

Bottlenecks and multiple introductions: population genetics of the vector of avian malaria in Hawaii

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Abstract

Avian malaria has had a profound impact on the demographics and behaviour of Hawaiian forest birds since its vector, *Culex quinquefasciatus* the southern house mosquito, was first introduced to Hawaii around 1830. In order to understand the dynamics of the disease in Hawaii and gain insights into the evolution of vector-mediated parasite–host interactions in general we studied the population genetics of *Cx. quinquefasciatus* in the Hawaiian Islands. We used both microsatellite and mitochondrial loci. Not surprisingly we found that mosquitoes in Midway, a small island in the Western group, are quite distinct from the populations in the main Hawaiian Islands. However, we also found that in general mosquito populations are relatively isolated even among the main islands, in particular between Hawaii (the Big Island) and the remaining Hawaiian Islands. We found evidence of bottlenecks among populations within the Big Island and an excess of alleles in Maui, the site of the original introduction. The mitochondrial diversity was typically low but higher than expected. The current distribution of mitochondrial haplotypes combined with the microsatellite information lead us to conclude that there have been several introductions and to speculate on some processes that may be responsible for the current population genetics of vectors of avian malaria in Hawaii.

Keywords: A + T-rich region, *Culex quinquefasciatus*, microsatellites, vector-mediated parasite–host interactions

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Introduction

Our view of parasite–host interactions has undergone a paradigm shift in the last two decades (May & Anderson 1979; Lenski & May 1994; Anderson 1995). Acknowledgement that populations of parasites and hosts exhibit variation in their capacity to exploit and susceptibility to being exploited, respectively, means that competition between parasites with different strategies can generate higher or lower levels of damage to the host (virulence). The direction of change depends on the available genetic variation and the balance between the benefit of higher exploitation vs. any decrease in parasite transmission. The theory is, however, best developed for interactions involving one parasite and one host (Anderson & May 1982). Vector-

mediated parasite–host interactions, that include some of the deadliest diseases to humans and wildlife (malaria, dengue, arboviruses), are commonly equated to simple parasite–host interactions mediated by water or air (Ewald 1987). The vector is thought to act as a neutral carrier of parasites from infected hosts to their next victim.

There are, however, many ways in which vectors may alter the relationship between the parasite and the other host or hosts. The simplest one may be the effect of the vector population structure on the genetic diversity of the parasite. Vectors that disperse widely, may move parasite strains between populations of the secondary host, and therefore increase the effective population size of the parasite. This in turn could reduce losses of genetic diversity due to genetic drift, or rescue highly virulent strains from local extinction. Likewise, a high degree of genetic diversity in the parasite may hinder the development of resistance (May & Nowak 1994). Key in this hypothesis is knowledge of the dispersal capacity of the vectors.

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In order to gain insight into the effect of vector dispersal in vector-mediated parasite–host interactions we are examining the population structure of the southern house mosquito, *Culex quinquefasciatus*, in the Hawaiian Islands. The interaction between the southern house mosquito, avian malaria (*Plasmodium relictum*) and endemic Hawaiian forest birds is relatively recent. Historical records tell us that *Cx. quinquefasciatus*, Say (Diptera: Culicidae) was introduced accidentally to Maui, Hawaii, between 1823 and 1830 (Dine 1904; Hardy 1960) and is the first mosquito reported in Hawaii. Although it is not clear when avian malaria was introduced to Hawaii, we know the disease could not be transmitted there until a vector was introduced. Currently both vectors and disease occur in all the main Hawaiian Islands and also in some of the North-Western Hawaiian Islands (Warner 1968; van Riper *et al.* 1986).

Once very abundant throughout the islands, populations of Hawaiian native birds, particularly of endangered forest species like Hawaiian honeycreepers (Drepanidinae), persist mainly at high altitudes (Warner 1968; Scott *et al.* 1986). The virtual absence of native birds from low and intermediate elevations, associated with the presence of large numbers of *Cx. quinquefasciatus* (Goff and van Riper *et al.* 1980), and the susceptibility of native birds to *Plasmodium* (van Riper *et al.* 1986; Atkinson *et al.* 1995) have led to the hypothesis that birds can only survive outside the mosquito zone. Although several factors may have contributed to the extinction and endangerment of the endemic species of Hawaiian honeycreepers, avian malaria transmitted uniquely by this mosquito (van Riper *et al.* 1986, D. LaPointe unpublished manuscript) is a very important factor, particularly today (Warner 1968; van Riper *et al.* 1986; Atkinson *et al.* 1995).

To determine the amount of gene flow among Hawaiian mosquito populations we opted to use molecular markers. Because all populations of an introduced species are recently derived from the same genetic stock, however, we needed to use genetic markers with either very high rates of mutation or high levels of polymorphism in the original population. Therefore we used microsatellite loci. Microsatellites are repeats of simple nucleotide motifs found in the genome of most organisms (Tautz 1989). Replication mistakes produce polymorphic populations with alleles varying in the number of motif repeats (Tautz *et al.* 1986). Microsatellite alleles are codominant and are therefore ideal to calculate heterozygosity. They have also been shown to have very high mutation rates ($> 10^{-4}$ per generation, Kruglyak *et al.* 1998) although there have been recent reports of low mutation rates in some microsatellite loci in *Drosophila* ($< 10^{-6}$ per generation, Schug *et al.* 1998). Prior to initiating this study we developed microsatellite primers for *Cx. quinquefasciatus*. We report details of the development, primer sequences, polymerase chain reaction (PCR) conditions, and GenBank accession numbers

elsewhere (Fonseca *et al.* 1998). Because this was only the second of a handful of mosquito species for which microsatellites have been developed and the only species outside the genus *Anopheles* (Walton *et al.* 1998), to allow comparisons with other studies we also sequenced a mitochondrial DNA locus (A + T-rich or control region).

In the present study we used both nuclear and mitochondrial markers to compare vector populations from most of the islands within the Hawaiian archipelago that harbour mosquitoes. On a more local scale, we compared several populations within the Big Island (Hawaii). Our objectives were to ascertain the current degree of mixing both among and within most of the Hawaiian Islands, and also to examine the types of processes that produced the current distribution of malaria vectors in Hawaii.

Materials and methods

Field collection and sample preparation

All specimens used in this study were either collected as adults or collected as eggs or larvae and reared to adults during the months of July and August 1997 and 1998. We used a standard rearing method (James Pecor personal communication, see protocol at <http://wrbu.si.edu>) where we kept groups of larvae from the same location in plastic cups and fed them fish food (Tetramin) in moderation every day. An inverted cup covering the larvae captured emerging adults. Adults were killed at -20°C and preserved in 80% ethanol within 1 day of emergence. When specimens were collected as larvae from small cavities a maximum of three individuals was analysed from each container to avoid the risk of repeatedly sampling within a single family. Also, at each location a minimum of three individual cavities was sampled. A genitalia analysis (Barr 1957) of a sample of males from each location was performed prior to DNA extraction. Mounted voucher specimens were entered in the mosquito collection at the Smithsonian Institution, National Museum of Natural History (accession # 2017083).

We obtained specimens from most of the main Hawaiian Islands from local branches of the Hawaiian Vector Control (HVC). Vector Control crews make collections every week throughout the year using New Jersey Light Traps (Mulhern 1942). Collections are made at several points around each island, particularly near population foci. Due to the concentration of urban development in the lowlands, all HVC collections are made at or near sea level. The specimens obtained from HVC were dry. When extracting DNA from adult females obtained from HVC we did not include the abdomens, where the spermathecas are located, to avoid contaminating female samples with male DNA. Specimens from Midway were collected as larvae from containers at the Navy Station there.

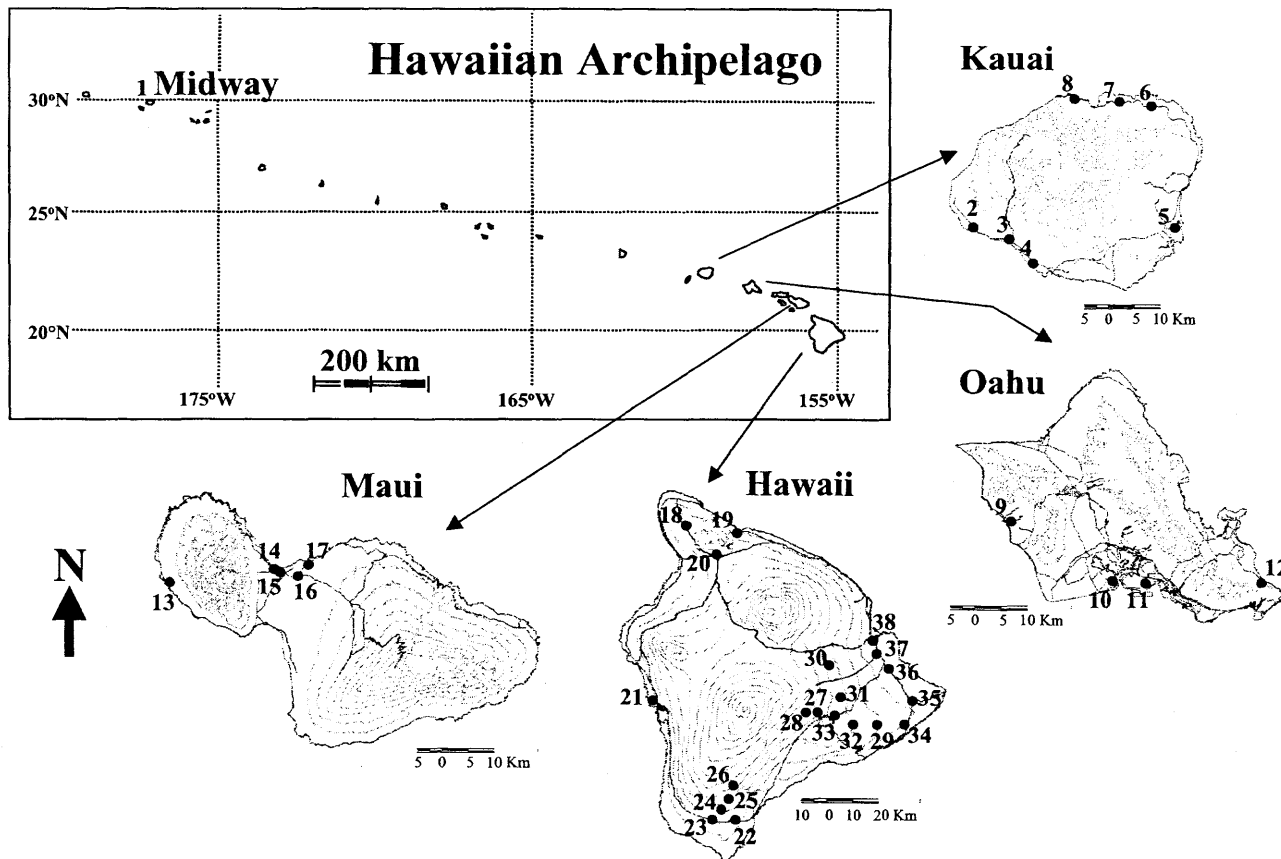


Fig. 1 The state of Hawaii. Dots mark locations on all islands where specimens were collected. Numbers correspond to No. in Table 1; see text for details.

To study population differentiation within a single island we sampled extensively at all altitudes in the island of Hawaii (Fig. 1, from here on designated 'Big Island' to avoid confusion with the name of the State). We collected larvae exclusively so that we could assign an exact source location. The most common sampling sites at low altitudes were flowerpots in cemeteries or roadside memorials, discarded tyres and other human-associated containers. At medium and high altitudes we collected larvae from small (up to 10 m × 10 m) artificial cattle ponds, cattle and Hawaiian goose water troughs and tree cavities. There were very few instances of standing water on the ground in the Big Island, mainly due to the porous volcanic nature of the soil in this very young island (0.43 Myr, Carson & Clague 1995), but when ground water was found no larvae were ever seen there. Deep in the Hawaiian rain forest it was common, however, to find larvae in cavities left by feral pigs after they had felled and eaten the starchy inside of tree ferns (*Cibotium chamissoi*). These are referred to as 'hapiu cavities' after the Hawaiian name for tree fern. A detailed description of location and number of specimens used from each is in Table 1.

DNA extraction

DNA was extracted using a phenol/chloroform method. Each specimen was homogenized in a buffer containing 100 mM NaCl, 100 mM Tris-HCl pH 7.5 and 100 mM ethylenediaminetetraacetic acid (EDTA) to which sodium dodecyl sulphate (SDS) and fresh proteinase K were added to a final concentration of 0.5% and 200 µg/mL, respectively, and left at 55 °C overnight. RNase was then added to 100 µg/mL with incubation for 15 min at 37 °C. We did a standard phenol/chloroform DNA extraction (Sambrook *et al.* 1989) at the end of which we added 400 µL of ice cold 95% ethanol to the supernatant. The mix was left at -20 °C for at least 2 h followed by centrifugation at 4 °C and 13 800 g for 10 min. The DNA pellet, sometimes invisible, was washed with 700 µL of 70% ethanol and air-dried under vacuum in a SpeedVac (Savant SC110, Stratagene). DNA pellets were suspended in TE (10 mM Tris-HCl, pH 7.5 + 0.1 mM EDTA) to a final concentration of 2–5 ng DNA/µL. We used 1 µL of extracted DNA for all PCR reactions.

Table 1 Locations and number of specimen (*n*) collected

No.	Population	Closest town or name of area	<i>n</i>	No.	Population	Closest town or name of area	Elevation	S	<i>n</i>
1	Midway	Midway	10	18	BI North	Kohala Mts	1200 m	H	12
2	Kauai	Kekaha	12	19	BI North	Waipio Valley	180 m	H	8
3	Kauai	Waimea	12	20	BI North	Waimea	300 m	H	15
4	Kauai	Gay & Robinson	12	21	BI South	Keahole	60 m	H	3
5	Kauai	Hanapepe	12	22	BI South	Manuka St Pk.	760 m	H	6
6	Kauai	Lihue	12	23	BI South	McCandless	610 m	N	21
7	Kauai	Kilauea	12	24	BI South	McCandless	1040 m	N	36
8	Kauai	Hanalei	12	25	BI South	McCandless	1189 m	N	21
9	Oahu	Waianae	15	26	BI South	McCandless	1341 m	N	29
10	Oahu	Pearl H. Pier 32	8	27	BI East	Keahou	365 m	N	3
11	Oahu	Pearl H. Pier 2	8	28	BI East	Keahou	1520 m	N	3
12	Oahu	Waimanalo	15	29	BI East	Kalapana	610 m	N	7
13	Maui	Lahaina	16	30	BI East	Kipuka	1220 m	N	22
14	Maui	Kahului	6	31	BI East	Puu Makaala	1120 m	N	13
15	Maui	Airport	6	32	BI East	Ainahou	1130 m	H	10
16	Maui	Paia	12	33	BI East	Volcano Nat Pk.	1220 m	H	3
17	Maui	Puu Nene	12	34	BI East	Rt 137 A	Sea level	H	7
				35	BI East	Rt.137 B	Sea level	H	8
				36	BI East	Pahoa	Sea level	H	10
				37	BI East	Zoo	Sea level	H	20
				38	BI East	Hilo	Sea level	H	3

Individuals from locations within each island or parts of the Big Island (BI North, South and East) were analysed together. S refers to the type of containers where larvae were collected: H = human source; N = natural source. All locations outside the Big Island are at sea level.

Species identification

Although Warner (Warner 1968) reported only finding mosquitoes at elevations below 600 m, currently there are breeding populations of *Culex quinquefasciatus* at elevations above 1500 m (Goff & van Riper 1980; van Riper *et al.* 1986, this study). Occurrence at high altitude is unusual for this tropical species and more akin to its temperate zone sibling species, *Cx. pipiens* (Barr 1957; Harbach *et al.* 1985). The two species are almost indistinguishable morphologically and only exceptionally experienced entomologists can tell the larvae or the females apart (EL Peyton, personal communication). To make sure we were working with only one species, we used a recently developed molecular method for species identification (Bourguet *et al.* 1998). Briefly, it entails amplifying a 700-base pair (bp) fragment of the acetylcholinesterase gene (Ace), a nuclear gene, restricting it with *ScaI*, and examining the results in 1.5% agarose gels. *Culex pipiens* specimens generate two bands while *Cx. quinquefasciatus* generate three. The protocol used is described in Bourguet *et al.* (1998).

Microsatellite DNA analysis

Although Fonseca *et al.* (1998) developed eight microsatellite primer pairs, in this study we use only four (CQ11, CQ26, CQ29 and CQ41). There are two reasons behind our

decision. First, when we tried using the remaining four primer sets we were faced with a very high occurrence of 'null alleles'. Indeed, we were commonly unable to amplify over half of the individuals from a population. Null alleles result from mutations in the primer region in the flanks of the microsatellite and seem to be quite common in mosquitoes (Walton *et al.* 1998). To confirm that the size of the DNA fragment corresponded to variation in the microsatellite repeat in the four loci used, we sequenced representatives of all alleles, including alleles of the same size in different populations. In all cases but one this was confirmed (data not shown). The exception was an allele of size 241 bp in CQ11 that results not from a reduction in the number of microsatellite repeats but from an 18-bp deletion in the flanking region. The microsatellite region is identical to that of a 259-bp allele. The deletion appears to be quite old since it also occurs in the sibling species *Cx. pipiens* (Fonseca, Mogi and Wilkerson *unpublished manuscript*). Second, because differences between populations in this study are almost exclusively in allelic frequencies it became very important to avoid obtaining allelic frequency distributions biased by small sample size artefacts. Therefore, we decided that it would be more profitable to analyse a higher number of individuals per population for a small number of independent loci than the converse (few individuals but more loci with null alleles). We made this decision after

having analysed the parents and 21 offspring from each of three families of mosquitoes from the Big Island and ascertaining that the four loci used were not in linkage disequilibrium, were not sex linked and were inherited in a Mendelian fashion (Fonseca *et al.* 1998). Previous analyses had also shown that the four loci exhibited a high number of alleles (six to nine alleles per locus for 123 individuals assayed), and had expected heterozygosities between 0.32 and 0.76 (Fonseca *et al.* 1998). To genotype individuals, PCR amplifications were performed in 20- μ L reaction volumes using 2–5 ng DNA, under the PCR conditions described in Fonseca *et al.* (1998). Both a positive and a negative PCR control were run each time. The PCR products were sized in 6% polyacrylamide gels with an ABI-373XL automated sequencer in the GENESCAN mode (PE Biosystems). A PCR positive control using the clone from which the microsatellite primers were designed as the template DNA was run in all gels to ensure accurate sizing.

Analysis of variation

We quantified differentiation among populations by testing for differences in allelic frequencies and by calculating fixation indices. All the loci used in this study have bimodal frequency distributions, suggesting the possibility that mutations at these loci may not follow the step-wise mutation model assumed in recent measures of population differentiation developed for microsatellites (e.g. R_{ST} , Slatkin 1995). Indeed because we include an allele created by a large flanking region deletion generating a trimodal allelic distribution in loci CQ11, the assumption of absolute stepwise mutation is not warranted. Besides since we used only four loci, F_{ST} values are more appropriate (Gaggiotti *et al.* 1999). Still, to allow comparisons with other studies, we present fixation and distance measures based both on infinite alleles (F_{ST}) and stepwise (R_{ST}) mutation models. We used GENEPOP ver 3.1d (Raymond & Rousset 1995) to test for differences in allele frequencies and heterozygote deficiencies, and FSTAT ver 1.2 (Goudet *et al.* 1996) to calculate and test whether F_{ST} and R_{ST} values differ significantly from zero. Other parametric and nonparametric statistical tests were performed with JMP ver 3.1 (SAS Institute Inc.).

Bottleneck analysis

Cornuet & Luikart (1996) developed tests based on the assumption that when populations experience a reduction in effective size, they generally have higher heterozygosity than predicted by their allelic diversity because allelic diversity is reduced faster than heterozygosity. This has only been demonstrated for loci evolving under the Infinite Allele Model (IAM, Maruyama & Fuerst 1985). As discussed above such a model or a mixed model appears

to be the most appropriate for our data. We used the program BOTTLENECK (Cornuet & Luikart 1996) to simulate the coalescent process of n genes under the IAM to produce the distribution of heterozygosity expected from the observed number of alleles given the sample size of each population under the assumption of mutation-drift equilibrium. This enables the calculation of the average expected heterozygosity, H_E , and a computation of the P -value for the difference between observed and expected heterozygosity at each locus. To determine whether a population exhibits a significant number of loci with heterozygosity excess we report both a 'sign test' (Cornuet & Luikart 1996) and a 'Wilcoxon sign-rank test' (Luikart *et al.* 1998). Furthermore, Luikart *et al.* (1998) argued that due to the probabilistic reduction in the number of rare alleles, bottlenecks might cause a characteristic mode-shift distortion in the distribution of allele frequencies, i.e. low numbers of low-frequency alleles. Because differentiation measures using polymorphic loci such as microsatellite are very sensitive to the effective size of the populations in question Hedrick (1999) advised the use of statistical tests to identify bottlenecks that use other than lowered heterozygosity. Therefore we tested for a 'mode-shift' in all populations also using Bottleneck.

Mitochondrial DNA variation

We sequenced the A + T-rich region of the mitochondrial DNA (mtDNA) because this is the only large noncoding region within the mitochondrial genome although the replication origin has been mapped there as in the case of the control region in vertebrates (Goddard & Wolstenholme 1980). Initially we used universal primers TM-N-193 (met-20) and SR-J-14233 (12sbi) from Simon *et al.* (1994) to amplify a product roughly 1.5 kilobases (kb) in length that included the entire A + T-rich region, most of the small ribosomal subunit (12s), the tRNAs for isoleucine, glutamine and most of the tRNA for methionine. We used 5 ng of genomic DNA in a 50- μ L reaction. The final concentrations of the PCR reagents were as follows: 1 $\times$ PCR buffer, 42 nM of each primer, 100 μ M of each dNTP, 2 mM $MgCl_2$, and 1 unit of *Taq* polymerase (PE Biosystems). The PCR amplification was preceded by a 5-min denaturation at 94 °C. The amplification consisted of 40 cycles of 30 s at 94 °C, 60 s at 50 °C and 90 s at 72 °C, and ended with a final extension step of 5 min at 72 °C. We sequenced approximately 200 bp and 400 bp at the 3' and 5' ends of the 1.5-kb PCR product, respectively, and used those sequences to design new primers that allowed us to amplify an 808-bp product almost exclusively in the A + T-rich region. The primers were DATrich1: 5'-GGGTATCTAATCCTAGTTTA-3'; DATrich2: 5'-GATCAC TGCGAATAAATAAAC-3'; and DILe-2: 5'-TCCTTTTAT CAGGCAATTC-3'. DATrich1 and 2 are forward primers and DILe-2 is a reverse primer

partly located in the tRNA isoleucine region. We also obtained the primers used by Guillemaud *et al.* (1997) and used them as internal primers under the same PCR conditions as described above but with an annealing temperature of 48 °C.

We sequenced the 808-bp region between DATrich1 and DILe-2 for a minimum of three individuals from several populations. After the initial PCR reaction, and following evaluation of 5 µL of the PCR product from each reaction in a 1% agarose gel, the PCR products were purified using a cleaning kit (QiaQuick, Qiagen) and resuspended in 30 µL of TE. Cycle sequencing was performed using a Dye Deoxy Terminator Cycle Sequencing Kit (PE Biosystems) and the samples were sequenced in a ABI 373XL Automatic Sequencer (PE Biosystems). Sequences were aligned with Sequencher (ver 3.01 Gene Codes Corporation).

Results

We found no evidence that *Culex pipiens* is present in Hawaii after examining 163 individuals from all the populations sampled.

Population differentiation — microsatellite data

Although we have specimens from several different locations in each island (Table 1, Fig. 1), mostly we present analyses considering all the mosquitoes from each island together. The only exception is the Big Island where we did extensive sampling with the specific objective of addressing within-island population subdivision. In the Big Island we divided our samples into three populations: North, South and East (Fig. 1). In the Eastern part of the island, we collected both from human-associated containers (e.g. flowerpots, tyres) and natural containers (almost exclusively hapuu cavities in the forest). Because we found no significant difference in either alleles or allelic frequencies between specimens collected in those separate types of containers (data not shown), we combined them into a single sample. Likewise the South population was originally divided into specimens collected at four different altitudes (Table 1), but we found no significant differences among them.

Table 2 shows the allelic frequencies and genetic characteristics of all loci for all seven populations surveyed. We found between eight and 11 alleles per locus with expected heterozygosities ranging from 0.45 to 0.81 (0.63 ± 0.10 , mean $\pm$ SD). Tests which simultaneously compared the frequency of alleles in each of the seven populations for each locus ($n = 4$ tests) were all highly significant ($P < 0.0001$ for each). Next, we compared allele and genotypic frequency distributions for all pair-wise combinations of populations at each locus ($n = 84$ tests). Because of the large number of tests performed we used the sequential Bonferroni procedure described by Rice

(1989) to adjust the α value for each test. The majority (85%) of the comparisons yielded significant differences and all pair-wise comparisons between populations were significant across all loci ($P < \alpha/k$, where $\alpha = 0.05$ and $k = 21$, Rice 1989). For each locus, most of the populations had observed heterozygosities that matched those expected under the assumption of Hardy–Weinberg equilibrium (Table 2). The exceptions are Maui and Big Island South for locus CQ26, and Maui and Oahu for locus CQ 41, which showed a heterozygote deficiency. Because decreasing statistical Type II error (Cohen 1988) was particularly important in this analysis we chose a conservative approach and did not use Bonferroni-adjusted α values. The departure from Hardy–Weinberg frequencies is significant at locus CQ26 in Maui but re-analysis of CQ26 variation in Maui revealed low heterozygosity in all locations so the homozygote excess is not likely to be caused by the Wahlund effect. It is therefore quite probable there may be at least one more allele at locus CQ26 in Maui.

We found a strikingly higher allele number (allelic diversity) in Maui when compared with the remaining islands (Fig. 2). Although only a small percentage of alleles are unique to Maui (from 0 to 12.5%, mean 9.8%, not counting putative nulls), the population from Maui has significantly more alleles than the other populations (Wilcoxon/Kruskal–Wallis Rank tests, $P = 0.018$). This pattern occurs although we collected neither extensively or intensively in Maui (Fig. 1 and Table 1). Therefore the number of individuals sampled per population does not appear to have had a significant effect on the number of alleles detected. Note also that Oahu, the most heavily populated island, with the only international airport in the State of Hawaii, has a significantly lower allelic diversity than Maui (Wilcoxon/Kruskal–Wallis Rank test, $P = 0.03$).

Fixation indices are measures of differentiation, which incorporate information on both the frequency and identity of the alleles. In this study, F_{ST} values ranged from a low of 0.01 between Maui and Oahu to a high of 0.1488 for the comparison between Big Island North and Maui (Table 3). The overall F_{ST} value of 0.056 is significantly different from zero, as are all pair-wise F_{ST} values ($P < 0.001$, FSTAT permutation procedure). R_{ST} values, which are the equivalent of F_{ST} under a stepwise mutation model, were very similar to F_{ST} values (Table 3). The mean R_{ST} averaged across all pair-wise population comparisons calculated as in Rousset (1996) was 0.039. All individual R_{ST} values were significantly greater than zero as none have 95% confidence intervals that overlap zero. The relationship between F_{ST} values and geographical distance is complex. If only populations from Maui and islands west of Maui are included in the analysis, there is a strong linear relationship between F_{ST} and distance ($r^2 = 0.89$). If

Table 2 Microsatellite allelic frequency distributions across the Hawaiian Islands and within the island of Hawaii. Frequencies larger than 10% within each population are in bold type

Alleles (bp)								Alleles (bp)							
MID	KAUAI	OAHU	MAUI	BIN	BIS	BIE		MID	KAUAI	OAHU	MAUI	BIN	BIS	BIE	
CQ11								CQ26							
241	0.150	0.267	0.141	0.202	0.157	0.143	0.260	212		0.022	0.022	0.010			
255				0.087		0.004		214				0.019	0.014	0.004	
257		0.078						216	0.250	0.333	0.359	0.240	0.443	0.599	0.577
259	0.100	0.089	0.283	0.279	0.114	0.457	0.274	218	0.200	0.333	0.337	0.308	0.314	0.078	0.216
261						0.004		220				0.038			
263		0.111		0.010		0.009		222				0.019		0.013	0.014
265	0.200	0.278	0.207	0.346	0.029	0.270	0.255	224	0.550	0.311	0.272	0.356	0.200	0.302	0.178
267	0.250	0.156	0.370	0.048	0.700	0.113	0.212	226			0.011	0.010	0.029	0.004	0.014
269	0.150	0.011		0.019											
271				0.010											
283	0.150														
No. of alleles	6	7	4	8	4	7	4	No. of alleles	3	4	5	8	5	6	5
2N =	20	90	92	104	70	230	208	2N =	20	90	92	104	70	232	208
H_E	0.86	0.81	0.73	0.75	0.45	0.69	0.75	H_{Ee}	0.63	0.69	0.71	0.73**	0.67	0.55*	0.59
H_O	0.60	0.76	0.83	0.75	0.31	0.68	0.77	H_O	0.60	0.82	0.69	0.40**	0.89	0.48*	0.60
H_{eq}	0.74	0.43	0.67	0.66	0.46	0.60	0.39	H_{eq}	0.45	0.52	0.43	0.62	0.54	0.52	0.47
P	0.004	0.005	0.049	0.265	0.463	0.336	0.000	P	0.117	0.122	0.034	0.242	0.196	0.494	0.303
CQ29								CQ41							
164				0.010		0.004		141			0.054	0.058		0.009	
168	0.250			0.029			0.010	143	0.150					0.013	
176		0.102	0.152	0.115		0.082	0.067	145		0.056	0.022	0.048		0.013	
178	0.150	0.466	0.500	0.519	0.257	0.418	0.269	147	0.100	0.022		0.010			
182				0.010				149	0.600	0.611	0.587	0.596	0.514	0.664	0.596
184							0.005	151			0.043	0.029			0.005
186	0.600	0.409	0.315	0.308	0.700	0.483	0.620	153	0.150	0.233	0.185	0.240	0.443	0.276	0.351
188		0.023	0.033	0.010	0.043	0.013	0.029	155		0.011	0.022	0.010	0.029	0.026	0.048
								157		0.067	0.087	0.010			
								159					0.014		
No. of alleles	3	4	4	7	3	5	6	No. of alleles	4	6	7	8	4	6	4
2N =	20	88	92	104	70	232	208	2N =	20	90	92	104	70	232	208
H_E	0.58	0.61	0.63	0.65	0.45	0.59	0.54	H_E	0.62	0.57	0.63**	0.57*	0.55	0.48	0.52
H_O	0.60	0.52	0.72	0.73	0.57	0.58	0.53	H_O	0.50	0.49	0.42**	0.46*	0.54	0.40	0.49
H_{eq}	0.45	0.45	0.52	0.61	0.36	0.45	0.52	H_{eq}	0.58	0.63	0.58	0.63	0.46	0.52	0.38
P	0.258	0.135	0.283	0.485	0.386	0.267	0.451	P	0.486	0.361	0.402	0.281	0.366	0.346	0.279

H_E and H_O = Hardy-Weinberg expected and observed heterozygosities, respectively. Islands are, from left to right, Midway (MID), Kauai, Oahu, Maui, Big Island (Hawaii) North (BIN), Big Island South (BIS), Big Island East (BIE). Significant differences between expected and observed heterozygosity are indicated by * ($P < 0.05$) or ** ($P < 0.001$). H_{eq} and P are values from a bottleneck analysis obtained from a simulation based on an Infinite allele model (see text for details).

the populations in the Big Island are included, however, the effect of distance on the exchange of alleles between populations is overwhelmed by high F_{ST} values stemming from comparisons with Big Island populations. Overall it is clear that irrespective of distance, Maui, Oahu and Kauai populations are more similar to each other than any of them to Big Island populations.

Bottleneck analysis

We found indication of recent bottlenecks in several populations (see Table 2 for details across the loci). 'Sign' tests are not significant but Wilcoxon Rank tests showed a significant heterozygous excess in Big Island East ($P = 0.03$), Big Island North ($P = 0.03$) and Midway

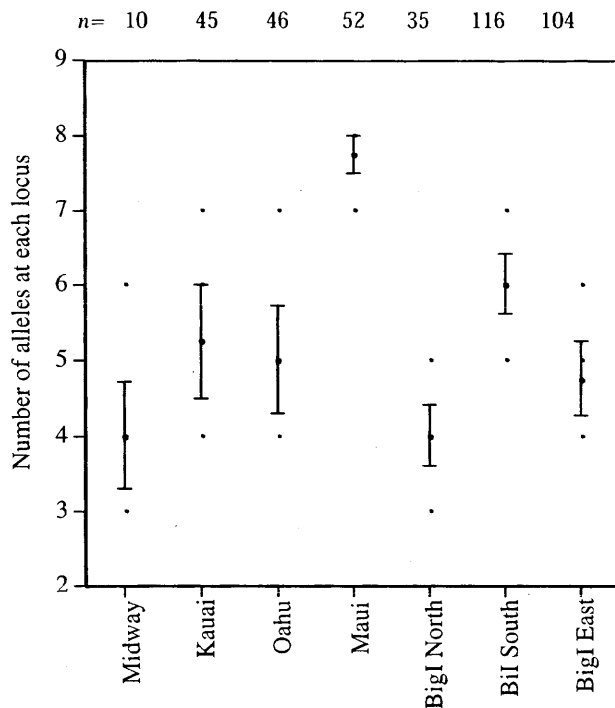


Fig. 2 Comparison of number of alleles at each locus in all the populations examined. Confidence intervals are one standard error of the mean.

($P = 0.03$). Furthermore, the allelic frequency distribution shows a significant 'mode-shift' (lack of low frequency alleles) in Midway.

mtDNA analysis

The 808-bp mtDNA fragment we sequenced has a 89.6% A + T bias including a row of 19 adenines starting at position 429 that prevented a large overlap between sequences started from both directions. Therefore we sequenced at least twice in each direction. A total of 38 individuals were sequenced and six different haplotypes were identified (Fig. 3). Most haplotypes differ only at

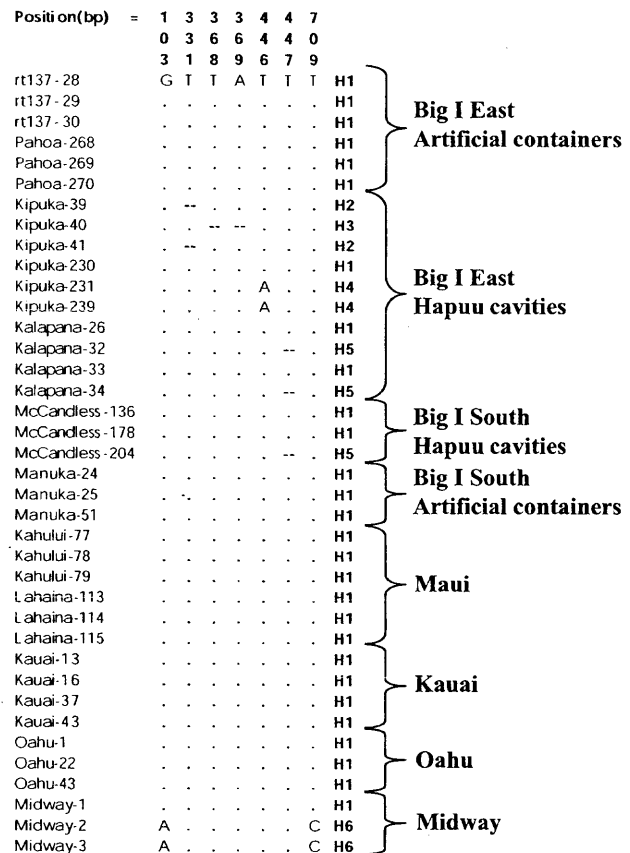


Fig. 3 Variable positions in the mtDNA sequence. Deletion = --; same character = .

one position or, as in the case of haplotype H3, differ by one in the number of repeats in a (TA)₇ microsatellite region. The nucleotide diversity is 0.0004 across all sites including deletions. The most common haplotype, H1, is found in all islands at all altitudes and in all types of habitat. Most of the mosquitoes collected at low altitudes in all islands had mtDNA haplotype H1. The only exception was the Midway population that, besides H1, had a unique haplotype, H6 that differs from all others at two positions,

Table 3 F_{ST} values (lower half) and R_{ST} values (upper half) for all comparisons between and within islands

Population	BIEast	BISouth	BINorth	Maui	Oahu	Kauai	Midway
BIEast		0.0137	0.0628	0.0434	0.0071	0.0532	0.1731
BISouth	0.0254		0.0576	0.0210	0.0065	0.0130	0.1335
BINorth	0.0673	0.1335		0.1224	0.0884	0.0475	0.0703
Maui	0.0651	0.0500	0.1488		0.0021	0.0289	0.1196
Oahu	0.0416	0.0560	0.1019	0.0106		0.0336	0.1324
Kauai	0.0512	0.0501	0.0823	0.0210	0.0161		0.0480
Midway	0.0681	0.0899	0.1032	0.0605	0.0447	0.0647	

two transitions. The most polymorphic population with respect to its mtDNA signature was Kipuka where four different haplotypes, H1, H2, H3 and H4, were identified. This population was collected from several hapuu cavities in a remote Hawaiian rain forest area located at approximately 1200 m altitude and isolated from other forested areas by lava flows. The remaining haplotype, H5, was identified in Kalapana and McCandless, both forest populations in remote areas (Fig. 1, Table 1).

Discussion

Microsatellite analysis

The most salient features of the comparison of microsatellite loci across the Hawaiian Islands are on the one hand the significant differences in allelic frequencies among all the populations examined, and on the other hand the significantly higher number of alleles in Maui (Table 2, Fig. 2). Differences between populations both in separate islands and within the Big Island result mainly from allelic frequency divergence. There are indeed very few unique alleles that occur at frequencies >0.1 except in Midway. Comparisons between Midway and the main Hawaiian Islands yielded some of the highest F_{ST} and R_{ST} values (Table 3). Although the small sample size (10 individuals) may have created a biased allelic frequency distribution, many of the differences between Midway and the other populations are due to alleles that occur with high frequency (Table 2). For example, allele 11-283 (locus CQ11, allele size 283 bp) occurs in three out of 10 individuals in Midway, but is absent from the 397 individuals examined in the other four islands. Another example is 29-168, which occurs in half of the individuals in Midway but only in four individuals from the other islands (50% vs. 1%). Combined with the high frequency of a unique mtDNA haplotype in Midway, these differences between Midway and the remaining populations seem to indicate a separate source for Midway mosquitoes. The presence of a Navy station on the island and high amount of traffic to and from Midway particularly during the Second World War, make a separate or secondary origin for Midway mosquitoes likely. The allelic frequency distribution indicates a recent bottleneck, and therefore a recent introduction, or reintroduction, although the small sample size makes this analysis tentative. It should be noted that the significant 'mode-shift' could simply be the result of a sample size of 10 that forces any alleles present to have a frequency of at least 0.05.

Separate introductions into Midway seem to agree with our original expectation of high contemporaneous traffic of mosquitoes in planes and boats (Raymond *et al.* 1991), however, the reduced allelic diversity in Oahu does not. Oahu has the only international airport in the state and

Pearl Harbor is a busy commercial port. Our collecting sites included locations near the international airport and the harbour, and the sampling effort in Oahu was similar to that in nearby Maui (Fig. 1), the island with the highest allelic diversity. Although the F_{ST} values between Maui, Oahu and Kauai are low, their allelic frequency distributions are significantly different and in particular the number of alleles in both Oahu and Kauai are significantly lower than in Maui (Fig. 2). It seems then that the rate of introductions from outside Hawaii is slow, and the exchange of mosquitoes between these islands is low. The low F_{ST} values may be due to recent common ancestry and reduced diversity of the founder event. They may also stem from the very high within-population heterozygosities (0.60 ± 0.15 , mean $\pm$ SD) that result from using highly polymorphic loci like microsatellites (Hedrick 1999). Still, the F_{ST} values between populations in Hawaii are higher than those found between populations of *Anopheles gambiae* between East and West Africa (Lehmann *et al.* 1996) or *An. maculatus* across Thailand (Rongnparut *et al.* 1999) although the population heterozygosities were comparable.

The picture that emerges from the microsatellite analysis is one of relatively isolated populations, particularly in the Big Island, that have evolved to quite different allelic frequencies. The prevailing winds and currents in Hawaii are from East to West, so that rain forests are located on the windward slopes of the mountains and deserts on the leeward slopes (Sohmer & Gustafson 1994). Therefore, if the introductions were into Maui or Oahu and Midway it may not be surprising that Big Island populations have reduced allelic diversity (Fig. 2).

mtDNA

Although by necessity of a much smaller scope than the microsatellite analysis, the mtDNA analysis provides us with a picture of the population structure of *Culex quinquefasciatus* in Hawaii that both partially agrees with the one provided by the microsatellite analysis and generates some tantalizing hypotheses. As mentioned before, the presence of unique haplotypes in Midway indicates a separate or mixed origin for those populations. On the other hand, although microsatellite allelic diversity is highest in Maui, only one mtDNA haplotype was found there. The same haplotype was found in Oahu and Kauai. In the Big Island, individuals from low elevations had also the same haplotype as those in the other main islands. The sources of new mtDNA haplotypes were mosquitoes from remote locations in the Big Island collected in hapuu cavities in the Hawaiian rainforest. Four unique haplotypes were found there.

Overall, the low diversity that was found in the A + T-rich region in Hawaiian *Cx. quinquefasciatus* was not

unusual. In their study, Guillemaud *et al.* (1997) sequenced individuals from Martinique, California, Thailand and Brazil and found only three haplotypes. Thailand and California had haplotype H1 (from Fig. 1), Martinique had haplotype H3 and Brazil had a haplotype not found in Hawaii. For a separate study we have also sequenced the A + T-rich region of *Cx. quinquefasciatus* from 16 populations across the World including Brazil, Florida and Mexico (Fonseca, Fleischer and Wilkerson, unpublished manuscript). We found the same haplotype in Brazil as Guillemaud *et al.* (1997), the common haplotype in Florida was H1 and in Jalisco, Mexico, was H4.

The low diversity in the mtDNA found in *Cx. quinquefasciatus* and also in *Aedes albopictus*, both mosquitoes with world-wide distributions, has been attributed to their recent human-aided expansion (Kambhampati & Rai 1991; Guillemaud *et al.* 1997). That is, range increase occurring sequentially through successive bottlenecks causing stochastic lineage survival and reduced haplotype diversity (Avise *et al.* 1984). Separate populations would have characteristic mtDNA haplotypes. The presence in Hawaii of five haplotypes plus the common H1 haplotype could then represent five separate introductions to the main Hawaiian Islands. The alternative to this hypothesis is to invoke high rates of convergent mutation in Hawaii. That may be the case for haplotype H3, which results from variation at a microsatellite in the mitochondrial DNA, but not a parsimonious hypothesis with respect to the other haplotypes. Furthermore a world-wide survey of microsatellite loci in *Cx. quinquefasciatus* also supports the hypothesis of multiple introductions to Hawaii (Fonseca, Fleischer and Wilkerson, unpublished manuscript).

In Midway both microsatellite and mtDNA data indicate that there has been a recent introduction from outside the Hawaiian Islands. However, Maui, the island with the highest microsatellite allelic diversity, only has mtDNA haplotype H1 and the remaining four mtDNA haplotypes occur almost exclusively in populations with low microsatellite diversity in the Big Island. Obviously because we have not yet sequenced mosquitoes from high elevations in Maui or any of the other islands, it is possible that some other mtDNA haplotypes may occur there. That does not detract from the paradox just delineated: that microsatellite diversity is high in areas with low mtDNA and some low microsatellite diversity areas have relatively high mtDNA diversity.

Although we cannot dismiss the possibility that unique mtDNA alleles at high elevation in the Big Island are simply the result of founder events maintained by limited dispersal and maternal inheritance, based on the microsatellite information there appear to be no restrictions to gene flow between high and low elevation. There may then be other equally or more parsimonious explanations for

this pattern. For example, *Cx. quinquefasciatus* mosquitoes are known to harbour multiple strains of *Wolbachia pipientis* (Yen & Barr 1973). This Rickettsia-like intracellular micro-organism is responsible for female-biased cytoplasmic incompatibility between mosquitoes harbouring different parasite strains (Laven 1967). A sweep involving *Wolbachia* can result in reduced mitochondrial diversity by hitch-hiking (Ballard *et al.* 1996). The introduction of a small *Wolbachia*-positive population could therefore have reduced mitochondrial diversity at low elevations without significantly changing nuclear genome patterns. Mitochondrial DNA diversity in high-elevation populations might then reflect better the history of introductions into the Hawaiian Islands than low-elevation populations. It should be noted that haplotype H4 found in two high-elevation individuals was the only haplotype found in Jalisco, Mexico by Fonseca, Fleischer and Wilkerson (unpublished manuscript). This population is near the putative original source of mosquitoes to the Hawaiian Islands (Dine 1904; Hardy 1960). Jalisco and Hawaii were the only localities out of 16 populations sampled across all continents where this haplotype has been found.

Conclusion

Patterns of microsatellite diversity in Hawaii show that populations of *Cx. quinquefasciatus* from the East and North sections of Big Island are relatively isolated from populations in other Hawaiian islands. There is indication of restricted movement of mosquitoes among populations in the Big Island and among populations in different Hawaiian Islands. These results raise the possibility that independent evolution in the different islands of *Plasmodium relictum* may have generated strains with widely varying degrees of virulence. We will be pursuing this avenue of research in the future.

Our studies also provide evidence of multiple introductions of *Cx. quinquefasciatus* into the Hawaiian Islands. Although morphologically very homogeneous, populations of this species are extremely variable both behaviourally and physiologically. Some populations are capable of laying eggs without acquiring a blood meal (autogeny) while others raised under the same conditions are incapable (Bushrod 1978). There is also variation across populations in refractoriness to avian malaria (Huff 1934) and insecticide resistance (Chevillon *et al.* 1997; Raymond *et al.* 1998). Although it is beyond the scope of this paper to dwell on it, reported changes in avian malaria virulence for Hawaiian birds (Goff & van Riper III 1980; van Riper *et al.* 1986; Atkinson *et al.* 1995), have been hard to interpret. It is tempting to speculate that they may result from unexplored effects of changes in predominant vector strains on the evolution of virulence. We hope that understanding the processes underlying the genetic landscape

of *Cx. quinquefasciatus* in Hawaii will aid in developing tools to both control avian malaria and understand the evolution of virulence in vector-mediated parasite–host interactions.

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