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# GENETIC DIVERSITY OF *WOLBACHIA* ENDOSYMBIONTS IN *CULEX QUINQUEFASCIATUS* FROM HAWAI'I, MIDWAY ATOLL AND AMERICAN SAMOA

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## ABSTRACT

Incompatible insect techniques are potential methods for controlling *Culex quinquefasciatus* and avian disease transmission in Hawai'i without the use of pesticides or genetically modified organisms. The approach is based on naturally occurring sperm-egg incompatibilities within the *Culex pipiens* complex that are controlled by different strains of the bacterial endosymbiont *Wolbachia pipientis* (*w*Pip). Incompatibilities can be unidirectional (crosses between males infected with strain A and females infected with strain B are fertile, while reciprocal crosses are not) or bidirectional (reciprocal crosses between sexes with different *w*Pip strains are infertile). The technique depends on release of sufficient numbers of male mosquitoes infected with an incompatible *w*Pip strain to suppress mosquito populations and reduce transmission of introduced avian malaria (*Plasmodium relictum*) and *Avipoxvirus* in native forest bird habitats. Both diseases are difficult to manage using more traditional methods based on removal and treatment of larval habitats and coordination of multiple approaches may be needed to control this vector. We characterized the diversity of *Wolbachia* strains in *C. quinquefasciatus* from Hawai'i, Kaua'i, Midway Atoll, and American Samoa with a variety of genetic markers to identify compatibility groups and their distribution within and between islands. We confirmed the presence of *w*Pip with multilocus sequence typing, tested for local genetic variability using 16 WO prophage genes, and identified similarities to strains from other parts of the world with a transposable element (*tr1*). We also tested for genetic differences in ankyrin motifs (*ank2* and *pk1*) which have been used to classify *w*Pip strains into five worldwide groups (*w*Pip1–*w*Pip5) that vary in compatibility with each other based on experimental crosses. We found a mixture of both widely distributed and site specific genotypes based on presence or absence of WO prophage and transposable element markers on Hawai'i Island (Volcano, Pu'u Wa'awa'a, Laupāhoehoe, Kaumana, Kahuku, Nīnole, and Maulua Gulch), Kaua'i Island (Kawaikōī, Mōhihi, Kalāheo, Lāwa'i and Hanapepe) and Midway Atoll. Genotypes from American Samoa were unique and formed their own clade. Based on analysis of ankyrin motifs, *w*Pip strains from Hawai'i, Kaua'i, and Midway Atoll were most similar to *w*Pip5 strains of Australasian origin. By contrast, *Wolbachia* strains from *Culex quinquefasciatus* collected in American Samoa were most similar to *w*Pip3 strains of American origin. We detected a single *Culex* mosquito from Pu'u Wa'awa'a on Hawai'i Island that was infected with a unique *w*Pip3 genotype. This discovery, plus a rarefaction analysis of genotypes from Kaua'i and Hawai'i Islands suggests that limited sampling may have underestimated diversity of *w*Pip in our study. Mosquitoes infected with *w*Pip5 and *w*Pip3 are bidirectionally compatible with each other based on prior studies, which would support their ability to coexist within the same population on Hawai'i Island. Available evidence from prior studies suggests that genotype *w*Pip4 from Africa, the Middle East, Europe, and Asia is bidirectionally incompatible with genotype *w*Pip5 and varies in compatibility with genotype *w*Pip3 depending on geographic origin. Since *w*Pip5 appears to be the most common compatibility group in Hawai'i based on limited sampling, logical next steps are to 1) expand the current survey to include additional islands and localities, 2) infect a laboratory colony of Hawaiian *Culex* with *w*Pip4 through tetracycline treatment of Hawaiian mosquitoes and backcross with *Culex* from Europe, North Africa, and the Middle East that are naturally infected with *w*Pip4, 3) conduct cage trials to confirm bidirectional incompatibilities between Hawaiian *Culex* infected with *w*Pip4 and *w*Pip5, and 4) conduct field trials to evaluate whether release of incompatible males can be applied at small scales to suppress local populations.

## INTRODUCTION

The introduction of a competent mosquito vector (*Culex quinquefasciatus*) and both *Plasmodium relictum* and *Avipoxvirus* to the Hawaiian Islands has had devastating effects on the population sizes and geographic distribution of endemic forest birds (Warner 1968, van Riper et al 1986). While Hawai'i 'amakihi (*Hemignathus virens*) have evolved some degree of tolerance to avian malaria and are expanding throughout lower elevations in the Puna District of Hawai'i Island (Spiegel et al. 2006, Atkinson et al. 2013), other species of native honeycreepers remain highly susceptible to introduced disease and restricted to high elevation habitats where mosquito populations and pox and malaria transmission are limited by lower temperatures (LaPointe et al. 2010, Ahumada et al. 2004). Current climate projections predict that these refugia may not exist at the end of this century (Liao et al. 2015, Fortini et al. 2015) and recent dramatic increases in both mosquito numbers and the prevalence of avian malaria on the Alaka'i Plateau of Kaua'i suggest that disease transmission is already moving to higher elevations (Atkinson et al. 2014, Glad and Crampton 2015). Transmission models for avian malaria on Hawai'i Island indicate that control might be feasible in areas where primary larval habitats are associated with feral animals if larval habitats can be reduced by more than 80% (Hobbelen et al. 2012). This approach, however, is not applicable to most montane wet forest habitats in the state where standing water and intermittent and permanent streams provide abundant habitat for larval mosquitoes.

Given anticipated changes in climate by the end of the century, projected expansion of year-round mosquito populations into high elevation habitats, and the possibility that many threatened and endangered species may not have enough genetic diversity to evolve tolerance to infection on their own (Atkinson and LaPointe 2009, Liao et al. 2015, Fortini et al. 2015), it is likely that integrated mosquito control based on a wide variety of approaches will be needed to sustain remaining species of forest birds into the next century (LaPointe et al. 2009). These include traditional methods based on source reduction and use of selected larvicides and adulticides, sterile male techniques based on release of male mosquitoes that have been sterilized by either radiation or chemical mutagens, and incompatible insect techniques based on cytoplasmic incompatibility (CI) between male and female mosquitoes infected with *Wolbachia.* Among species of *Aedes* mosquitoes, infection with *Wolbachia* strains derived from *Drosophila* (*w*Mel) (Walker et al. 2011) or closely related mosquitoes can interfere with transmission of arboviruses through activation of mosquito innate immunity (Rainey et al. 2014) or induce CI (Brelsfoard et al. 2011). By contrast, naturally occurring *w*Pip infections in *Culex* mosquitoes may have little or no effects on development of the sporogonic stages of avian *Plasmodium* (Zélé et al. 2014), possibly because of co-evolutionary adaptations that might be expected in natural vector pathogen associations.

Genetic modification (GMO, genetically modified organisms) of male and female mosquitoes to induce sterility or insert genes that reduce vectorial capacity for specific pathogens is an emerging technology that may be used alongside more traditional approaches. This technology has advanced rapidly in recent years and reached the stage of field testing for control of *Aedes* mosquitoes (Alphey et al. 2014, Burt et al. 2014). While promising, GMO techniques are controversial because of public fears (Enserink 2010, Alverez 2015) and ethical and environmental concerns about using gene drive mechanisms to spread modified genes that could alter entire wild populations (Ledford 2015). Some of these concerns can be addressed by establishing scientific standards, clear regulations, and well defined safety checks for the use of

genetically modified insects, but these issues have not been fully vetted by regulatory agencies and public discussion (Reeves et al. 2012).

GMO technology for control of mosquitoes in Hawai'i may ultimately prove to be the most cost effective method for reducing or controlling disease transmission, but it may require years of public outreach and education and complicated regulatory approval (Lavery 2008, Ramsey et al. 2014). Since incompatible insect techniques using *Wolbachia* do not require use of biotechnology to alter mosquito hosts or bacterial endosymbionts and rely on natural reproductive incompatibilities within the *Culex pipiens* complex, their use avoids regulatory issues associated with GMO technology (Ostera and Gostin 2011).

Our primary goal was to explore feasibility of incompatible insect control techniques in Hawai'i. We first confirmed the presence of *w*Pip in Hawaiian *C. quinquefasciatus* (the only species of *Culex* that has been introduced to the islands) with multilocus sequence typing (MLST). We then investigated local genetic diversity of *w*Pip across the state, Midway Atoll, and American Samoa with Polymerase Chain Reaction (PCR) primers to 16 WO prophage genes. Finally, we identified compatibility groups and geographic relationships of Hawaiian strains of *w*Pip with other global populations based on a transposable element *tr1* (Duron et al. 2005) and ankyrin motifs (*ank2* and *pk1*; Atyame et al. 2014).

## METHODS

### Field Collection and DNA Extraction

Adult female *Culex* mosquitoes and egg rafts were collected between August, 2010 and November, 2014 at 19 different locations on Hawai'i Island, Kaua'i, Midway Atoll (Sand Island) and American Samoa (Tutuila and Ta'u Islands; Table 1). Mosquitoes were captured using gravid traps (Model 1712, John W. Hock Company Gainesville, FL) baited with an infusion of alfalfa rabbit chow and a yeast protein/lactalbumin mixture and placed into 100% ethanol after removal from the trap. We also collected egg rafts from stock tanks and pans of water containing the alfalfa/yeast/lactalbumin mixture at several field sites and placed these directly into 100% ethanol. All specimens were stored at -20°C in 100% ethanol until extraction.

For DNA extractions, egg rafts or adult mosquitoes were transferred from tubes containing ethanol and placed individually in 1.5 ml centrifuge tubes containing 180 µl of phosphate buffered saline, pH 7.2. An electric homogenizer (Fisher Scientific Pellet Pestle, #12-141-361, Fisher Scientific, Pittsburgh, PA) with disposable pestles was used to grind individual mosquitoes to break the exoskeleton and disrupt tissues. We added 20 µL of proteinase K (Qiagen DNeasy Blood and Tissue Kit, Valencia, CA) and 200 µL Qiagen AL Buffer to the sample, mixed briefly on a vortex mixer, and incubated the sample for 10 min at 70°C. The remainder of the DNA extraction process followed instructions for soft bodied insects that were provided with the extraction kit. DNA concentrations and purity were measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). All extracts were stored at -20°C until use.

Table 1. Field sites, collection dates, and sample sizes for adult *Culex* mosquitoes and/or *Culex* egg rafts that were collected during the study.

| Location | Island | Locality | Elevation (m) | Dates | N |
|---|---|---|---|---|---|
| Hawai'i | Hawai'i | Kahuku | 1200 | Apr, 2014 | 19 |
| | | Volcano | 1150 | May, 2012 | 2 |
| | | | | Jan, 2014 | 4 |
| | | | | May, 2014 | 17 |
| | | | | Sep, 2014 | 4 |
| | | Kaumana | 180 | Apr, 2014 | 21 |
| | | Nīnole | 350 | Apr, 2012 | 3 |
| | | | | May, 2012 | 2 |
| | | | | Feb, 2014 | 8 |
| | | | | Sep, 2014 | 8 |
| | | Maulua Gulch | 30 | Apr, 2012 | 9 |
| | | Laupāhoehoe | 1200 | Oct, 2013 | 2 |
| | | | | Feb, 2014 | 3 |
| | | | | Aug, 2014 | 5 |
| | | | | Oct, 2014 | 8 |
| | | Pu'u Wa'awa'a | 930 | Apr, 2014 | 21 |
| Kaua'i | Kaua'i | Hanapepe | 50 | Nov, 2013 | 1 |
| | | Lāwa'i | 150 | Dec, 2013 | 5 |
| | | Kalāheo | 200 | Nov, 2013 | 4 |
| | | | | Dec, 2013 | 9 |
| | | | | Nov, 2014 | 2 |
| | | Kawaikōī | 1200 | Oct, 2009 | 10 |
| | | Poipu | 50 | Dec, 2013 | 6 |
| | | Mōhihi | 1300 | May, 2012 | 1 |
| | | Halepa'akai | 1400 | Oct, 2013 | 2 |
| Midway Atoll | Sand Island | Septic Tanks | 10 | Apr, 2012 | 19 |
| | | | | Apr, 2014 | 1 |
| American Samoa | Tutuila | Olo Ridge | 250 | Mar, 2011 | 8 |
| | | Amalau Valley | 30 | Mar, 2011 | 2 |
| | Ta'u | Faleasao | 130 | Aug, 2010 | 5 |
| | | | | Jan, 2011 | 4 |
| Total (all Locations) | | | | | 215 |

Table 2. PCR primers that were used in this study with expected product sizes and original citations.

| Primer | Direction | Sequence (5'-3') | Citation | Product (bp) |
|---|---|---|---|---|
| gatB_F1 | Forward | GAKTTAAAYCGYGCAGGBGTT | Baldo et al. 2006 | 369 |
| gatB_R1 | Reverse | TGGYAAYTCRGGYAAAGATGA | Baldo et al. 2006 | |
| coxA_F1 | Forward | TTGGRGCRATYAACTTTATAG | Baldo et al. 2006 | 402 |
| coxA_R1 | Reverse | CTAAAGACTTTKACRCCAGT | Baldo et al. 2006 | |
| hcpA_F1 | Forward | GAAATARCAGTTGCTGCAAA | Baldo et al. 2006 | 444 |
| hcpA_R1 | Reverse | GAAAGTYRAGCAAGYTCTG | Baldo et al. 2006 | |
| ftsZ_F1 | Forward | ATYATGGARCATATAAARGATAG | Baldo et al. 2006 | 435 |
| ftsZ_R1 | Reverse | TCRAGYAATGGATTRGATAT | Baldo et al. 2006 | |
| fbpA_F1 | Forward | GCTGCTCCRCTTGGYWTGAT | Baldo et al. 2006 | 429 |
| fbpA_R1 | Reverse | CCRCCAGARAAAAYYACTATTC | Baldo et al. 2006 | |
| wsp_F1 | Forward | GTCCAATARSTGATGARGAAAC | Baldo et al. 2006 | 546 |
| wsp_R1 | Reverse | CYGCACCAAYAGYRCTRTAAA | Baldo et al. 2006 | |
| Gp1b | Forward | AAGTGGCTGGAAAATGTATAAC | Duron et al. 2006b | 307 |
| Gp1b | Reverse | TGAGTTTGCTATTTACTGCTAG | Duron et al. 2006b | |
| Gp2a | Forward | GCAAATATTTTAGGTGAGGCGC | Duron et al. 2006b | 363 |
| Gp2a | Reverse | ACGGAGTTCTCCACAAAGTACT | Duron et al. 2006b | |
| Gp2b | Forward | CGTAGTGGCATTGAATTTAACC | Duron et al. 2006b | 642 |
| Gp2b | Reverse | ACGGAGTTCTCCACAAAGTACT | Duron et al. 2006b | |
| Gp2d | Forward | AGAACACCCTGGTGAAAATACC | Duron et al. 2006b | 586 |
| Gp2d | Reverse | ACGGAGTTCTCCACAAAGTACT | Duron et al. 2006b | |
| Gp2e | Forward | TTCTACAACAGATGATCAAACG | Duron et al. 2006b | 306 |
| Gp2e | Reverse | CATCATCGGCCTACATAGCCA | Duron et al. 2006b | |
| Gp3a | Forward | AAGTGGGTTTGATGAAAAATGT | Duron et al. 2006b | 1339 |
| Gp3a | Reverse | TACATCATCATGCGGAATGTGC | Duron et al. 2006b | |
| Gp3b | Forward | CAGAGGTCTTTCAATTGAAAAG | Duron et al. 2006b | 428 |
| Gp3b | Reverse | GCGGTTATAAAATTTAAATGCA | Duron et al. 2006b | |
| Gp3c | Forward | CAGAGGTCTTTCAATTGAAAAG | Duron et al. 2006b | 196 |
| Gp3c | Reverse | AAGAACTTCAGTACGATACTTG | Duron et al. 2006b | |
| Gp3d | Forward | AAGTGGGTTTGATGAAAAATGT | Duron et al. 2006b | 361 |
| Gp3d | Reverse | AAGAACTTCAGTACGATACTTG | Duron et al. 2006b | |
| Gp7d | Forward | AAAAGGTTCTACAAGATTTTTGAA | Duron et al. 2006b | 423 |
| Gp7d | Reverse | CCTTTATAACCTCTTGGCATTGT | Duron et al. 2006b | |
| Gp9a | Forward | TTTTGCCATTGCAGAGTTACAG | Duron et al. 2006b | 220 |
| Gp9a | Reverse | TGATAACTCTCCCAATGGT | Duron et al. 2006b | |
| Gp9b | Forward | GATTTCAGAGCTGAATAGGAAG | Duron et al. 2006b | 332 |
| Gp9b | Reverse | GCTTCTCTTTATCATATAACAGT | Duron et al. 2006b | |
| Gp15a | Forward | TGTGACTACTAATGCTTCAGGA | Duron et al. 2006b | 296 |
| Gp15a | Reverse | CTTCCTCACAGTATTCGAGTTT | Duron et al. 2006b | |
| Gp15b | Forward | GTAGAAGCAAAAGAGTTTGTTG | Duron et al. 2006b | 484 |
| Gp15b | Reverse | CTTCCTCACAGTATTCGAGTTT | Duron et al. 2006b | |
| Gp24a | Forward | AGGTAAGTATGGTAAGCTTGGG | Duron et al. 2006b | 706 |
| Gp24a | Reverse | AGAGCAAATGGGAATAACTGCA | Duron et al. 2006b | |
| Gp24b | Forward | ATAAGCTACTTGGATTTACCAC | Duron et al. 2006b | 398 |
| Gp24b | Reverse | GGAATCCATAGGCATAACTGCA | Duron et al. 2006b | |

| | | | | |
|---|---|---|---|---|
| Tr1e-F1 | Forward | ACTTTAGAGGGGTGCTTTCT | Duron et al. 2005b | 303-1221 |
| Tr1e-R1 | Reverse | TTCATGGAGCTGAAGGTAT | Duron et al. 2005b | |
| ank2-F | Forward | CTTCTTCTGTGAGTGTACGT | Atyame et al. 2011b | 313-511 |
| ank2-R2 | Reverse | TCCATATCGATCTACTGCGT | Atyame et al. 2011b | |
| pk1F | Forward | CCACTACATTGCGCTATAGA | Duron et al. 2007 | 1328 |
| pk1R | Reverse | ACAGTAGAACTACACTCCTCCA | Duron et al. 2007 | |
| pk1iF | Forward | TACTCCATTTAGCTGCACAAAG | (this study) | 564 |
| pk1iR | Reverse | GGTGTCTGATTTCGTGCATC | (this study) | |

**Multilocus Sequence Typing**

We used MLST based on five highly conserved housekeeping genes, *gatB* (glutamyl-tRNA amidotransferase, subunit B), *coxA* (cytochrome c oxidase, subunit I), *hcpA* (conserved hypothetical protein), *ftsZ* (cell division protein), and *fbpA* (fructose-bisphosphate aldolase) and the *Wolbachia wsp* (outer surface protein) gene to verify identity of *w*Pip in specimens of *C. quinquefasciatus* collected from Hawai'i Island and Midway Atoll (Baldo et al. 2006). Reaction conditions, primers, and cycling conditions followed those described in Baldo et al. (2006) (Appendix 1). PCR products were visualized on 1.5% agarose gels containing ethidium bromide, cleaned with ExoSAP-IT (Affymetrix Inc, Cleveland, OH) and sequenced in both directions on an Applied Biosystems 3500 Genetic Analyzer (University of Hawaii, Hilo EPSCoR Evolutionary Genomics Core Facility). All sequences were aligned and trimmed to *w*Pip reference sequences available on GenBank using Sequencer 5.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA) (http://www.genecodes.com/) and Geneious R8 (Biomatters LTD, Auckland, New Zealand) (http://www.geneious.com, Kearse et al., 2012). Representative isolates from Hawai'i Island and Midway Atoll were typed based on similarities to *w*Pip reference sequences available from the *Wolbachia* MLST Database (http://pubmlst.org/wolbachia/). Representative sequences were deposited in the *Wolbachia* MLST Database.

**Fine Scale Genetic Differences**

All samples were tested for the presence or absence of 16 unique WO prophage gene products (Duron et al. 2006a, 2006b). These bacteriophage mobile genetic elements are widespread in *Wolbachia* spp. that infect arthropods, are capable of high rates of transfer between divergent lineages, and are useful markers for characterizing polymorphism and diversity of these bacterial endosymbionts (Bordenstein and Wernegreen 2004, Gavotte et al. 2004, Duron et al. 2006b). PCRs for WO prophage genes were carried out in 25 µl volumes with 0.5 U of GoTaq Polymerase (Promega, Madison, WI) in a master mix made with 1X Green GoTaq Flexi Buffer (Promega, Madison, WI) and primers specific for each gene product (Table 2, Appendix 2). Products were visualized on 2.0% agarose gel containing ethidium bromide and size was established through comparison with a 100bp DNA fragment ladder (Promega Corporation, Madison, WI). Digital pictures of the gels were taken with a Gel Logic 100 digital camera mounted on an ultraviolet (UV) transilluminator and analyzed using Carestream MI SE software (Carestream Healthcare, Rochester, NY). Samples were declared positive for specific WO prophage gene products if there was a peak with the expected fragment size and a signal to noise ratio of 5:1 or greater.

**Transposable Element Polymorphism**

We used a set of primers that amplify unique open reading frames based on the bacterial transposable element *tr1* to provide additional information on similarities of Hawaiian strains of *w*Pip to other *w*Pip isolates from around the world (Duron et al. 2005, 2006b, Atyame et al.

2011b). PCRs for *tr1* were carried out as described earlier for WO prophage genes with primers Tr1e-F1 and Tr1e-R1 which amplify a 1,321 bp fragment containing the *tr1* transposable element (Duron et al. 2005) (Table 1, Appendix 3). Absence of the *tr1* insert is determined by amplification of a smaller 404 bp PCR product, while restriction fragment length polymorphism (RFLP) analysis of the full 1,321 bp PCR product can distinguish four additional worldwide groups of *w*Pip (Duron et al. 2005).

**Worldwide Compatibility Groups**

*Wolbachia* infections were classified based on similarities with other *w*Pip strains and assigned to cytoplasmic compatibility groups based on sequence data and RFLP analysis of two genes exhibiting ankyrin motifs: *pk1* and *ank2* (Duron 2007, Dumas et al. 2013). PCRs were performed as described above for the WO prophage gene products with primers specific for *ank2* and *pk1* (Table 1), but cycling conditions were modified (Appendix 4). *HinfI* digestion of *ank2* PCR products allowed discrimination of five alleles with different worldwide distributions. Allele a was distinguished by presence of a single, 313 bp RFLP fragment; allele b was distinguished by presence of 217, 195 and 98 bp fragments; allele c was distinguished by presence of 293 and 217 bp fragments, allele d was distinguished by presence of 217 and 195 bp fragments; allele e was distinguished by presence of a single 415 bp fragment (Dumas et al. 2013). We sequenced complete *pk1* PCR products (1,328 bp) from a small subset of representative samples from different locations to establish relationships with *pk1* reference sequences for alleles a, b, c, d, and e (Dumas et al. 2013). We amplified and sequenced a smaller 564 bp fragment of the *pk1* gene for a larger number of samples with primers pk1iF and pk1iR (Table 1, Appendix 4). This fragment was internal to the larger 1,328 bp *pk1* product and had sufficient variability to allow us to distinguish among the five *pk1* alleles described by Dumas et al. (2013). We also sequenced examples of *ank2* alleles. Representative sequences of partial *ank2* and *pk1* alleles were deposited in Genbank.

**Data Analysis**

We conducted a rarefaction analysis to evaluate diversity of WO prophage genotypes on Hawai'i, Kaua'i, Midway Atoll and American Samoa with EstimateS version 9.1.0 (Colwell 2013). This program uses a statistical approach to link rarefaction and extrapolation curves (Colwell et al. 2012), computes 95% confidence intervals, and allows comparison of sample richness between both large and small sample sizes without the need to discard data.

We converted presence/absence data for each of the 16 WO prophage gene products for each mosquito into a concatenated genotype composed of A (present) and T (absent) nucleotides for each of the individual gene products. All unique genotypes were aligned with ClustalW and used to construct a consensus tree with MrBayes 3.1 (Ronquist and Huelsenbeck 2003) to help visualize relationships across geographic areas that were sampled. The model was run for 3,000,000 generations with a sampling frequency of every 500 generations using a Markov chain Monte Carlo (MCMC) algorithm and a JC69 Model that assumes base frequencies are equal and rate substitutions between states are the same (Huelsenbeck and Ronquist 2005). The first 25% of trees were discarded as burn-in. We selected an arbitrary support threshold of 10% for generating a final consensus tree based on visual inspection of the data. This value minimized number of branches while preserving those with the most consensus support.

To establish relationships among our samples and five worldwide compatibility types that have been described previously, we used ClustalW in Geneious 8 to align both complete (1,328 bp) and partial (564 bp) sequences of *pk1* reference alleles with sequences from a representative

subset of samples from American Samoa, Midway Atoll, Kaua'i, and Hawai'i Islands. Phylogenetic trees were constructed with the MrBayes plugin for Geneious 8 after alignments that were trimmed to the shortest sequence (1,198 bp for 1,328 bp product, 498 bp for 564 bp product) were tested with jModeltest version 2.1.4 (Darriba et al. 2012, Guindon and Gascuel 2003) to identify the model of DNA substitution with the best support based on AIC (Aikake Information Criterion) value. For the Bayesian analysis we ran 3,000,000 generations with a sampling frequency of every 500 generations using a MCMC algorithm and a HKY85 model. The first 25% of trees were discarded as burn-in.

## RESULTS

### Multilocus Sequence Typing

We were able to type MLST housekeeping genes from an individual *C. quinquefasciatus* collected on Sand Island, Midway Atoll and one individual collected at 1,200 m in the Kahuku Unit of Hawai'i Volcanoes National Park (Table 3). We obtained partial genotypes from seven additional individuals from these two locations (Table 3). Sequences of all five housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) were 100% identical to *Wolbachia* Sequence Type (ST) 9, represented in the MLST database by *C. pipiens* collected in New York and California (identification numbers 29 and 30) and *C. quinquefasciatus* collected in Kenya (identification number 498). *Wsp* gene sequences from mosquitoes collected at Midway and Kahuku were also 100% identical to the *Wsp* gene sequences from *C. pipiens* collected in New York and California, including all four hypervariable regions (HVR1–HVR4) (Table 3). Isolate information is available in the *Wolbachia* MLST Database (Isolates 1808, 1809; http://pubmlst.org/wolbachia/).

Table 3. Partial and complete MLST genotypes of 9 representative *C. quinquefasciatus* from Sand Island (Sand Is.), Midway Atoll and the Kahuku Unit of Hawai'i Volcanoes National Park. Numbers correspond to allele numbers in the Wolbachia MLST database (http://pubmlst.org/wolbachia/). Colored cells indicate missing data. We were able to obtain complete MLST data on two individuals (6782, 6763). gatB = glutamyl-tRNA amidotransferase, subunit B); coxA = cytochrome c oxidase, subunit I; hcpA = conserved hypothetical protein; ftsZ = cell division protein; fbpA = fructose-bisphosphate aldolase; wsp =Wolbachia outer surface protein; HVR = hypervariable region of wsp.

| Sample | Location | Site | *gatB* | *coxA* | *hcpA* | *ftsZ* | *fbpA* | *wsp* | HVR1 | HVR2 | HVR3 | HVR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6782 | Hawai'i | Kahuku | 4 | 3 | 3 | 22 | 4 | 10 | 10 | 8 | 10 | 8 |
| 6763 | Midway | Sand Is. | 4 | 3 | 3 | 22 | 4 | 10 | 10 | 8 | 10 | 8 |
| 6762 | Midway | Sand Is. | 4 | 3 | | 22 | | 10 | 10 | 8 | 10 | 8 |
| 6764 | Midway | Sand Is. | 4 | | 3 | | | | | | | |
| 6775 | Hawai'i | Kahuku | | | 3 | | | 10 | 10 | 8 | 10 | 8 |
| 6778 | Hawai'i | Kahuku | 4 | | 3 | | | 10 | 10 | 8 | 10 | 8 |
| 6779 | Hawai'i | Kahuku | 4 | 3 | 3 | | | 10 | 10 | 8 | 10 | 8 |
| 6780 | Hawai'i | Kahuku | 4 | 3 | 3 | | | | 10 | 8 | 10 | |
| 6786 | Hawai'i | Kahuku | | 3 | 3 | | | | | | | |

**Fine Scale Genetic Differences**

PCR amplification of WO prophage genes using 16 primer sets (GP1a–GP24b) amplified products of expected sizes when they were present. We did not amplify GP15a, GP24a and GP24b, and detected GP15b in only two mosquitoes from American Samoa (Table 4). Remaining markers had variable patterns of amplification, dependent on island and source population.

We detected 40 different concatenated genotypes in a sample of 215 mosquitoes from Hawai'i Island (7 locations), Kaua'i (7 locations), Midway Atoll (1 location) and American Samoa (3 locations) based on presence or absence of 16 WO prophage gene products and the transposable element *tr1* (Table 4). Some geographic structure was evident from Bayesian analysis of concatenated genotypes (Figure 1). Genotypes from American Samoa (J, M, K, L, I) were unique to this archipelago and formed their own clade in the analysis (Figure 1, Group 3). Genotype M was present on both Tutuila and Ta'u Islands while genotypes J, K, L, and I were unique to either Tutuila (K, L) or Ta'u (J, I) (Figure 2). Remaining genotypes from Hawai'i, Kaua'i, and Midway Atolls were widely distributed across the Hawaiian archipelago (Figures 3–5) as an admixture of both locally unique genotypes and those that were shared both between and within islands. Geographic localization of particular genotypes was most evident for east Hawai'i Island (Figure 1, Group 1) and Midway Atoll (Figure 1, Group 2), but both areas had genotypes that were present elsewhere in the archipelago, e.g. genotypes C and MM, Figures 3, 4. Some genotypes were particularly widespread and shared between Hawai'i, Kaua'i, and Midway (MM) or shared more commonly between Hawai'i and Kaua'i (MM, LL, HH, II, V, Y and BB). All localities had unique genotypes that were not found elsewhere (Figures 2–5). Rarefaction analysis of genotypes from Hawai'i, Kaua'i, Midway Atoll and American Samoa indicates that our sampling on the larger islands of Hawai'i and Kaua'i only partially documented the full range of genotypes that are likely to be present (Figure 6, A and B dotted lines) and that our sample sizes were not able to distinguish significant differences in diversity of genotypes on Kaua'i and Hawai'i based on 95% confidence intervals (Figure 7). Numbers of possible genotypes present in American Samoa and on Midway Atoll appear to be much smaller based on rarefaction curves and our limited sampling appears to have documented most of the diversity of genotypes present in those locations (Figure 6, C and D dotted lines).

**Transposable Element Polymorphism**

We typed mosquitoes with primers Tr1e-F1 and Tr1e-R2 to provide an additional marker for identifying relationships between Hawaiian *w*Pip isolates and those from other parts of the world. We amplified a 404 bp product from almost all mosquitoes indicating absence of the 1,321 bp *tr1* transposable element. PCR products were not detected in four mosquitoes—two from Olo Ridge in American Samoa, one from Laupāhoehoe on Hawai'i Island, and one from Halepa'akai on Kaua'i Island in spite of high quality DNA and positive results for WO prophage genetic markers (Table 4). Mosquitoes that were missing the *tr1* insert were classified as group 3 based on criteria from Duron et al. (2005), with similarities to *w*Pip isolates that are worldwide in distribution but absent from North America.

Table 4. Presence or absence of WO gene products (Gp–Gp24) and the mobile genetic element *tr1* based on PCR reactions with primers listed in Table 2.
Numerical entry for *tr1* indicates *w*Pip group defined by Duron et al. (2005) based on presence/absence of a 404 bp PCR product. We identified 40 different genotypes (A–NN) based on presence (+) or absence (-) of each marker. N = number of mosquitoes with each genotype.

| Genotype | N | Gp1b | Gp2a | Gp2b | Gp2d | Gp2e | Gp3a | Gp3b | Gp3c | Gp3d | Gp7d | Gp9a | Gp9b | Gp15a | Gp15b | Gp24a | Gp24b | Tr1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | - | - | - | - | - | - | - | - | + | - | + | - | - | - | - | - | 3 |
| B | 1 | - | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - | 3 |
| C | 5 | - | - | - | - | - | - | + | - | + | + | + | - | - | - | - | - | 3 |
| D | 1 | - | - | - | + | - | - | - | + | + | + | + | + | - | - | - | - | 3 |
| E | 2 | - | - | - | + | - | - | + | - | + | - | + | - | - | - | - | - | 3 |
| F | 3 | - | - | - | + | - | - | + | - | + | + | + | - | - | - | - | - | 3 |
| G | 1 | - | - | - | + | - | + | + | - | + | + | + | - | - | - | - | - | 3 |
| H | 2 | - | + | - | - | - | - | + | - | + | + | + | - | - | - | - | - | 3 |
| I | 2 | - | + | - | + | + | - | + | - | + | + | + | + | - | - | - | - | 3 |
| J | 1 | - | + | + | + | + | + | + | + | + | - | + | + | - | + | - | - | 3 |
| K | 2 | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - |
| L | 5 | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | 3 |
| M | 9 | - | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | 3 |
| N | 1 | + | - | - | - | - | - | + | - | + | + | - | - | - | - | - | - | - |
| O | 1 | + | - | - | + | - | - | - | - | - | - | + | - | - | - | - | - | 3 |
| P | 1 | + | - | - | + | - | - | - | - | - | + | + | - | - | - | - | - | 3 |
| Q | 2 | + | - | - | + | - | - | - | - | - | + | + | + | - | - | - | - | 3 |
| R | 5 | + | - | - | + | - | - | - | + | - | + | + | + | - | - | - | - | 3 |
| S | 5 | + | - | - | + | - | - | - | + | + | + | + | + | - | - | - | - | 3 |
| T | 1 | + | - | - | + | - | - | + | - | - | - | + | - | - | - | - | - | 3 |
| U | 5 | + | - | - | + | - | - | + | - | + | - | + | - | - | - | - | - | 3 |
| V | 3 | + | - | - | + | - | - | + | - | + | + | + | - | - | - | - | - | 3 |
| W | 1 | + | - | - | + | - | - | + | - | + | + | + | + | - | - | - | - | 3 |
| X | 1 | + | - | - | + | - | + | - | - | - | - | - | - | - | - | - | - | 3 |
| Y | 6 | + | - | - | + | - | + | - | - | + | - | + | - | - | - | - | - | 3 |
| Z | 1 | + | - | - | + | - | + | - | - | + | - | + | + | - | - | - | - | 3 |
| AA | 3 | + | - | - | + | - | + | - | - | + | + | + | - | - | - | - | - | 3 |
| BB | 2 | + | - | - | + | - | + | - | - | + | + | + | + | - | - | - | - | 3 |
| CC | 1 | + | - | - | + | - | + | - | + | + | + | + | - | - | - | - | - | 3 |
| DD | 5 | + | - | - | + | - | + | - | + | + | + | + | + | - | - | - | - | 3 |
| EE | 2 | + | - | - | + | - | + | + | - | - | + | + | - | - | - | - | - | 3 |
| FF | 1 | + | - | - | + | - | + | + | - | + | - | + | - | - | - | - | - | 3 |
| GG | 3 | + | - | - | + | - | + | + | - | + | + | - | - | - | - | - | - | 3 |
| HH | 28 | + | - | - | + | - | + | + | - | + | + | + | - | - | - | - | - | 3 |
| II | 5 | + | - | - | + | - | + | + | - | + | + | + | + | - | - | - | - | 3 |
| JJ | 4 | + | - | - | + | - | + | + | + | - | - | - | + | - | - | - | - | 3 |
| KK | 1 | + | - | - | + | - | + | + | + | - | + | + | + | - | - | - | - | 3 |
| LL | 20 | + | - | - | + | - | + | + | + | + | + | + | - | - | - | - | - | 3 |
| MM | 71 | + | - | - | + | - | + | + | + | + | + | + | + | - | - | - | - | 3 |
| NN | 1 | + | - | - | + | - | + | + | + | + | + | + | - | - | - | - | - | - |

Figure 1. Geographic structure of mobile genetic element genotypes based on Bayesian analysis of concatenated genotypes. Geographic structure is evident for Group 1 (East Hawai'i), Group 2 (Midway Atoll) and Group 3 (American Samoa), with remaining genotypes widely shared among locations and islands.

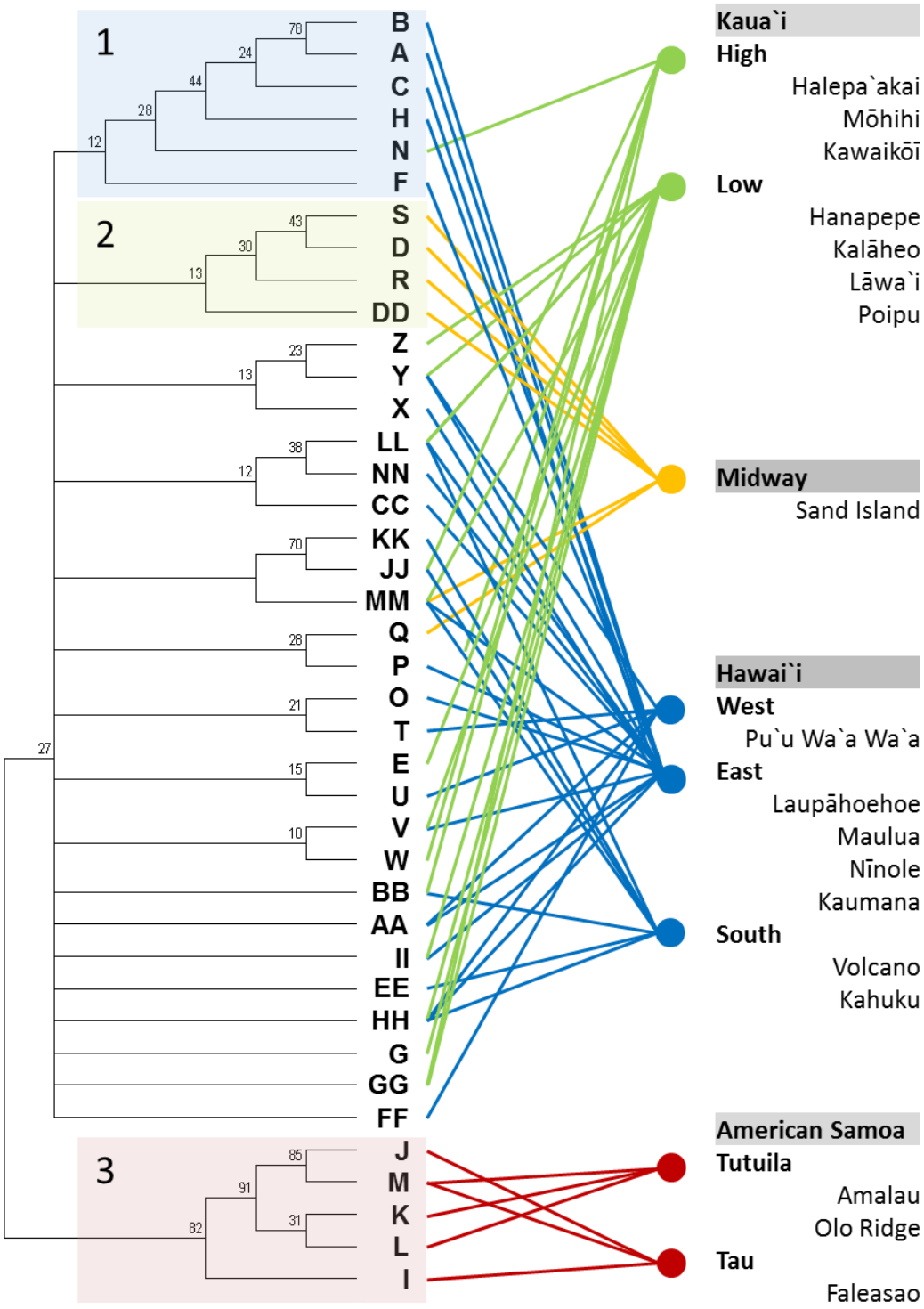


Figure 2. Distribution of mobile genetic element genotypes in American Samoa. Shared genotypes between Tutuila and Ta‘u Islands have the same color in pie diagrams. Unique lineages, i.e. K, L, I, J, are dark grey. None of the genotypes detected in American Samoa were common with those in the Hawaiian Islands.

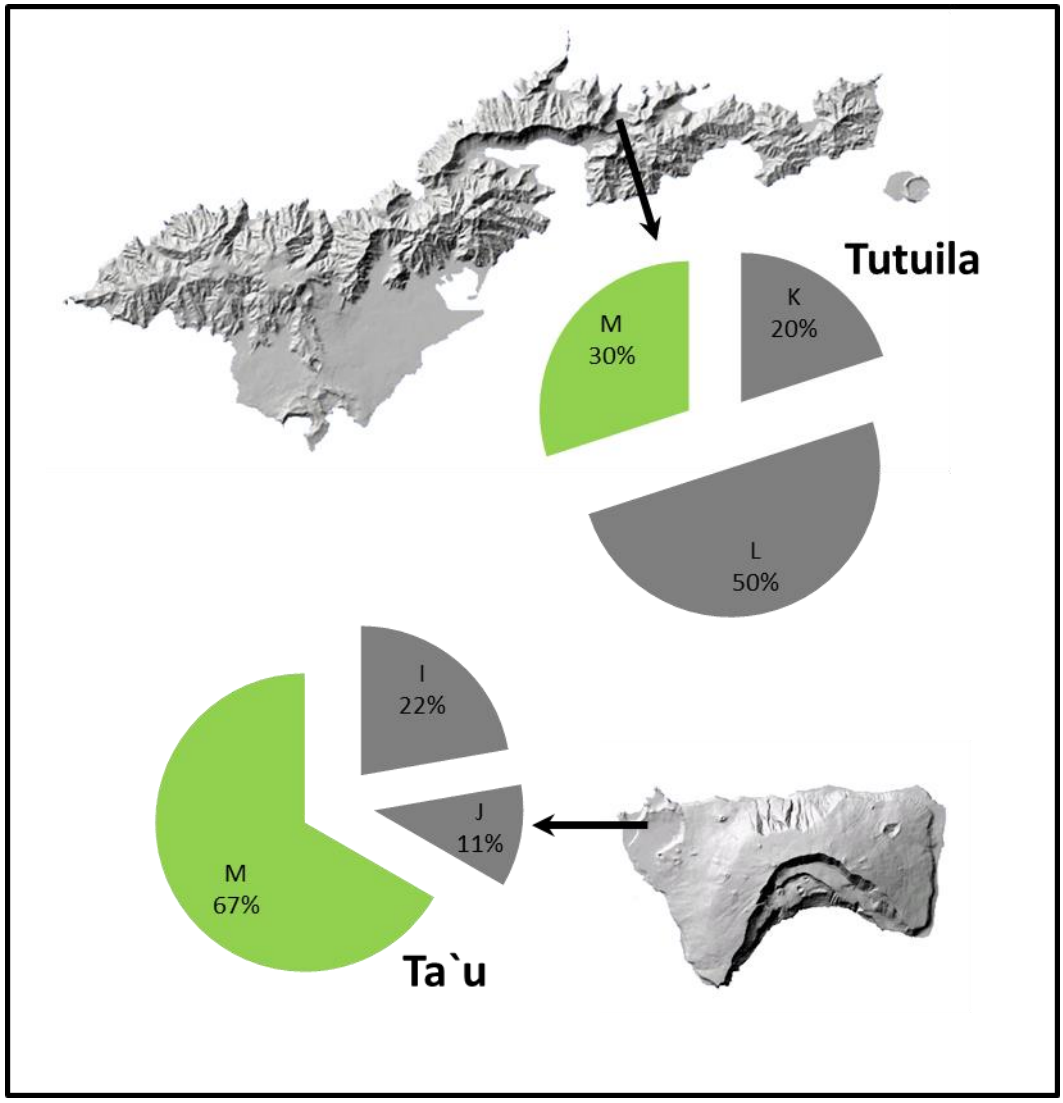


Figure 3. Summary of mobile genetic element genotypes from Hawai'i (A), Kaua'i (B) and Midway Atolls (C). Shared genotypes between and within Hawai'i, Kaua'i, and Midway Atolls have the same color in pie diagrams. Unique lineages, e.g. S, D, DD, R, and Q from Midway (C), are dark grey. Cross hatched lineages C and GG occurred at more than one location on either Hawai'i (C) or Kaua'i Islands (GG).

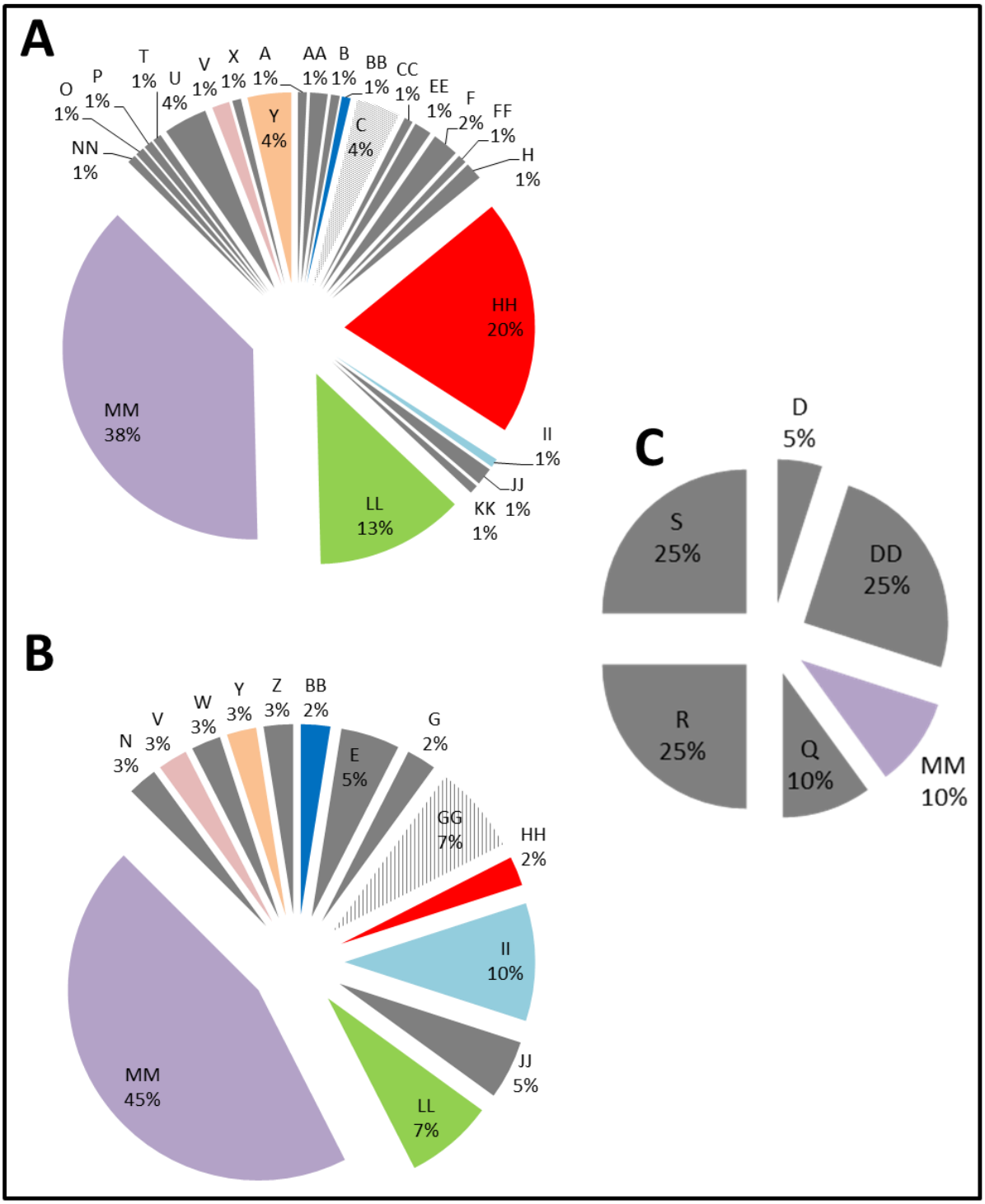


Figure 4. Distribution of mobile genetic element genotypes on the Island of Hawai'i. Shared genotypes between Hawai'i, Kaua'i, and Midway Atolls have the same color in pie diagrams. Unique lineages, e.g. A, B, H, F from Site E, are dark grey. Cross hatched lineage C occurred at more than one location on Hawai'i Island but was not found elsewhere. A = Kahuku B = Volcano; C = Kaumana; D = Nīnole; E = Maulua; F = Laupāhoehoe, 1200 m; G = Pu'u Wa'awa'a.

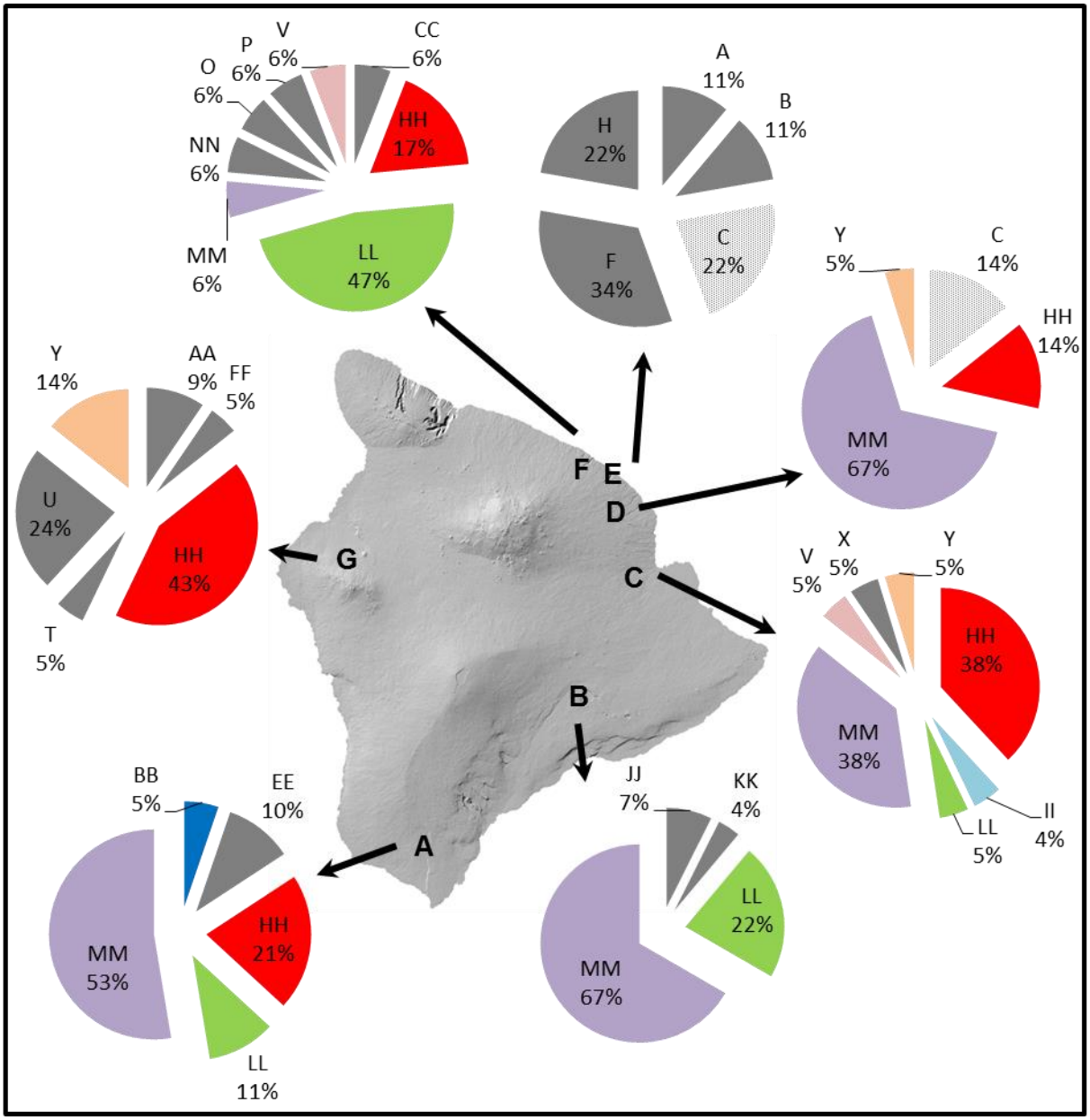


Figure 5. Distribution of mobile genetic element genotypes on the Island of Kauaʻi. Shared genotypes between Hawaiʻi, Kauaʻi, and Midway Atolls have the same color in pie diagrams. Unique lineages, e.g. N and W, are dark grey. Cross hatched lineage GG occurred at more than one location on Kauaʻi Island but was not found elsewhere. Because of small sample sizes, locations were grouped into high elevation sites (A = Kawaikōī; B = Mōhihi; C = Halapaʻakai Stream) and low elevation sites (D = Hanapepe, E = Kalāheo; F = Lāwaʻi; G = Poipu).

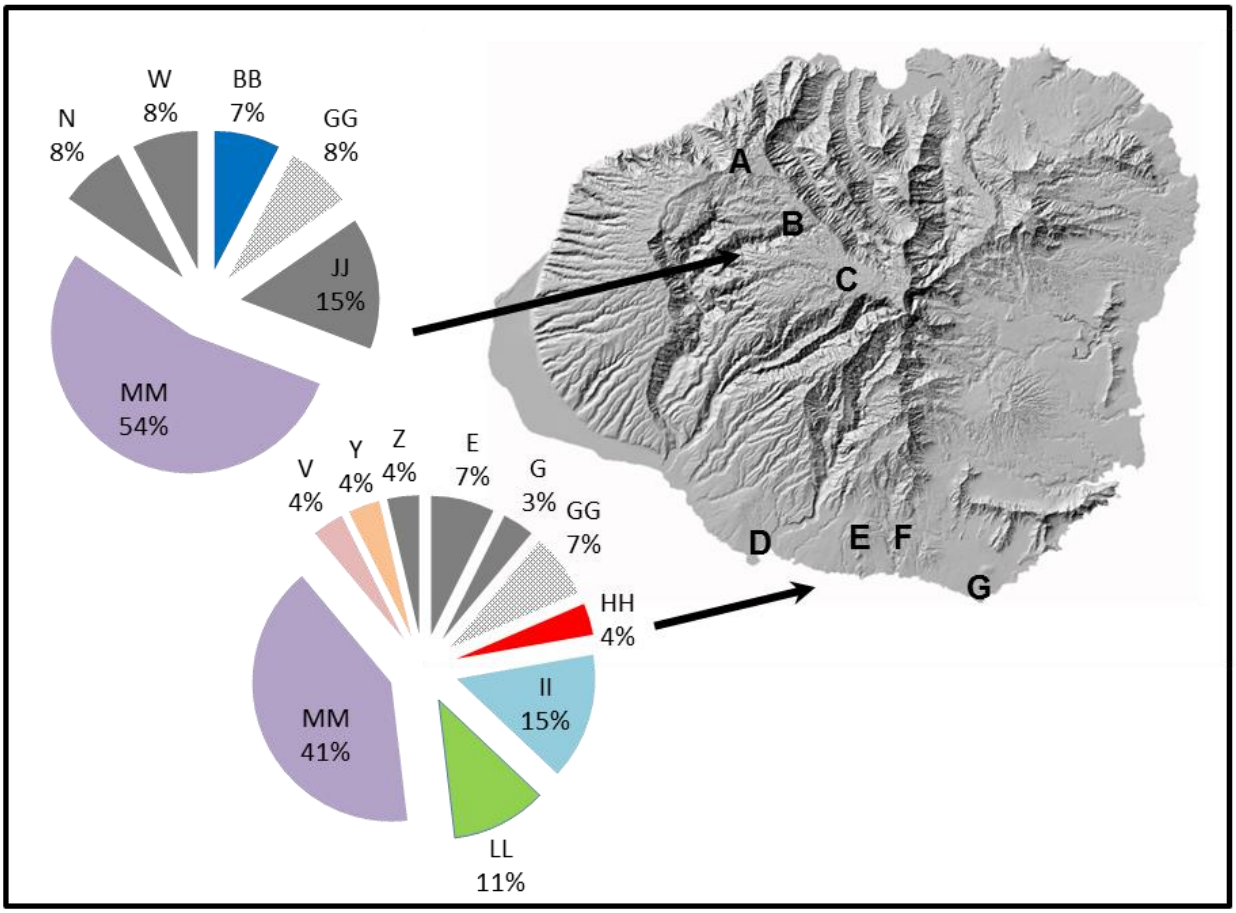


Figure 6. Rarefaction analysis of mobile genetic element genotypes from Hawai'i (A), Kaua'i (B), Midway Atoll (C), and American Samoa (D). Black dots indicate reference samples (i.e. actual number of genotypes that were detected) from each island or group of islands and dotted lines indicate extrapolated values based on sample sizes of 500 from each location.

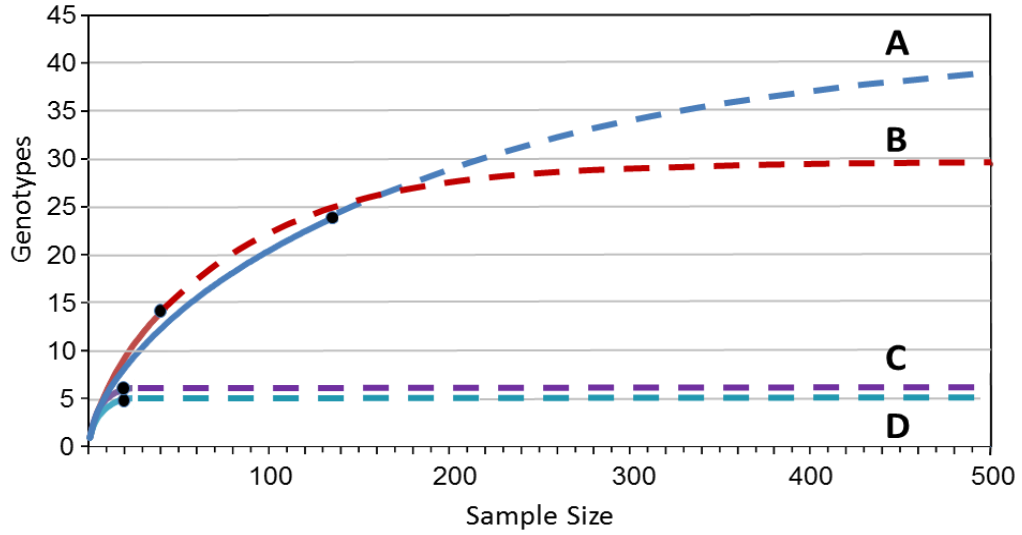
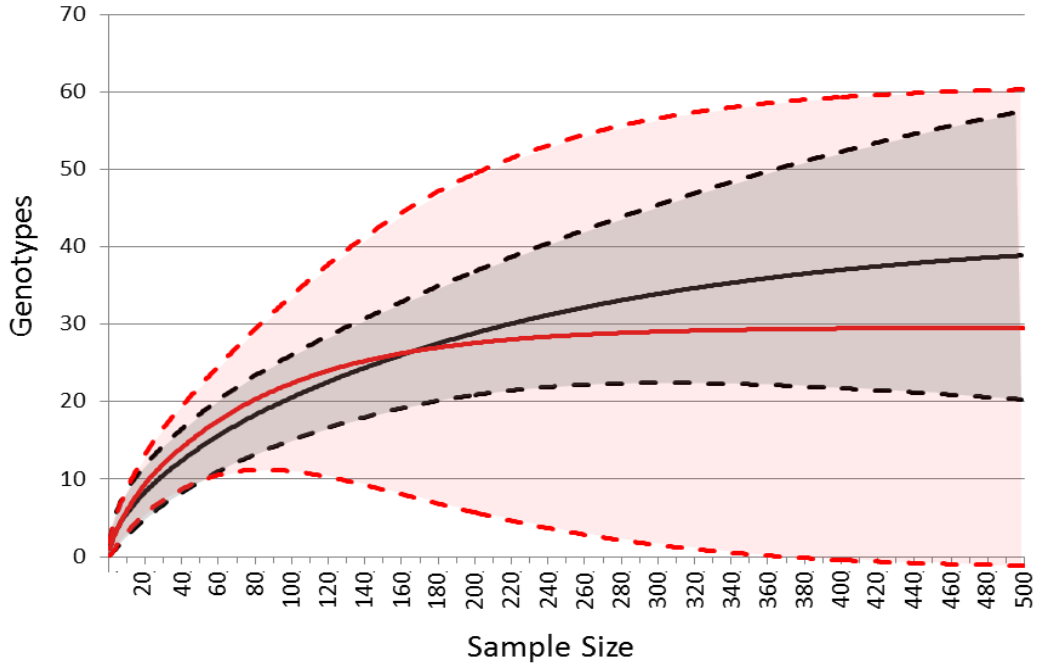



Figure 7. Rarefaction curves (solid lines) and 95% confidence intervals (dotted lines and shaded areas) for number of predicted genotypes from Hawai'i (black) and Kaua'i Islands (red) based on reference samples of 136 and 40 mosquitoes, respectively. No significant difference in number of genotypes was evident based on limited sample sizes.



**Worldwide Compatibility Types**

We determined worldwide compatibility types (*w*Pip1–*w*Pip5) based on sequences of 1,198 and 498 bp fragments of the *Wolbachia pk1* gene (Genbank Accession Numbers KU291877–KU2911878) (Table 5, *w*Pip group; Figures 8, 9) (Dumas et al. 2013) and identified regional *ank2* alleles based on sequences of representative samples (Genbank Accession Numbers KU291879–KU2911880) and RFLP digests of PCR products (Table 5). All mosquitoes collected in American Samoa plus a single mosquito collected at Pu'u Wa'awa'a on Hawai'i Island had *pk1* allele b and fell within group *w*Pip3 (Table 5, Figures 8, 9). All other mosquitoes from Midway Atoll, Kaua'i, and Hawai'i Islands had *pk1* allele e and fell within group *w*Pip5 (Table 5, Figures 8, 9). We identified *ank2* alleles a and d based on RFLP digests. Allele a was present in all mosquitoes from American Samoa that were screened and approximately half (4/7) mosquitoes from Midway Atoll that were tested. By contrast, allele d was present in approximately half (3/7) of mosquitoes from Midway Atoll that were screened and all mosquitoes that we tested from the main Hawaiian Islands (Table 5).

Based on *ank2*/*pk1* genotypes and locality data provided by Dumas et al. (2013), the majority of mosquitoes we tested from Kaua'i and Hawai'i Islands had *Wolbachia* genotypes that were Australasian in origin (genotype d/e—Australia, Philippines, China, Russia), while mosquitoes from American Samoa had *Wolbachia* genotypes that were Mexican or Costa Rican in origin (genotypes d/b, a/b, Mexico, Costa Rica). Mosquitoes collected on Midway Atoll had genotypes that were of Japanese (a/e) and Australasia origin (d/e—Australia, Philippines, China and Russia). A single mosquito collected at Pu'u Wa'awa'a on Hawai'i Island had a unique combination of *ank2* and *pk1* genotypes that was most similar to *Wolbachia* genotypes from Costa Rica (genotype d/b) (Table 5).

Table 5. Allelic profiles of ankyrin domain genes *ank2* and *pk1* and *w*Pip groups and localities based on sequence data and RFLP patterns as described by Dumas et al. (2013). Ank2/pk1 genotypes a/b have been detected in Mexico. Ank2/pk1 genotypes a/e have been detected in Japan. Ank2/pk1 genotypes d/b have been detected in Costa Rica. Ank2/pk1 genotypes d/e have been detected in China, the Philippines, Australia, and Russia (C/P/A/R).

| ID | Location | Site | *ank2* | *pk1* | *w*Pip Group | Locality |
|---|---|---|---|---|---|---|
| 9138 | American Samoa | Olo Ridge | a | b | *w*Pip3 | Mexico |
| 9124 | American Samoa | Olo Ridge | a | b | *w*Pip3 | Mexico |
| 8194 | American Samoa | Faleasao | a | b | *w*Pip3 | Mexico |
| 8200 | American Samoa | Faleasao | a | b | *w*Pip3 | Mexico |
| 7336 | Midway | Sand Island | d | e | *w*Pip5 | C/P/A/R |
| 7341 | Midway | Sand Island | d | e | *w*Pip5 | C/P/A/R |
| 7343 | Midway | Sand Island | d | e | *w*Pip5 | C/P/A/R |
| 9443 | Midway | Sand Island | a | e | *w*Pip5 | Japan |
| 7337 | Midway | Sand Island | a | e | *w*Pip5 | Japan |
| 9447 | Midway | Sand Island | a | e | *w*Pip5 | Japan |
| 6766 | Midway | Sand Island | a | e | *w*Pip5 | Japan |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7809 | Kauaʻi | Lāwaʻi | d | e | *w*Pip5 | C/P/A/R |
| 7805 | Kauaʻi | Kalāheo | d | e | *w*Pip5 | C/P/A/R |
| 7686 | Kauaʻi | Kalāheo | d | e | *w*Pip5 | C/P/A/R |
| 7683 | Kauaʻi | Kalāheo | d | e | *w*Pip5 | C/P/A/R |
| 7801 | Kauaʻi | Poipu | d | e | *w*Pip5 | C/P/A/R |
| 7810 | Kauaʻi | Lāwaʻi | d | e | *w*Pip5 | C/P/A/R |
| 7441 | Kauaʻi | Kawaikōī | d | e | *w*Pip5 | C/P/A/R |
| 7786 | Kauaʻi | Kawaikōī | d | e | *w*Pip5 | C/P/A/R |
| 6771 | Hawaiʻi | Kahuku | d | e | *w*Pip5 | C/P/A/R |
| 8356 | Hawaiʻi | Kahuku | d | e | *w*Pip5 | C/P/A/R |
| 7092 | Hawaiʻi | Volcano | d | e | *w*Pip*5* | C/P/A/R |
| 7091 | Hawaiʻi | Volcano | d | e | *w*Pip*5* | C/P/A/R |
| 9438 | Hawaiʻi | Volcano | d | e | *w*Pip5 | C/P/A/R |
| 9435 | Hawaiʻi | Volcano | d | e | *w*Pip5 | C/P/A/R |
| 7875 | Hawaiʻi | Kaumana | d | e | *w*Pip5 | C/P/A/R |
| 7881 | Hawaiʻi | Kaumana | d | e | *w*Pip5 | C/P/A/R |
| 7815 | Hawaiʻi | Kaumana | d | e | *w*Pip5 | C/P/A/R |
| 7500 | Hawaiʻi | Nīnole | d | e | *w*Pip5 | C/P/A/R |
| 9585 | Hawaiʻi | Nīnole | d | e | *w*Pip5 | C/P/A/R |
| 7235 | Hawaiʻi | Maulua | d | e | *w*Pip5 | C/P/A/R |
| 9841 | Hawaiʻi | Laupāhoehoe | d | e | *w*Pip5 | C/P/A/R |
| 9844 | Hawaiʻi | Laupāhoehoe | d | e | *w*Pip5 | C/P/A/R |
| 6768 | Hawaiʻi | Laupāhoehoe | d | e | *w*Pip5 | C/P/A/R |
| 7852 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7982 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7932 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7938 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7937 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7935 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7936 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7979 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7981 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7983 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7984 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip*5* | C/P/A/R |
| 7985 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| 7986 | Hawaiʻi | Puʻu Waʻawaʻa | d | e | *w*Pip5 | C/P/A/R |
| | | | | | | |
| 7851 | Hawaiʻi | Puʻu Waʻawaʻa | d | b | *w*Pip3 | Costa Rica |

Figure 8. Unrooted Bayesian cladogram that infers relationships among a 1,198 bp alignment of *pk1* sequences from Hawaiian and Samoan *w*Pip isolates and *pk1* reference alleles from Genbank (Dumas et al. 2013). Reference alleles and corresponding Genbank Accession number are red. Node values represent consensus support (%).

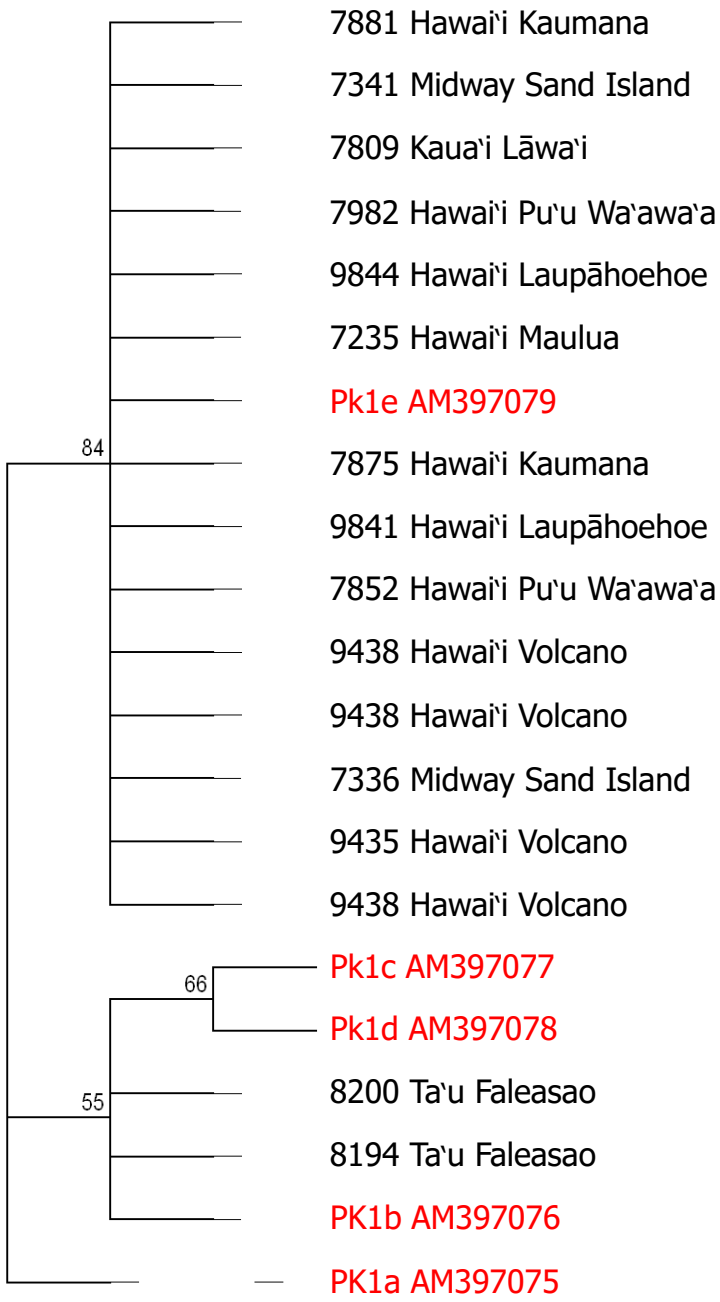
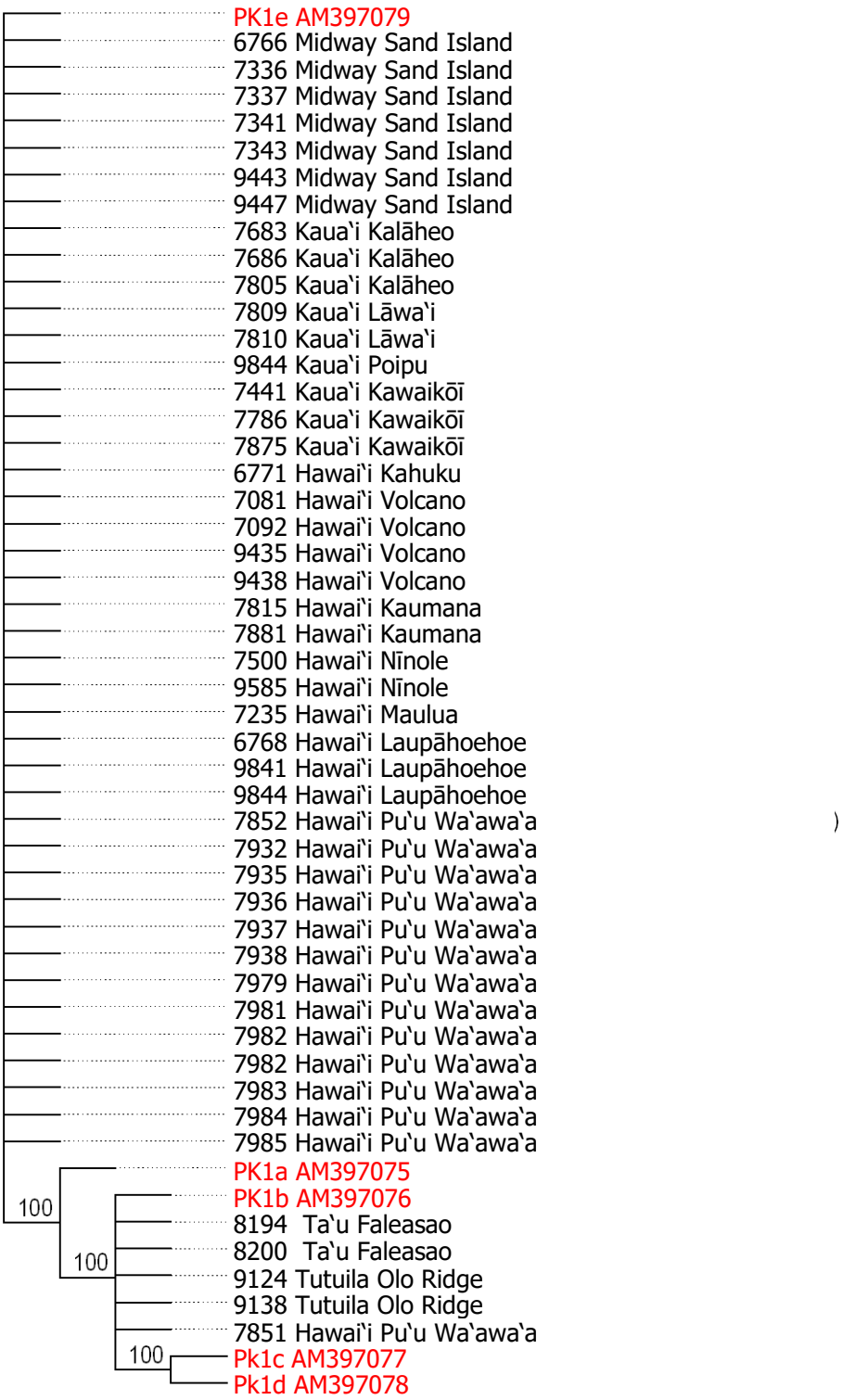


Figure 9. Unrooted Bayesian cladogram that infers relationships among a 498 bp alignment of *pk1* sequences from Hawaiian and Samoan *w*Pip isolates and *pk1* reference alleles from Genbank (Dumas et al. 2013). Reference alleles and corresponding Genbank Accession numbers are red. Node values represent consensus support (%).



## DISCUSSION

### Multilocus Sequence Typing and *w*Pip Diversity

We verified presence of *w*Pip in Hawaiian *C. quinquefasciatus* by MLST genotyping (Baldo et al. 2006). Portions of five housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA* ) and one cell surface protein (*wsp*) were identical to sequences reported from *w*Pip isolates from *C. pipiens* and *C. quinquefasciatus* in North America and Africa (http://pubmlst.org/wolbachia/). This agrees with other studies that document the monophyletic origin and absence of worldwide diversity in highly conserved regions of the *w*Pip genome (Duron et al. 2006b, Atyame et al. 2011b). By contrast, more than 20% of the *Wolbachia* genome is composed of highly polymorphic mobile genetic elements that include prophages and transposable elements as well as an unusually high number of genes encoding proteins with ankyrin motifs that may play important roles in mediating protein-protein interactions (Atyame et al. 2011b, Klasson et al. 2008, Wu et al. 2004). A relatively complex marker system based on PCR amplification of WO prophage genes combined with RFLP and sequence based analysis of transposable elements and ankyrin motifs has been developed over the past 10 years to examine fine scale genetic diversity of *w*Pip and to find specific markers for predicting compatibility among different *w*Pip strains (Duron et al. 2005, 2006a, 2006b, 2007, Atyame et al. 2014). We used a subset of these markers to fit Hawaiian strains of *w*Pip into this overall system of classification that groups strains by both geographic distribution and cytoplasmic compatibility.

### Fine Scale Genetic Diversity

We detected a relatively high amount of fine scale genetic diversity in *w*Pip isolates from the Hawaiian archipelago based on detection of 35 different genotypes within a sample of only 196 individual *C. quinquefasciatus* from Hawai'i, Kaua'i and Midway Atoll (17.9 genotypes per 100 samples). The number of genotypes we detected is approximately six times greater than what has been observed from *C. quinquefasciatus* on Réunion Island in the Indian Ocean (3.3 genotypes per 100 samples) (Atyame et al. 2011c), but less than half of what has been reported in Europe (47.6 genotypes per 100 samples, Duron et al. 2006b) and North America (47.2 genotypes per 100 samples, Morningstar et al. 2012). While some genotypes were widely distributed, the majority (77%, 27/35) were very site specific. This was particularly true for mosquitoes from northeastern Hawaii Island and Midway Atoll which had unusually high numbers of closely related *Wolbachia* genotypes that were not found elsewhere. As might be expected because of their isolation, *w*Pip strains from American Samoa were the most unique and formed a separate, well supported clade that was independent from Hawaiian strains of the endosymbiont (Figure 1).

These findings parallel the high genetic diversity of *C. quinquefasciatus* in the Hawaiian Islands and presence of unique alleles and allele frequencies in mosquitoes from Midway Atoll based on nuclear and mitochondrial markers (Fonseca et al. 2000, 2006). The Hawaiian Archipelago is a transportation crossroads in the central Pacific and independent introductions of mosquitoes from commercial and military traffic may explain the relatively high genetic diversity that is found here (Fonseca et al. 2000, 2006). Studies of the population genetics of *C. quinquefasciatus* across the archipelago suggest that the introductions are infrequent, with limited genetic exchange between populations on other islands as well as among populations within individual islands (Fonseca et al. 2000, Fonseca et al. 2006, Keyghobadi et al. 2006). Limited dispersal of mosquitoes after they become established plus recombination of WO prophage genes may lead to emergence of unique *w*Pip genotypes over time (Bordenstein and Wernegreen 2004, Wu et al. 2004). The presence of both widespread and locally unique *w*Pip

genotypes suggests that movement of mosquitoes through either natural dispersal or anthropogenic activities followed by local genetic recombination in populations once they become established may be responsible for some of the patterns we observed. This is evident for clusters of related genotypes at Maulua Gulch in northeastern Hawai'i Island and at Midway Atoll, although nodes were poorly supported in the analysis (consensus support < 50%, Figure 1). Since WO prophage markers are inserted randomly in the genome and appear to be independent of genetic mechanisms associated with CI, there is no evidence to suggest that this diversity affects compatibility among different populations.

Rarefaction analysis of samples we collected on Kaua'i and Hawai'i Islands indicates that our sampling efforts were not extensive enough to detect all possible genotypes. Rarefaction curves suggest that as many as 60 different genotypes may be present on Hawai'i and Kaua'i Islands based on 95% confidence intervals (Figure 7). By contrast, we may have detected most of *w*Pip diversity on the much smaller islands of American Samoa and Midway Atoll with sample sizes that were as few as 20 individual mosquitoes. Given the diversity of genotypes on Kaua'i and Hawai'i Islands, more extensive sampling in additional habitats and sampling on Oahu, Maui, Moloka'I, and Lana'i will help to provide a more complete picture of the *w*Pip diversity across the archipelago.

**Compatibility Groups and Worldwide Diversity**

In addition to fine scale genotyping, we identified alleles present in a subset of samples based on sequence analysis of portions of the *Wolbachia pk1* gene. This allowed us to place Hawaiian *w*Pip within an overall worldwide framework of five *w*Pip compatibility groups (Dumas et al. 2013, Atyame et al. 2014). *w*Pip5 (*pk1* allele e) is the primary compatibility group on Kaua'i, Hawai'i Island and Midway Atoll based on our analysis of *pk1* alleles. *w*Pip5 is common in Australasia (Dumas et al. 2013) where it is believed that more recent introductions of *C. quinquefasciatus* to Hawai'i originated (Fonseca et al. 2006). Detection of a single specimen with *w*Pip3 (*pk1* allele b) on Hawai'i Island is particularly interesting since this compatibility group is common in North and South America and Europe (Dumas et al. 2013). Its presence might represent remnants of the original introduction of *C. quinquefasciatus* from Mexico to Maui (Fonseca et al. 2006), but this remains speculative until more in-depth sampling can be pursued. While one compatibility group usually occurs within any given region, mixed groups have been detected in Western Europe (*w*Pip2/*w*Pip3), Italy (*w*Pip2/*w*Pip4, *w*Pip1/*w*Pip4), North Africa (*w*Pip1/*w*Pip4), South America (*w*Pip1/*w*Pip3) and Eastern Asia (*w*Pip1/*w*Pip5) (Dumas et al. 2013). At least one study has shown stable persistence of two incompatible *w*Pip groups along a contact zone as a result of limited dispersal and isolation of *Culex* mosquitoes within restricted habitats (Atyame et al. 2015b). Limited experimental data indicates that *w*Pip5 and *w*Pip3 are bidirectionally compatible with each other (Atyame et al. 2014), which can explain their apparent ability to co-exist within the same *Culex* population on Hawai'i Island. Interestingly, *w*Pip3 was the only compatibility group that we detected in American Samoa, but our sample sizes are limited.

We detected two *ank2* alleles in the Hawaiian Islands and American Samoa. Allele d was present in all *Wolbachia* genotypes collected on Kaua'i and Hawai'i Islands and approximately half of those collected on Midway Atoll. Allele a was present in all *Wolbachia* genotypes collected in American Samoa and approximately half of those collected in Midway Atoll. The mixture of alleles d and a on Midway Atoll is interesting given the small size of the Atoll and supports the idea that *C. quinquefasciatus* may have been introduced from different source populations through movement of military ships and airplanes when it was an active naval base

(Fonseca et al. 2000). The combined presence of *ank2* allele a and *pk1* allele e in *w*Pip from American Samoa, suggests that origins of *C. quinquefasciatus* and introductions to the southwestern Pacific may differ from the main Hawaiian Islands.

Based on genotypes that combine both *ank2* and *pk1* alleles, *w*Pip from collections of *C. quinquefasciatus* from Kaua'i, and Hawai'i Islands was most similar to strains that have been detected in *Culex* mosquitoes from China, the Philippines, Australia, and Russia (*ank2*/*pk1* genotypes d/e, mating group *w*Pip5; Dumas et al. 2013). By contrast, *w*Pip genotypes from American Samoa were most similar to strains that have been detected in *Culex* mosquitoes from Mexico (*ank2*/*pk1* genotypes a/b, mating group *w*Pip3; Dumas et al. 2013). The single *w*Pip infection that we detected from Pu'u Wa'awa'a, had *ank2*/*pk1* genotype d/b (mating group *w*Pip3) and was most similar to *w*Pip genotypes from Costa Rica (Dumas et al. 2013). The presence of the *ank2* d allele in this sample, common in Hawaiian samples, but absent in samples from American Samoa, suggests that this is not laboratory contamination or mixed infection.

We did not find evidence of the *tr1* insertion in any of the mosquitoes we tested. Based on a limited worldwide analysis of *tr1* genotypes, our samples were most similar to a genotype (Duron et al. 2005) that is common throughout many parts of the world (South America, Africa, Europe, Asia, Oceania) but absent in North America.

We were able to confirm the only prior report of *w*Pip from specimens of *C. quinquefasciatus* that were collected in 2001 from Lihue, Kaua'i (Dumas et al. 2013). Dumas et al. (2013) identified *ank2* allele b and *pk1* allele e (group *w*Pip*5*) in 11 specimens. While we also found *pk1* allele e in all samples that were screened from Kaua'i, we did not detect *ank2* allele b in samples from the southern and central parts of the island (Figure 5). Lack of detection of *ank2* allele b in our study may reflect a very local distribution of allele b on Kaua'i, temporal shifts in the abundance of allele b in the decade since samples were collected by Dumas et al. (2013), or the limited number of locations and specimens that were sampled across the island.

**Applications in Hawai'i**

Successful use of *w*Pi-mediated CI to suppress wild populations of *C. quinquefasciatus* was reported more than 50 years ago in an early attempt to control filariasis transmission in southeast Asia (Laven 1967), but it is only recently that a better understanding of the genetic diversity of *w*Pip has led to renewed interest in this approach (Atyame et al. 2011a). Several recent studies of *C. quinquefasciatus* on Réunion Island and other islands in the Indian Ocean by Atyame and colleagues (Atyame 2011a, 2015a) suggest that incompatible insect techniques may be possible on islands where overall diversity of *w*Pip genotypes is low relative to mainland locations and where geographic isolation may restrict movement of mosquitoes from outside of treatment areas. Atyame et al. (2011a, 2015a) typed *C. quinquefasciatus* on Réunion Island as *w*Pip1 and established that they were bidirectionally incompatible with an Istanbul reference colony of *C. quinquefasciatus* infected with *w*Pip4. They were able to produce a colony of Réunion mosquitoes infected with *w*Pip4 by treating Réunion males with tetracycline to clear *w*Pip infections and backcrossing the uninfected males with Istanbul females and their hybrids for multiple generations (Atyame et al. 2011a). Réunion males infected with *w*Pip4 were able to compete successfully with wild males in cage mating trials under semi-field conditions (Atyame et al. 2015a), suggesting that control *C. quinquefasciatus* on Réunion Island may be possible with this technique.

A similar approach may be feasible in the Hawaiian Islands where *w*Pip5 appears to be the dominant mating type and where overall *w*Pip diversity is less than mainland locations. It may be particularly effective in locations where *Culex* populations are fragmented by stream canyons, mountain ridges, or restricted to isolated atolls. *Culex* mosquitoes infected with *w*Pip5 are bidirectionally incompatible with mosquitoes infected with *w*Pip4 (Atyame et al. 2014) and techniques for introducing a *w*Pip4 strain into Hawaiian *C. quinquefasciatus* are relatively straightforward. The single detection of *w*Pip3 on Hawai'i Island suggests that more intensive screening is needed to verify frequency and distribution of this compatibility type. Compatibilities between *Culex* infected with *w*Pip3 and *w*Pip4 are highly variable and need to be determined empirically through cage trials (Atyame et al. 2014). Thus, it may be possible to find one or more *w*Pip4 isolates from Europe, North Africa, or the Middle East that are incompatible with both *w*Pip3 and *w*Pip5 from Hawai'i (Atyame et al. 2014).

Successful application of this technique depends on rearing large numbers of mosquitoes infected with an incompatible *w*Pip strain and separating males from females for release. Prior studies have depended on size based mechanical methods for separating male pupae from female pupae with accuracies as high as 97–100%, depending on aperture size in the apparatus (Sharma et al. 1972). A high degree of accuracy is needed, because released females will be fully compatible with released males. As wild populations decline, relative abundance of released females increases, potentially leading to complete replacement of the wild *w*Pip strain with the release strain. This risk can be reduced if pupae are irradiated after mechanical sexing at a dose that is sufficiently high to induce female sterility without affecting male fertility or competitiveness (Atyame et al. 2015a, Zhang et al. 2015).

Midway Atoll is a promising location for evaluating this technology because of small size, isolation, and presence of substantial infrastructure for supporting mosquito rearing and release. If coupled with other traditional mosquito control technologies, including treatment or elimination of larval habitats, it may be possible to eradicate *C. quinquefasciatus* from Sand Island where substantial populations still persist. Besides providing a proof of concept, it would reduce *Avipoxvirus* transmission in seabird colonies on the islands, prevent avian malaria from becoming established in both native and non-native passerine birds on the atoll, and have immediate conservation benefits (LaPointe et al. 2014).

In conclusion, incompatible insect techniques for control of *C. quinquefasciatus* in Hawai'i are promising. Over the short term, CI approaches may have major advantages over GMO-based methods. There are few regulatory obstacles since it is classified as a bio-pesticide and no major technical developments are necessary. While some public controversy is expected given that male mosquitoes will be reared and released, it may not reach levels associated with release of genetically modified organisms. Mass rearing techniques for this mosquito have been developed and the most significant technical obstacle may be the refinement of accurate and efficient methods for separating males from females. This technology can then lay the groundwork for eventual application of GMO methods when they become available. An integrated approach using CI may reduce disease transmission in remote forest habitats if combined with habitat restoration, selective application of larvicides and newer GMO technologies, but field trials are necessary to establish costs and effectiveness. Logical next steps include more extensive surveys of *w*Pip diversity, particularly from Hawaiian Islands that were not sampled as part of this study, infection of Hawaiian *C. quinquefasciatus* with one or more isolates of *w*Pip4, confirmation of incompatibility through cage trials, tests of

competiveness of laboratory reared males with wild males, and evaluation of feasibility and costs for rearing and separating male mosquitoes for release.

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APPENDIX 1. PCR master mix and cycling conditions for Multilocus Sequence Typing.
All reactions used 50 ng of DNA template per reaction in 40 µl reaction volumes. *gatB* = glutamyl-tRNA amidotransferase, subunit B; *coxA* = cytochrome c oxidase, subunit I; *hcpA* = conserved hypothetical protein, *ftsZ* = cell division protein; *fbpA* = fructose-bisphosphate aldolase; *wsp* = outer surface protein

| | $MgCl_2$ (mM) | dNTP (mM) | Forward Primer (µM) | Reverse Primer (µM) | Taq (Units) |
|---|---|---|---|---|---|
| MLST | | | | | |
| *gatB* | 1.5 | 0.2 | 1 | 1 | 0.5 |
| *coxA* | 1.5 | 0.2 | 1 | 1 | 0.5 |
| *hcpA* | 1.5 | 0.2 | 1 | 1 | 0.5 |
| *ftsZ* | 1.5 | 0.2 | 1 | 1 | 0.5 |
| *fbpA* | 1.5 | 0.2 | 1 | 1 | 0.5 |
| *wsp* | 1.5 | 0.2 | 1 | 1 | 0.5 |

| | Target | Step | Temp (°C) | Time (min:sec) |
|---|---|---|---|---|
| Program 1 | *gatB, coxA* | 1 | 94 | 2:00 |
| | *hcpA, ftsZ* | 2 | 94 | 0:30 |
| | | 3 | 54 | 0:45 |
| | | 4 | 72 | 1:30 |
| | | 5 | Go to 2 | 37 times |
| | | 6 | 72 | 10:00 |
| Program 2 | *wsp, fpbA* | 1 | 94 | 2:00 |
| | | 2 | 94 | 0:30 |
| | | 3 | 58 | 0:45 |
| | | 4 | 72 | 1:30 |
| | | 5 | Go to 2 | 37 times |
| | | 6 | 72 | 10:00 |

APPENDIX 2. PCR master mix and cycling conditions for WO prophage genes (Gp markers). All reactions used 50 ng of DNA template per reaction in 25 µl reaction volumes.

| | $MgCl_2$ (mM) | dNTP (mM) | Forward Primer (µM) | Reverse Primer (µM) | Taq (Units) |
|---|---|---|---|---|---|
| WO Prophage | | | | | |
| Gp1b | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp2a | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp2b | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp2e | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp3a | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp3b | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp3c | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp3d | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp7d | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp9a | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp9b | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp15a | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp15b | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp24a | 1 | 1.5 | 0.8 | 0.5 | 0.5 |
| Gp24b | 1 | 1.5 | 0.8 | 0.5 | 0.5 |

| | Target | Step | Temp (°C) | Time (min:sec) |
|---|---|---|---|---|
| Program | All | 1 | 95 | 2:00 |
| | | 2 | 94 | 0:30 |
| | | 3 | 52 | 0:30 |
| | | 4 | 72 | 1:00 |
| | | 5 | Go to 2 | 31 times |
| | | 6 | 72 | 5:00 |

APPENDIX 3. PCR master mix and cycling conditions for transposable element *tr1*.
All reactions used 50 ng of DNA template per reaction in 25 µl reaction volumes.

| | $MgCl_2$ (mM) | dNTP (mM) | Forward Primer (µM) | Reverse Primer (µM) | Taq (Units) |
|---|---|---|---|---|---|
| *tr1* | 1 | 1.5 | 0.8 | 0.5 | 0.5 |

| | Target | Step | Temp (°C) | Time (min:sec) |
|---|---|---|---|---|
| Program | All | 1 | 95 | 2:00 |
| | | 2 | 94 | 0:30 |
| | | 3 | 50 | 0:30 |
| | | 4 | 72 | 1:30 |
| | | 5 | Go to 2 | 30 times |
| | | 6 | 72 | 5:00 |

APPENDIX 4. PCR master mix and cycling conditions for ankyrin domain genes *pk1* and *ank2*.
All reactions used 50 ng of DNA template per reaction in 25 µl reaction volumes.

| | $MgCl_2$ (mM) | dNTP (mM) | Forward Primer (µM) | Reverse Primer (µM) | Taq (Units) |
|---|---|---|---|---|---|
| Ankyrin genes | | | | | |
| *pk1* | 1.5 | 0.2 | 1 | 1 | 0.5 |
| *ank2* | 1.5 | 0.2 | 1 | 1 | 0.5 |

| | Target | Step | Temp (°C) | Time (min:sec) |
|---|---|---|---|---|
| Program 1 | *pk1* | 1 | 94 | 5:00 |
| | | 2 | 94 | 0:30 |
| | | 3 | 52 | 0:30 |
| | | 4 | 72 | 1:00 |
| | | 5 | Go to 2 | 36 times |
| | | 6 | 72 | 2:00 |
| Program 2 | *ank2* | 1 | 94 | 5:00 |
| | | 2 | 94 | 0:30 |
| | | 3 | 52 | 0:30 |
| | | 4 | 72 | 1:30 |
| | | 5 | Go to 2 | 36 times |
| | | 6 | 72 | 2:00 |