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Molecular Phylogenetics and Evolution 47 (2008) 1173–1180
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Population structure and genetic diversity in two species of Hawaiian picture-winged *Drosophila*

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Received 24 September 2007; revised 10 December 2007; accepted 4 February 2008
Available online 21 February 2008

Abstract

Over the last several decades many picture-winged *Drosophila* have become less common in both geographical distribution and local population size (pers. obs., Foote pers. comm., Montgomerey pers. comm.). Here we report on a study of two Hawaiian *Drosophila* species, *D. engyochracea*, and *D. hawaiiensis*, to determine the impact that changes in population sizes over the past thirty years have had on the genetic diversity of these species. *D. engyochracea* is known from only two locations on the Island of Hawai'i (Kipuka Ki and Kipuka Pua'ulu), while *D. hawaiiensis* is currently more wide spread across Hawai'i Island. We collected 65 *D. hawaiiensis* and 66 *D. engyochracea* from two forest patches (kipuka) isolated by a 400 year old volcanic ash deposit. DNA sequence data for 515 bases of the mitochondrial gene COII was analyzed for both species to estimate relative total genetic diversity as well as inter-kipuka gene flow. The more wide spread species, *D. hawaiiensis,* has more genetic diversity (23 vs. 11 unique haplotypes) than the rarer species, *D. engyochracea*. The distribution of haplotypes in the kipuka is consistent with more gene flow in *D. engyochracea* than in *D. hawaiiensis*. Phylogenetic analysis indicates a small number of individuals morphologically identified as one species but have DNA sequence diagnostic for the other species. These results are consistent with these individuals being descendant from hybrids between species.

Keywords: Hawaiian *Drosophila*; Genetic diversity; Population decline; Population genetics; Bottleneck effects

1. Introduction

The Hawaiian *Drosophila*, comprising more than 500 known species (Markow and O'Grady, 2006), is among the best examples of adaptive radiation. Today many of these species are experiencing dramatic declines in population size and many populations are now threatened by extinction. An early sign of danger for a species is the reduction in population size within a geographical location. One of the challenges in documenting a decline in population size is the lack of historical census data. In one Hawaiian picture-winged *Drosophila* species, *Drosophila engyochracea*, Fontdevila and Carson (1978) estimated the population size in Kipuka Ki, an isolated forest patch in Hawai'i Volcanoes National Park on the South East slope of Mauna Loa on the Island of Hawai'i. In a week long series of capture, mark, release, and recapture conducted in 1974 the Kipuka Ki population was estimated to be approximately 40,000 individuals. While apparently robust, this population was, and is, one of only two known populations of this species of picture-winged fly. The other population is found in Kipuka Pua'ulu a smaller (292,000 m^2 vs. 575,000 m^2) kipuka approximately 2 km and about 200 m downslope from Kipuka Ki. These kipuka were isolated from each other by a lava flow about 1000 years ago followed by a substantial ash deposit approximately 400 years ago (references in Fontdevila and Carson, 1978). The *Sapindus* tree (*Sapindus saponaria*), on which *D. engyochracea* oviposits, is found only in these two kipuka and in one other verified stand (Montgomery, 1975). Repeated efforts to collect *D. engyochracea* in the other *Sapindus* stands have been unsuccessful (Foote pers. observation).

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doi:10.1016/j.ympev.2008.02.007

In the years since Fontdevila and Carson's (Fontdevila and Carson, 1978) study the abundance of *D. engyochracea* has appeared to decline and we undertook to repeat the Fontdevila and Carson study to determine the current population size. We broadened the scope of the original study in order to document the genetic diversity, and intraspecific levels of gene flow between Kipuka Ki and Kipuka Pua'ulu. Our study involved a repetition of the original protocol with the inclusion of a closely related sympatric species *D. hawaiiensis* (Fig. 1), a second kipuka, and five (one every three months) repetitions of the collection over twelve months. In this paper, we focus on the genetic data and address three questions: (1) What is present level of genetic diversity estimated from Cytochrome C Oxidise Sub-Unit II (COII) sequence? (2) What level of gene flow is detectable between Kipuka Ki and Kipuka Pua'ulu? (3) Is there evidence of gene flow and hybridization between species?

1.1. Study species and study sites

Drosophila engyochracea and *D. hawaiiensis* are found only on the Island of Hawai'i and likely speciated after a founder colonization from the Maui Nui (the islands including Maui, Molokai, Lanai, and Kaho'olawe) complex. *D. hawaiiensis* is wide spread on the Island of Hawai'i and breeds on bark and flux found on Koa trees (*Acacia koa*) in sub-alpine mesic forests (Montgomery, 1975). *D. engyochracea* breed on the bark of the *Sapindus* (*S. saponaria)* only in Kipuka Ki and Kipuka Pua'ulu (Fig. 2), though apparent mating behavior has been observed on other trees (Martin pers. comm.). The life span for both species of picture-winged fly is approximately three months (Steiner, 1974). While the kipuka in this study have both host plants, the intervening landscape, a 400-year-old partially revegetated ash deposit, lacks *Sapindus* trees but has a patchy distribution of Koa. Both kipuka have an average year-round temperature between 15 and 18 °C, annual rainfall of 1600 mm, and the relative humidity one meter above the forest floor is about 80% (references in Fontdevila and Carson 1978). The local climate profiles of the two kipuka differ significantly in their stability. While Kipuka Ki fluctuates continuously throughout the day, Kipuka Pua'ulu is much more stable in temperature, gradually warming then cooling over the day (Fig. 2).

2. Methods

In September of 1974, Fontdevila and Carson began a capture, mark, release, and recapture study of *D. engyochracea* in two 3700 m^2 plots in Kipuka Ki. The plots are separated by a park road, which was proposed as a possible barrier to dispersal of the flies. Different color florescent dusts were used to test for movement across the road, which was found not to be an important barrier to the movement of the flies (Fontdevila and Carson, 1978). Their sampling protocol included an initial 8 h collection followed on six consecutive days by 3 h collections. Flies were attracted to sponges that were covered with sterilized banana (banana baby food), and captured by placing a one inch glass vial over them. The flies were kept cool by covering the vials in the leaf litter.

2.1. Sampling methods

We replicated the search methods of Fontdevila and Carson (1978), with a few minor modifications, so as to make the results of these two studies comparable. Some modifications of the protocol were made in order to broaden the scope of the study. First, we included a second, sympatric species, *D. hawaiiensis* in order compare the population size estimates of ecologically similar species whose geographical distribution is more wide spread across Island of Hawai'i. We collected each afternoon (days 2–7) for 4–5 h and also expanded the total collection to include five repetitions of the week-long collection over the course of a year in order to normalize estimates of population size over seasonal fluctuations. We used fresh mashed bananas mixed with water and brewer's yeast on the bottom one-half of the attraction (bait) sponge and on the top one-half we sprayed the liquid strained from a mixture of fermented mushrooms and yeast (found to be most effective bait combination). Since the previous study demonstrated that the road did not act as a barrier to dispersal we chose not to

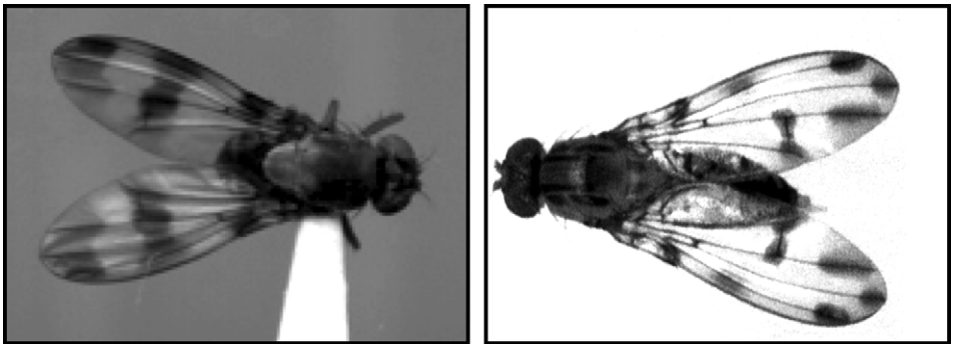

Fig. 1. *Drosophila hawaiiensis* (left), and *D. engyochracea* (right). Diagnostic wing patterns distinguish the two species: solid bars for *D. hawaiiensis* and peripheral spots for *D. engyochracea*.

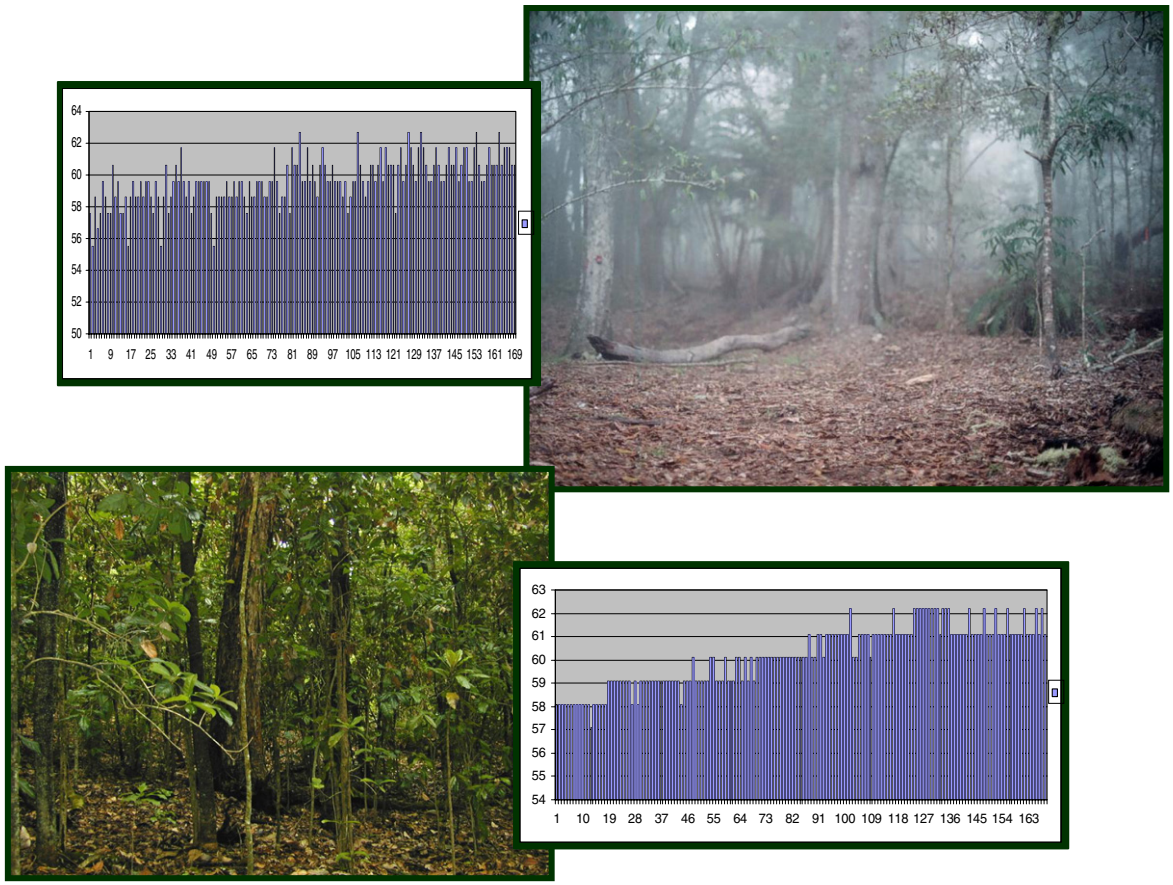

Fig. 2. Kipuka Ki (top) and Kipuka Pua'ulu (bottom) with representative temperature profiles shown in 15 min intervals collected from 4am to 6pm. The "*Y*" axis in both graphs are in Fahrenheit degrees and the numbers along the "*X*" axis indicate the 15 min interval over the time period. Notice that the temperature in Kipuka Ki fluctuates much more than does the temperature in the more densely treed Pua'ulu.

use different color dusts for different sides of the road. We did however use different color dust for each collection day.

We extended our study to examine the potential for the intervening habitat between Kipuka Ki and Kipuka Pua'ulu to act as a barrier to dispersal. We collected *D. hawaiiensis* and *D. engyochracea* from Kipuka Pua'ulu one month after the second Kipuka Ki collection using the same week long capture protocol. We kept samples of both species from Kipuka Ki and Kipuka Pua'ulu so that they could be included in the measurement of genetic diversity and gene flow between kipuka.

2.2. Genetic analysis

We collected 66 *D. engyochraea* and 65 *D. hawaiiensis* individuals for genetic analysis. DNA for phylogenetic analysis was extracted from the heads of recently captured individuals in order to avoid possible contamination from non-specific sperm in the case of females. DNA was extracted using DNeasy kit according to manufacturer's suggested protocol. Whole genomic DNA extracts were used as template for PCR amplification of COII using primers L3034 and H3796 (Simon et al., 1994). PCR cocktails included 1 mM $MgCl_2$, Taq buffer (50 mM KCL, 10 mM TRIS–HCl, .1% Triton X-100, .2 mM primer, and 1 U of Taq polymerase (Qiagen). The primers used for amplification are from Simon et al. (1994): L3034 5′ TAA TAT GGC AGA TTA GTG CA, H3796 5′ ACT ATT AGA TGG TTT AAG AG and the thermocycler conditions were ((95 C/1 min, 43 C/1 min, 72 C/1 min) × 10, (94 C/1 min, 46 C/1 min, 72 C/1 min) × 25, 72 C/7 min). PCR products were agarose gel isolated and purified using GeneClean kit according to recommended protocol. IRD dye labeled primers from LiCor were used in cycle sequencing with USB thermosequenase. Sequence gels were read using LiCor/NEN gel reader and DNA sequences were aligned using AlignIR DNA sequence alignment software. All sequence reads and alignments were checked and verified manually against the gel image. DNA sequences were assessed for the best fit model to explain sequence evolution using Modeltest (Posada and Crandall et al., 1998), which identified the K3P model as the best fit. DNA sequence analysis was performed with Arlequin 2.0 (Schneider et al., 2000), and PAUP*4b10 (Swofford, 2003) using both distance (K3P, K2P, HKY85, F84 as the top four evolutionary models), and likelihood criteria and 300 bootstrap replicates using *D. yakuba* as an outgroup. Phylogenetic trees were constructed using PAUP*4b10 under both distance (Fig. 3) and likelihood criteria with 300 bootstrap replicates and show no conflict

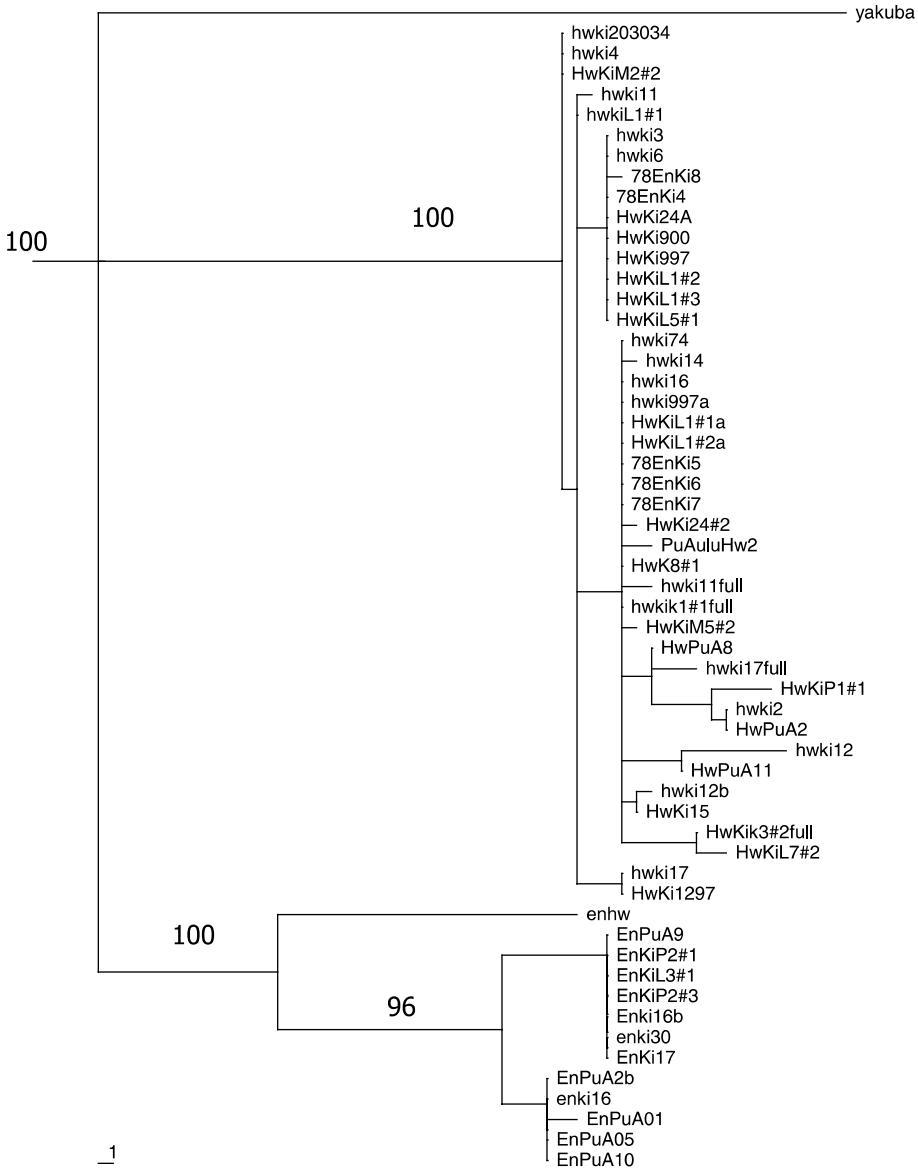

Fig. 3. Neighbor-joining tree using K2-P model distance data shows insignificant difference in topology from K3-P, F84, HKY=85, or ML (F84, HKY-85) criteria. Individuals with Hw identifiers are *D. hawaiiensis*, while EN indicates *D. engyochracea* haplotypes. There are individuals identified morphologically (wing pattern and body color) as one species that share sequence identity with haplotyes carried by the other species. The individuals that have the "wrong" haplotype may be the female descendants of hybrids that back-crossed to the species of the father. The individual "enhw" was identified when .captured as having some intermediate phenotypes (wing pattern and body color) has an intermediate haplotype, which is consistent with descendance from an individual that had recombined bi-parentally inherited (paternal leakage) mtDNA from both species. Major branch points have bootstrap values of 100 as indicated. The topology of internal branches are also well supported though not particularly important to arguments of this manuscript.

with the distribution of haplotypes across a minimum spanning network (MSN) construction (Fig. 4).

3. Results

A total of 515 bases of the mitochondrial COII gene were sequenced for *65 D. hawaiiensis*, and 66 *D. engyochracea* specimens for this study. The sequence for both species had GC content of approximately 32%. The greatest pair-wise distance found in comparing DNA sequence from the two species is an inter-specirfic comparison with a distance of .151. The greatest distance within a species is .035 (for *D. hawaiiensis* and .020 for *D. engyochracea*) accounting for only 23% of the maximum pair-wise distance. Three tests of selective neutrality were used to test the gene for freedom from selective constraint. Differences in the values between *D. hawaiiensis* and *D. engyochracea* for the three tests are attributed to population growth, based on unpublished data (Steiner pers. comm.) indicating much lower population size in *D. hawaiiensis* during the time period of Fontdevila and Carson (1978) study, and gene flow since the two species share the same habitat

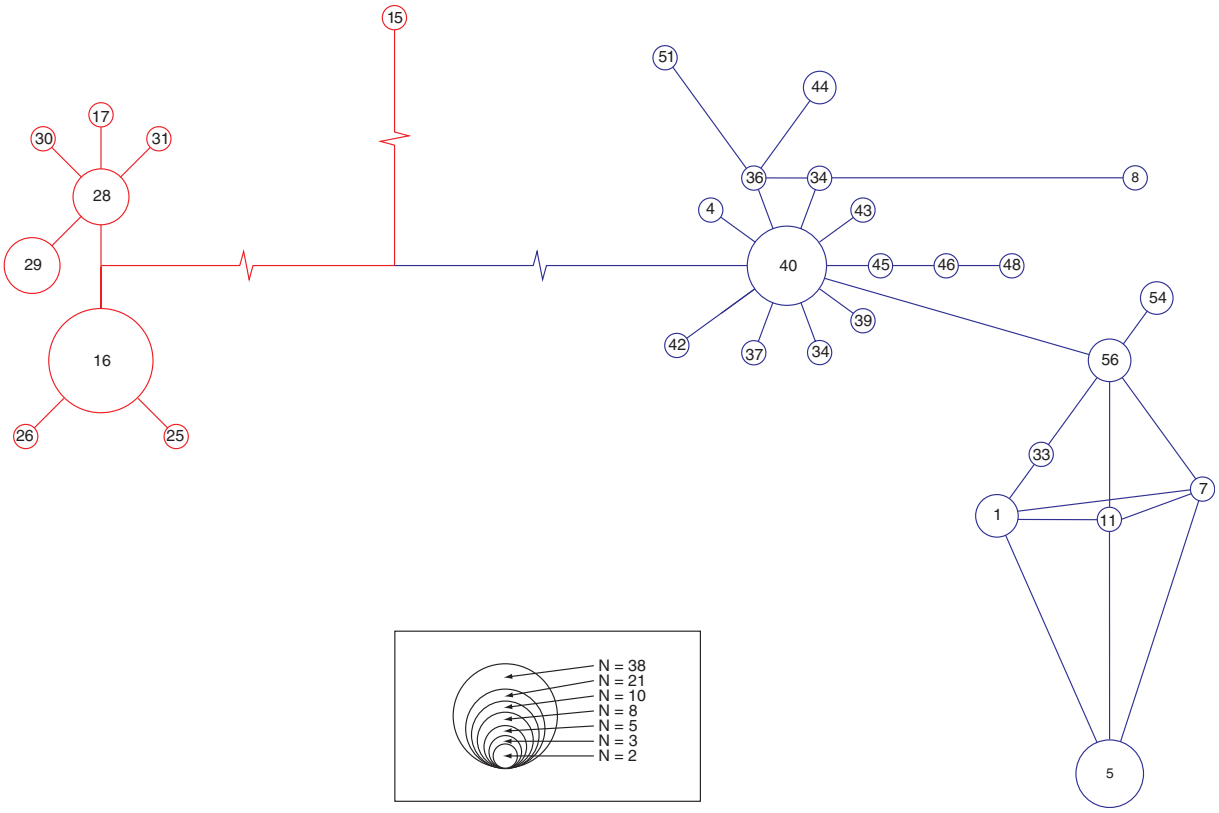

Fig. 4. Minimum Spanning Network (MSN) of haplotypes (blue, *D.hawaiiensis*; red, *D. engyochracea*) constructed with K3P constructed pair-wise distance data. Circle size is roughly proportional to frequency of the haplotype. Branch length, except for the branches with the distance breakers indicating they are longer than indicated, is roughly proportional to genetic distance between haplotypes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

and presumably experience the same selective constraints. Ewen–Watterson's (Ewens 1972; Slatkin 1994, 1996; Watterson 1978, 1986) is an infinite alleles model test and gives very close expected vs. observed values (0.065 and 0.087, respectively, see Table 1) for F (homozygosity test; Watterson 1978, 1986) in *D. hawaiiensis* indicating a neutrally evolving site while the difference in *D. engyochracea* is larger (0.186 vs. 0.47 for expected and observed values, respectively, see Table 1). The inter species difference is mirrored in Chakraborty's test (Chakraborty, 1990) of expected vs. observed number of haplotyes (*D. hawaiiensis* 20.7 and 23, and in *D. engyochracea* 3.97 and 11, respectively, see Table 1) which Chackraborty (1990) contended was more sensitive to population amalgamation than homozygosity. Both differences are consistent with a higher level of gene flow in *D. engyochracea* supported by differences in the F_{ST} for *D. hawaiiensis* and *D. engyochracea* (0.163 and 0.049). Tajima's test for selective neutrality (Tajima, 1989), an infinite sites model that compares number of segregating sites to mean pairwise difference, gives negative values for both *D. engyochracea* and *D. hawaiiensis,* which indicates an increased number of segregating sites and is consistent with gene flow or population growth. The Tajima *D* value is larger for *D. hawaiiensis* than for *D. engyochracea* (−2.197 and −1.083), which we believe is the result of population expansion in *D. hawaiiensis* from earlier population sizes observed by Steiner (pers. Comm.) concurrently with the Fondevilla and Carson study. Moreover, the *D. hawaiiensis* population in Kipuka Ki may have immigrants from populations other than Kipuka Pua'ulu since the species is wide spread across the Island of Hawai'i while *D. engyochracea* is endemic to the studied kipuka. We found 11 haplotypes among the *D. engyochracea* sequences all of which were shared (Fig. 3) by more than a single individual of those sampled. Interestingly, three of these haplotypes are shared with *D. hawaiiensis* individuals and may have been possessed by the maternal descendants of a hybrid of these species. Funk and Omland (2003) point out, on the other hand, that inter-species distribution of an allele may be the result of incomplete lineage sorting (ILS) to which young Hawaiian *Drosophila* species may be

Table 1
Values for phylogenetic tests run on data

Test	*D. hawaiiensis*	*D. engyochracea*
Ewen–Watterson *F*	Obs. = .47125 Exp. = .18581	Obs. = .47125 Exp. = .18581
Chackraborty allele number	Obs. = 23 Exp. = 20.7	Obs. = 11 Exp. = 3.97
Tajima's *D*	−2.197 P (*D* random < *D* Obs.) .00448	−1.083 P (*D* random < *D* Obs.) .14609

particularly susceptible. In contrast, there were 23 haplotypes, all of which were shared by more than one individual, among the *D. hawaiiensis* studied.

The difference in topology of the phylogeny for two species (Figs. 3 and 4) stands out in particular on recalling that the N for both clades is equal. The difference in the number of haplotypes coincides predictably with the difference in population size showing the more populous species, *D. hawaiiensis* harboring more haplotypes. While a decrease in the diversity, as measured by the number of halpotypes, accompanying a drop in population size of the magnitude that we found (unpublished data) is expected, we lack additional supporting evidence. We have neither any evidence that, prior to the drop in the population size of *D. engyochracea*, that the two species had similar levels of genetic diversity, nor do we have any reason to think that the two closely related, ecologically similar, species living in the same forest would have very different diversity in populations of similar size. It does appear that among the small number of haplotypes found in *D. engyochracea* there appears to be a paucity of intermediate haplotypes. All phylogenetic constructions show significantly more genetic diversity in *D. hawaiiensis* than in *D. engyochracea* in terms of numbers of haplotypes. Using a different measure of diversity, maximum pairwise distances between haplotypes, we find a smaller difference between the two species.

The geographic distribution of haplotypes reveals that there are haplotypes shared by individuals in both kipuka. The haplotype distribution could be a relict from a time prior to the isolation of the kipuka. However, the kipuka have been separated for well over 1000 generations (kipuka most recently separated 400 years ago with ~3 generations per year Foote ers. Comm.). A comparison of heterozygosity differences between populations for both species supports a higher level of migration in *D. engyochracea* than with *D. hawaiiensis*. Both the phylogenetic and MSN constructions have an intermediate haplotype, hwen or 15, The hwen haplotype is combination of SNP's that are otherwise diagnostic for both species. While paternal leakage of mitochondria involving a rare hybrid leading to inter-species recombinant mitochondrial haplotype may help to explain the intermediate haplotype, we have no other similar sequence and the distribution of the "diagnostic SNP's" does not indicate a single cross over point. Interestingly, when the individual with this haplotype was captured it was remarked that the phenotype seemed to include aspects of both species.

4. Discussion

There were more than three times as many *D. hawaiiensis* as *D. engyochracea* in Kipuka Ki (unpublished data). There is a notable difference in the level of genetic diversity between species, and not surprisingly, *D. hawaiiensis,* with the currently larger population, has more diversity than *D. engyochracea*. One explaination for the difference in the genetic diversity of the two species is that *D. engyochracea* did not have a higher level of diversity prior to the decline in the population size that we have studied. We, however, believe that a drop in diversity that accompanies a severe decline in population size (unpublished data) is easier to explain than two closely related and ecologically similar species living in the same environment having naturally very different levels of genetic diversity.

While one measure of genetic diversity is the number of unique genotypes/haplotypes resident in a population, another measure is a survey of the overall pairwise distances. Both types of diversity are very important for a population and both are likely to be affected by a bottleneck in slightly different, but important ways. A population that is subject to a very short lived but severe bottleneck is likely to loose many alleles, but since the loss will be relatively random, there may continue to be pairwise differences that are similar to that of the pre-bottlenecked population. If, on the other hand, the bottleneck is long lived, drift is likely to eliminate many more haplotypes leading to eventual reduction of pairwise differences among haplotypes. The range of pairwise differences may then be an indicator of the duration of bottleneck through which a population has emerged (Price et al., unpublished manuscript). While the number of *D. engyochracea* haplotypes is much reduced (less than ½ of the haplotypes), the overall pairwise distance is more similar (~2/3 maximum pairwise distance) to that of *D. hawaiiensis*. The diversity profile for *D. engyochracea* is therefore consistent with a shorter lived bottleneck.

The maximum pairwise distance, excluding putative hybrid descendants, is similar for *D. hawaiiensis* and *D. engyocrhracia*, despite *D. engyocrhracia* having fewer haplotyes (diversity). This indicates that there were similar rates of mutations accepted into the populations. The similarity in maximum pair wise distance in conjunction with a significant difference in haplotype numbers (distance vs. diversity) indicates that the *D. engyochracea* population has recently lost diversity. These results do not provide good support for the possibility that *D. engyochracea* lost diversity a long time ago or that it never had higher genetic diversity. The contrast in diversity findings indicates that both species have accumulated similar numbers of mutations since they became reproductively isolated as species. The difference is that *D. engyochracea* appears to have lost a large number of the haplotypes, compared to *D. hawaiiensis*, which is likely due to a recent, dramatic decline in population size. Since haplotypes are lost randomly due to drift, one would not expect more divergent haplotypes to have been lost preferentially.

Our study included collections from both Kipuka Ki and Kipuka Pua'ulu. In the original study Fontdevila and Carson (1978) determined that the road that bisects Kipuka Ki did not interrupt dispersal of the flies across the road. Our study concurs with this conclusion. We regularly found marked flies released on one side of the road had traveled to the other side by the following day. Furthermore, the geographic distribution of haplotypes across

the two kipuka was not discrete. Several haplotypes were shared between flies in both kipuka and F_{ST} values for both species indicate a higher level of gene flow between the kipuka in *D. engyochracea* than with *D. hawaiiensis*. We speculate that the difference in migration rates may be due to reduced mate availability in the *D. engyochracea* that has smaller current population size. In this scenario, *D. engyochracea* moves greater distances in search of a mate and may be more likely to venture over habitat not containing the host material (as is the case with the intervening habitat between the kipuka in this study). It is possible that the F_{ST} values reflect the movement of individuals among the meta-population prior to the isolation of the kipuka by an ash deposit 400 years ago. However, the 1000+ generations that have passed in those 400 years (approximately 3–4 generations per year) seems sufficient to have resulted in greater lineage sorting in the absence of gene flow especially given the small sizes of the populations recently.

There are individuals that appear in both phylogenetic constructions on the "wrong" branch. This indicates that there are six morphologically identified *D. engyochracea* that share more sequence similarity with *D. hawaiiensis* than their morphological con-specifics. Similarly, there is one individual that is morphologically identified as *D. hawaiiensis* that shares more sequence similarity with *D. engyochracea* than with *D. hawaiiensis*. These putative hybrid descendents are evidence for low levels of natural hybridization, consistent with observations in other sibling species of Hawaiian picture-winged *Drosophila* (Kaneshiro 1990; Price and Muir, 2008).

Hybridization is typically seen as destructive, resulting in offspring that have reduced fitness or, potentially, the extinction of one or both hybridizing species. Reduced fitness or inviability has traditionally been seen as a necessary outcome of an interspecies cross, since it is assumed that the species are the result of either isolation, or divergent selection and protracted reproductive isolation. When two closely related species live sympatrically, their integrity is thought to be maintained by assortative mating. But, assortative mating may not be complete and thus interspecific gene flow can still occur. Nevertheless, Lande (1999), and Porter (2002) among others have shown that species can remain distinct despite low levels of gene flow. In fact, the introgression of advantageous alleles from one species into another my result from hybridization followed by recombination and selection (Wu 2001; Price and Muir, 2008). An alternate explanation is that there has been incomplete lineage sorting since the common ancestor of the two species. While *D. hawaiiensis* and *D. engyochracea* are not sister taxa, they do belong to an unusually speciose clade. Thus the close relation that may allow for hybridization, may on the other hand be why the haplotypes are incompletely sorted among the lineages. It would be useful to determine the distribution of haplotypes among the *grimshawi* clade since there may be other cases of inter-specifically shared sequence, which as a whole may be more elucidating. If the suspect haplotypes are a consequence of incomplete lineage sorting rather than hybridization, it does seem conspicuous that there is an absence of closely related haplotypes. In other words, one might expect that haplotypes that have survived in a species since its beginning would have given rise to other haplotypes as a result of mutations. These mutations would for the most part be single nucleotide substitutions so the collection of mutants would cluster around the ancestral haplotype due to the short genetic distances.

Drosophila engyochracea appears to have suffered a large decline in population size since the Fontdevila and Carson study, and we are concerned for two reasons. First, *D. engyochracea* has little genetic diversity for the gene that we studied compared with *D. hawaiiensis*. With such a high inbreeding potential, we are concerned that genetic drift may further erode what little appears to be left of the population's genetic diversity. Second, *D. engyochracea* is limited in range to Kipuka Ki and Kipuka Pua'ulu and local extirpation would mean extinction for the species. That the different number of haplotypes in the two species is related to changes in the two population sizes presupposes that the number of alleles in the two species was the same before the decline in *D. engyochracea.* We have no data for the historic genetic diversity of either species but believe that the simplest model is one in a which two closely related and similar species living in the same environment with similar population sizes would have similar population genetic characteristics and that the decline in the size of one population would be coincident with a loss of allelic diversity.

Acknowledgments

Mahalo to David Foote who managed the mark-recapture study to estimate population size. We gratefully acknowledge the assistance of Karin, Kiko Camp (ne. McMurray), Will, Dena Cutler, financial support from NSF Career Award (DEB0114405) to D.P., financial support from USGS to D.F. This manuscript has benefited from the helpful comments of at least three anonymous reviews.

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