wolbachia*-infected males are almost always unviable, and offspring of *Wolbachia*-infected females are viable regardless of the male infection status (Hoffmann and Turelli, 1997). Because CI greatly reduces the relative fitness of uninfected females when *Wolbachia*-infected males are common, it drives establishment of *Wolbachia* in isolated mosquito populations (Caspari and Watson, 1959). However, *Wolbachia* infection also imposes some fitness cost in the host, such as reduced larval competitive ability (Ross *et al.,* 2016). The interaction between costs and benefits produces a critical frequency of *Wolbachia* infection ($\hat{p}$)

53 that needs to be exceeded for *Wolbachia* to invade the mosquito population (Caspari and Watson,
54 1959; Hoffmann *et al.,* 1990; Turelli, 2010).

55 Releases of *w*Mel-infected *Ae. aegypti* into two sites near Cairns, Australia, have confirmed
56 that *Wolbachia* can establish stably in quasi-isolated habitat patches (Hoffmann *et al.*, 2011; 2014). A
57 subsequent study with releases centred within continuous mosquito habitat demonstrated the
58 successful spread of the invasion into surrounding habitat when releases were conducted over a large
59 enough area, so that the infection region grew ≈ 70 – 85% over the ensuing 18 months (Schmidt *et al.,*
60 2017). However, this rate of spread of approximately 100 – 200 m per year is slow compared with the
61 much more rapid spread observed in some natural infections of other insects of 100 km per year or
62 more (Kriesner *et al.* 2013; Turelli and Hoffmann, 1991), which could reflect lower fitness costs
63 associated with natural *Wolbachia* infections.

64 Slow spread of *w*Mel through *Ae. aegypti* in Cairns may be a product of various biological and
65 environmental factors (Barton and Turelli, 2011; Turelli and Barton, 2016), three of which are
66 investigated in this study: i) the presence of barriers to mosquito dispersal; ii) a leptokurtic dispersal
67 kernel skewed towards long-distance movement of *Ae. aegypti* adults; and iii) the occasional loss of
68 *Wolbachia* from mother to offspring (μ). Understanding the factors that produced slow *w*Mel spread
69 in Cairns is important for designing future *Wolbachia* release strategies that maximise the area invaded
70 while minimising both the cost and the time taken to achieve invasion.

71 This study evaluates dispersal barriers and leptokutically-biased dispersal, as well as loss of
72 *w*Mel through maternal transmission, in the *Ae. aegypti* population from Cairns. We screened *Ae.*
73 *aegypti* collected from Cairns in 2015 for the *w*Mel transinfection and genotyped individuals at
74 genome-wide single nucleotide polymorphisms (SNPs) using double-digest restriction-site associated
75 DNA sequencing (ddRADseq; Peterson *et al.,* 2012). SNP datasets produced with ddRADseq have been
76 used to elucidate genetic structure in *Ae. aegypti* at broad geographic scales (Rašić *et al.,* 2014a; 2014b;

77 2015a; 2015b) as well as within cities (Rašić *et al.,* 2015a; 2015b), and have power superior to
78 microsatellites when inferring relationships between *Ae. aegypti* individuals and populations (Rašić *et*
79 *al.,* 2014a).

80 Dispersal of *Ae. aegypti* by flight is generally thought to be limited to short distances, and
81 highways have been proposed as likely barriers to *Ae. aegypti*'s dispersal. For example, patches
82 separated by a 120 m-wide highway in Trinidad were found to have different frequencies of
83 mitochondrial haplotypes (Hemme *et al.*, 2010). A Mark-Release-Recapture (MRR) study in Cairns with
84 releases centred next to a ≈ 20m-wide road recorded lower recapture rates at sites across the road
85 (Russell *et al.,* 2005). Indirect inference of an inhibitive effect of highways on dispersal was made from
86 the observed dynamics of *Wolbachia* invasions in the vicinity of highways. Namely, *Wolbachia* failed
87 to invade a region across a highway in Gordonvale, Queensland after several years (Turelli and Barton,
88 2016), and similar dynamics were recently observed in urban Cairns (Schmidt *et al.,* 2017). Here we
89 explicitly tested the hypothesis that *Ae. aegypti's* genetic structure in Cairns is affected by highways
90 acting as barriers to dispersal. This was tested against the alternative hypotheses of isolation-by-
91 distance (IBD; Wright, 1943) and a pattern of patchy and asynchronous releases of *Wolbachia*-infected
92 mosquitoes in the region.

93 If host dispersal distances follow a leptokurtic distribution, *Wolbachia* spread is expected to
94 be slower than if the distribution is Gaussian (Turelli and Barton, 2016). This is because under
95 leptokurtic dispersal, long-distance migrants with *Wolbachia* infection will be too infrequent in a new
96 location to be able to initiate a *Wolbachia* spread. MMR studies have produced a wide range of
97 estimates for female flight distance in *Ae. aegypti*, ranging from 50 - 100m (McDonald, 1977; Muir and
98 Kay, 1998; Harrington *et al.,* 2005; Russell *et al.,* 2005; Maciel-de-Freitas *et al.,* 2007; 2010) to over 800
99 m within a single gonotrophic cycle (Shannon and Davis, 1930; Reiter *et al.,* 1995; Honório *et al.,* 2003).
100 *Aedes aegypti* females lay batches of eggs through multiple acts of oviposition (Reiter, 2007) and over

multiple gonotrophic cycles (Christophers, 1960). This means that full-siblings can be found at different sites. The distance of separation between sampled full-siblings is indicative of female flight ranges (or passive movement such as following entry into vehicles) within one or two gonotrophic cycles. In this study we can compare the distance between full-siblings to those previously obtained from the MRR studies with fluorescent dust marking.

A slower than anticipated and heterogeneous *Wolbachia* spread, such as that recorded in Cairns, Australia (Schmidt *et al.,* 2017), can also result from a non-perfect vertical transmission of *Wolbachia.* The loss of *w*Mel during maternal transmission has not been detected in laboratory populations of *Ae. aegypti* (μ = 0) (e.g. Walker *et al.,* 2011; Ross *et al.,* 2016). However, recent studies showed that laboratory populations subjected to high fluctuating temperatures exhibited considerable decreases in *w*Mel infection density (Ross *et al.* 2017, Urlich *et al*. 2016), and this could lead to reduced transmission fidelity of the infection to offspring (Clancy and Hoffmann, 1998; Ikeda *et al.,* 2003). We looked for evidence of maternal transmission failure in *Ae. aegypti* from Cairns (μ > 0) by comparing the infection status of all individuals belonging to the same matrilineage. If some members of a single matrilineage have *w*Mel infection and others do not, we can assume that individuals without the *w*Mel infection have failed to inherit it.

Methods

Study site and sample collection

We deployed 110 ovitraps within the properties of consenting householders in Cairns, Australia between 13 and 16 April, 2015. The traps covered a 3300 x 1900 m region of central Cairns, which we partitioned into 6 “plots” for reference: Cairns North West (CNW), Cairns North East (CNE), Parramatta Park North (PPN), Parramatta Park South (PPS), Westcourt (WC) and Bungalow (BN) (Fig 1). Partitions

were established based on geographic location, location of highways, and the release history of *w*Mel. Each of these had three possible assignments: the locational groupings of Cairns North [CNW, CNE], Parramatta Park [PPN, PPS] and Westcourt/Bungalow [WC, BN]; the highway groupings of southeast of Bruce Highway [PPS, BN], west of both highways [CNW, PPN, WC], or northeast of Captain Cook Highway [CNE]; and the *Wolbachia* release groupings of releases in 2013 [PPN, WC], releases in 2014 [CNW, BN], or no history of releases [CNE, PPS] (see Schmidt *et al.,* 2017).

Within each plot, ovitraps were deployed in a quasi-random pattern. at bungalows, two-storey Queenslander-style houses, multi-storey apartment complexes and local businesses. Our sampling period of mid-April was at the end of the region's monsoonal wet season with high abundance of *Ae. aegypti*. Each ovitrap consisted of a black plastic bucket filled halfway with an infusion of water and alfalfa (lucerne) pellets to attract gravid female *Ae. aegypti* (Ritchie, 2001), which oviposit on strips of red felt clipped to the bucket and extending into the liquid. With a generation time of > 14 days in *Ae. aegypti* (Christophers, 1960), we allowed only a brief window of time for oviposition to ensure that our sampling did not spread over multiple mosquito generations. Traps were left in place for 5 - 7 days, then the felt strips were removed and dried. Dried strips of mosquito eggs were transferred into the laboratory and hatched by immersing all strips from each trap into vessels filled with reverse osmosis water, with 2 – 3 grains of yeast and one quarter of a crushed tablet of TetraMin tropical fish food (Tetra, Melle, Germany). The water, food and yeast were replaced after three days. Emerging virgin adults were transferred to freezing ethanol and stored at -20°C until DNA extraction.

Genomic DNA was extracted using Roche DNA Isolation Kit for Cells and Tissues (Roche, Pleasanton, CA, USA), with an additional step of RNAse treatment. Of the 110 ovitraps deployed, 74 produced adult *Ae. aegypti*, from which we selected 161 individuals for sequencing. As we expected ovitraps to contain many full-siblings from the same oviposition (Apostol *et al.,* 1993; Hoffmann *et al.,* 2014; Rašić *et al.,* 2014a), which can bias analyses of population structure (Goldberg and Waits,

2010), we limited the number of samples per ovitrap to three individuals. This ensured that, after removing closely related individuals, we retained a large enough sample for a powerful analysis of genetic structure.

SNP discovery

Double-digest RADseq library preparation

We applied the method of Rašić *et al.,* (2014a) for our double-digest RAD-seq (ddRAD-seq) library preparation, but selected a smaller size range (350–450 bp) of genomic fragments to accommodate a larger number of mosquitoes per library. An initial digestion of 100 ng of genomic DNA from each individual was performed in a 40 µL reaction, using 10 units each of *NlaIII* and *MluCI* restriction enzymes (New England Biolabs, Beverly MA, USA), NEB CutSmart® buffer, and water. Digestions were run for 3 hours at 37°C with no heat kill step, and the products were cleaned with 60 µL Ampure XP™ paramagnetic beads (Beckman Coulter, Brea, CA). These were ligated to modified Illumina P1 and P2 adapters overnight at 16°C with 1000 units of T4 ligase (New England Biolabs, Beverly, MA, USA), before undergoing heat-deactivation at 65°C for 10 minutes.

Size selection of fragments was performed using a Pippin-Prep 2% gel cassette (Sage Sciences, Beverly, MA). Final libraries were created by pooling eight 10 µL PCR reactions per library, each consisting of 1 µL size-selected DNA, 5µL of Phusion High Fidelity 2× Master mix (New England Biolabs, Beverly MA, USA) and 2 µL of 10 µM standard Illumina P1 and P2 primers, run for 12 PCR cycles. These were cleaned and concentrated using an 0.8× concentration of Ampure XP™ paramagnetic beads (Beckman Coulter, Brea, CA) to make the final libraries. Three libraries containing a total of 161 *Ae. aegypti* were sequenced in three Illumina HiSeq2500 lanes using 100 bp paired-end chemistry.

171 *Sequence data processing*

172 Raw fastq sequences were processed within a customized pipeline (Rašić *et al.,* 2014a), with reads
173 filtered based on a minimum phred score of 13 and trimmed to the same length of 90 bp. High-quality
174 reads were aligned sequentially to the *Ae. aegypti* reference nuclear genome AaegL1 (Nene *et al*.,
175 2007), and the *Ae. aegypti* reference mitochondrial genome (Behura *et al*. 2011), using the program
176 Bowtie (Langmead *et al.,* 2009). We allowed for up to three mismatches in the alignment seed, and
177 uniquely aligned reads were analysed with the Stacks pipeline (Catchen *et al.,* 2013), which we used
178 to call genotypes at RAD stacks of a minimum depth of five reads.

179 The Stacks program *populations* was used to export VCF files that were then further
180 manipulated with the program VCFtools (Danecek *et al.,* 2011). We first removed individuals with >
181 20% missing data, leaving 134 individuals. We then extracted the 42,183 SNPs that were present in at
182 least 75% of individuals. We applied further filtering to retain only those loci that were at Hardy-
183 Weinberg Equilibrium and with minor allele frequencies of ≥ 0.05. To avoid using markers in high
184 linkage disequilibrium, data were thinned so that no single SNP was within 250 kbp of another. As
185 *Aedes* genome is thought to contain approximately 2.1 megabases per cM (Brown *et al.*, 2001), 250
186 kbp roughly corresponds to eight SNPs per map unit, a sampling density that has been shown to largely
187 eradicate the effects of linkage in SNPs (Cho and Dupuis, 2009). Our final dataset had 3,784 unlinked
188 and informative SNPs for analyses of relatedness and genetic structure.

189

190 **Assessing barriers to dispersal**

191 *Landscape resistance modelling*

192 We tested the hypotheses that highways had a significant effect on *Ae. aegypti* genetic structure in
193 Cairns (H_1), and that genetic structure was significantly affected by recent releases of *Wolbachia* (H_2).

For H_1, we considered each individual as occupying a position southeast of Bruce Highway, west of both highways, or northeast of Captain Cook Highway (Fig 1), and from this assigned them a score of [0, 1, 2] respectively, so that the distance between two individuals' scores represented the number of highways between them; we called this variable "Highways". We approached H_2 similarly, but with the distances representing the time since *Wolbachia* releases were commenced in the area (Fig 1). Thus each individual also received a score of [0, 1, 2], depending on whether the plot it occupied underwent releases in 2013, 2014 or had never been treated; we called this variable "Releases". Plots with 2013 releases were considered more 'distant' from those never experiencing releases than those with 2014 releases, as potential structuring effects such as selection or reductions in diversity would presumably display a greater effect over time. Treating the geographical or temporal separation of individuals as additive variables analogous to landscape resistance surfaces avoids issues associated with detecting discrete barriers (Landguth *et al.,* 2010). Analyses of population structure are generally thought to be biased when full-siblings are included in analyses (Goldberg and Waits, 2010; Porras-Hurtado *et al.,* 2013), though specific testing of this effect has produced ambiguous results (Peterman *et al.,* 2016). For the distance-based redundancy analyses (dbRDA, Legendre and Anderson, 1999), we sampled one individual from each group of full-siblings with the smallest percentage of missing data, retaining 100 individuals.

We performed partial dbRDA to test H_1 and H_2 as explicit hypotheses, while controlling for the potentially confounding effects of IBD and latitudinal and longitudinal clining. We placed potentially confounding variables (Easting and Northing UTM coordinates and a binary variable representing *Wolbachia* infection status for each individual) inside a conditional matrix. The dependent variable was a distance matrix of Rousset's *a* scores (Rousset, 2000) calculated for each pair of individuals using the program SPAGeDi.

All remaining model procedures were performed in the package VEGAN (Oksanen *et al.*, 2007). The dbRDA models were built using the function *capscale*. For all models, we applied the effects of the conditional matrix described above, and assessed the significance of "Highways" and "Releases". We built three models: one implementing both predictor variables and the other two implementing each variable in isolation. We assessed the statistical significance of predictor variables with the function *anova.cca,* using 99999 permutations to test for the marginal significance of each term after accounting for the effects of the others. The entire procedure was then repeated with the binary variable representing *Wolbachia* infection status removed from the conditional matrix and used as a predictor variable alongside "Highways" and "Releases". While constructing models, we calculated variance-inflation factors (VIF) to check for multicollinearity between predictor variables, using the function *vif.cca*. All VIFs were < 1.1, so none were rejected.

We tested the sensitivity of the variables used to investigate H_1 and H_2 by performing additional dbRDAs that followed the above procedure but with modifications made to "Highways" and "Releases". In each case, instead of a given barrier being 100% the strength of the other, it was assigned strengths of 150% and 200%. Thus, instead of scores of [0, 1, 2] for a given variable, these scores were [0, 1.5, 2.5] or [0, 1, 2.5] for strengths 150%, and [0, 2, 3] or [0, 1, 3] for strengths 200%.

Type I error testing

Although ordination methods such as dbRDA are thought to detect genetic structure with greater power than traditional Mantel tests (Legendre and Fortin, 2010; Cushman *et al.,* 2013), they also suffer from elevated risk of Type I error (Kierepka and Latch, 2015a). Specifically, Type I errors can be made if hypotheses of geographical structure are proposed against only null hypotheses of panmixia, rather than against alternative geographical hypotheses (Cushman *et al.,* 2006; Cushman and Landguth, 2010). We considered IBD an alternative hypothesis of genetic structure. A Mantel test performed with

241 the function *mantel* in the R package VEGAN showed slight correlation between geographical and
242 genetic distances ($r = 0.047$, $P < 0.05$), and high correlations between geographical distance and both
243 "Highways" ($r = 0.424$, $P < 0.001$) and "Releases" ($r = 0.305$, $P < 0.001$). When genetic structure exhibits
244 IBD, spatial dependence of barrier variables and inadequately extensive sampling can lead to
245 significance being observed when no barrier effect exists (Kierepka and Latch, 2015b). Therefore, in
246 order to confirm any significance observed among variables in dbRDA, further analyses were required
247 to eliminate IBD as a cause of genetic structure.

248 We adapted the method of Kierepka and Latch (2015b) to evaluate whether the spatial
249 distribution of our traps relative to barriers would lead to a high risk of Type I error. We used CD-POP
250 to simulate the field site without any highways, *Wolbachia* infections, or mosquito release histories,
251 thus creating an artificial environment in which only IBD could explain the pattern of structuring. We
252 designated 1000 locations as the positions of individual mosquitoes, which included the locations of
253 the 100 individuals used in dbRDA and 900 new locations placed at random throughout our field site.
254 We used parameters coding for high recruitment and high mortality, and negative exponential
255 dispersal approximating a leptokurtic dispersal kernel with no maximum limit to dispersal, but where
256 dispersal over 300 m was relatively rare. Dispersal was unbiased by sex, given that *Ae. aegypti* males
257 generally disperse more readily early in their lives, and females tend to travel greater distances over
258 their lifetimes (Sheppard *et al.,* 1969; McDonald, 1977). The CD-POP input file listing all relevant
259 parameters is supplied in Supplementary Information A.

260 We allowed CD-POP to generate genotypes for each individual. As this process sets initial allele
261 frequencies so that populations are initially at maximum genetic diversity, we used fewer loci than the
262 3,784 in our data set so that the real and simulated data would have similar numbers of effective
263 alleles. Effective allele counts have been found to correspond well with analytical power across
264 different types of genetic data (Wang, 2006; Hauser *et al.,* 2011). We used the method of Kimura and

Crow (1964) to calculate the number of effective alleles in our 3,784 loci set, which gave 5,618 effective alleles, corresponding to 2,809 biallelic loci at maximum diversity.

We constructed 100 simulations and ran each for 80 discrete generations, after which we sampled the genotypes of the mosquitoes now present at the original 100 locations. The number of generations was chosen iteratively, to achieve similar values of the Mantel correlation coefficient in the simulated and empirical datasets ($r_{simulated}$, $\bar{x}$ = 0.082, σ = 0.022, all $P < 0.05$; $r_{empirical}$ = 0.047, $P < 0.05$). For each sample we constructed new dbRDA models with the same parameters as the observed data, and calculated the marginal significance of "Highways" and "Releases" with ANOVAs. We considered that more than 5 of the 100 simulated samples showed significance for a variable is an indication of an elevated risk of Type I error.

Assessing the long-distance host movement and loss of *Wolbachia* infection

Estimating relatedness/kinship

We used the program SPAGeDi (Hardy and Veckmans, 2002) to calculate Loiselle's *k* (Loiselle *et al.,* 1995) among individuals. First degree kin relations (full-sibling or parent/offspring) can be ascertained with only hundreds of SNPs (Tokarska *et al.,* 2009; Cramer *et al.,* 2011; Sellars *et al.,* 2014; Weinman *et al.,* 2015), and with the thousands of SNPs available through ddRADseq this confidence can be extended to include second-degree relations (half-sibling or grandparent/grandchild) (Rašić *et al.,* 2014a; Bateson *et al.,* 2016; Munshi-South *et al.,* 2016; Shultz *et al.,* 2016). Considering that our cross-sectional study design provided a period for sampling (7 days) shorter than that required for mosquito development (> 14 days; Christophers, 1960), we assumed that related individuals were of the same generation, and thus pairs with first degree levels of relatedness were considered to be full-siblings and those with second degree relatedness were considered half-siblings.

288 The estimated pairwise kinship $k > 0.1875$ represented putative full-siblings, and $0.1875 > k >$
289 0.09375 represented putative half-siblings, with each pair being assigned the most likely kinship
290 category (Iacchei *et al.,* 2013). We considered half-siblings to be paternal because polyandry is much
291 rarer than polygyny in wild *Ae. aegypti* (Richardson *et al.,* 2015). Therefore, we assumed full-siblings
292 to come from the same matrilineage, and half-siblings from different matrilineages.

293 As our investigations of long-distance mosquito movement and *Wolbachia* loss both require
294 precise assignment to kinship categories, we also used the program ML-Relate (Kalinowski *et al.*, 2006)
295 to run specific hypothesis tests of putative relationships. These were run for all pairs of putative full-
296 siblings with different infection statuses, and for all individual pairs with $k > 0.09375$ collected across
297 different ovitraps. For each pair we ran one standard test that estimated the relationship assuming
298 that the kinship category assigned using *k* was more likely than the next most likely kinship category,
299 followed by a conservative test that assumed that the kinship category assigned using *k* was less likely
300 to be correct. All tests were run using 10,000,000 simulations.

301 We estimated the distance of separation between full-siblings as an indication of female
302 movement range. Full-siblings found within a single ovitrap would likely be from the same gonotrophic
303 cycle, while those further apart would likely be from one or two gonotrophic cycles. The distance of
304 separation between half-siblings is more difficult to characterise, but could reflect the total movement
305 of the male and two females involved. Although passive dispersal of mosquitoes by humans is usually
306 thought to occur at the egg rather than adult stage, we could not rule out human interference in the
307 dispersal of adults. Importantly, the distinction between active and passive adult dispersal is irrelevant
308 for the purpose of our study, given that both types contribute to a *Wolbachia* invasion. Our measure
309 of flight range is therefore a composite of active mosquito flight and potential passive movement, and
310 is more informative for understanding the *Wolbachia* invasion success than active dispersal alone.

311

312 *Wolbachia infection screening*

313 Mosquitoes were screened for *Wolbachia* using the protocol of Lee *et al.* (2012). The infection
314 is diagnosed with polymerase chain reactions (PCR) run on the Roche LightCycler® 480 system (384-
315 well format). For each mosquito, PCR was performed using three primer sets, *Aedes* universal primers
316 (*mRpS6*_F/*mRpS6*_R), *Ae. aegypti*-specific primers (*aRpS6*_F/*aRpS6*_R) and *Wolbachia*-specific
317 primers (*w1*_F/*w1*_R). A sample was scored as *Wolbachia* positive when there was robust
318 amplification of all three primer sets *mRpS6*, *aRpS6* and *w1,* while an *Ae. aegypti* sample that was
319 *Wolbachia* negative amplified only *mRpS6*, and *aRpS6.* Each PCR was run using three positive
320 *Wolbachia* controls and three negative *Wolbachia* controls. *Wolbachia* titre, defined as the ratio of
321 *Wolbachia* gene copies to host gene copies, was estimated using $2^{[\mathrm{cp(A)}-\mathrm{cp(W)}]}$, where cp(A) is the
322 crossing point of the *aRpS6* marker and cp(W) is the crossing point of the *w1* marker (Lee *et al.*, 2012).

323 To provide a comparison between lab-raised and field-raised mosquitoes, we also assayed 23
324 field-caught adult mosquitoes from Gordonvale, Australia. This small town 23 km south of Cairns was
325 subjected to intensive *Wolbachia* releases in 2011 (c.f. Hoffmann *et al.,* 2011). The sample was taken
326 in January 2013, after the *Wolbachia* invasion had successfully established and remained at near-
327 fixation (Hoffmann *et al*., 2014). Adult mosquitoes were collected using BG-Sentinel traps (Biogents
328 AG, Weissenburgstr. 22, 93055, Regensburg, Germany), and were processed and assayed in a manner
329 identical to those from Cairns. Field-caught adult Gordonvale mosquitoes were expected to exhibit
330 broader variation in age and physiological state (Williams *et al.,* 2006) compared with our Cairns
331 sample.

332 For each individual from Cairns and Gordonvale we performed three PCR replicates to confirm
333 its *w*Mel infection status. No inconsistencies in assigning *Wolbachia* infection status were observed
334 across replicates of samples and controls, and similar titres were also observed across replicates (single
335 factor ANOVA *F*-value = 0.341, *P* = 0.711). Within individuals, only 11.3% had a maximum titre more

than twice the magnitude of their minimum titre. In comparison, the lowest-scoring replicate from the individual with the highest mean titre had a titre 86.8 times larger than the highest-scoring replicate from the infected individual with the lowest mean titre. Considering the relative consistency of titres across replicates, we used the mean of the three scores to attain final estimates of *Wolbachia* titre for each individual.

From our *Wolbachia*-infection screening and relatedness analysis, we identified *w*Mel-infected matrilineages as being any group of full-siblings containing at least one individual infected with *Wolbachia*. An uninfected individual within an infected matrilineage was considered to have either failed to inherit the infection from its mother or to have lost it during development. We estimated the rate of infection loss per generation (μ) by comparing the number of these individuals with the total number of full-siblings from infected matrilineages.

Results

Barriers to dispersal

Landscape Resistance Modelling and Type I Error Assessment

ANOVAs performed on partial dbRDA models showed that only the "Highways" variable was predictive of genetic structure (Table 1; $1.55 \leq$ F-value ≤ 1.64; $P < 0.05$). "Highways" was a significant effect regardless of "*Wolbachia* infection" being included or not. Based on the partial Eta squared (η_p^2, Tabachnick and Fidell, 1996), this variable explained 1.7% of the variance in each model. Supplementary Information B describes additional analyses of genetic structure among groups, with results that are in accordance with these findings. Of the 100 CD-POP simulations only two showed significant structuring by "Highways" at the $P < 0.05$ level, and inclusion of the "Releases" variable in

358 the models did not change this observation. This suggests that the effect of "Highways" observed in
359 the real data is unlikely a Type I error caused by sampling bias or residual autocorrelation.

360 Sensitivity analyses of "Highways" and "Releases" showed similar results for different relative
361 resistances. Increasing the relative barrier strength of Captain Cook Highway to 150% increased the
362 variance explained in each model to 1.82%, and increasing it to 200% increased the variance explained
363 to 1.90%. Conversely, increasing the relative barrier strength of Bruce Highway to 150% and 200%
364 decreased the variance explained to 1.59% and 1.54% respectively, and the variable was not
365 statistically significant at 95% confidence when Bruce Highway was 200% the barrier strength of
366 Captain Cook Highway. Sensitivity analyses of "Releases" showed no statistical significance for any
367 variation in relative strengths.

368 Although an effect size of η_p^2 = 1.7% suggests only a slight reduction in dispersal across
369 highways, *Wolbachia* invasions with bistable dynamics are particularly sensitive to barriers (Barton and
370 Turelli, 2011) and dispersal does not need to be completely disrupted to slow or stop an advancing
371 wave (Turelli and Barton, 2016). To demonstrate this, we used a patch-based simulator derived from
372 Nemo2 (Guillaume and Rougemont, 2006) and empirical data from Schmidt *et al*. (2017) to simulate
373 the spread of *w*Mel in the absence of barriers. Parameters, methodology and results are detailed in
374 Supplementary Information C. Our simulations showed that the patches separated by highways that
375 do not pose any barriers to dispersal tended to have higher *w*Mel frequencies than what was observed
376 in the field (Schmidt *et al*. (2017). The average overestimation in simulations was 6.5%. Importantly,
377 no discrepancy between the simulated and field data (i.e. overestimation in simulations) was detected
378 for patches that are not separated by highways. Overall, simulation of the *Wolbachia* invasion progress
379 south of Bruce Highway (Supplementary Information C) indicated that barrier strength corresponded
380 to an added 30 - 35 m of separation.

381

382 ***Estimates of Long-distance movement***

383 We detected 31 putative full-sibling groups that contained 43 full-sibling pairs, 41 of which were found
384 within single ovitraps. Two pairs were spread between different plots (Fig 2): one was split between
385 CNW and CNE (239 m separation, k = 0.203); the other between CNW and PPS (1312 m, k = 0.235).
386 Standard maximum-likelihood simulations gave strong support to both pairs being full-siblings (both P
387 < 0.0001). However, conservative simulations were unable to reject the hypotheses that either pair
388 represented half-siblings (P < 0.0001 and P < 0.02 respectively), but rejected them as unrelated (both
389 P = 1).

390 Eight of the 27 putative half-sibling pairs ($0.0989 \leq k \leq 0.187$) were found across multiple traps
391 (47 - 560 m separation), though none of these were located in different plots. There was a gap in k
392 scores (Δk = 8.6%) between the most closely related half-siblings (k = 0.187) and the most distantly
393 related full-siblings (k = 0.203), indicating that the cut-off point in relatedness between full-siblings and
394 half-siblings (k = 0.1875) is valid. This 8.6% difference between the two categories is much greater than
395 the difference between the top two half-sibling pairs (Δk = 0.9%) and the bottom two full-sibling pairs
396 (Δk = 2.9%). However, conservative maximum-likelihood simulations could not assign these pairs
397 exclusively to one or the other kinship category. Of the eight putative half-sib pairs found in different
398 traps ($0.0989 < k < 0.1412$), only the most closely related pair (k = 0.1412, 54 m separation, P < 0.07)
399 was confidently assigned as half-siblings and not assigned as unrelated using conservative maximum
400 likelihood estimation.

401

402 ***Loss of Wolbachia***

403 Triplicate runs of real-time PCR assay for *w*Mel detection indicated that 60 out of 161 individuals from
404 Cairns were infected with *Wolbachia.* The six samples from PPN were all infected, none of PPS and CNE

were infected; and the remaining plots had a mixture of infection rates (Fig 1). All individuals caught in the 2013 Gordonvale sample were infected with *w*Mel. *Wolbachia* titre scores in Gordonvale ($\bar{x}$ = 18.4, σ = 10.8) were almost three times higher than those of Cairns ($\bar{x}$ = 6.6, σ = 3.8; t-test with unequal variances, P < 0.001), but were also three times as variable (Fig S1). One infected mosquito from Gordonvale had a titre of just one gene copy for every three *Aedes* gene copies, while the lowest titre in Cairns was more than three times this amount. The highest titre in Gordonvale was 56 *Wolbachia* gene copies for each *Aedes* gene copy, more than double the highest recorded in Cairns (Fig S1).

We detected 10 full-sibling groups from the *Wolbachia*-infected matrilineages (containing 21 individuals) and 21 full-sibling groups from uninfected matrilineages. Importantly, we recorded a single case of infection loss in CNW. The sample from this matrilineage consisted of a pair of putative full-siblings (*k* = 0.376), one of which carried the infection (titre = 6.15) and one of which did not. Maximum likelihood simulation provided strong support for the hypothesis that this pair represented full-siblings (P < 0.0001) and subsequently rejected the hypotheses that they were either half-siblings or unrelated (both P = 1). We calculated a tentative probability of infection loss among offspring within infected matrilineages, giving a likelihood of loss of one in 21 (μ = 0.048), although the 95% binomial confidence intervals around this estimate were very wide (0.001, 0.238).

Discussion

Our study produced three main findings: i) highways exert a small but significant influence on *Ae. aegypti* genetic structure and the expected spread of *Wolbachia;* ii) some *Ae. aegypti* females may oviposit at distances spanning > 1 km; and (iii) the *w*Mel infection in *Ae. aegypti* in Cairns may show <100% maternal transmission rate (μ > 0). Each phenomenon is expected to have a slowing effect on *w*Mel invasion (Barton and Turelli, 2011; Turelli and Barton, 2016). Because ii) and iii) were based on

428 single observations, we cannot confidently predict how common they are among *Ae. aegypti* in Cairns.
429 However, field observations of a slow spread of *w*Mel through Cairns (Schmidt *et al*., 2017; Turelli and
430 Barton, 2016) are congruent with some long-distance movement operating in Cairns and may also
431 reflect occasional imperfect transmission.

432

433 *Major Cairns roads act as dispersal barriers*

434 While previous population genetic studies suggested that major roads could be barriers to *Ae. aegypti*
435 dispersal (e.g Hemme *et al.,* 2010), we provide the first evidence for such an effect through explicit
436 hypothesis testing within a landscape genetics framework. We detected a minor but statistically
437 significant barrier effect of highways, corresponding to 1.7% of dbRDA variance in genetic distance
438 between individuals. Our simulations of the *Wolbachia* invasion progress south of Bruce Highway
439 (Supplementary Information C) showed that barrier strength corresponded to an added 30-35 m of
440 separation. The *w*Mel invasion observed at PP was slow (100-200 m per year) (Schmidt *et al.,* 2017)
441 with the infection frequencies at the wave front being only slightly above the critical threshold ($\hat{p} \approx$
442 0.35 for *w*Mel in *Ae. aegypti* (Turelli and Barton, 2016)). Therefore, an added "cost" to cross the
443 highway could prove to be influential in halting the invasion. If the restrictive effect of highways on
444 dispersal increase with highway width and traffic levels, then many urban highways in cities earmarked
445 for future *w*Mel releases would likely be effective barriers to spread.

446 On the other hand, the restrictive effects of highways on dispersal can strengthen the invasion
447 within the area they enclose. The habitat patches along these highway boundaries will have increased
448 infection frequencies relative to patches in regions that are not subdivided, and could fortify the local
449 *Wolbachia* frequency from the counteracting effect of uninfected immigrants. Following the 2013
450 *Wolbachia* releases in Cairns, the largest and most successfully invaded release site at Edge
451 Hill/Whitfield recorded a large influx of uninfected *Ae. aegypti* at the start of the 2014/2015 wet

452 season (Schmidt *et al.,* 2017). This was likely in part due to the greater connectivity of Edge
453 Hill/Whitfield with surrounding uninfected regions. Prospective release sites in areas with such
454 characteristics should be positioned adjacent to dispersal barriers such as highways, as this may help
455 reduce the threat of reinvasion of uninfected mosquitoes. Additionally, the slow rate of spread (100-
456 200 m/year, Schmidt *et al.,* 2017) observed at Cairns emphasizes the need to focus on area-wide
457 releases rather than rapid regional invasions, and denser release points even in regions that are not
458 subdivided (Turelli and Barton, 2016).

459

460 *Movement range of* Aedes aegypti *in Cairns*Analyses of local patterns of kinship have provided several
461 useful inferences regarding *Ae. aegypti* ecology. We have recorded two pairs of probable full-siblings
462 ($k > 0.18375$, 28% larger than the category boundary) that were 1312 m apart, This suggests a single
463 female moving 1312 m between oviposition events, which represents either the extreme end of the
464 flight range in *Ae. aegypti* or some combination of active and passive dispersal. Previous maximum
465 dispersal estimates of gravid *Ae. aegypti* females have been less than a kilometre in a single
466 gonotrophic (egg producing) cycle (Reiter *et al.,* 1995; Honório *et al.,* 2003). In our study, seven days
467 passed between deployment and sampling of the two ovitraps (1312 m apart), which is long enough
468 for an additional unobserved egg deposition somewhere between them (Christophers, 1960). If we
469 assume this distance to reflect active dispersal, an additional gonotrophic cycle is likely. The length of
470 a single such cycle is rarely more than a few days, particularly when temperatures exceed 30°C
471 (Christophers, 1960) as they did during our sampling. If the distance was crossed in a single gonotrophic
472 cycle, an average daily speed would have to be almost an order of magnitude greater than the previous
473 estimates of average female flight speed in Cairns (Muir and Kay, 1998). Therefore, it is likely that a
474 passive, human-assisted transport occurred. With relatively heavy traffic along Bruce and Captain Cook
475 Highways and an increased frequency of re-entering vehicles in commercial areas, the two highways

476 may act as conduits for passive female dispersal. After allowing for mosquito "hitchhiking" along
477 highways, the total distance between the ovitraps (measured from each trap to the nearest highway
478 segment) is reduced to 277 m.

479 Using a continuous coefficient of relationship such as *k* to assign pairs of individuals to discrete
480 kinship categories can be problematic when scores are close to the critical cut-off values. Pairing this
481 method with conservative maximum likelihood estimation resolved some of the clearer distinctions
482 (i.e. the full-siblings exhibiting *Wolbachia* loss) but not others (i.e. the putative full-siblings exhibiting
483 long-distance movement). However, the *k* scores of putative full-siblings and putative half-siblings
484 were clearly separated from each other relative to the variability in *k* scores within each category. This
485 reflects the power of genome-wide SNPs for inferring relationships (Blouin, 2003; Tokarska *et al.,* 2009;
486 Cramer *et al.,* 2011; Sellars *et al.,* 2014; Weinman *et al.,* 2015). Inferring dispersal from relatedness
487 also avoids potential biases resulting from lab-raised *Ae. aegypti* used in MRR studies failing to develop
488 experience in local conditions that will inform their future oviposition choices (Kaur *et al.*, 2003;
489 Ruktanonchai *et al*., 2015). Our findings are broadly consistent with the results of several MRR studies
490 in *Ae. aegypti* adults (Shannon and Davis, 1930; Reiter *et al.,* 1995; Honório *et al.,* 2003; Reiter, 2007),
491 and suggest that this method provides an alternative to MRR for studying dispersal.

492

493 *Non-perfect Wolbachia transmission could occur in Aedes aegypti in Cairns*

494 We found the first evidence in support of maternal transmission failure (μ = 4.8%) in the *Ae.*
495 *aegypti*/*w*Mel system, albeit from a single data point. It is worth noting that a comparable loss of 3.3%
496 was recorded for the *w*AlbA infection in the field-collected *Ae. albopictus* (Kittayapong *et al.,* 2002).
497 Transmission of *w*Mel in *Ae. aegypti* has previously been estimated as perfect or quasi-perfect in the
498 laboratory (Walker *et al.,* 2011) or in the field (Hoffmann *et al.,* 2014), but this was based on assaying
499 eggs that were oviposited in the laboratory (Hoffmann *et al.,* 2014). By comparison, samples in this

500 study were eclosed from eggs that had spent days in the field before collection, potentially exposing
501 them to stressors such as high heat fluctuations, which have been observed to affect *w*Mel
502 transmission in laboratory populations (Ross *et al.,* 2017).

503 Another line of evidence in support of the influence of the rearing conditions on *Wolbachia*
504 titres comes from the considerable disparity in *Wolbachia* titre between Cairns and Gordonvale
505 samples. Namely, the Gordonvale sample had titres that were both higher on average and more
506 variable than the Cairns sample. The Gordonvale sample was collected from BG-Sentinel traps and
507 consisted of field-raised adults of variable age and physiological state (Williams *et al.,* 2006). In
508 contrast, the Cairns sample was collected from ovitraps, where mosquitoes eclosed in the laboratory
509 and were stored within a day of eclosion as virgin adults. The inclusion of blood-fed females in the
510 Gordonvale sample may partially explain the occurrence of very high titres, as *w*Mel titres within *Ae.*
511 *aegypti* are known to double in blood-fed individuals (Frentiu *et al.,* 2014), which approximates the
512 difference between the maximum titres from Gordonvale and Cairns. The very low titres recorded for
513 some Gordonvale individuals, on the other hand, might result from high temperature fluctuations
514 during development in the field (Ross *et al.,* 2017), or from a reduction in titre with age, which has
515 been recorded for *w*AlbA in *Ae. albopictus* males (Tortosa *et al.,* 2010).

516 The *w*Mel strain successfully invaded *Ae. aegypti* populations in Gordonvale and Yorkey's
517 Knob, two quasi-isolated release sites near Cairns, in 2011 (Hoffmann *et al.,* 2011) and these areas
518 have maintained infection rates close to 95% for years without reaching fixation (Hoffmann *et al.,*
519 2014). Under the assumptions of $\mu = 0$, high migration rates of uninfected gravid females would be
520 required to maintain the observed frequencies of infection (0.03 into Gordonvale, 0.06 into Yorkey's
521 Knob) (Hoffmann *et al.,* 2014). Alternatively, the failure to reach fixation in these areas could be due
522 to a combination of migration and infection loss, which would mean that migration rates of uninfected,
523 gravid females may be lower than 0.06.

524

525 **Conclusions**

526 Our study has provided empirical evidence for the processes predicted to slow down the spread of
527 *w*Mel in *Ae. aegypti*, using a landscape genomics analytical framework and molecular assays of
528 *Wolbachia* infection. This approach could be extended to other host/*Wolbachia* systems that are
529 increasingly considered for the biocontrol of disease vectors and pests. Non-perfect maternal
530 transmission of *w*Mel in *Ae. aegypti* may not occur in other *Wolbachia* strains such as *w*AlbB, whose
531 density shows greater constancy under fluctuating high temperatures (Ross *et al.*, 2017). Also, it is as
532 yet unclear whether the observed transmission failure of *w*Mel occurs at a high enough frequency to
533 affect invasion dynamics, and a more extensive field test of transmission fidelity will be necessary to
534 derive an accurate estimate of μ. On the other hand, regardless of the *Wolbachia* strain deployed,
535 presence of barriers like highways and leptokurtic dispersal are potential problems for any *Wolbachia*
536 invasion strategy requiring spatial spread (Turelli and Barton, 2016).

537

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545

546

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548

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766 laboratory population. *Science* **310**, 326-328.
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769 **Data Archiving**

770 Demultiplexed fastq files have been deposited at NCBI SRA under [name].

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772 **Author Contributions**

773 A.A. Hoffmann, G. Rašić, and T. L. Schmidt conceived of and designed the study.

774 T. L. Schmidt, G. Rašić, and I. Filipović collected and dried the samples.

775 T. L. Schmidt performed the laboratory work and conducted the analyses, with assistance from G.
776 Rašić, and computational support from I. Filipović.

777 T. L. Schmidt wrote the manuscript with assistance from A.A. Hoffmann and G. Rašić.

778

779 **Table and Figure Captions**
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781

782 **Table 1:** Results of ANOVAs testing marginal significance of "Highways" and "Releases" variables in
783 dbRDA. The two variables were each analysed in isolation in separate models, then together in a single
784 model. In every case, "Highways" was predictive of genetic structure while "Releases" was not. Partial
785 Eta squared (η_p^2) showed that "Highways" accounted for 1.7% of the variation within each model.

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787

788 **Figure 1:** Sampling locations of the mosquitoes analysed with ddRADseq, set within the six sampling
789 plots. Each sample was assigned a *Wolbachia* infection status, a score indicating its position relative
790 to the two highways, and a score indicating when *Wolbachia* releases were carried out in the area.
791 Plot abbreviations are: CNW (Cairns North West), CNE (Cairns North East), PPN (Parramatta Park
792 North), PPS (Parramatta Park South), WC (Westcourt) and BN (Bungalow). (The underlying road
793 network is derived from "Australia Oceania Continent Roads" made available by MapCruzin.com and
794 OpenStreetMap.org under the Open Database License
795 [https://opendatacommons.org/licenses/odbl/1.0/].)

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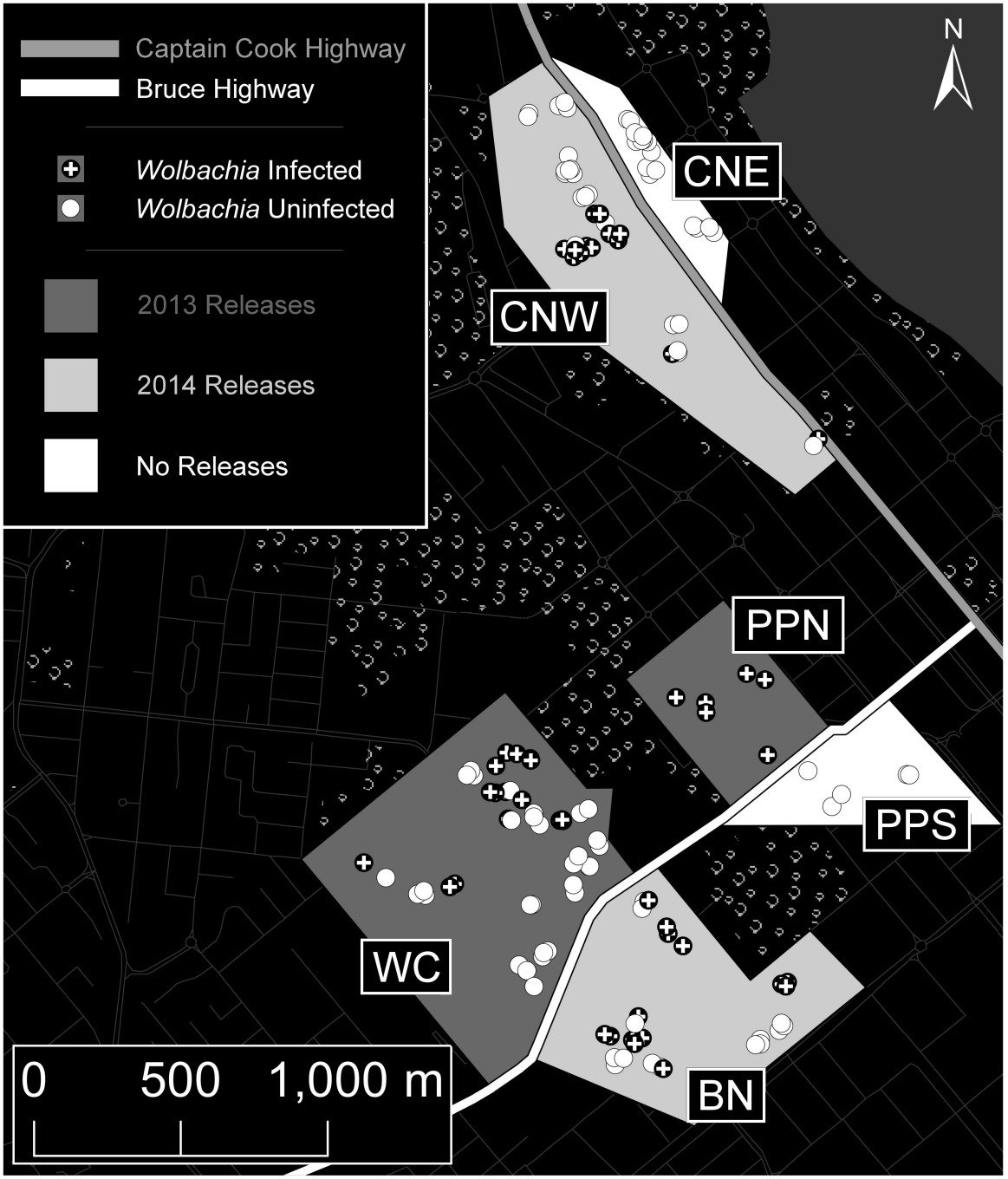
798 **Figure 2:** Loiselle's *k* estimates for sample pairs of relatedness $k > 0.046875$. Pairs of $0.09375 < k <$
799 0.1875 are most likely half-sibs, those of $k < 0.1875$ are most likely full-sibs. Most related pairs were
800 found within the same trap, but separation distances of up to 1312 m were observed.

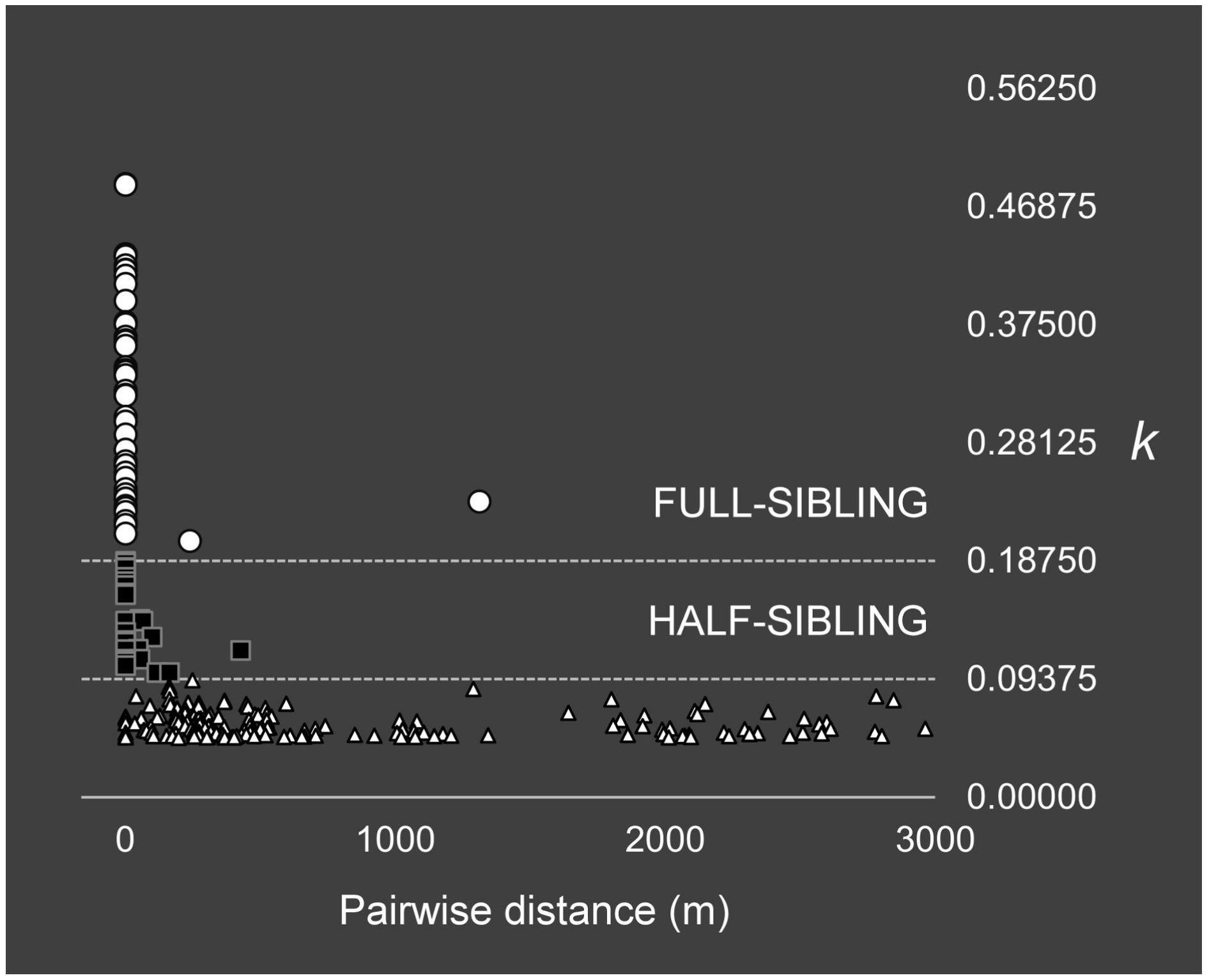
801

Table 1: Results of ANOVAs testing marginal significance of "Highways" and "Releases" variables in dbRDA

		sum of squares	F-value	P	η_p^2
analysed in isolation	HIGHWAYS	0.021	1.635	**0.021**	0.017
	RELEASES	0.016	1.241	0.163	0.013
analysed together	HIGHWAYS	0.020	1.555	**0.032**	0.017
	RELEASES	0.015	1.165	0.231	0.012

The two variables were each analysed in isolation in separate models, then together in a single model. In every case, "Highways" was predictive of genetic structure while "Releases" was not. Partial Eta squared (η_p^2) showed that "Highways" accounted for 1.7% of the variation within each model.


Captain Cook Highway
Bruce Highway
Wolbachia Infected
Wolbachia Uninfected
2013 Releases
2014 Releases
No Releases
N
CNE
CNW
PPN
PPS
WC
BN
0
500
1,000 m


0.56250
0.46875
0.37500
0.28125
k
FULL-SIBLING
0.18750
HALF-SIBLING
0.09375
0.00000
0
1000
2000
3000
Pairwise distance (m)