

Diversity and phylogenetic relationships of *Wolbachia* in *Drosophila* and other native Hawaiian insects

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Wolbachia is a genus of parasitic alphaproteobacteria found in arthropods and nematodes, and represents one of the most common, widespread endosymbionts known. *Wolbachia* affects a variety of reproductive functions in its host (e.g., male killing, cytoplasmic incompatibility, parthenogenesis), which have the potential to dramatically impact host evolution and species formation. Here, we present the first broad-scale study to screen natural populations of native Hawaiian insects for *Wolbachia*, focusing on the endemic Diptera. Results indicate that *Wolbachia* infects native Hawaiian taxa, with alleles spanning phylogenetic supergroups, A and B. The overall frequency of *Wolbachia* incidence in Hawaiian insects was 14%. The incidence of infection in native Hawaiian Diptera was 11% for individuals and 12% for all species screened. *Wolbachia* was not detected in two large, widespread Hawaiian dipteran families—Dolichopodidae (44 spp screened) and Limoniidae (12 spp screened). Incidence of infection within endemic Hawaiian lineages that carry *Wolbachia* was 18% in Drosophilidae species, 25% in Calliphoridae species, > 90% in Nesophrosyne species, 20% in *Drosophila dasyncemia* and 100% in *Nesophrosyne craterigena*. Twenty unique alleles were recovered in this study, of which 18 are newly recorded. Screening of endemic populations of *D. dasyncemia* across Hawaii Island revealed 4 unique alleles. Phylogenetic relationships and allele diversity provide evidence for horizontal transfer of *Wolbachia* among Hawaiian arthropod lineages.

Introduction

Wolbachia is an endosymbiotic genus of inherited, transovarially transmitted alphaproteobacteria that infects the major arthropod orders and nematodes.^{1,2} Previous studies have estimated that the incidence of arthropod infections ranges from 20–75% in different systems and up to 65% worldwide.^{1,3–6} While these estimates are based on the criteria of a positive infection rate of > 1% for individuals of a given species and are corrected for low sample size, they demonstrate that *Wolbachia* is one of the most abundant and widespread endosymbionts known.^{1,3–6} *Wolbachia* is characterized by its varied and potentially dramatic effects on host reproduction, causing cytoplasmic incompatibility, feminization of genetic males and male killing.¹ In contrast, the bacterium also provides mutualistic benefits to insect hosts, including wasps, bedbugs and *Drosophila*.^{7–9} Due to *Wolbachia*'s pervasiveness and potential influence on host population demographics, it has been proposed as a possible driver of speciation in highly diverse insects.⁶

Wolbachia has been a subject of intense interest due to its unusual diversity and potential role in insect diversity. Recent research has investigated the applied science of *Wolbachia*, by infecting natural populations of Mosquito vectors in an effort to

control human disease such as Dengue fever.^{10,11} However, fundamental questions regarding the mechanisms of *Wolbachia*'s global persistence and its net effects on speciation remain open questions, with many large geographic areas and insect taxonomic groups remaining to be studied.¹ In order to better answer these basic questions, a more complete understanding of *Wolbachia* occurrence across novel environments and taxonomic groups is required. This will help elucidate the role of this bacterium in host evolution, teasing it apart from other demographic, ecological and geographic factors that may drive local and global insect diversity. This study expands previous work on *Wolbachia* by conducting the first broad scale screening of environmentally sampled, endemic Hawaiian insects, including multiple lineages of Diptera (Drosophilidae, Dolichopodidae, Limoniidae, Calliphoridae) and Hemiptera (Cicadellidae).

The Hawaiian Islands and their native biota present a unique and simplified system to explore questions regarding *Wolbachia* persistence and spread between interacting arthropod lineages that are both distantly and closely related. The archipelago is isolated by 4000km of oceanic water, with constituent islands arranged in a linear geologic chronology. As a result, arthropod lineages are taxonomically disharmonic with respect to mainland sources, and are typically high in species diversity.^{12–14} In order

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for Wolbachia to persist in Hawaiian arthropods, it must first be present in colonizing individuals, and subsequently persist through repeated colonization of neighboring islands and explosive intra-island host radiations. The pattern of repeated intra-island founder events, and resultant population bottlenecks, may purge Wolbachia infections. Thus, long-term persistence may further require extensive horizontal transfer from infected to uninfected lineages to spread and persist.

Sampling in this study focuses on Hawaiian Diptera, one of the most diverse and dominant components of the native Hawaiian fauna. The endemic genus *Drosophila* is a classic example of adaptive radiation in nature, comprising over 1,000 species and representing 10% of the endemic Hawaiian insect fauna.¹⁵ To contrast potential results from Hawaiian Diptera, we further include a number of non-native lineages found on Hawaii and other Pacific locations (Australia and South Pacific Islands), and several species of the large, native leafhopper genus *Nesophrosyne* (Hemiptera: Cicadellidae). This strategy offers both a broad taxonomic and ecological sampling to compare distantly related lineages that share similar geographic constraints (*Drosophila* vs. *Nesophrosyne*, which co-occur in similar habitats and share host plants), span a wide variety of habitat types (mesic forest, rainforest, cloud forest, semi-aquatic habitats, invaded ecosystems), encompass different degrees of ecological specialization (generalists and specialists), and occupy different ecological niches (predators, saprophagous taxa, plant-feeding insects).

Results from this study demonstrate that Wolbachia is present in native Hawaiian insects, a novel result since previous screens in Hawaiian arthropods were based on either non-native flies, produced negative results, or were limited in taxonomic diversity and based on long-standing laboratory cultures.^{16,17} The incidence of Wolbachia in native Hawaiian insects was 14%, but was lower for native Hawaiian Diptera (11%). Several large groups including the dipteran families Dolichopodidae (Hawaii), Limoniidae (Hawaii) and Mycodrosophila (Oceania Region) showed no evidence of infection. Overall, Wolbachia incidence was generally low (< 20%). Sequenced Wolbachia alleles are placed in both the A and B supergroups, with most alleles from endemic hosts placed in the B supergroup. Twenty alleles were recovered in this screen, including 18 newly discovered, and evidently known only from Hawaii. Phylogenetic relationship of Wolbachia *wsp* alleles from endemic insects form clades with other Hawaiian taxa or infections from Japan. Concordant with previous results, Hawaiian Wolbachia show evidence of horizontal transfer.^{4,18}

Results

Incidence of Wolbachia infection and allelic diversity. Table 1 lists the positive results for taxa screened in this study, including Wolbachia supergroup placement and allelic designations (Table 3 provides a full breakdown of all taxa screened). Table 2 lists the percent frequency of Wolbachia occurrence in the different categories of insects screened (e.g., all taxa, Native Diptera, *Drosophila dasyncnemias*, etc.). The incidence of Wolbachia in all individuals and species screened was 11%. Native Hawaiian taxa

showed a 14% incidence for both species and individuals, with endemic Diptera showing lower individual and species level incidences of 11% and 12%, respectively. Of the four major dipteran families screened, Drosophilidae (18%) and Calliphoridae (25%) yielded positive infection results. In contrast, Dolichopodidae and Limoniidae yielded no Wolbachia despite the relatively large number of taxa screened ($n = 120$; Table 3). The native Hawaiian leafhoppers (*Nesophrosyne*) gave positive results for 12 of the 13 individuals screened (92% infection rate), and 100% infection incidence for all species screened.

Wolbachia incidence within species varied. Of the 58 *Drosophila dasyncnemias* taxa screened, 12 (21%) individuals were infected. The widespread, non-native *Drosophila suzukii* and *D. kikkawai* demonstrated relatively low within-species Wolbachia incidence of 13% and 5%, respectively. In *Nesophrosyne* species, a 100% infection incidence was observed for the five individuals of *N. craterigena*, three individuals *N. giffardi* and two individuals of *N. n.sp9*.

Table 1 lists the Wolbachia alleles discovered in this study. Twenty unique alleles were sequenced, of which 18 are novel and sequenced from native Hawaiian insects. Several species share alleles, including *Drosophila dasyncnemias*, *D. prodita* and *D. redunca* (wDasA); *D. forcifata* and *D. dorsigera* (wFor); *D. ancyla*, *D. nr.fundita*, *Nesophrosyne giffardi interrupta* and *N. n.sp9* (wGin); *D. nigrocirrus*, *D. nr.semiflava* and *D. longiestosa* (wEla); *Nesophrosyne n.sp9* and *N. giffardi* (wGiff). Individuals of the species, *Nesophrosyne craterigena*, have either allele wCraA or wCraB. Of the multiple individuals screened for *D. dasyncnemias*, four unique alleles were recovered (wDasA–wDasD).

Phylogenetic relationships. Global Maximum Likelihood (ML) results for the 284 Wolbachia *wsp* sequences included in this study are shown in Figure 1 ($-lnL = 8396.93$). Sequences were placed in the Wolbachia phylogenetic supergroup core clades, A and B, with high bootstrap support (BS = 95). Hawaiian alleles are distributed between the supergroups A and B, but placed predominantly in the B supergroup (38 of 45 sequenced results). The global tree is split into six different sections for ease of discussion; hereafter referred to as Hawaiian subgroups 1–6 (Figs. 1–4). Support for relationships range from moderate to high support (Fig. 2, subgroup 2; BS = 60–97) to poor or no support (subgroup 1; BS < 50), and are summarized below:

Hawaiian subgroups 1 and 2 are placed within the Wolbachia B supergroup (Figs. 1–3; BS = 95). The closest relatives of these alleles are primarily other Hawaiian sequences, and Lepidoptera and Heteroptera from Japan and Africa. Subgroup 1 (Fig. 2) includes infections sequenced from two *Drosophila* species, *D. kikkawai* and *D. apicipuncta* (BS = 95) and three *Nesophrosyne* species. Relationships of Wolbachia sequences in this clade are generally poorly supported, however *Nesophrosyne* infections represent a highly supported clade (BS = 97). Wolbachia alleles from *Drosophila* are placed sister with high support (BS = 95). The closest alleles to the Hawaiian infections are Heteroptera from Japan, although support is low (BS < 50)

Hawaiian subgroup 2 (Fig. 3) contains four well-supported clades (BS = 71–97). The largest clade is composed of Wolbachia from *Drosophila dasyncnemias* mixed with *D. redunca* and

Table 1. Taxonomic information for Hawaiian insect hosts sequenced *Wolbachia* wsp

Family	Genus	Species	<i>Wolbachia</i> supergroup	Allele	GenBank accession (Study barcode)	
Drosophilidae	<i>Drosophila</i>	<i>dasyncnemias</i>	B	wDasA	JX134910 (85w)	
			B	wDasA	JX134911 (135w)	
			B	wDasB	JX134912 (136w)	
			B	wDasA	JX134913 (137w)	
			B	wDasA	JX134914 (138w)	
			B	wDasD	JX134921 (139w)	
			B	wDasA	JX134916 (140w)	
			B	wDasB	JX134920 (141w)	
			B	wDasA	JX134918 (201w)	
			B	wDasA	JX134917 (202w)	
			B	wDasA	JX134919 (268w)	
			B	wDasC	JX134915 (269w)	
			B	wKik	JX134922 (277w)	
			B	wApi	JX134926 (276w)	
			<i>kikkawai</i>	B	wGin	JX134924 (200w)
		B		wGin	JX134925 (87w)	
		B		wFun	JX134929 (86w)	
		B		wEur	JX134927 (142w)	
		B		wBas	JX134934 (81w)	
		B		wBas	JX134933 (82w)	
		B		wDasA	JX134932 (83w)	
		B		wDasA	JX134931 (84w)	
		B		wFor	JX134928 (149w)	
		B		wFor	JX134935 (150w)	
		B		wTet	JX134936 (281w)	
		A		wEla	JX134930 (42w)	
		A		wRi	JX134952 (70w)	
		A		wRi	JX134953 (71w)	
		<i>Scaptomyza</i>		<i>flava</i>	A	wFla
			A		wEla	JX134939 (43w)
A	wEla		JX134923 (58w)			
Calliphoridae	<i>Dyscritomyia</i>	<i>obscura</i>	B	wObs	JX134937 (199w)	
Cicadellidae	<i>Nesophrosyne</i>	<i>n.sp9</i>	B	wGin	JX134949 (143w)	
			B	wGiff	JX134945 (283w)	
		<i>giffardi interrupta</i>	B	wGin	JX134950 (270w)	
			B	wGiff	JX134946 (284w)	
			B	wGiff	JX134947 (285w)	
		<i>giffardi</i>	B	wGiff	JX134948 (286w)	
			B	wSil	JX134944 (282w)	
		<i>craterigena</i>	B	wCraA	JX134942 (154w)	
		<i>craterigena</i>	B	wCraB	JX134951 (151w)	
		<i>craterigena</i>	B	wCraA	JX134943 (155w)	
<i>craterigena</i>	B	wCraB	JX134940 (153w)			
<i>craterigena</i>	B	wCraB	JX134941 (152w)			

Table 2. Incidence of Wolbachia for taxonomic groups screened for the *wsp* loci (individual/species)

Geographic area	Groups	Positives	Total screened	% Infected
Pacific				
	Total	45/25	419/230	11%/11%
	Diptera	33/20	406/220	8/9%
Hawaii				
	Native Hawaiian Insects	41/23	288/161	14%/14%
	Native Diptera	29/18	275/156	11%/12%
	Native Drosophilidae	27/17	149/94	18%/18%
	Calliphoridae	1/1	4/4	25%
	Dolichopodidae	0	46/50	0%
	Limoniidae	0	12/76	0%
	<i>Nesophrosyne</i>	13/5	12/5	92%/100%
	<i>Drosophila dasyncnemiea</i>	12	56	20%
	<i>Drosophila suzukii</i>	3	24	13%
	<i>Drosophila kikkawai</i>	1	22	5%
	<i>Nesophrosyne craterigena</i>	5	5	100%

D. prodita from across Hawaii Island (BS = 84). Wolbachia from two distantly related species, *D. spnr.basimacula* and *D. eurypeza*, are placed sister with moderate support (BS = 71). Wolbachia sequenced from *Nesophrosyne* species are placed in two separate clades: a monophyletic *N. craterigena* sister to Japanese and African Lepidoptera (BS = 94), and a *Drosophila*–*Nesophrosyne* mixed clade (BS = 97). Several Wolbachia alleles are unplaced with no supported relationships, including infections from *Dyscritomyia obscura* (wObs, Calliphoridae), *Drosophila ancyla* (wGin), *D. nr.fundita* (wGin), *D. fundita* (wFun), *Nesophrosyne giffardi interrupta* (wGin) and *N. n.sp9* (wGin).

Hawaiian subgroups 3 (BS = 80) and 5 (BS = 70; both shown in Fig. 4) and two native drosophilids are placed in the taxonomic supergroup A. Subgroup 3 comprises the widespread *Drosophila* species *D. suzukii* from Hawaii, *D. ananassae*, *D. simulans* and *D. auria* (BS = 100). Subgroup 5 is moderately supported (BS = 70), containing three Hawaiian sequences from both native Hawaiian drosophilid genera *Scaptomyza* (*S. nr.semiflava* and *S. longiestosa*) and *Drosophila* (*D. nigrocirrus*). Hawaiian sequences are placed within a moderately supported clade (BS = 73), comprising infections from a Japanese Heteroptera and Hymenoptera parasitoides (*Spalangia cameroni*).

Finally, Wolbachia from *Drosophila tetraspilota* (subgroup 4; BS > 97) and *Scaptomyza flava* (subgroup 6; BS = 100, results not shown, but see Fig. 1) are placed individually in supergroup B and A, respectively. Neither shares alleles with other Hawaiian *Drosophila*.

Discussion

Incidence of Wolbachia on Hawaii. This study is the first to conduct broad-scale screening for Wolbachia in naturally occurring populations of native Hawaiian insects. Our results demonstrate that Wolbachia is present in the native Hawaiian Drosophilidae (> 1,000 species), Calliphoridae (25 species), and Cicadellidae

(> 200 species). This result is significant, as previous studies have failed to find Wolbachia in lab reared native and non-native *Drosophila* from Hawaii,^{16,17} and it is the first to show Wolbachia infecting other Hawaiian families. Eighteen of the recovered haplotypes are known only from native Hawaiian taxa. This suggests the intriguing possibility that Hawaii may host endemic Wolbachia lineages, but further Pacific-wide screening is required to confirm this. An equally interesting outcome of this study is the apparent absence of Wolbachia from the endemic fly families Dolichopodidae (> 120 species) and Limoniidae (12 species), despite the large numbers of individuals screened (see Table 3). Several of these groups represent some of the largest Hawaiian insect radiations, and they are all common components of the Hawaiian entomofauna, sharing habitats throughout the islands.

Previous studies have estimated that local and global species level incidence of Wolbachia ranges from 20–75%, however these estimates are potentially biased due to incomplete taxon sampling and the inconsistent number of individuals screened per species.^{4,6,20,21} Thus, direct comparisons of Wolbachia incidence between geographic localities, or to worldwide estimates, are difficult to interpret directly. Among the Hawaiian insects screened in this study, the overall incidence of Wolbachia at the species level is 14%, with the endemic Hawaiian Diptera demonstrating an even lower incidence of 12% for species screened (see Table 2). Contrary to this finding, the native *Nesophrosyne* Hawaiian leafhoppers exhibited a high incidence of Wolbachia of 100% for species screened. The exceptional Wolbachia occurrence in *Nesophrosyne* needs to be further examined, since sampling is taxonomically limited and all specimens were acquired from the same geographic location—Kipuka Puauulu, Hawaii Island. This result may represent a localized infection with an unusually high level of infection. A closer examination of Wolbachia in individual groups demonstrate a higher infection incidence of ~20% at the family level (18% for drosophilids, including the genera *Drosophila* and *Scaptomyza*) and the species level

Table 3. Summary of taxon sampling arranged by taxonomic groups and geographic locality

Order	Family	Genus	Group	Species	Locality	Samples screened	Individuals infected	Species infected
Diptera	Calliphoridae	<i>Dyscritomyia</i>		4 spp	Hawaiian Islands, USA	4	1	1
	Dolichopodidae	<i>Adachia</i>		1 sp	Hawaiian Islands, USA	1	0	0
		<i>Arciellia</i>		4 spp	Hawaiian Islands, USA	4	0	0
		<i>Campsicnemus</i>		26 spp	Hawaiian Islands, USA	26	0	0
		<i>Chrysotus</i>		2 spp	Hawaiian Islands, USA	2	0	0
		<i>Dolichopus</i>		2 spp	Hawaiian Islands, USA	2	0	0
		<i>Eurynogaster</i>		8 spp	Hawaiian Islands, USA	8	0	0
		<i>Sweziellia</i>	<i>tergoprolixa</i>		Hawaiian Islands, USA	1	0	0
		<i>Tachytrechus</i>	<i>angustipennis</i>		Hawaiian Islands, USA	1	0	0
		<i>Uropachys</i>	<i>fusticercus</i>		Hawaiian Islands, USA	1	0	0
	Drosophilidae	<i>Dichaetophora</i>		<i>plana</i>	Australia	1	0	0
		<i>Drosophila</i>	<i>dispar</i>	<i>dispar</i>	Australia	1	0	0
			<i>melanogaster</i>	<i>kikkawai</i>	Pacific Region	22	1	1
				<i>suzukii</i>	Pacific Region	24	3	1
			<i>immigrans</i>	<i>immigrans</i>	Pacific Region	15	0	0
				<i>nasuta</i>	Pacific Region	3	0	0
				<i>nasutoides</i>	Pacific Region	2	0	0
			Hawaiians, <i>spoon tarsus</i>	<i>dasyncnemia</i>	Hawaiian Islands, USA	56	12	1
			Hawaiians, <i>Drosophila</i>	81 spp	Hawaiian Islands, USA	81	13	13
			Hawaiians, <i>Scaptomyza</i>	12 spp	Hawaiian Islands, USA	12	3	3
		<i>Hirtodrosophila</i>		4 spp	Pacific Region		0	0
		<i>Leucophenga</i>		<i>scutellata</i>	Australia		0	0
		<i>Mycodrosophila</i>		47 spp	Pacific Region		0	0
		<i>Paramycodrosophila</i>		<i>pictifrons</i>	French Polynesia		0	0
		<i>Samoaiia</i>		2 spp	Samoa		0	0
		<i>Scaptodrosophila</i>		<i>albifrontata</i>	Australia		0	0
		<i>Sphaerogastrella</i>		<i>novaguine-nensis</i>	New Guinea		0	0
		<i>Tambourella</i>		<i>endiandrae</i>	Australia		0	0
		<i>Zygothrica</i>		4 spp	Pacific Region		0	0
	Limoniidae	<i>Dycranomyia</i>		12 spp	Hawaiian Islands, USA		0	0
Hemiptera	Cicadellidae	<i>Nesophrosyne</i>		5 spp	Hawaiian Islands, USA		12	5
Totals				230 spp		419	45	25 spp

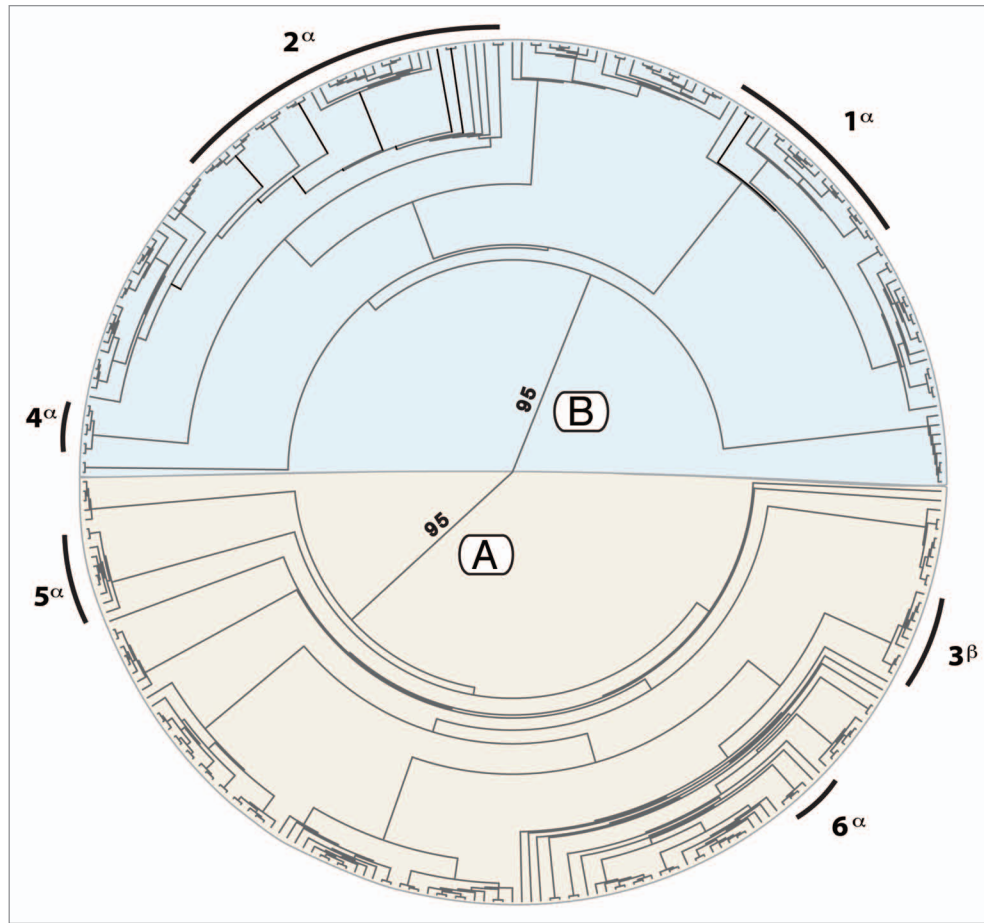


Figure 1. Maximum-likelihood phylogeny of 284 *Wolbachia* isolates for surface protein gene *wsp* conducted using RAxML-HPC2 v7.2.7.^{51,52} Phylogeny is mid-point rooted, and color-coded according to the (A) (red) and (B) (blue) supergroup systematic classifications. Inset numbers correspond to bootstrap support values. Numbers 1–6 demarcate subsections of the phylogeny containing *Wolbachia* sequenced from Hawaiian insects in this study. Symbols α and β indicate groups that contain endemic Hawaiian taxa and those that do not, respectively. Hawaiian subsections are enlarged to show relationships and endemicity of constituent taxa in Figures 2–4.

(21% within *Drosophila dasyncnemis*).⁶ Further, the non-native *D. kikkawai* and *D. suzukii* show an incidences of 5% and 13%, respectively.

The depressed incidence and complete absence of *Wolbachia* in some Hawaiian lineages suggests that the dynamics of *Wolbachia* may be different than for other geographic regions.^{6,19,22} A plausible hypothesis for the observed or general absence of *Wolbachia* from some groups may be due to the remote location of the Hawaiian Islands: Hawaii is isolated from the nearest continent by over 4,000 km of water, with an estimated successful arthropod colonization every 175,000 y.²³ The rarity of these events, combined with the global finding that few if any lineages have a 100% *Wolbachia* incidence,^{4–6,19,20} suggests that infections may simply be left behind as uninfected colonizers disperse to Hawaii. Similarly, after establishment on the archipelago, lineages typically disperse to neighboring islands, speciate in allopatry, and experience resultant reductions in effective population size and genetic diversity.^{24,25} As lineages colonize new islands within the Archipelago, infection may be further reduced or purged through population bottlenecks and sorting events. The interplay between these processes could explain the low incidence and

complete absence of *Wolbachia* in some Hawaiian groups (e.g., Dolichopodidae and Limoniidae). Thus, *Wolbachia*, in order to persist, must survive multiple rounds of colonization or find alternative routes to spread, such as horizontal transfer.

Relationships of Hawaiian *Wolbachia*. Native Hawaiian *Wolbachia* alleles were placed predominantly in the B supergroup (~75% of the species screened), with only four isolates placed in the A supergroup (*Drosophila flava*, *D. nigrocirrus*, *Scaptomyza nr. semiflava* and *S. longiestosa*). The non-native Hawaiian *Drosophila*, *D. suzukii* and *D. kikkawai*, were also placed in the A supergroup along with other widespread species (Fig. 4), indicating a potentially shared infection for these species. While the A and B supergroups are known to primarily infect insect hosts, little is known about the phylogenetic split between them or basic biological roles of either group.

Phylogenetic reconstructions tend to cluster Hawaiian *Wolbachia* together, along with the Japanese and African Heteroptera and Lepidoptera infections (Figs. 3 and 4). The close association of Hawaiian alleles suggests that *Wolbachia* is maintained through speciation or potentially transferred between related hosts (e.g., horizontal transfer). Several of the

infected *Drosophila* species are closely related and share the same or closely related *Wolbachia* alleles. For example, *D. ancyla* and *D. nr.fundita* share the wGin alleles, and both are placed in the *split tarsus* species subgroup along with *D. fundita*, which carries the closely related *Wolbachia* allele, wFun (Fig. 3).²⁶ Similarly, sister species *D. redunca* and *D. prodita* both share the wDasA allele. (Fig. 3) The close relationships between these species suggest that *Wolbachia* infections may persist through cladogenesis. On the other hand, closely related species share distantly related alleles and vice versa. The wGin allele shared among *split tarsus* species, is also shared with native Hawaiian leafhoppers (Fig. 3). The shared allele between *D. redunca* and *D. prodita*, is also carried by the distantly related *D. dasyncnemias*. Finally, *Nesophrosyne* species in subgroup 1, which share the wGiff and closely related wSil alleles, are not closely related (Fig. 2). Rather, these species only occur in sympatry, but occupy different host plants and are members of unrelated species groups (Bennett, unpublished). This result provides evidence for other mechanisms of *Wolbachia* spread and persistence, such as horizontal transfer, which is described in more detail below.

It is not possible to propose biogeographic hypotheses regarding the origins of Hawaiian *Wolbachia* at this time. Geographic and taxonomic sampling of *Wolbachia* is biased toward certain taxonomic groups and geographic areas. While the associations with Japanese Heteroptera and Lepidoptera *Wolbachia* alleles are intriguing,^{19,22} significant regions of the Western Pacific basin have not been sampled, nor have many of the dipteran groups. It is possible that the shared alleles found in this study include widely dispersed, pan-pacific *Wolbachia* lineages. In this case, the *Wolbachia* lineages recovered here may have arrived to Hawaii from other sources not yet screened. Or, the sister relationships between Japanese and Hawaiian *Wolbachia* is merely an artifact of incomplete taxon sampling. In order to robustly test the potential origins of *Wolbachia* infection on Hawaii, broad scale taxonomic sampling across the Pacific region that targets known outgroups and related genera is required.

Horizontal transmission of *Wolbachia* among Hawaiian lineages. *Wolbachia* in lineages with relatively low incidence rates (< 20%) may rely on other mechanisms to sustain infection, including mutualistic benefits to hosts, interactions with other endosymbionts, or widespread horizontal transfer.²⁷⁻²⁹ Our results provide several lines of evidence for extensive horizontal transmission of *Wolbachia* between Hawaiian taxa at multiple taxonomic scales, which is congruent with other studies.^{1,30-32} At the ordinal level, nearly identical *Wolbachia* alleles are shared between Diptera species (e.g., *Drosophila forficata*, and *D. spnr. dorsigera*) and Hemiptera (*Nesophrosyne craterigena*: Fig. 3). A plausible mechanism for this scenario is explained by the reliance of both *Drosophila* and *Nesophrosyne* species on shared host

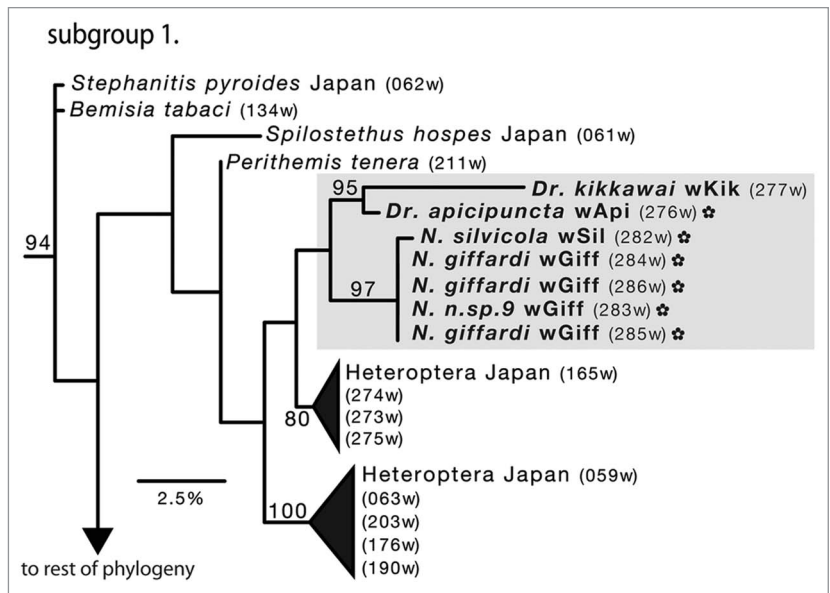


Figure 2. Hawaiian *Wolbachia* subgroup 1 (supergroup B) from the maximum-likelihood phylogenetic reconstruction presented in Figure 1. The gray box delineates Hawaiian sequences, with endemic taxa starred. Hawaiian genera abbreviations are as follow: *Dr.* = *Drosophila* (Diptera: Drosophilidae), *Dy.* = *Dyscritomyia* (Diptera: Calliphoridae), *S.* = *Scaptomyza* (Diptera: Drosophilidae), and *N.* = *Nesophrosyne* (Hemiptera Cicadellidae). Branch numbers correspond to bootstrap values (BS < 50 not shown). Names correspond to the taxonomic placement of the host species and unique allele name (e.g., wGiff); parenthetical three-digit barcode corresponds to Table S1.

plants across their ranges (e.g., both feed from and lay their eggs directly into their host plants).³³⁻³⁵ These results corroborate the findings of Sintupachee et al.³⁶ that hosts spanning multiple insect orders, which rely on the same host plants, tend to share closely related *Wolbachia* alleles. It is notable that in contrast to this result, the uninfected dolichopodid and limoniid flies do not rely on host plants, as they are predatory and semi-aquatic or aquatic, respectively.^{37,38} This difference in habitat use and life strategy may eliminate potential mechanisms for horizontal *Wolbachia* transmission.

Within the Drosophilidae family, there is evidence for horizontal transmission of *Wolbachia* between species. Shared alleles emerge between distantly related species (*Drosophila eurypeza* and *D. nr.basimacula*), and between the widely sampled *D. dasyncnemias*, *D. redunca* and *D. prodita*. The shared alleles between *D. dasyncnemias* and *D. redunca*, which are endemic to different islands (Hawaii Island and Molokai, respectively), suggests that horizontal transmission has occurred somewhat recently and potentially through a network of interacting species. The native and non-native drosophilid lineages placed in the A supergroup also share similar alleles (*D. suzukii*, *D. ananassae*, *D. simulans* and *D. auria*; and, *D. nigrocirrus*, *Scaptomyza. nr.semiflava* and *S. longiestosa*: Fig. 4) both with other widespread taxa and parasitoid wasps from other studies (see Table S1 for references). This close association suggests parasitoids as a second potential mechanism for *Wolbachia* horizontal transmission between native and non-native Hawaiian *Drosophila*. There is substantial evidence that wasps transfer *Wolbachia* among the hosts they

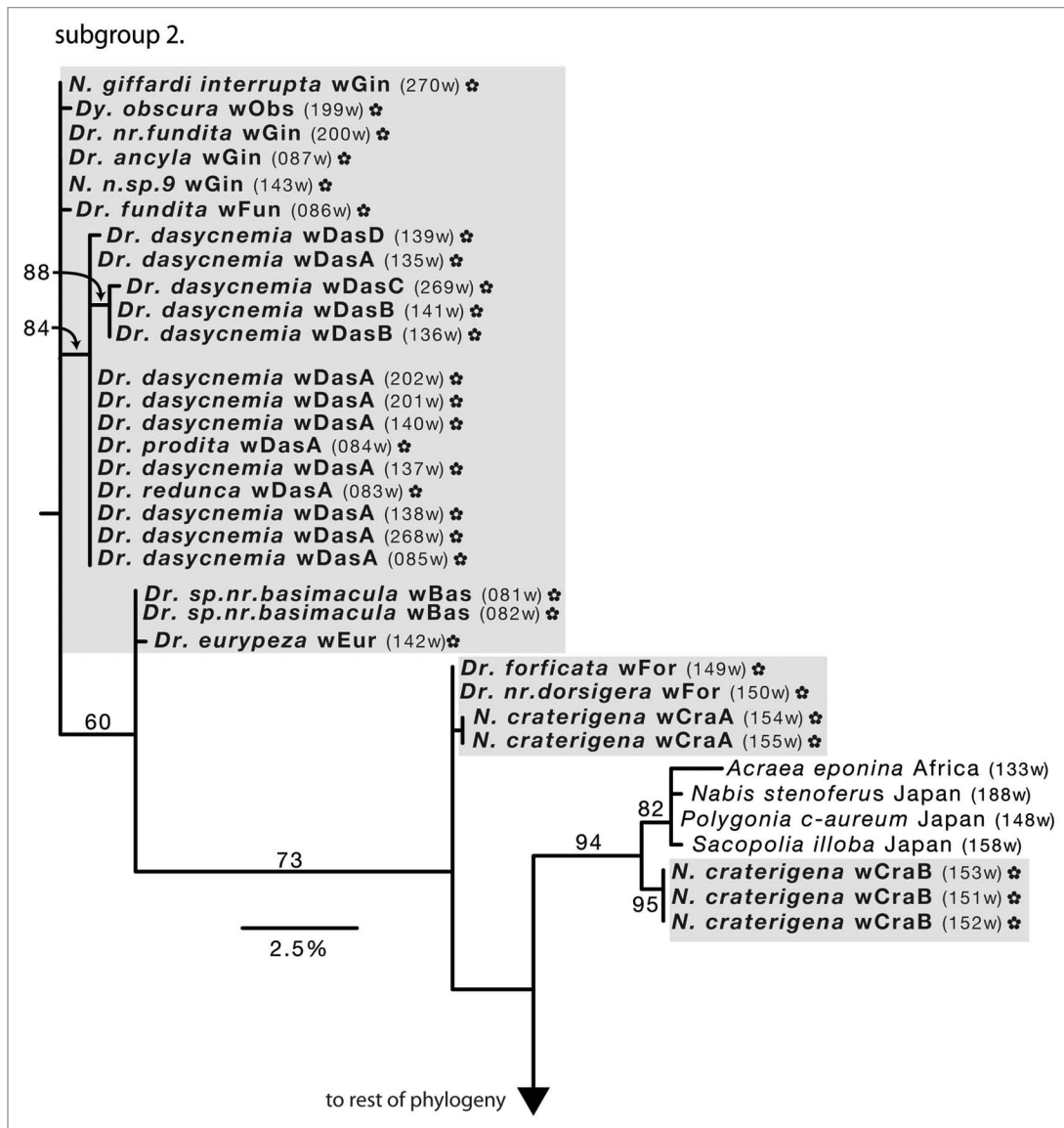


Figure 3. Hawaiian Wolbachia subgroup 2 (supergroup B). See **Figure 2** legend for explanation and interpretation of species abbreviations, branch support, DNA barcodes and other symbols.

provision themselves with, and especially *Drosophila*, which are susceptible to parasitism.^{32,39,40} Parasitoid relationships within *D. dasyncnemia*, *D. redunca* and *D. redunca* are currently unknown. However, Hawaii is home to a large diversity of endemic and introduced parasitoid wasps known to attack both native and non-native insects.⁴¹ This mechanism may allow for newly colonizing lineages to introduce and spread novel Wolbachia alleles to previously uninfected Hawaiian arthropods.

Materials and Methods

Taxon sampling and DNA extraction. Taxonomic sampling included 419 specimens, focusing on Diptera in four families (*Drosophilidae*, *Limoniidae*, *Calliphoridae*, *Dolichopodidae*) and 22 genera (see **Table 3** for the taxonomic placement of all species screened for this study). Hawaiian Diptera sampling

consisted primarily of native flies, but included several introduced *Drosophila* in the *D. melanogaster* and *D. immigrans* species groups, and Pacific-wide species not known to occur in Hawaii (e.g., *Drosophila dispar* species group and *Mycodrosophila*). Thirteen native Hawaiian leafhopper (*Nesophrosyne*) individuals in five species were also included from an ongoing project examining endosymbiont associations in this genus. To examine the potential rate of infection within a single population, 56 individuals of native *Drosophila dasyncnemia* from Hawaii Island were screened for Wolbachia. Field collected material was placed in 95% ethanol for preservation and then identified to species. DNA was extracted from whole insects specimens using the DNeasy Tissue Kit (QIAGEN) following the manufacturer's protocol.

Wsp screening, PCR and DNA sequencing. Presence of Wolbachia was determined by screening potential dipteran and

hemipteran hosts, using primers specific to the *wsp* gene. Despite some criticisms of the single use of the *wsp* gene,⁴³ few studies have screened Pacific islands for Wolbachia infections, and the available *wsp* sequence data provides the best opportunity for geographic contextualization of potential infections in Hawaiian taxa. The *wsp* gene has been one of the most widely used in Wolbachia identification and systematics (~2200 worldwide GenBank sequences), including extensive sampling of Japanese Lepidoptera and Heteroptera.^{19,22} As well, the *wsp* gene provides adequate terminal resolution to assess relationships of Wolbachia alleles among Hawaiian taxa. The *wsp* locus was sequenced for the forward and reverse directions using Polymerase Chain Reaction (PCR) primers: *wspF* 5'-TGG TCC AAT AAG TGA TGA AGA AAC TAG CTA G-3' and *wspR* 5'-AAA AAT TAA ACG CTA CTC CAG CTT CTG CAC-3'.¹⁷ A general touchdown PCR protocol was implemented: 3 min at 94°C; 5 cycles of 30 sec at 94°C, 30 sec at 65°C and 90 sec at 72°C; 5 cycles of 30 sec at 94°C, 30 sec at 60°C and 90 sec at 72°C; 25 cycles of 30 sec at 94°C, 30 sec at 55°C and 90 sec at 72°C; 5 min at 72°C. Positive infections were determined from successful PCR amplifications with the *wsp* primers, which were then cleaned using ExoSap (Stratagene) following the manufacturer's protocol and sequenced at the U.C. Berkeley, Barker Sequencing Center. Sequenced specimens gave clean wave profiles, indicating single Wolbachia infections of screened Hawaiian Insects. New sequences derived from this study were submitted to GenBank (see Table 1 for accession numbers).

Sequence editing and alignment. Sequenced Hawaiian taxa were imported into Geneious Pro v5.0.4,⁴⁴ which was used to build and edit contigs for forward and reverse sequence fragments. Nucleotide BLAST searches were conducted with Hawaiian sequences to identify the most closely related *wsp* sequences, which were then imported into a large alignment. All individual sequences were given a four-digit barcode (e.g., 001w–286w) for reference, which corresponds to host taxonomic information, geographic collection locality and references (see Table S1).

A total data matrix containing 284 *wsp* sequences (45 are new to this study) was aligned using Muscle V.3.5.⁴⁵ Two taxa obtained from GenBank (006w and 007w; see Table S1) were removed from further analyses due to difficulty in aligning. The total alignment was then exported to MacClade v4.08,⁴⁶ translated into amino acid sequences, and checked against the amino acid conceptual translation for the *wsp* gene acquired from GenBank. Two ambiguously aligned regions between 239–250 and 572–589 (30 base pairs) were removed from analyses, yielding a total gene length of 605 base pairs. The edited alignment is available from the O'Grady lab website (<http://www.drosophilaevolution.com/index.html>). All sequence data generated for this study has been deposited to the GenBank database and assigned sequence accession number JX134910–JX134954 (see Table 1).

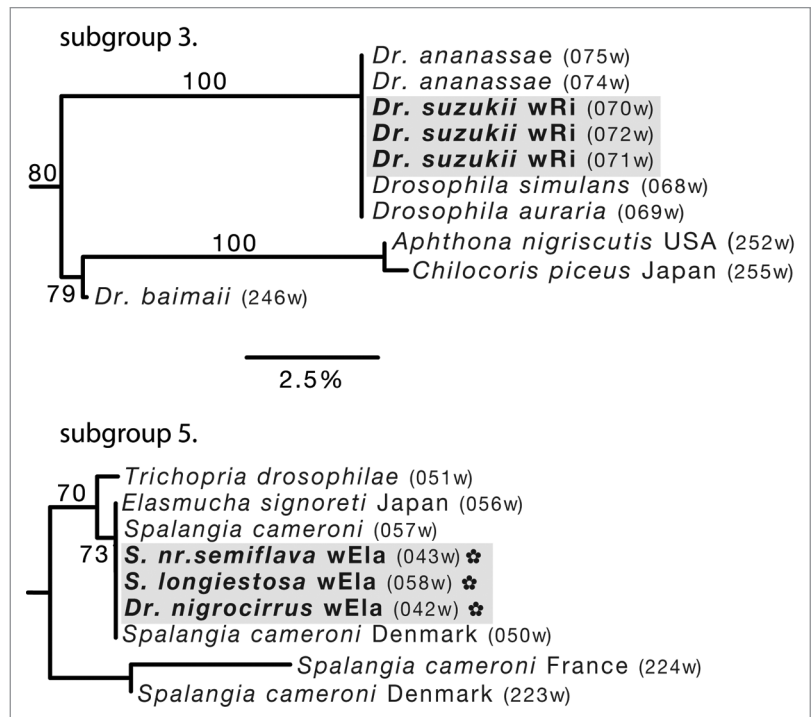


Figure 4. Hawaiian Wolbachia subgroups 3 and 5 (supergroup A). See Figure 2 legend for explanation and interpretation of species abbreviations, branch support, DNA barcodes and other symbols.

Phylogenetic analyses. Phylogenetic analyses were performed using Maximum Likelihood methods. A likelihood model of nucleotide substitution for the *wsp* gene data set was estimated using Modeltest v3.7 run in PAUP* and determined via Akaike Information Criterion,⁴⁷⁻⁴⁹ which approximated GTR+inv+gamma as the best fit model. In order to successfully reconstruct a statistical phylogenetic inference of Wolbachia relationships for all 284 taxa (Parsimony and Bayesian analyses failed to finish), RAxML-HPC2 v7.2.7 was employed through the CIPRES portal on the ABE server.⁵⁰⁻⁵² RAxML was run with the rapid bootstrapping option for 1000 bootstrap iterations with a GTRCAT model of nucleotide substitution. A best-scoring maximum likelihood tree was then estimated under a GTRGAMMA model, with the suggested default of 25 rate categories. Resultant trees were exported into FigTree v1.3.1 for viewing and editing, and rooted at its mid-point.⁵³

Conclusion

Results from this study provide the foundation for understanding Wolbachia infections on the remote Hawaiian Archipelago. The local Wolbachia incidence for Hawaiian groups is low (generally less than 20%), suggesting that the direct effects on the sexual reproduction and population dynamics of endemic lineages may be reduced or even absent.^{28,42} Thus, the overall role of Wolbachia in speciation of native Hawaiian insects may not be significant, however this remains to be directly tested. While horizontal transfer appears to be common in native Hawaiian taxa, broad

scale screening of different native lineages is required to confirm its overall role in the persistence of *Wolbachia* among endemic Hawaiian arthropods. Phylogenetic evidence indicates two potential, testable mechanisms may contribute to the observed horizontal transmission of *Wolbachia*: shared host plants and parasitoid interactions. Future studies should screen not only insect lineages sharing particular host plants and habitats, but also the shared host plant tissues and parasitoids known to occur or provision themselves with geographically overlapping species. The Hawaiian drosophilids and cidradellids present tractable systems to examine these and other mechanisms in future studies, as sister species in these groups are geographically isolated between islands and volcanoes, species are members of large intra-island radiations within discreet island boundaries, and much is known about the age and ecology of their species.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
<http://www.landesbioscience.com/journals/fly/article/21161/>

33. Bennett GM, O'Grady PM. Review of the native Hawaiian leafhopper genus *Nesophrosyne* (Hemiptera: Cicadellidae: Deltocephalinae) with description of eight new species associated with *Broussaisia arguta* (Hydrangeaceae). *Zootaxa* 2011; 2805:1-25.
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