# PATHWAYS OF EXPANSION AND MULTIPLE INTRODUCTIONS ILLUSTRATED BY LARGE GENETIC DIFFERENTIATION AMONG WORLDWIDE POPULATIONS OF THE SOUTHERN HOUSE MOSQUITO 

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#### Abstract

The southern house mosquito Culex quinquefasciatus is a principal vector of human lymphatic filariasis, several encephalitides (including West Nile virus), avian malaria, and poxvirus, but its importance as a vector varies considerably among regions. This species has spread with humans and is ubiquitous in tropical urban and suburban environments. This was the first mosquito to reach Hawaii and we performed a worldwide genetic survey using microsatellite loci to identify its source. Our analyses showed divergent Old World and New World genetic signatures in Cx. quinquefasciatus with further distinctions between east and west African, Asian, and Pacific populations that correlate with the epidemiology of human filariasis. We found that in Hawaii south Pacific mosquitoes have largely replaced the original New World introduction of Cx. quinquefasciatus, consistent with their reported expansion to higher elevations. We hypothesize worldwide pathways of expansion of this disease vector.


## INTRODUCTION

A select group of insect vectors of disease have expanded their ranges radically in association with humans. ${ }^{1}$ Although their introduction to new areas has sometimes heralded disease outbreaks, e.g., yellow fever epidemics in the New World after the introduction of Aedes aegypti, ${ }^{2}$ the distribution of disease vectors does not always correlate with the distribution of the diseases they transmit. ${ }^{3}$ For example, nocturnal periodic lymphatic filariasis (Wuchereria bancrofti) is transmitted primarily (some say exclusively) by Culex (Culex) quinquefasciatus Say in urban eastern Africa and in Asia. ${ }^{4}$ However, although Cx. quinquefasciatus is omnipresent and very common across all tropical and subtropical regions of the world, ${ }^{5}$ the primary vectors of nocturnal filariasis in rural and western Africa are several Anopheles species, while in the Pacific islands the nocturnal form of $W$. bancrofti is virtually absent and diurnal/sub-periodic forms are transmitted by local Aedes species. ${ }^{4}$ Across the world Cx. quinquefasciatus is also a locally important vector of St. Louis encephalitis and West Nile virus, as well as of avian malaria and pox viruses. ${ }^{6}$

New or modified vector-mediated host/parasite interactions occur when natural or human-assisted introductions of vectors and parasites are made into new ranges. ${ }^{1,7}$ However, in addition to possible new vector-disease-host combinations, the vector itself may undergo local selection, genetic drift, or hybridizations that can modify their ability to transmit a disease. ${ }^{8,9}$ Our capacity to predict, prepare, and react to emerging arthropod-borne diseases depends not only on our understanding of emerging disease organisms, but also their vectors. As a model system we have been focusing on one of the best-known mosquito introductions, that of Cx. quinquefasciatus to the Hawaiian Islands where as the sole vector of avian malaria it has contributed to the endangerment of many endemic forest bird species. ${ }^{10}$ T. R. Peale, an American naturalist, found no mosquitoes when he visited the islands in $1823,{ }^{11}$ so as the first introduced mosquito, Cx. quinquefasciatus was conspicuous to natives, explorers, and missionaries.

[^0]The most detailed and possibly the most speculative description of the introduction is that of Reverend William Richards ${ }^{12}$ who indicts the crew of the "Wellington" for releasing larvae of the southern house mosquito with old drinking water obtained in San Blás, Mexico, while at port in Lahaina, Maui, in 1826. Other authors provide fewer details, but because Cx. quinquefasciatus was then not likely present in the Pacific, ${ }^{13}$ the mosquito source is always the New World. ${ }^{11,14}$ The epidemiology of avian malaria (Plasmodium relictum) in the Hawaiian Islands is often cited as a classic example of co-evolution subsequent to the introduction of disease to a highly susceptible, isolated wildlife population. ${ }^{15}$ However, the role of the vectors has not been previously considered. The objective of our study was to examine the history of introductions of $C x$. quinquefasciatus to Hawaii. While doing so we uncovered evidence of multiple introductions diagnosed by an unexpected degree of genetic differentiation worldwide.

## MATERIALS AND METHODS

Samples and microsatellite genotyping. In an attempt to obtain a snapshot of the genetic signature of Cx. quinquefasciatus across the world, we obtained specimens from as many different locations as possible with the aid of local entomologists (PHS permit no. 99-05-0660) or from colleagues (Table 1). For an updated yet still incomplete map of the distribution of $C x$, quinquefasciatus, see the report by Smith and Fonseca. ${ }^{16}$ We excluded samples from Shanghai, China, because they showed considerable hybridization with $C x$. pipiens pallens, an east Asian member of the Cx. pipiens complex. ${ }^{16}$ To achieve representative sample sizes, we chose to combine close-by samples (within each country or geographic location). The exceptions are Hawaii, where we examined separately the current (Oahu) and historic (Maui) main entry ports because we had ample access to specimens and one of our objectives was to understand better the timing of introductions. We also decided not to combine specimens from the Midway Atoll (Hawaii) with other Hawaiian specimens because we had prior knowledge of separate introductions to this atoll, ${ }^{17}$ which was the site of extensive naval traffic during World War II. We also did not combine specimens from

TABLE 1
Collection location, samples sizes, stage from which DNA was extracted, date of collection, and sources of Culex quinquefasciatus analyzed*

|  | Location | No. | Stage | Date ${ }^{\dagger}$ | Source |
| :---: | :---: | :---: | :---: | :---: | :---: |
| 1 | Makurdi and Zaria, Nigeria | 20 | A | June 1999 ${ }^{1}$ | Light traps near houses |
| 2 | Dongola, Sudan | 28 | L | Jan 2003 ${ }^{2}$ | Three larval dips within city limits |
| 3 | Kisumu and Malindi, Kenya | 31 | A | May $1999^{3}$ | Collected inside houses in five villages |
| 4 | Kochi, India | 9 | A | May $2000^{4}$ | Hand collected inside medical compound |
| 5 | Depok (near Jakarta), Java, Indonesia | 16 | A | 1999, $2003{ }^{5}$ | Adults reared from larvae collected within city limits |
| 6 | Makassar, Sulawesi, Indonesia | 8 | A | Aug $2003^{5}$ | Adults reared from larvae collected within city limits |
| 7 | Kupang, West Timor, Indonesia | 32 | A | 1999, $2003{ }^{5}$ | Adults reared from larvae collected within city limits |
| 8 | Okinawa, Japan | 24 | A/L | April $1999{ }^{6}$ | Light traps and larval dips |
| 9 | Cairns, Australia | 22 | A | $2004{ }^{7}$ | Adults raised from > 7 egg rafts |
| 10 | Kingswood and Glandore, Australia | 25 | A | April $1999{ }^{8}$ | Adults raised in the lab from $>100 \mathrm{egg}$ rafts |
| 11 | Auckland, New Zealand | 35 | L | Jan $1999{ }^{9}$ | Larval dips. Three separate sites within city limits |
| 12 | American Samoa | 14 | L | $1999{ }^{10}$ | Larval dips |
| 13 | Midway, HI, USA | 10 | L | $1998{ }^{10}$ | Larvae in rainwater pooled in plastic covers |
| 14 | Oahu, HI, USA | 25 | A | $1998{ }^{11}$ | Light traps in airport, Pearl Harbor, and Honolulu |
| 15 | Maui, HI, USA | 53 | A/L | $1997{ }^{12}$ | Lihue, Lahaina, Airport, and cattle puddle up Haleakala |
| 16 | Chino, CA, USA | 46 | A | Oct $2002{ }^{13}$ | Light traps within city limits |
| 17 | Jalisco, Mexico | 44 | A | $1998{ }^{14}$ | Light traps at Estacion Biologica de Chamela |
| 18 | Tapachula, Chiapas, Mexico | 24 | A | July $1998{ }^{15}$ | Light traps near Centro de Investigacion de Paludismo |
| 19 | Santa Cruz, Galapagos Islands, Ecuador | 29 | A | May $2004{ }^{16}$ | Oviposition trap next to house (27 egg rafts), Puerto Ayora |
| 20 | Manta, Ecuador | 17 | A | Dec $2004{ }^{17}$ | Light traps within city limits |
| 21 | New Orleans, LA, USA | 24 | L | March $1999{ }^{18}$ | Larval dips at sewage treatment plant |
| 22 | Archer, FL, USA | 24 | L | Sept $1998{ }^{19}$ | Collected from 5 scrap tires |
| 23 | Bermuda, UK | 44 | L | Dec $2004{ }^{20}$ | Human containers (boat, tires, etc.) across the island |
| 24 | George Town, Cayman Islands, UK | 24 | A | June $2003{ }^{21}$ | Light traps within city limits |
| 25 | Savanna la Mar, Jamaica | 22 | A | Dec $2004{ }^{1}$ | Adults reared from larvae collected with oviposition traps |
| 26 | Trujillo, Venezuela | 30 | L | Mar $2004{ }^{22}$ | Larval dips into large cemetery flower pot (multiple cohorts) |
| 27 | Amapá, Macapá, Brazil | 13 | A | May $1997{ }^{22}$ | Hand-net collection of swarming specimens inside restaurant |
| 28 | São Paulo, Brazil | 42 | A | Dec $2002{ }^{23}$ | Aspirations from ferns, Alto do Pinheiro, next to houses |
|  | Total | 735 |  |  |  |

* The numbers in the left column correspond to those in Figures 1 and 2 and in the allelic frequency tables in the Supplementary Materials. $\mathrm{A}=$ adult; $\mathrm{L}=$ larva
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mainland Ecuador and the Galapagos Islands so we might investigate the sources of the recent introduction of $C x$. quinquefasciatus to the Galapagos Islands. Furthermore, we extracted DNA from five-dried museum specimens collected between 1919 and 1944 in Hawaii (Bishop Museum collections).

All specimens were field collected as adults or larvae and they were sent to us either dry or in ethanol. Larval collections were made from multiple oviposition sites in each location to prevent a few families from influencing the results. A morphologic examination, including genitalia analysis of males ${ }^{18}$ to confirm species, was performed prior to DNA extraction. In later samples, rapid polymerase chain reaction (PCR)-based assays were used to confirm species identification. ${ }^{16}$ DNA was extracted using a standard phenol/ chloroform method ${ }^{17}$ and $1 \mu \mathrm{~L}$ of the DNA was used in each PCR. To prevent contamination with male DNA, female abdomens were not used. The DNA extraction and PCR preparations involving the five dried museum specimens were performed in a separate room away from the main laboratories. We examined twelve microsatellite loci: CQ11, CQ26, CQ29, CQ41, CQ46, pGT12, pGT46, pGT51, and qGA12, qGT4, qGT8, and qGT17 (Table 2). Analyses of mosquito families have showed that all the microsatellite loci used in this study are inherited in a Mendelian fashion and are not sexlinked. ${ }^{19-21}$ Microsatellite loci were amplified and sized as described by Smith and others. ${ }^{21}$

Statistical analyses. Tests of Hardy-Weinberg equilibrium were conducted using GENEPOP 1.2, ${ }^{22}$ and allelic richness was calculated with FSTAT 2.9.3 ${ }^{23}$ using a rarefaction index $(2 \mathrm{~N}=10)$ to account for different sample sizes. We used Cavalli-Sforza and Edwards chord distance as well as Nei's genetic distance as a measures of population differentiation, ${ }^{24}$ which were implemented using the programs MSA3.0, ${ }^{25}$ fol-

TABLE 2
Microsatellite loci used in this study*

| Locus name | $\mathrm{H}_{\mathrm{e}}$ | No. of alleles |
| :--- | :---: | :---: |
| CQ11 | 27 |  |
| CQ26 $^{\mathrm{A}}$ | 0.68 | 14 |
| CQ29 | 0.61 | 7 |
| CQ41 | 0.41 | 26 |
| CQ46 | 0.64 | 11 |
| qGA12 $^{\mathrm{B}}$ | 0.74 | 19 |
| qGT4 $^{\mathrm{B}}$ | 0.70 | 10 |
| qGT8 $^{\mathrm{B}}$ | 0.58 | 24 |
| qGT17 $^{\mathrm{B}}$ | 0.68 | 21 |
| pGT12 $^{\mathrm{C}}$ | 0.68 | 8 |
| pGT46 | 0.36 | 15 |
| pGT51 | 0.65 | 20 |

* The summary statistics were calculated for all populations combined ( $\mathrm{N}=735$ ), except for CQ46. The information for CQ46 refers only to American populations, where the locus is in Hardy-Weinberg equilibrium. For all loci, expected heterozygosity ( He ) was calculated as $[2 n /(2 n-1)]\left[1-\operatorname{sum} \mathrm{p}_{\mathrm{i}, \mathrm{k}}\right]$, where $n$ is the number of individuals, $k$ is the number of distinct alleles, and $p_{i}$ is the relative frequency of allele $i$. Subscripts after the name of each locus refer to membership to one of three multiplexes.
lowed by NEIGHBOR and CONSENSE in Phylip $3.573 \mathrm{c}^{26}$ with 1,000 replicate bootstraps. Trees were assembled with TreeViewPPC. ${ }^{27}$ Furthermore, we assigned individuals to clusters (strains) based on their multilocus genotypes with a maximum likelihood algorithm implemented in the program Structure 2.0. ${ }^{28}$ We used 20,000 burn-in steps and $1,000,000$ runs with a model of uncorrelated allele frequencies allowing admixture (gamma $=0.34$, calculated at $\mathrm{K}=1,{ }^{29}$ ). In this analysis, the origin of each specimen is not disclosed but the number of clusters ( K ) is decided a priori for each run. To assess the consistency of the analysis, we performed an exhaustive comparison of 10 runs at each K scoring the similarity coefficient described by Rosenberg and others. ${ }^{30}$ Formal estimation of admixture proportions (i.e., calculation of the putative contribution of introductions from different locations to the current Hawaiian populations of Cx. quinquefasciatus) was performed with the Markov Chain Monte Carlo method LEADMIX, a superior maximum likelihood based method that allows for more than two source populations and can incorporate admixture due to non-simultaneous migration from different source populations. ${ }^{31}$


## RESULTS

We obtained 735 specimens from 28 locations across the world always near or inside human dwellings (Table 1). Although some collecting devices were placed in areas less impacted by humans, those did not yield $C x$. quinquefasciatus. Of the specimens examined, $70 \%$ were adults, but less than $40 \%$ of those were collected with light traps or other methods that mostly collect females. Many larvae were reared to adults giving us access to known mixes of males and females. Furthermore, as mentioned before, the microsatellite loci used in this study are not sex-linked.

Even after we redesigned primers, ${ }^{21}$ two of the 12 microsatellite loci used in the analyses (CQ46 and CQ41) had significant heterozygote deficits in several populations (see Tables in Supplemental Material). Thus, they were excluded from the analyses. Neighbor-joining distance trees (CavalliSforza and Edwards chord distance and Nei's genetic distance produced nearly identical trees) using the remaining 10 microsatellite loci showed two well-supported geographically structured groups (Figure 1): a Pacific group and a New World group. The samples from east Africa and those from Southeast Asian locations also clustered together, but samples from both Nigeria (West Africa) and Japan clustered with the New World populations. Because of DNA degradation, we only attempted to amplify three loci (CQ26, CQ29, and CQ41) from the museum specimens. Two specimens (both collected in the 1920s) did not yield products. The remaining three specimens $(1919,1931,1944)$ yielded 11 alleles but only the specimen from 1944 had an allele unique to Pacific populations (CQ29-186, Table A3). Table A3 appears online at www.ajtmh.org.

The results of the multilocus genetic structure analysis, which combines all individual multilocus genotypes and separates them into distinct clusters analogous to the hierarchical branching of tree diagrams, ${ }^{32}$ gave similar results to the distance analysis but with a higher resolution (Figure 2). The similarity of results across 10 replicates at each number of inferred clusters (K) was high (0.9-0.99) for K from 2 to 4 . At $\mathrm{K}=2$, the 735 specimens of $C x$. quinquefasciatus examined cluster into two clearly defined groups: Hawaii plus Old


Figure 1. Unrooted consensus nearest-neighbor tree depicting the relationships between populations used in this study (to decrease sampling related artifacts, we excluded populations with less than 14 specimens from this grouped analysis). Numbers on branches indicate bootstrap percentages. The numbers before each name correspond to those in Figure 2. All populations group first according to geographic proximity except for Nigeria and Japan, which consistently group with the American populations. Isl = Islands.

World and New World, although similar to earlier findings in this report, Nigeria has New World ancestry. At K $=3$, Austral and Pacific specimens separate from the Old World cluster, while Brazilian, Nigerian, and Japanese specimens have a mixed signature (Old and New World). Finally, at $\mathrm{K}=4$, populations along the Atlantic Coast of the Americas, as well as Japan and Nigeria, all separate into a distinct group. Throughout the analysis, Hawaiian populations cluster with Australia and New Zealand although Maui has more specimens with New World ancestry than does Oahu.

Admixture analyses showed that Hawaiian populations have an uneven mixture of alleles from New World and Old World populations (less than $20 \%$ input from New World populations), which agrees with our other analyses that assign a predominantly South Pacific ancestry to current Hawaiian populations. The concordance between the different analyses is not surprising because a few microsatellite alleles that are common in the Americas (e.g., qGA12-157) occur in Hawaiian populations but are absent from the South Pacific (Tables A1-A11), while many alleles common in the south Pacific and also common in Hawaii are conspicuously absent from the Americas (e.g., CQ26-220, CQ29-186). American popula-


Figure 2. Results of a Bayesian cluster analysis of multilocus microsatellite genotypes. Each of the 735 individuals included in the analysis is represented by a thin vertical line, partitioned into colored segments that represent the individual's probability of belonging to one of each of the genetic clusters. Although the origin of each specimen is not used in the analysis, in this figure specimens were grouped by location (separated by a vertical line). The geographic locations of all samples with associated location numbers are shown in the world map and are the same as in Figure 1. Included are two populations with samples of less than 14 specimens: India $(\mathrm{n}=9)$, Midway Island $(\mathrm{n}=10)$, and Amapá, Brazil $(\mathrm{n}=13)$, which were excluded from the distance analysis. Their location numbers are 4,13 , and 27 , respectively.
tions, especially those on the Pacific Coast, had significantly ( $P<0.05$ ) lower allelic richness than Old World populations. The highest allelic richness was found in Asian and east Africa populations $(P<0.05)$ and the lowest was found in west Africa.

## DISCUSSION

Although we were able to obtain specimens from multiple locations in some countries but not from others, those events are randomly distributed across the samples and do not reflect the allelic richness encountered. For example, in both Nigeria and Kenya, specimens came from a range of collection sites but those two locations in our study have the lowest and one of the highest allelic richness, respectively. The similarity between Hawaiian and South Pacific populations is unexpected (based on historical accounts) and significant because South Pacific populations of Cx. quinquefasciatus are adapted to cold southern hemisphere environments. In New Zealand, where winter temperatures are occasionally below their putative survival minimum, larvae are found from July through September, the southern winter months. ${ }^{33}$ The introduction and genetic swamping of such populations could explain the apparent recent expansion of $C x$. quinquefasciatus to higher elevations in Hawaii. ${ }^{7,34}$ The fact that Maui contains many more admixed specimens argues that the nonAustralasian introduction occurred when Maui was the main port of entry to Hawaii (in the 1800s), which supports the original accounts of the first introduction of mosquitoes. These analyses also indicate that most of the dozens of $C x$. quinquefasciatus arriving monthly in Oahu in aircrafts from both Asia and the Americas ${ }^{35}$ do not reproduce, an important
observation from a control standpoint. The presence of a unique South Pacific allele only in the specimen from 1944 gives us a date by which we know South Pacific $C x$. quinquefasciatus had already arrived in Hawaii, although the small number of specimens available does not allow us to reject the possibility they had arrived earlier.

It is not known when Cx. quinquefasciatus arrived in Australia. Marks argued that it arrived with or shortly after the colonial First Fleet in 1788, ${ }^{1,36}$ while others have attributed its arrival to the opening of Australian ports to American whalers in $1831 .{ }^{37}$ Its introduction to New Zealand appears to be recent because there are suggestions that it is just starting to penetrate inland. ${ }^{38}$ Supporting the idea that an Australian strain of Cx. quinquefasciatus could have remained localized, the introduction of Ae. australicus (a species native to Australia) to New Zealand is well documented and occurred only during the last $50-80$ years. ${ }^{37}$ The expansion of Cx. quinquefasciatus to the smaller Pacific islands is thought to be even more recent and linked to events during World War II. ${ }^{13}$ Such introductions are supposedly linked to the increased connectivity between Australia and the Pacific islands because of the intense traffic of whaling boats and later passenger airplanes and warships. ${ }^{37}$

The low diversity of New World populations of Cx. quinquefasciatus agrees with assertions that it is a recently introduced species. So does its only recent arrival to the Galapagos Islands and the similarity between mainland Ecuador and Galapagos specimens. However, the source of New World Cx. quinquefasciatus is unclear. Its arrival from west Africa as claimed ${ }^{39}$ is unlikely because the species was reported absent there before $1942 .{ }^{40}$ The similarity between Nigerian and American populations instead indicates that the former were
introduced from the New World, which is supported by the even lower allelic richness of Nigerian specimens. The differences between Pacific and Atlantic coasts in the New World are consistent across latitude (Figure 2) and close examination of the allelic frequencies (Tables A1-A11) and the higher allelic richness in Atlantic Coast populations supports the hypothesis that the extensive boat traffic across the Caribbean and the Atlantic may have led to extensive mixing.

East African and Asian populations cluster together (Figure 2). Although the heavy human traffic across the Indian Ocean might be the reason, if, as it has been proposed, $C x$. quinquefasciatus originated in Africa, ${ }^{41}$ African populations would have ancestral polymorphism. Instead, both the east African and Asian populations we examined had the highest allelic richness across all populations examined. The very different signatures of east and west African populations, as well as the uniqueness of the interaction between $C x$. quinquefasciatus and its sibling species Cx. pipiens in Africa, ${ }^{42}$ hint at a complex origin and distribution of the species there and will require further sampling in the continent. Critically, our findings agree with the epidemiology of nocturnal filariasis described earlier and are supported by vector competence studies showing low susceptibility of $C x$. quinquefasciatus from west Africa and Polynesia to W. bancrofti. ${ }^{43,44}$

The presence of distinct strains of $C x$. quinquefasciatus across the world was unexpected because previous studies examining loci involved in insecticide resistance concluded this species is undergoing a human-aided expansion with nontrivial levels of gene flow between populations. ${ }^{45}$ Our results, however, show that recombination may break the connection between selected and neutral loci very quickly, maintaining the integrity of the microsatellite signature while allowing the penetration of useful insecticide resistance alleles. This result is a critical example of cryptic introgression of useful genes, which may be a common phenomenon with very broad consequences. ${ }^{46}$

In conclusion, we found that worldwide populations of $C x$. quinquefasciatus are significantly genetically differentiated and correlated to known W. bancrofti vector competence, and their pathways of expansion are remarkably similar to those inferred for Ae. aegypti, another vector species associated with humans. ${ }^{2}$ Furthermore, we found there has been at least a second introduction of Cx. quinquefasciatus into Hawaii. This conclusion is significant because changes in the dynamics of avian malaria in Hawaii, especially the perceived increase in the altitudinal range of the mosquitoes ${ }^{7,34}$ as well as changes in parasite virulence, ${ }^{34}$ may be associated with this secondary introduction. We are currently examining differences in vector competence to avian malaria of the various genetic strains. Multiple introductions may be a fundamental evolutionary agent in invasive species ${ }^{47}$ and in disease vectors they may impact important epidemiologic parameters.

Received June 8, 2005. Accepted for publication September 28, 2005.
Acknowledgments: We thank all our collaborators for field support or for providing mosquito samples. We also thank Gordon Nishida and the Bishop Museum in Hawaii for granting us access to rare specimens from the early 1900s. Nusha Keyghobadi, Carolyn Bahnck, and Jon Beadell greatly improved the manuscript with their comments.

Financial support: This work was supported by the Smithsonian Institution, Walter Reed Army Institute of Research, the Friends
of the National Zoo, National Institutes of Health grant NIH R01GM063258, and grant CDC/NIH\#U50/CCU220532.

Disclaimer: This material reflects the views of the authors and should not be construed to represent those of the Department of the Army or the Department of Defense.

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Table A3
A1-A11 Allelic frequencies at 11 microsatellite loci in Culex quinquefasciatus*

Table A3
Continued

Table A3


Table A3
Continued


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