Genetic Variation, Population Structure, and Morphology of an Endemic Bat, *Lasiurus cinereus semotus* (Chiroptera: Vespertilionidae) Across the Hawaiian Islands

A thesis submitted to the graduate division of the University of Hawai`i at Hilo
In partial fulfillment of the requirements for the degree of

Master of Science
In
Tropical Conservation Biology and Environmental Science

August 2019

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Keywords: genetic diversity, populations, cytochrome oxidase, microsatellites, lasiurus, *L. cinereus semotus*, Hawai`i, endangered species, bat

Acknowledgments

This research and thesis would not have been possible without the support of many bat loving friends and family. Mahalo nui loa. I am deeply grateful for the opportunity to study `Ōpe`ape`a while working in the field of conservation biology across my home state. I owe this to my amazing mentor Frank Bonaccorso. I would also like to thank the USGS PIERC and Hawai`i Cooperative Studies Unit at the University of Hawai`i at Hilo for collaboration and support throughout my graduate program. I recognize my outstanding co-workers for their friendship during the challenging times of graduate school, Kristina Montoya-Aiona and Christopher Todd. The biggest hurdle in this study was obtaining tissue samples from an endangered species across several islands, and I am indebted to a long list of colleagues, including local federal and state agencies, who made this a reality. Many people put in effort mist netting with our project, sent me samples, or allowed me to collect information from preserved bats at their facilities! Thank you for field work assistance R. Bernard, Y. Castaneda, N. Cortez-Delgado, A. Hubancheva, K. Lahaela, D. Johnston, J. Johnson, A. Miles, G. Parish, R. Moseley, G. Reyes, B. Yuen, V. Zrncic, and T. Zinn. Thank you for facilitating with carcass sample collection G. Akau, R. Breeden, J. Charrier, M. Craig, J. Dutton, F. Duvall, S. Franklin, M. Hagemann, K. Olival, D. Sether, M. Stelmach, M. VanZandt, and T. Work. Lab work was just as puzzling for me, and I thank Anne Veillet for sharing her knowledge at the bench and generous time with processing bat samples. I thank Seth Judge for his time teaching me about the magic of document formatting. Standing by me for as long as it takes to graduate, I appreciate the firm direction and understanding offered by my TCBES committee members, Donald Price, M. Renee Bellinger, Patrick Hart, and Rebecca Ostertag. Behind those who love to research bats - stand those who love bat researchers - and deal with their crazy schedules. I deeply thank my husband, Craig Mitchell, for his unwavering heart and behind the scenes strength, when bats turned our lives upside down. And Mum, can't forget my devoted mother and number one fan, Deborah Pinzari.

Abstract

Determining the connections between islands and assessing subpopulations are required to effectively manage an endemic, seasonally migrant bat species with an observed archipelago wide distribution. An innovative technique to characterize the connectivity among populations is to evaluate the genetic similarity between individuals sampled from among and within islands. By combining mitochondrial and nuclear DNA markers (or genetic variants), we can identify how island groups may differ between populations, sexes, and estimate relative abundances. One mitochondrial gene and six nuclear microsatellite loci were used to explore genetic connectivity among and within three islands inhabited by the endangered Hawaiian hoary bat (*Lasiurus cinereus semotus*). Employing the resources of an existing collection of bat tissue samples (~140) from the four major islands (Kaua`i, Hawai`i, Maui, and O`ahu) and applying classical population genetics analyses, I tested for population structure; quantified levels of genetic variation, genetic distance, and gene flow in bats among and within the Hawaiian Islands; estimated both historical long-term female effective population size, and contemporary effective population size; and examined the data for patterns of past bottleneck events. In order to accurately measure degree of population structure and phenotypic variation with respect to sex, I conducted genetic sex determination tests on bat samples from both live and desiccated specimens. I also examined the morphological characteristics of bat skull and wing size on 23 individuals to determine differences with respect to island, mitochondrial clade, and sex. This project provides the most current data set of population level information, describing the genetic diversity and geographic structure of Hawai`i's only endemic terrestrial land mammal. This study contributes demographic information, sex determination techniques, and banking of diverse DNA samples available for future genomic sequencing, to support management and recovery of an endangered species. Research results may provide support to state and federal agencies tasked with balancing the demands of sustainable wind generated energy and wildlife conservation in Hawai`i.

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Conservation of Island Bats

The power of flight has enabled bats to distribute and diversify around the globe. They have spread across vast continental land masses and have even colonized extremely distant oceanic islands. Bats usually represent most of the native mammal diversity on isolated islands. Over half of all bat species live on islands, and a quarter of them are island endemics, with a little less than 10% being single-island endemic species (Jones *et al.* 2009). Evolutionary processes on islands have been responsible for unique species diversity in bats and thus many tropical environments rely on bats to provide valuable ecosystem services such as pollination, seed dispersal, and insect predator supression. Unfortunately, about half of all threatened bat species are island endemics, and within that group, almost half are found on single islands (Jones *et al.* 2009). Threatened bats face many risks: habitat loss, intrinsic factors (population, dispersal, and reproduction), harvesting, human disturbance, natural disasters (hurricanes, volcanoes), invasive species, and persecution (pest control). The loss of island bats is of great concern to global biodiversity and ecosystem function, and the study of biogeographic and evolutionary science (Fleming & Racey 2009).

Island endemic bats are also a poorly studied group when it comes to allocation of research in conservation. Conenna *et al.* (2017) reviewed the global research patterns for island endemic bat species, geographic distribution, and the Red List conservation status of the International Union for Conservation of Nature (IUCN). The review identified species and area-based priorities for future research, and found that although island endemic bats are significantly more threatened than non-island endemics, research focusing on these bats is scarce. Their analyses revealed that species of highest research priority included threatened and data deficient species from Pacific Islands (Conenna *et al.* 2017).

The Hawaiian hoary bat, *Lasiurus cinereus semotus*, is listed in the United States and the State of Hawai`i as an endangered subspecies of the North American Hoary Bat, *Lasiurus cinereus*. Although the only endemic terrestrial land mammal in the Hawaiian archipelago, *L. cinereus* is found on every high volcanic island (Tomich 1986, Kepler & Scott 1990). Generally, it is solitary, insectivorous, tree-roosting, and seasonally active at different elevations (Baldwin 1950, Findley & Jones 1964, Whitaker & Tomich 1983). Like other island bat species, detailed information regarding its biology, ecology, and current population size is largely unknown, and thus conservation management is a challenging task when facing anthropogenic threats to island habitats.

Hawaiian Hoary Bat Life History

The genus *Lasiurus*, belongs to the large and diverse bat family Vespertilionidae, and is comprised of "hairy-tailed" bats, 17 loosely yet morphologically distinct species, and several lesser known sub-species (Simmons 2005). The Hoary bat group (*L. cinereus sensu lato*) consists of three sub-species, the North American hoary bat (*L. c. cinereus*), the South American hoary bat (*L. c. vilosissimus*) and the Hawaiian hoary bat (*L. c. semotus*). Only the first sub-species is well studied. Hoary bats have established populations on remote oceanic islands; *L. c. cinereus* has been documented on Iceland, Bermuda, and Caribbean Islands, while the Galapagos is inhabited by an extension of *L. c. vilosissimus*. *L. c. semotus* is found in the Hawaiian archipelago. North America hoary bats (*L. c. cinereus*) are exclusively insectivorous and are specifically associated with tree and foliage roosting behaviors (Shump & Shump 1982). They are highly migratory by season and movement patterns are sex specific (Cryan 2003, Cryan *et al.* 2004). Winter for both sexes is spent in warmer geographic locales close to oceanic coasts, migration to the continental interior begins in spring first by females who distribute to regions with favorable summer conditions for raising young, autumn sees the widest distribution of hoary bats with males and females overlapping during the breeding season (Hammerson *et al.* 2017, Hayes *et al.* 2015). They are even able to track lepidopteran prey resources during

migration to summer breeding grounds (Valdez & Cryan 2009). Long distance seasonal movements greater than 1,000 kilometers (Cryan *et al.* 2014, Weller *et al.* 2016), as well as oceanic travel routes with stop overs on coastal islands have been observed in North American hoary bat migration behaviors within California (Cryan & Brown 2007).

Recent genetic exploration into biogeographic history of Hawai`i's bat suggests that contemporary Hawaiian populations of *Lasiurus* may be the result of more than one separate founding event. The origin of colonizing ancestors were North American hoary bats, who most likely were swept off migratory routes from the coast of California by storm events or navigation errors many thousands of years ago (Bonaccorso & McGuire 2013, Baird *et al.* 2015, Russell *et al.* 2015). Bonaccorso & McGuire (2013) used flight modeling and empirical data to support their hypothesis that hoary bats with full fat and water stores typical of preparation for migration, assisted or not by prevailing tail winds, could theoretically reach the Hawaiian Islands from off the coast of California in less than four days (but quicker with substaintal wind assistance). Analysis into the timing of the separate founding events and the distinctness of lineages they have produced in Hawai`i has offered two differing scientific opinions. Russell *et al.* (2015) used one mitochondrial and two nuclear gene markers with Bayesian modeling to date arrivals and population expansions of a founding clade at 10,000 years ago and a second clade at 800 years ago. Baird *et al.* (2015) used three mitochondrial and one Y-chromosome marker and calculated a much older colonization time of about one million years ago. Present analysis by Baird *et al.* (2017) incorporated data from the Russell *et al.* (2015) study and examined 4 mitochondrial and two nuclear markers; phylogenetic results suggested an initial colonization by the ancestor of today's *L. c. semotus* about 1.35 million years ago, and more recent multiple arrivals of *L.c. cinereus*. A new estimate of population expansion places growth of *L. c. semotus* at 20,000 years ago, possibly due to colonization waves to new islands or release after a bottleneck event (Baird *et al.* 2017). Recent genetic data also support recognition of two distinct species of Hawaiian hoary bats with a divergence of 4.2%, *L. c. semotus* whose mitochondrial

haplotypes are unique to Hawai`i, and *L. c. cinereus*, of the more recent founding(s) whose haplotypes are parallel to North American Hoary bat haplotypes (Baird *et al.* 2017).

New arrivals hoary bats from overseas, in theory may have overlapped for thousands of years with a now extinct cave-dwelling bat species *Synemporion keana* (Ziegler *et al.* 2016) on all five of the major Hawaiian Islands. Described from skeletal remains found in lava tubes around the late Pleistocene and Holocene, *S. keana* represents a monotypic genus of vespertilionid bat from an even older lineage arriving in Hawai`i at 320,000 years before present. Speculation on the reason for its extinction around a thousand years ago include those for all other extinct endemic bird species: habitat loss from founding Hawaiian human populations and predation from introduced species (Ziegler *et al.* 2016).

Regardless of how many species of hoary bats exist in Hawai`i today, considerable effort has gone into scientific research on its habitat distribution and ecology in order to conserve this endangered bat across the state. According to acoustic occupancy studies, which calculate the occupancy or probability of bat detection and activity rates via echolocation calls (Gorresen *et al.* 2008, Gorresen *et al.* 2018), bats are present on all five major Hawaiian Islands: Kaua`i, O`ahu, Maui, Molokai, and Hawai`i (Jacobs 1994, Fullard 1989, Gorresen *et al.* 2013, 2015; Fraser *et al.* 2007, Pinzari *et al.* 2019, Todd *et al.* 2016). Between 2016 and 2017, the first acoustic survey conducted on Kahoʻolawe Island documented seasonal presence of bats between April and November (J. Bruch, personal communication). On Hawai`i Island, bat surveys have detected use of foraging habitats at coastal wetlands and fish ponds (Pinzari *et al.* 2016, Montoya-Aiona *et al.* 2019), across lava fields and native forests (Fujioka & Gon 1988), and as high as 3,000 meters above sea level around lava tube entrances on Mauna Loa volcano (Bonaccorso *et al.* 2016). No information exists on the frequency of inter-island movements.

Acoustic surveys documenting the seasonal shifts in bat calls relative to sunset reveal their altitudinal movement patterns on Hawai`i Island. They occupy low elevations during the

summer months and move, to high elevations during the winter (Menard 2001). This seasonal trend in bat occurrence is concordant with detections from lower-elevation acoustic surveys which show they are most common during the late spring and summer months, highest in the fall, and rare during the winter months (Gorresen *et al.* 2013, Bonaccorso *et al.* 2016). This altitudinal migration behavior may be due to changes in prey resources (Todd 2012), heavy winter rains dampening foraging opportunities, or the physiological needs of female bats for gestation, birth, and rearing of young (McGuire & Boyle 2013). Reproducing bat populations are known on the islands of Hawai`i and Kaua`i, and more recently new records of lactating and pregnant females have come from mist net capture studies on O`ahu (2013) and Maui (2017) (personal observation). Hawaiian hoary bats give birth to twins in June, and lactation continues through mid-September when pups fledge and leave the roost. Hawaiian hoary bats are thought to be habitat generalists, yet only one published study to date has attempted to quantify bat core use areas and home ranges. Bonaccorso *et al.* (2015) found that adult male bats in east Hawai`i Island had highly variable foraging ranges that were very large and smaller core use areas with little overlap, and reflected a structured use of individual space that was most likely dependent on local weather, habitat, and food resources.

A substantial body of research on the acoustic signatures and foraging behaviors of Hawaiian hoary bats has uncovered great diversity and evolution in their echolocation characteristics and diets. Jacobs (1993) proposed that *L. c. semotus* experienced "character release" upon evolving in isolation of the islands, with no competitive bat species to constrain peak frequency of echolocation calls used to navigate and forage (at least following the extinction of *S. keana*). The calls of *L. c. semotus* have also evolved to compensate for a tropical environment with high vegetative clutter; recordings made on Hawai`i and Kaua`i document bats using higher frequency and shorter duration calls that those exhibited by North American hoary bats (Jacobs 1996, Barclay *et al.* 1999, O'Farrell *et al.* 2000). Lepidoptera and Coleoptera make up the majority of Hawaiian hoary bat diet as presently understood, but bats also consume

arthropods from Diptera, Homoptera, Isoptera, and other arthropods (Whitaker & Tomich 1983, Bernard 2011, Bernard & Mautz 2016, Pinzari *et al.* 2019). Bats have been noted to have seasonal and altitudinal foraging patterns based on prey abundances (Todd 2012) and respond to foraging opportunities such as lepidopteran outbreaks in native forests during defoliation events (Banko *et al.* 2014). Recent research into Hawaiian hoary bat echolocation and foraging activity by Gorresen *et al.* (2017, 2018), which correlates bat acoustic detection with thermal imagery, revealed that Hawaiian hoary bats often navigate sans vocalization, flying in silence, and this raises new questions about past local survey efforts to document bat distribution with acoustic-only methodologies.

Nonetheless, there are still many biological questions central to the conservation of Hawaiian hoary bats that remain unanswered. These include inquiries into adult life span, pup survival rates, roost characteristics, sex and seasonal specific dietary needs, identification of insect prey species, winter distribution and potential torpor use, and most importantly to wildlife managers, population size and connectivity.

Hawaiian Hoary Bat Management Issues

The Hawaiian hoary bat received federal and state protection as an endangered species in 1970, due to the lack of available information on abundance, distribution, critical habitat needs and population status (USFWS 1998). In 2015, the State of Hawai`i declared the Hawaiian hoary bat as the official state terrestrial land mammal, in hopes of enhancing public knowledge and raising awareness of local conservation efforts. In Hawai`i's modern environment, threats to the bat's recovery include timber harvest forestry practices during the pupping season, entanglement on barbed-wire fencing, poisoning from pesticides, and most recently fatal collisions with wind turbines (USFWS 2011).

In the last decade, wind turbines have emerged as an extinction level threat to several species of migratory tree bats, particularly the North American Hoary Bat, *L. cinereus* (Frick *et*

al. 2017). Hoary bats make up to 44% of the documented bat kills at wind farms in the United States (Arnett & Baerwald 2013). It is estimated that between 655, 579 thousand and 1,318,743 million bats have been removed from the population pool in North America between 2000 and 2011(Arnett & Baerwald 2013), with 80% of the fatalities being migratory tree bats. These large, demographic losses among hoary bats may be detrimental on both ecological and biological scales, especially given the absence of acuurate population estimates (Kunz *et al.* 2007, Hein & Schirmacher 2016).

Hawaiian hoary bats are at present are experiencing unprecedented annual collisions with wind turbines on the islands of Hawai`i, Maui, and O`ahu (Mykleseth 2017). Difficult terrain encountered in searching for bat carcasses, high rates of decay in the tropical climate, coupled with predator removal of carcasses makes it difficult for managers to calculate accurate estimates of fatalities. Model estimators are used that rely on features related to searching for carcasses, predator removal rates of trial carcasses, days to deterioration, and probability of discovery (Huso *et al.* 2016). Some facilities even employ canines to more effectively locate bat carcasses during search attempts (Mathews *et al.* 2013). Regardless of these well-meant efforts to estimate removal of individuals from the local population, it is still necessary to know initial relative population sizes and subpopulation structure for accurate assessment of the cumulative impact of wind energy on Hawaiian hoary bats. It is not known if wind turbines pose population level threats for the endangered Hawaiian hoary bat on islands with operational wind energy facilities (Gorresen *et al.* 2015).

There are direct benefits of genetic information to management of this endangered bat in Hawai`i. Results could potentially be applied to management objectives in Habitat Conservation Plans, contribute to efforts in resource equivalency area modeling, and verify sexual identification of bats found at wind facilities, increasing the validity of take assessments and cumulative effects on bat populations. Critically important research questions that require study

for effective management of the Hawaiian hoary bat were recommended by a five-year review of the species by the U.S. Fish and Wildlife Service (2011) and include: What are the effective population sizes of O`ahu, Maui, Kaua`i, and Hawai`i Islands? Do one or more distinct populations of hoary bats exist in Hawai`i? What level of connectivity through gene flow occurs across the Hawaiian Islands?

Bat Populations and the Use of Molecular and Morphological Tools

There are numerous reasons to apply molecular genetic techniques to understand Hawaiian hoary bat demography and distribution. Population census and dynamics are not reflected through acoustic survey methods because it remains impossible to count how many individual bats are recorded. Monitoring for population trends on an island wide scale, using occupancy metrics alone, to detect significant declines, may prove to be both logistically and financially unrealistic. Low rates of recapture using capture/recapture methodology typically found in hoary bats coupled with the cryptic nature of tree roosting, makes these studies impractical and expensive. Schorr *et al.* (2014) estimated that in order to detect the smallest population decline (~10-15%) in the annual survival of migratory tree bats, one would need to mark and recapture around 50,000 individuals per year. These issues are confounded when the species in question is endangered, and rare in certain seasons or habitats. However, it is feasible to use available tissue banks and apply genetic techniques to answer population level questions (Schwartz *et al.* 2006). The USGS Pacific Islands Ecosystems Research Center on Hawai`i Island holds a collection of ~200 Hawaiian hoary bat biological samples from live captures and carcasses gathered from across the state. Post-construction fatality monitoring required of wind facilities has also produced ~75 bat carcasses as of October 2017. Tissue samples from these carcasses now make possible that bat populations on O`ahu and Maui, where genetic samples were historically sparse, be included in a current genetics study.

Genetic Sex Determination

The factors contributing to bat collisions with wind turbines are not well understood (Cryan & Barclay 2009). Current research evaluating the sexual readiness of hoary bats killed at wind turbines, pinpoints reproductive maturity to be the precursor to migration in North America, and suggests that bats may be attracted to the physical structures of wind turbines as they resemble natural lekking structures (Cryan & Brown 2007; Cryan *et al.* 2012). Effective mitigation and minimization strategies may benefit from understanding of potential sex specific vulnerability or seasonal pattern in wind turbine associated deaths (Hein & Schirmacher 2016). It appears that across North America, female hoary bats migrate greater distances than males during the summer in order, to reach preferred habitats and climates for birth and rearing of pups (Cryan 2003, Cryan *et al.* 2004). It is not known if such sex specific migration or movement patterns exist in Hawaiian hoary bats, or if there is frequent inter-island migration.

Identification of carcass sexes discovered during routine fatality searches can be of great assistance in documenting the impact of wind energy on local bat populations and calculating the best assessments of take as required by state and federal endangered species management laws. Unless a bat carcass is less than 1-2 days old, the sex of decayed or scavenged carcasses are difficult or nearly impossible to identify from external observation alone, leading to a male biased dataset with females often being identified as unknown (Korstian *et al.* 2013). A simple and reliable genetic method now exists to accurately determine the sex of vespertilionid bat carcasses, and has been validated for the North American hoary bat (Korstian *et al.* 2013). Testing the sex chromosome markers developed by Korstian *et al.* (2013) and optimizing this method of sex determination in Hawaiian hoary bats would greatly benefit genetic studies in the species, allowing carcass tissue samples to be used in sex specific population questions, such as if there are differences in male and female gene flow between islands or if one sex if more prone to inbreeding depression.

Population Genetic Approaches

A population genetic approach has been used to identify conservation priorities and delineate management units for endangered island bat species (Brown 2011, Salgueiro *et al.* 2004, Salgueiro *et al.* 2007), and more specifically has been applied to understanding potential population structure in *L. c. cinereus* across North America (Korstian *et al.* 2016, Pylant *et al.* 2016, Baerwald 2015). The primary goal of this approach is to measure and preserve genetic diversity, so that species and populations have an improved chance to persist and adapt to environmental changes (Frankham 2005).

For species at risk of extinction, measures of genetic variability and population differentiation can be used to estimate effective population sizes. Effective population size (N_E) is maximized when a population under genetic drift experiences fully random mating, has equal numbers of males and females with reproductive ability contribute genetic diversity to the next generation, and a stable or growing population size over time. The effective population size is generally much lower than actual census size. N_E is quickly decreased by any demographic deviations that could affect ideal conditions, such as a bottleneck or change in sex ratio of mating individuals. Populations with low effective sizes will experience losses in genetic diversity rapidly, and begin to decline in number, compared to populations buffered by large N_E. Effective size can be estimated for both historic and contemporary time frames and be useful for conservation purposes (Wang 2005). In some cases, where N_E is very large, contemporary estimates may not be precise due to widening confidence intervals. This trend has been observed for continental populations of Lasurine bats (Vonhof and Russell 2015), including hoary bats (Pylant *et al.* 2016, Korstain *et al.* 2016). No robust measures of Hawaiian hoary bat effective population size (historic or contemporary) have been produced with molecular analysis for Hawaiian hoary bats, due to uneven tissue sampling efforts across islands. However, if a large number of individuals from each island could be analyzed, estimates of population N_E for each

island could be used to understand relative subpopulation structure and potential extinction risk.

The relationship between population size and genetic structure is important to conservation strategies (Franklin & Frankham 1998, Waples 2002). Migratory bats with great dispersal abilities tend to show less population structure than non-migratory species (Burns & Broders 2014), yet dispersal can still be affected by barriers created by sexual behavior or physical boundaries (such as oceanic channels). Island populations of bats often show population structure while there can be varying patterns for continental bat populations (Ditchfeild 2000). Behavior such as female roost philopatry, present in bats that return to the same maternal roost areas for pupping, could potentially weaken natural gene flow and contribute to local genetic structure in populations. If genetic structure exists between islands due to female philopatry and females tend to remain on the island of birth, this would be especially important to understand on islands where wind facilities are concentrated and impact local population numbers.

Classical genetic methods for determining population structure of bats involve developing DNA polymorphic loci (both mtDNA and nuclear) and assessing the levels of heterozygosity between alleles across the loci at the individual and population scale. Early molecular work involving *L.c. semotus* was restricted to a single individual in the studies by Baker *et al.* (1988) and Morales & Bickham (1995) and focused on phylogenetic classification of the genus *Lasiurus*, and placement of the subspecies of hoary bats. Morales & Bickham (1995) suggested a North American ancestry for *L.c. semotus* and found a low level of divergence (1.8%) between hoary bats in Hawai`i and North America. Phylogenetic history of the Hawaiian hoary bat is still an evolving topic, with new studies producing higher divergence rates and evidence for distinct lineages (Baird *et al.* 2015, Russell *et al.* 2015, Baird *et al.* 2017). However, these studies focus on questions pertaining to the source and timing of founding events and

population expansions, and do not offer robust information on inter-island population structure or estimates of effective population sizes.

Advances in molecular techniques, the innovation of "conservation genetics", and the crucial need to understand populations of migratory tree bats impacted by wind energy threats across North America has stimulated the development of microsatellite marker resources for many species within the genus *Lasiurus* and more specifically for the hoary bat and red bat which could be cross amplified and sequenced in the Hawaiian hoary bat (Baerwald 2015, Korstian *et al.* 2015, Pylant *et al.* 2016, Sovic *et al.* 2016, Vonhoff & Russell 2015).

As stated earlier, because no practical techniques exist for the direct census of populations for Hawaiian hoary bats, an alternative course to estimate population size is available through molecular genetics (Luikart *et al.* 2010). For migratory species that are capable of long-distance movement and exhibit cryptic behavior, indirect methods provide a practical approach to infer population patterns (Moussy *et al.* 2013). Characterizing the genetic variation of individuals across the Hawaiian Islands can provide critical information on population structuring and degree of connectivity between islands. When populations experience restricted gene flow, genetic drift and mutation are predicted to cause divergence of populations into distinct genetic clusters. Maternally inherited mitochondrial DNA can infer female site fidelity and the extent between breeding sites, while the analysis of polymorphic nuclear microsatellite DNA provides complementary information for both sexes (Vonhoff & Russell 2015).

Morphological Character Analysis

Hawai`i's hoary bats have undergone divergence from mainland populations resulting in smaller body size and unique pelage color. Coloration of coat in *L. c. semotus* has been suggested as an indicator of species significance by Sanborn & Crespo (1957) and Tomich (1974). Individuals on Hawai`i Island have fur that appears more reddish in coloration with much less

intensity of white frosting than seen in typical North American hoary bats. Previous research has also explored how lack of ecological competition, diet shifts, and insular evolutionary forces may have shaped morphological differences found in *L. c. semotus* compared to their North American hoary ancestors (Jacobs 1993, 1994). Jacobs quantified size differences between North American and Hawaiian hoary bats and found that Hawaiian counterparts were about 30% smaller in size (based on weight, forearm length, and wing loading) and observed sexual dimorphism with females being larger than males. He also found cranial and mandible size changes in the skulls of smaller Hawaiian hoary bats and suggested a shift in diet from moths to hard-bodied beetles. However, Jacob's morphological studies (1993, 1996) were solely limited to a small sample of bats from one population in the southern region of Hawai`i Island, and it is unknown how these character states exist in other island populations or how they relate to genotype.

Cryptic species can be thought of as two or more species existing in sympatry that have been classified incorrectly as a single species due to superficial similar morphology. Cryptic species are often uncovered after comparing DNA sequence data, and later confirmed by more refined morphological and ecological data (Bickford *et al.* 2007). Cryptic speciation is found within many tropical and temperate bat taxa, and has been revealed through careful comparisons of intraspecific mitochondrial lineages, echolocation characteristics, morphology, and additional genetic markers with different modes of inheritance such as nuclear DNA and sex chromosomes (Clare 2011). Cryptic species require special consideration in conservation planning, especially if they exist within an already endangered species complex. Depending on the abundance and behaviors of each, different conservation strategies may be necessary. Multiple lineages have been suggested for Hawaiian hoary bats, and the presence of two distinct mitochondrial clades on the islands of O`ahu and Maui have been confirmed by genetic studies (Baird *et al.* 2015, 2017, Russell *et al.* 2015), however no morphological or ecological data was

examined in conjunction with genotyping in these studies, and it is unclear if any differences in phenotypic characters support the claim of the Baird group, of two, possibly cryptic, species.

To my knowledge, no local studies to date have combined the above mentioned techniques for Hawaiian hoary bats on a multi-island scale. An investigation that describes potential population structure, measures genetic diversity on each island, and attempts to relate morphological size characteristics to genetic data across several islands would certainly aid in conservation and management benefits for this species.

Chapter II
Genetic Structure and Morphology in the Hawaiian Hoary Bat, *Lasiurus cinereus semotus* (Chiroptera: Vespertilionidae) Across the Hawaiian Islands

INTRODUCTION

Island bat species are at disproportionately at risk of extinction due to both natural and human disturbances to their ecosystems (Fleming & Racey 2009). Hawaiian Island biota epitomizes the struggle of endangered species, with hundreds of endemic and native species facing declines due to loss of resources, changes in climate and habitat, competition from invasive species, over harvest, loss of pollinators, and disease (Fortini *et al.* 2016, Paxton *et al.* 2018). The Hawaiian Islands are home to only one extant native bat today, the Hawaiian hoary bat (*Lasiurus cinereus semotus*). This species boasts a fascinating colonization history, with current research suggesting multiple founding events via flights of *Lasiurus cinereus* to the Hawaiian Island chain from the coast of North America (Baird *et al.* 2015, Russell *et al.* 2015). Like its North American sister species, *L. c. semotus* is a solitary, insectivorous, tree roosting bat; however, long isolation and restriction to an island system have led to curious differences in body size patterns, flight behavior, and echolocation in this sub-species (Jacobs 1993, Barclay *et al.* 1999, O'Farrell *et al.* 2000).

Technically a sub-species of the North American Hoary Bat, the Hawaiian hoary bat has received federal and state protection as an endangered species since 1970. It was listed as endangered because of the lack of available information on abundance, distribution, critical habitat needs and population size (USFWS 1998). Emerging and current threats to the bat's recovery include timber harvest forestry practices during the pupping season, entanglement on barbed-wire fencing, poisoning from pesticides, and most recently fatal collisions with wind turbines (USFWS 2011). Efforts to preserve the species across the state include mitigation through habitat conservation and applying acoustic survey techniques to grasp current distribution and seasonal use of habitat.

Over the past decade, considerable local research has been conducted across the state regarding acoustic monitoring surveys to characterize seasonal distributions (Gorresen *et al.* 2008, 2013; Todd *et al.* 2016), altitudinal movements and estimation of home ranges (Menard 2001; Bonaccorso *et al.* 2015), and prey availability and dietary composition (Bernard 20011, Todd 2012, Pinzari *et al.* 2019). However, population size and structure remain unknown since contemporary acoustic methods cannot enumerate the number of individuals encountered in passive samples. It is unclear if bat populations are geographically segregated by island with little migratory exchange or if the archipelago hosts one large, panmictic population of bats that regularly traverse among islands. Cryptic tree roosting behavior and the solitary nature of the Hawaiian hoary bat also makes mark-recapture studies difficult. Field capture rates for Hawaiian hoary bats are low regardless of mist-netting effort and rate of recaptures yet even lower. The low power of detection yields diminishing returns, because to detect a slight population decline in migratory tree bats would require mark-recapture of many thousands of bats on an annual basis (Schorr *et al.* 2014).

Past genetic studies of the Hawaiian hoary bat have had limited geographic sampling, because protected species laws restrict field opportunities to capture and sample tissues from wild bats, and few museum specimens are available for analyses. Thus, the ability to incorporate genetic data from a wide variety of resources is necessary to collect samples and assemble datasets capable of addressing population level questions. In this matter, carcasses collected by state and federal agencies from such places at wind farm facilities have greatly improved sample sizes for islands where little bat research has taken place.

Because no practical techniques exist for the direct census of populations for Hawaiian hoary bats, an alternative method is through molecular genetics to estimate effective population size (Luikart *et al.* 2010). For migratory species capable of long-distance movement and that exhibit cryptic roosting behavior, indirect methods provide a practical approach to infer population patterns (Moussy *et al.* 2013). Characterizing the genetic variation of individuals

across the Hawaiian Islands can provide critical information on population structuring and degree of connectivity between islands. When populations experience restricted gene flow, genetic drift and mutation may cause divergence into distinct genetic clusters. Maternally inherited mitochondrial DNA can infer female site fidelity and the distance between breeding sites, while the analysis of polymorphic nuclear microsatellite DNA provides complementary information for both sexes (Vonhoff & Russell 2015). Genetic identification of sex is valuable to species conservation because sex ratios are often used to monitor the persistence and health of populations at risk of decline.

The present study characterizes the population structure and estimated effective sizes of Hawaiian hoary bats across three main Hawaiian Islands, with an emphasis on understanding genetic diversity and gene flow on the islands of Hawai`i, O`ahu, and Maui, populations where bats are presumed to be impacted by sustainable energy development. This study is also the first to determine sex using genotyping techniques in the Hawaiian subspecies of hoary bat, and expands the knowledge base of morphological information on sexual dimorphism. Research goals were to: 1) use known molecular genetics techniques to build a dataset of mitochondrial and nuclear DNA sequences and determine sex in Hawaiian hoary bats; 2) document morphological differences in body size; and 3) conduct population genetics analyses on Hawaiian hoary bat sequence data.

Inferences from levels of genetic variation and population structure across the archipelago will provide information on population boundaries useful for conservation of Hawaiian hoary bats, should management actions be warranted. In order to meet these objectives, I tested the following hypotheses: 1) More than one distinct population of hoary bats exists across the state of Hawai`i, 2) Migration rates are low between islands and estimated effective population sizes (Ne) are proportionate to island size, 3) There is morphological variation in body size among islands, between mitochondrial clades, and between sexes.

METHODS

Hawaiian Hoary Bat Tissue Collection

The USGS-PIERC Hawaiian hoary bat research group initiated collection of biological samples from bats in 2007, and an extensive group of tissue (wing membrane and muscle) samples representing approximately 200 individuals (male, female, and unknown sex) across four major Hawaiian Islands (Hawai`i, Kaua`i, O`ahu and Maui) are held in tissue banks (Figure 1). Bat tissue samples were obtained from USGS during mist-netting field research as well as from donated carcasses from private wind energy facilities, Hawai`i State wildlife offices, and the U.S. Geological Survey's National Wildlife Health Center Honolulu Field Station. New samples are received every few months, and this study incorporated samples deposited through 2016 (Table 1).

Live bats were captured using four-shelf, nylon mist nets approximately 2 meters high and ranged from 6 to 18 meters in length (Kunz & Parsons 2009). Nets were opened during sunset in corridors (roads, trails, gulches, ponds) where bats had been observed flying. On some occasions, playback of Hawaiian hoary bat social calls was used to lure bats into mist nets. Nets were checked every 15 mintures and bats were removed from the net by USGS permitted personnel. Bats were then placed individually in cloth holding bags until processing. Biological data collection for each individual included; age (juvenile or adult), sex, reproductive condition, forearm length (mm), and body mass (g) (Kunz & Parsons 2009). A sterile 3mm biopsy punch was used to obtain tissue samples from each wing. Bats were released at the site of capture immediately after processing.

USGS procedures are consistent with the guidelines for capture, handling, and care of mammals according to the Institutional Animal Care and Use Committee (IACUC) through the University of Hawai`i at Hilo and the American Society of Mammologists (#04-039-12) (Sikes *et al.* 2011). Biological samples from bats were collected under the following federal and state of

Hawai`i endangered species collection and handling permits: USFWS TE003483-31; DLNR-DOFAW WL 16-04.

Carcasses were refrigerated or frozen upon discovery and tissue samples from necropsies were stored at -20 °C. Carcasses were assessed for external sexual morphology. Small tissue samples were taken from soft wing membrane with a sterile 3mm circular biopsy tool. In some cases, fresh muscle tissue was cut away from the breast area with a sterile scalpel.

All tissue samples were stored in 1.5 ml tubes containing a preservation solution of NaCl-saturated 20% DMSO or on silica gel desiccant beads at ambient temperature in the field, and then frozen at -20 °C until DNA extraction.

Morphological Measurements

To evaluate differences in bat body size, I used linear measurements from preserved voucher skull specimens of *Lasiurus cinereus semotus* in the Vertebrate Collection of the Bernice Pauahi Bishop Museum (Table 2), and several different wing measurements recorded from both live bats and fresh carcasses (Figure 2). Skull and wing datasets included adult female and male bats from the islands of Hawai`i, Kaua`i, O`ahu, and Maui.

I measured the linear lengths of several skull features; basal or total length (B), condylobasal length (CB), mastoid (M), zygomatic width (ZW), palatal width (PW), and mandibular tooth (MT) in millimeters (mm) using calipers following the methods in Jacobs (1996). I used the linear lengths of the forearm (FA), length of the 5th finger without wrist (D5), length of the 3rd finger without wrist (D3), length of the 1st phalanx of the 4th finger (P4.1), and the length of the 2nd phalanx of the 4th finger (P4.2), in millimeters made with calipers following the methods in Dietz *et al.* (2006). Each measurement was repeated three times and averaged to account for variation. To obtain a single measurement to reflect "wing size", I calculated the wing area for bats using the method of Blood and MacFarlane (1998); where the area of one wing = FA x D5 + 0.5 (D5 x D3).

Molecular Techniques

DNA extraction

DNA was isolated from bat tissues using a Qiagen DNeasy Blood and Tissue kit according to the manufacturer's protocol for purification of total DNA from animal tissues. After extraction, DNA quantity was estimated, and quality assessed with two different methods. First, 2 µL of the DNA sample was electrophoresed through a 1.5% agarose gel and visualized under UV illumination against a 1 kb bp ladder after staining with SYBR Safe (Life Technologies). Second, UV absorbance was measured using spectrometry in 3µL of each DNA sample on a NanoDrop 1000 machine. Isolated DNA was used to amplify one mitochondrial marker, six nuclear microsatellites, and two genes on the X and Y sex chromosomes of Hawaiian hoary bats.

Mitochondrial DNA

A 657-bp region of the mitochondrial gene cytochrome c oxidase I (COI) was amplified using the forward primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and the reverse primer LCOI490 (5'-GGTCAACAAATCATAAAGATATTG-3') (Folmer *et al.* 1994), with standard polymerase chain reactions (PCR) and Sanger sequencing. PCR was conducted using Illustra Hot Start mix PCR beads (GE Healthcare) in 25 µL reaction volumes; containing 20.5 µL sterile water, 0.5 µL of each primer (10 µM), and 2.5 µL of genomic DNA template. PCR cycling conditions consisted of 1 cycle at 94 °C for 1 minute; 5 cycles of 1 minute at 94 °C, 1 minute 30 seconds at 45 °C, 1 minute 30 seconds at 72 °C; 35 cycles of 1 minute at 94 °C, 1 minute 30 seconds at 50 °C, 1 minute at 72 °C, and a final extension period of 5 minutes at 72 °C (Folmer *et al.* 1994) and was carried out on an Eppendorf Pro S Thermal Cycler. PCR products were checked for desired fragment size using gel electrophoresis and a 100 bp ladder. PCR products were cleaned of excess nucleotides and primers using Exo-Sap (Affymetrix) according to manufacturer's protocol and quality checked using UV spectrometry. Sanger sequencing of both the forward and reverse primer PCR products was performed on an ABI Prism 3500 Genetic

Analyzer (Applied Biosystems) at the UH Hilo Core Genomics Facility. Sequence chromatograms were manually trimmed, edited, and contigs formed in Sequencher v5.2.4 (Gene Codes 2014). All sequences retrieved were checked against GenBank using a BLAST search to confirm hoary bat sequence match percent. After editing and confirmation, sequences were combined with 59 COI Hawaiian hoary sequences from the Russell *et al.* (2015) study, downloaded from public resources on GenBank. The combined data set was aligned and trimmed by eye using MEGA v6 software (Tamura *et al.* 2011).

Microsatellites

A suite of six nuclear microsatellite loci developed, tested, and published for the North American hoary bat (*L. cinereus*) were amplified in Hawaiian hoary bat samples (Table 3) using multiplex methods for PCR and genotyping (Korstian *et al.* 2014, Korstian *et al.* 2015).

Loci were amplified in two multiplex groups, E (LcM, CotoG12, LcO) and F (LcP, LcU, LbG) using Qiagen's Multiplex Reaction Kit standard protocol and cycling conditions. PCR consisted of 10 µL volume reactions each containing 50 ng template DNA, 0.2 µM of each primer, 1X Multiplex Master Mix with HotStarTaq, Multiplex PCR Buffer with 3mM $MgCl_2$ pH 8.7, and dNTPs. Cycling parameters were 1 cycle at 95 °C for 15 minutes, then 30 cycles of 30 seconds at 94 °C, 90 seconds at 60 °C, 90 seconds at 72 °C, with a final extension at 60 °C for 30 minutes and were carried out on Eppendorf Pro S Thermal Cycler. Multiplex products were also checked for desired fragment size using gel electrophoresis and a100 bp ladder. For each microsatellite marker, the forward PCR primer was fluorescently labeled on the 5' end. Each group (E or F) consisted of 3 different colored markers, one for each locus (i.e. Group F: Lcp Blue, Lcu Green, and LbG Black). Multiplexes of microsatellites were diluted in sample loading solution with red labeled size standard (CEQ 8000 DNA standard kit 500 Beckman Coulter), and electrophoresed on an automatic Beckman Coulter CEQ 8000. Genotypes were scored and

sized using the Beckmann fragment analyzer package and binned using TANDEM (Matschiner & Salzburger 2009).

Genetic Sex Determination

Genotyping for sex was performed on 70 bat samples, including a subset of known sex (10 females, 10 males), identified by external morphology, to validate the utility of this method for Hawaiian hoary bats. I used two primer sets that amplified regions on the *Zfx* and *Zfy* introns, and produced sex-specific size variants, which were developed and validated for North American hoary bats using carcasses salvaged from wind facilities (Korstian *et al.* 2013).

The sexing primers were developed to be multiplexed and consist of two pairs (Korstian *et al.* 2013). The first pair for the region within the Zfx intron ~245 base pairs in length (F-ZFXBat: AGTCAAGGGRTGTCCATCR, R-ZFXBat: GTTTGYASACCAGGTTCCTC) and the second pair for the region within the Zfy intron ~80 base pairs in length (F-ZFYBat: GGTRAGDGCACAYRAGTTCCACA, R-ZFYBat: TGCYATTACAAAACCTTTRTAGATAC). The primers were amplified using Qiagen's Multiplex Reaction Kit standard protocol. PCRs were 10 µL volume reactions each containing 20 ng template DNA, 0.5 µM of each X-primer, 0.35 µM of each Y-primer, 1X Multiplex Master Mix with HotStarTaq, Multiplex PCR Buffer with 3mM $MgCl_2$ pH 8.7, and dNTPs. Cycling parameters were 1 cycle at 95 °C for 15 minutes, then 30 cycles of 30 seconds at 94 °C, 15 seconds at 57 °C, 30 seconds at 72 °C, and were carried out on an Eppendorf Pro S Thermal Cycler. PCR products were checked for desired fragment size using gel electrophoresis and UV light visualization; a 1% agarose gel at 110 volts for 30 minutes and stained using SYBRSafe gel dye, with a100 bp ladder for reference. Bat sex was determined based on the number of bands present for the individual in the gel lane. Males produced two bands, one resulting from the X chromosome intron, and another from the Y chromosome intron. Females produced only one large, very bright band, from the X-chromosome intron. X-chromosome bands appeared at ~245 bp product size, and Y-chromosome bands at ~80 bp.

Data Analysis

Genetic Diversity and Structure

Classical population genetic methods were employed to assess geographic structuring of mitochondrial COI and nuclear microsatellite DNA. Datasets were considered by the influences of island, mitochondrial clade, and sex. Mitochondrial genetic diversity was estimated from the number of haplotypes, number of polymorphic sites, haplotype diversity, per site nucleotide diversity (π), Watterson's θ and Tajima's D. Sequences were collapsed into unique haplotypes using DnaSP v5.10.1 (Librado & Rozas 2009). Microsatellite genotype summary statistics (e.g., number of alleles, observed (H_O) and expected (H_e) heterozygosity, deviation from Hardy-Weinberg equilibrium, and linkage disequilibrium) were calculated using Arlequin v 3.5.2.2 (Excoffier & Lischer 2010). Genotyping errors from allelic dropout, stuttering error, and null alleles in microsatellites were screened using Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2014).

Relationships among unique COI haplotypes at 657 bp in length were analyzed using a maximum parsimony TCS network (Clement *et al.* 2000) and visualized using PopArt (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz). A maximum likelihood phylogenetic tree of all Hawaiian derived sequences plus one *Lasiurus cinereus cinereus* (downloaded from GenBank), was selected with the model test feature and constructed in Mega v6, using a T92+1 model of evolution (Tamura 3 parameter) and 1,000 bootstraps to assess support at each node (Tamura *et al.* 2011). The tree was rooted in FigTree v1.4.3 (http://tree.bio.edu.ac.uk/software/figtree). Sequences from *L. xanthinus* and *L. intermedius* (available from GenBank) were used as outgroups. Relationships among nuclear microsatellite genotypes were analyzed simply with a neighbor-joining tree of Nei's standard genetic distance using the software Populations v.1.2.32 (O. Langella, http://bioinformatics.org/populations) and visualized with FigTree v1.4.3.

Differentiation of COI haplotypes among and within island and mitochondrial clade was assessed using molecular variance (AMOVA) and pairwise F_{ST} in Arlequin v 3.5.2.2 (Excoffier & Lischer 2010). Average pair-wise genetic differences were calculated between and within clades and islands using MEGA v6 software (Tamura *et al.* 2011). Differentiation of microsatellite allele frequencies among and within islands was computed Arlequin v 3.5.2.2 and additionally, the program FreeNA (Chaupis & Estoup 2006) was used to correct positive bias that may have appeared in estimates of microsatellite pairwise F_{ST} values due to the presence of null alleles. Alternative diversity measures for microsatellite loci – harmonic mean of population size (Ñ), Nei's within-subpopulation heterozygosity (H_{s_est}), total subpopulation heterozygosity (H_{T_est}), relative differentiation (G_{ST_est}), genetic differentiation (G''_{ST_est}), and Jost's actual differentiation (D_{est}) were calculated using the "nearly unbiased" estimation program SMOGD (Crawford 2010). For each island population I used GenAlEx v.6.5.03 (Peakall & Smouse 2006) to calculate G''_{ST} and Jost's D measures. Both of these alternative measures of differentiation overcome issues caused by highly polymorphic loci; Jost's D is based on effective number of alleles (Jost 2008), and G''_{ST} is standardized by the expected heterozygosity and corrected sampling in small numbers of populations.

Structure 2.3.4 (Pritchard el al. 2000) was used to test for the presence of more than one genetic subpopulation. Structure uses a Bayesian approach to estimate ancestry coefficients based on an *a priori* designated number of clusters (K), and to proportionally assign ancestry to individuals to evaluate cluster membership. The program was run to allow for admixture and for allele frequencies to be correlated across clusters, and the admixture parameter α, to be inferred from the microsatellite data. I used a range of 1-10 for values of K, and 4 iterations per K, with a burn in period of 10,000 steps of the MCMC chain, followed by analysis over the next 100,000 steps. Structure output was processed using Structure Harvester (Earl & Vonholdt 2012) to compute the ΔK, an ad hoc estimate of the most probable number of K-clusters based on the

rate of change in the log probability of the data between consecutive K-values (Evanno *et al.* 2005). Ancestry coefficients were visualized using Structure Plot v 2.0 (Ramasamy *et al.* 2014).

Estimating Gene Flow

In order to detect potential differences in dispersal between female and male bats, inbreeding coefficient (F_{IS}), F_{ST}, gene diversity (H_S), relatedness, mean assignment index (mAIc), and variance of the assignment index (vAIc) were quantified separately for both sexes using microsatellite loci across islands, with the program FSTAT v 2.9.3 (Goudet 1995, Goudet *et al.* 2002). Statistical significance was determined from the randomization method implemented in FSTAT (10,000 permutations).

To determine if gene flow is restricted by the oceanic passage between islands, I tested the correlation of distance with population structure by using an isolation by distance analysis (IBD) on both mitochondrial clade and microsatellite data with Mantel tests in the web based program IBDWS v 3.32 (Jensen *et al.* 2005). IBD can illustrate the relationship between calculated pairwise genetic distance (F_{ST}) and pairwise geographic distance (km) between islands.

To measure contemporary migration rates between the islands of Hawai`i, Maui, and O`ahu I used modeling software BayesAss v 3.0.3 (Wilson & Rannala 2003). Treating each of the three islands as single populations, I ran a BayesAss for 50 million generations with a burn-in of 10 million, sampling every 1,000 generations. The step sizes of migration rates were adjusted to m= 0.1, allele frequencies to alpha= 0.3, and in-breeding coefficients to f = 0.8, so that the acceptance rate of proposed changes to these parameters remained between 40 and 60% through the duration of the run, in accordance with the recommendations from the BayesAss manual. I used Tracer v 1.6 (Rambaut *et al.* 2014) to visualize sampled parameter values to ensure that adequate mixing through generations and for convergence prior to the end

of the burn in period. In addition to estimating migration rate between populations, BayesAss also provides the posterior probability of the migrant history of each individual.

Estimating Historic, Contemporary Effective Population Size, Bottlenecks

Long-term historical female effective population size (N_{EF}) was estimated from mitochondrial sequences using the equation $\theta=2*N_e*u$ where θ is the Watterson's estimator calculated in Arlequin and *u* is the mutation rate per sequence per generation. Mutation rates at COI are not available for the genus *Lasirurus*, so I used published substitution rates calculated for cytochrome b in the family Vespertilionidae from Korstian *et al.* (2015), which were published in recent population estimates for the North American hoary bat. I adjusted mutation rates (per sequence, per year) to my COI sequence length (657 bp); the values used were 8.06 x 10^{-6} for the "low mutation rate" and 1.08 x 10^{-4} for the "high mutation rate". I reported both rates because they were quite different. Long-term effective population size was also calculated using the average expected heterozygosity of the microsatellite loci for the infinite allele model (IAM) and stepwise mutation model (SMM) using the equations: SMM $N_e = ((1/1-H)^2-1)/8\mu$, IAM $N_e = H/4\mu\ (1-H)$ (Nei 1987). The infinite allele model (IAM) assumes that each mutation is unique, an infinite number of them can arise. The stepwise mutation model (SMM) follows the changes in length of the mutation, allowing a step up or down by one at each mutation. Mutation rates for microsatellite loci were averaged from the IAM and SMM using 10^{-3} and 10^{-5}, following the methods in Korstian *et al.* (2015).

Estimates of contemporary effective population size (N_e) for each island were estimated using the microsatellite linkage disequilibrium method in the program NeEstimator V2 (Do *et al.* 2014) on the three suitable microsatellite loci that demonstrated Hardy-Weinberg equilibrium. The linkage disequilibrium single-sample method relates a decrease to Ne with an increase in genetic drift, linkage disequilibrium increases because small populations increase in non-random association of alleles. Discrete generations are assumed using this method, so I

included all years of bat collected during the analysis. Random mating was assumed, and alleles with frequencies below 0.02 were screened out. Estimates of Ne are reported with ± 95% confidence intervals determined with jackknife re-sampling.

With only six potential microsatellite loci, I was limited in statistical approaches available to examine if each island population showed genetic signatures of a population bottleneck. I calculated modified Garza-Williamson M-ratios using three loci for each island population using Arlequin (Excoffier & Lischer 2010). The M-ratio test compares the number of alleles at a locus with the allelic size range and assumes that during a bottleneck event, the number of alleles will be reduced compared to the allelic size range. The M-ratio in Arlequin is modified to avoid dividing by zero in monomorphic populations. A critical value of 0.68 generally indicates a recent population bottleneck, but the Critical M program (Garza & Williamson 2001) can be used to simulate specific critical M values and determine the significance of M-ratios. Specific critical M values were generated for my dataset varying θ from 0.1 to 10 which encompassed all estimates of θ produced by the mitochondrial sequences, and used the following values for the TPM parameters, the probability of changes greater than one step (p_g=0.1) and the size of one-step changes (Δ_g =3.5).

To further assess signs of possible bottlenecks or expansions in island populations and mitochondrial clades, I conducted a neutrality test in DnaSP for Tajima's D (Tajima 1989). This tests a null hypothesis that the number of rare and common mutations is equal in a stable population, while the alternative is an increase in low frequency mutations for a population experiencing an expansion or rapid growth.

Morphological Patterns

Using only adult bats with known sex, I used principal components analysis (PCA) on skull and wing measurements to understand the relative contribution of each measurement to

the total variation observed in the dataset. Since the true number of *L.c. semotus* species in Hawai`i has been debated (Ziegler *et al.* 2016, Baird *et al.* 2017), I used a conservative statistical approach of analyzing all morphological data as one unit. PCA does not take into account any difference between groups based on *a priori* classification of the samples. Discriminant analysis was used to retain principal components, and optimal number of principal components identified using an α-score. Variation in size of the measured lengths was summarized by the first axis of the PCA. Loadings describe the direction and magnitude of measurements with their respective axis. The method assigns membership probability for each individual to different clusters. Within each skull and wing measurement analysis, I examined the distribution of clusters for patterns in mitochondrial clade membership, island, and sexual dimorphism.

A two-way analysis of variance (ANOVA) was used to investigate the main effects of sex and clade, and their potential interaction on forearm length, and skull length and width. For each sex, I used one-way ANOVAs to test for differences between islands for bat skull length, skull width, and forearm measurements. A post-hoc analysis tested for separation in means between groups was conducted in tandem with each ANOVA, using least square means for multiple comparisons

Descriptive statistics (mean, standard deviation, and range) on body size were obtained for all individuals. All statistical tests were performed in R Studio using the program R, version 3.2.0. (R Core Team 2015). Figures to display PCA output graphically were created with the assistance of R packages “ggplot2” (Wickham 2009) and “ggbiplot” (Vu 2011).

RESULTS

Mitochondrial sequence characteristics and diversity

The maximum likelihood mitochondrial COI phylogenetic tree indicated reciprocal monophyly of the Hawai`i clade "Clade 1" (containing bats from Hawai`i, O`ahu, Maui, and Kaua`i) and the Hawai`i/NA clade or "Clade 2" (containing bats from Maui, O`ahu, and the North American Hoary bat sequence) with bootstrap support of 100 (Figure 3). Clade 2, containing the *L. c. cinereus* sequence shows many individuals on Maui and O`ahu to share a closely related mitochondrial sequence and thus their matriarchal lineage may be more closely related to the North American hoary bat; however there also appears to be a well-supported node within this clade which forms a separate group of bat sequences unique to Maui. Clade 1 appears to have some sequence divergence by island, but the nodes are not highly supported by bootstrap analysis.

Comparisons of nucleotides resulted in varying pairwise genetic distances between and within clades as well as islands (Table 4). The greatest divergence (2.95%) was found between the clades, with very small differences (0.25%, 0.31%) within 1 and 2. When genetic distance was analyzed by island, O`ahu and Kaua`i showed to be most divergent from Hawai`i and Maui within Clade 1. Within Clade 2, genetic distance within Maui was slightly larger than genetic distance bewtween Maui and O`ahu.

The total COI data assembled for this study of 140 individual bats from four islands had 31 polymorphic sites and an average nucleotide diversity (π) of 0.0148 with a range of 0.0006 to 0.104 (Table 5). I found 15 unique haplotypes and an average haplotype diversity (h) of 0.80 with a range of 0.39 to 0.68. Hawai`i had the highest number of haplotypes (7), followed by Maui (6), O`ahu (4). Only three haplotypes were shared across islands, while most were privately found on individual islands (Figure 4). Kaua`i only contained one single haplotype which was shared with other islands. Haplotype diversity was highest on Maui and O`ahu.

When 92 bats belonging to clade 1 were analyzed separately, I found 11 polymorphic sites in Hawaiian hoary bats across four islands and an average nucleotide diversity (π) of 0.0019 with a range of 0.0017 to 0.0002 (Table 6). There were 11 unique haplotypes and overall haplotype diversity (h) of 0.68 ± 0.03 with a range of 0.08 to 0.53. Hawai`i had the highest number of haplotypes (7), followed by Maui (3), O`ahu (2), and Kaua`i (1). Haplotypes were shared across islands, with Kaua`i containing only 1.

When 45 bats belonging to clade 2 were analyzed separately, I found 6 polymorphic sites in Hawaiian hoary bats across two islands and an average nucleotide diversity (π) of 0.0035 with a range of 0.002 to 0.0037 (Table 6). There were 4 unique haplotypes and an overall haplotype diversity (h) of 0.52 ± 0.06 with a range of 0.22 to 0.55. Haplotypes were shared across Maui and O`ahu islands.

Microsatellite diversity

Ninety-three Hawaiian hoary bats across three islands (Hawai`i = 21, Maui = 34, and O`ahu = 38) were genotyped at 6 microsatellite loci; one locus (LcO) was monomorphic in Hawai`i and O`ahu populations but variable in the Maui population (Table 7). The presence of a shared monomorphic locus between two island populations suggests they arose from a common ancestral population, although loss of heterozygosity from genetic drift cannot be ruled out. Eighty-five Hawaiian hoary bats were genotyped at all 6 loci, 5 individuals amplified at 5 loci, 1 individual amplified at 4 loci, and 2 individuals amplified at 3 loci. The number of alleles per locus ranged from 2 to 14 (Table 6). The islands all showed similar levels of average observed heterozygosity, although Maui trended towards the lowest value (Hawai`i = 0.50 ± 0.29, Maui = 0.46 ± 0.21, O`ahu = 0.56 ± 0.17). Initial multiple comparison tests on five loci showed that linkage disequilibrium was present in 7 of 45 of comparisons (15%) Consistent linkage disequilibrium was present between CotoG12 and LcP, indicating they were not independent in the O`ahu and Maui populations. Overall, three loci (CotoG12, LcM, and LbG) showed significant deviations from Hardy-Weinberg Equilibrium in more than one island population,

and contained null alleles (Table 6). These loci were discarded from further analysis. Deviations from Hardy-Weinberg Equilibrium are likely due to a combination of null alleles and the Wahlund effect, which is the reduction in observed heterozygosity caused by subpopulation structuring within the group being tested. Of the three suitable loci, multiple comparisons showed no significant linkage disequilibrium, and they were considered independent.

Alternative diversity measures from SMOGD (Table 8) for the three microsatellite loci used in population structuring analyses show a range of high to low values for heterozygosity and differentiation. With the loci LcU being the most differentiated, followed by LcP, and lastly LcO.

Mitochondrial DNA structure

In all sampled Hawaiian hoary bats, pairwise F_{ST}s revealed significant genetic distance between all island pairs with the exception of O`ahu and Kaua`i (Table 9). Pairwise differences ranged from 0.656 between the islands Hawai`i and Kaua`i, to 0.351 between Maui and O`ahu. Midrange values of 0.576, 0.469, and 0.459 described separation between Hawai`i and O`ahu, Hawai`i and Maui, and Maui and Kaua`i respectively. When only bats from Clade 1 were considered in analysis, a similar pattern of population separation between islands persisted. However, the greatest population differentiation was between Maui and O`ahu. F_{ST} values were higher for all other island comparisons, except for Hawai`i and Kaua`i which remained the same (Table 10). This suggests that clade 1 has a greater degree of mitochondrial historical separation between populations across the islands. When only bats from Clade 2 were tested, the pairwise F_{ST} value between O`ahu and Maui was the lowest out of all comparisons but remained significant, and thus continues to suggest some population structure is maintained in clade 2 mitochondrial DNA between these islands.

AMOVA tests for mitochondrial haplotype differences among and within islands for all bats (both clades together) exhibited more variance among islands (65%) than within islands (35%) (Table 11). When grouped by clade, Clade 1 bats showed the greatest genetic variance

among islands (80%) than within islands (20%). An opposite pattern occurred in Clade 2 group between O`ahu and Maui, where almost 90% of the variance in mitochondrial haplotype was found within islands (due to high haplotype diversity) and only about 10% was found among the islands.

Microsatellite structure

In Hawaiian hoary bats, pairwise F_{ST}s of three nuclear microsatellites in Arlequin showed low but significant genetic distance between Hawai`i and O`ahu, and Hawai`i and Maui (Table 12). When $F_{ST}$ was corrected for null alleles using FreeNA, these values increased, again indicating greater genetic differentiation between the islands of Maui and Hawai`i, and Maui and O`ahu, and less for Hawai`i and O`ahu (Table 12). The alternative measures of $G'_{ST}$ and Jost's $D_{EST}$ found similar significant population structuring results to $F_{ST}$ tests for comparisons between islands (Table 13). Hawai`i and Maui populations have the most allele frequency genetic differentiation at the loci examined, followed by O`ahu, while the same alleles do not differ greatly between populations of bats on O`ahu and Maui.

An AMOVA conducted in Arlequin for 3 microsatellite loci showed very small percent of variance due to differences between islands (~3%), a negative value (~-10%) for variance within islands, and over 100% of the variance due to the differences within individual bats (Table 11).

The neighbor-joining tree of nuclear genotypes for three microsatellite loci shows mixture between bats with mitochondrial clades 1 and 2, as well as mixture of alleles across islands (Figure 5).

The characterization of genetic structuring with Bayesian modelling in Structure, for three microsatellite loci across Hawai`i, O`ahu and Maui, determined that four genetically distinct clusters had the highest probability. A large slope increase from K=3 to K=4 shows that the greatest probability occurs at K=4 (Figure 6). However, genetic clusters did not directly correspond to island, instead genetic assignments of individuals to K=4 revealed that each

island is comprised of individuals from all four genetic clusters, and that ancestry is fairly admixed in each individual bat (Figure 6).

Gene flow

There were no significant differences found in the statistical descriptors (F_{IS}, F_{ST}, H_S, mAIc, and vAIc) between the sexes calculated with microsatellite loci data from 46 females and 47 males pooled across the three islands (Table 14). Inbreeding coefficients (F_{IS}) were negative for both males and females, indicating high heterozygosity and outbreeding (since all populations were pooled), with no apparent bias in dispersal. Pooling the island populations likely violated neutral model expectations in this analysis, since sub-structure was demonstrated between islands. Even though it was not significant, the F_{IS} for males (-0.0720) was about 68% less than the value calculated for females (-0.1062). Negative values of F_{IS} indicate individuals in a population are less related than expected under a model of random mating, as expected for a pooled population with known sub-structure.

The Mantel test using pooled clade data indicated no significant correlation between F_{ST} and geographic distance for mitochondrial DNA ($r = 0.62$, $p = 0.12$) or microsatellites ($r = -0.23$, $p = 0.67$) (Figure 7). When mitochondrial data was separated according to clade and analyzed, there was still no correlation in Clade 1 ($r = 0.34$, $p = 0.36$). A Mantel test for Clade 2 could not be conducted since this clade only occurred on two islands. Comparing the regressions conducted between mitochondrial and microsatellite DNA revealed a greater, positive relationship between mitochondrial DNA and pairwise F_{ST} and geographic distance, and a weaker, negative relationship for microsatellite DNA.

Within the past generation sampled in this study, migration rates calculated from BayesAss suggest that dispersal events have occurred between the islands of Hawai`i, Maui, and O`ahu and that there is some evidence for directional dispersal from O`ahu to Maui to Hawai`i (Figure 8). The highest measured proportion of individual migrants (0.50) indicates greatest levels of movement occur directionally from Maui to Hawai`i. There are similar dispersal rates

(0.15) from O`ahu to Maui and from O`ahu to Hawai`i. Dispersal from Hawai`i to Maui or O`ahu is low (0.08), as is the dispersal from Maui to O`ahu (0.11). However, the 95% confidence intervals associated with most of these migration rates include zero (with the exception of Maui to Hawai`i) thus numbers should be interpreted with caution and not taken as absolutes.

Estimates of population size and bottlenecks

Long-term female historical population estimates from COI sequence data varied widely and were sensitive to the mutation rate used in each analysis (Table 15). Estimates calculated using the low mutation rate showed wide ranges in values for single island populations, between 33,061 and 359,932 bats. Low mutation rate estimates for Clade 1 + 2 pooled were between 81,094 and 359,932 bats. Low mutation rate estimates for Clade 1 were between 33,061 and 99,808 bats. Low mutation rate estimates for Clade 2 were 74,856 for Maui and 137,236 for O`ahu. Values were considerably lower when calculations were made using the high mutation rate, between 2,434 and 26,501 bats. High mutation rate estimates for Clade 1 + 2 pooled were between 5,971 and 26,501 bats. High mutation rate estimates for Clade 1 were between 2,434 and 7,349 bats. High mutation rate estimates for Clade 2 were 5,511 for Maui and 10,104 for O`ahu. When both clades were grouped together, population size of Hawai`i was the lowest and those on Maui and O`ahu were almost five times greater. Within Clade 1 the historical size for Maui was the largest and O`ahu the smallest, with Hawai`i in between those values. Clade 2 historic population sizes showed an opposite trend with O`ahu populations twice that of Maui. These results should be interpreted with caution because of lack of confidence intervals to measure precision of estimates.

Long-term Ne estimated from the microsatellite data using the Stepwise Mutation Model (SMM) and the Infinite Allele Model (IAM) varied widely and were also sensitive to the mutation rate used with each model (Table 16). For both models, the low mutation rate

estimated larger long-term effective population sizes (Ne). SMM estimates for the low mutation rate were between 10,773 and 17,834 bats. SMM estimates for the high mutation rate were between 108 and 178 bats. IAM estimates for the low mutation rate were between 9,112 and 13,945. IAM estimates for the high mutation rate were between 91 and 139 bats. Estimates from averaging results between the model types were lowest for Hawai`i and higher for Maui and O`ahu for both mutation rates. The lower mutation rate gave estimates that overlapped with the estimates produced with the COI sequences, and like the COI results, should be interpreted with caution because of lack of confidence intervals to measure precision of estimates.

Estimates of contemporary effective population size of Hawaiian hoary bats on each island were variable and imprecise, with a negative value for Hawai`i and jackknife confidence intervals with upper bounds of infinity for Hawai`i and Maui populations (Table 17). Positive estimates for effective population size were almost 800 individuals for Maui, but were very low less than 20 individuals for O`ahu. O`ahu was the only estimate to produce a fully bounded confidence interval, and the maximum bound was near 50 individuals based on my dataset.

I detected slight signatures of genetic bottlenecks in the overall bat population, and specifically within populations on Hawai`i and O`ahu, where modified M-ratios were at or lower than the critical value of 0.68, but not Maui (mean ± SD; Hawai`i = 0.58 ± 0.30, Maui = 0.92 ± 0.14, O`ahu = 0.69 ± 0.39, overall = 0.73 ± 0.28). Modified M-ratios for Hawai`i and O`ahu, as well as the overall M-ratio for all populations, were also within or lower than the critical values of M generated between $\theta = 0.1$ (M_crit = 0.75) and $\theta = 10$ (M_crit =0.65).

Tajima's D testing in all bats was variable across islands (Table 5). It was significantly negative for Hawai`i, and positive for Maui and O`ahu. The patterns were different when bats were separated by mitochondrial clade (Table 6). For clade 1, all islands showed negative Tajima's D values, with Hawai`i and O`ahu being significantly different from the neutral model. For clade 2, only O`ahu had a significantly negative D value, and it was the highest of all values across clades.

Genetic sex determination

The sex was identified successfully in 66 (out of 70 attempted) Hawaiian hoary bat individuals by genotyping the X chromosome intron (Zfx) and the Y chromosome intron (Zfy). Samples that did not amplify during PCR were due to extremely low DNA quantity and poor quality extractions, and could be attributed to tissue samples from carcasses with the most advanced decomposition. Thirty-five tested males produced two bands, one resulting from the X chromosome intron, and another from the Y chromosome intron. Thirty-one tested females produced only one large, very bright band, from the X-chromosome intron. X-chromosome bands appeared at ~245 bp product size, and Y-chromosome bands at ~80 bp (Figure 9). Genotyping tests for known individuals (males and females) had 100% confirmation between sex genotype observed from electrophoresed PCR product bands and sex observed from external genitalia.

Patterns in bat body size

The PCA of wing measurements revealed overlap among individuals from Hawai`i, Maui, and O`ahu islands, sexes, and clades to some degree. For this mixed dataset, principal component (PCA) 1 (length of forearm) explained 40.5% of the variation observed, while PCA 2 (length of the 5th digit or D5) explained 34.9% of the variation (Figure 10, Table 18). All five wing measurements were correlated with PC1 to a similar extent and direction. The wing measures of D5, D3, and wing area were more correlated with PC2. The overlap patterns in island and clade were very similar with Clade 1 mimicking O`ahu and Clade 2 mimicking Maui. Male and female bats showed the least degree of overlap, suggesting there may be differences in wing area due to sex.

The PCA of skull length measurements revealed a much lesser degree in overlap between islands, sexes, and clades. For this mixed dataset, PCA 1 (basal length) explained 65.9% of the variation observed, while PCA2 (condylobasal length) explained 26.3% of the variation (Figure 11, Table 18.). All three skull length measurements were correlated with PC1 in extent and

direction. The mandibular length measurement was more correlated with PC2. The individuals on Kaua`i clustered together and were separate from Maui, but still fell within Hawai`i space. There was very little overlap between Clade 1 and Clade 2. Male and female bats showed overlap but it may be likely due to misidentification of sex, since the clusters seem generally distinct.

The PCA of skull width measurements also revealed less overlap between sexes and clades, but not islands. General patterns were similar to those of skull length. For this mixed dataset, PCA 1 (mastoid width) explained 51.9% of the variation observed, while PCA2 (zygomatic width) explained 39.4% of the variation (Figure 12, Table 18). All three skull width measurements were negatively correlated with PC1 in extent and direction. The palatal width was negatively correlated with PC2. There was very little overlap between Clade 1 and Clade 2 and male and female bats.

Morphological variation in bat body size was examined by using the linear measurements characteristics that explained the most variation in skull (basal and mastoid) and wing (forearm) PCAs. The effects of sex and clade were assessed together, as well as their potential interaction, using two-way ANOVAs. I found that females had significantly longer forearms than males ($F = 29.05$, df =1, $P < 0.0001$) (Figure 13). Clade was found to be weakly significant ($F = 2.83$, df =1, $p < 0.1$.) There was no interaction between sex and clade for forearm length. I found that skull length was significantly longer in bats from Clade 2 than Clade 1 ($F=5.01$, df =1, $p < 0.05$) (Figure 14). I did not find an interaction between sex and clade for skull length. I also found that females had significantly wider skulls than males ($F=4.75$, df =1, $p < 0.05$) (Figure 15). There was no difference between clades or interaction with sex for skull width.

Since sexual dimorphism was found to be the most significant factor for differences underlying morphology, I separated the sexes and conducted a series of one-way ANOVAs on forearm, skull length and width to look for any differences due to island population. No significant differences were discovered between island for female or male bats, confirming skull

and wing sizes are generally similar across islands for their respective sexes (Figure 16). However, this comparison may be compromised by pooling bats from Clade 1 and 2 in the Maui and O`ahu island groups. Mean, standard deviation, and range of morphological measures were reported for individuals by island for each sex (Tables 19 and 20).

DISCUSSION

Knowledge of genetic diversity and population structure are important baseline measures for an endangered species with an unknown census size and contribute to the effectiveness of management actions which aim to mitigate for loss of individuals due to human impacts. Although solitary, tree roosting, migratory bats are difficult to count, we can begin to comprehend some key features about their demographic histories, movements, and population patterns through the use of molecular genetic tools. In this study I was able to quantify genetic diversity, identify subtle population structure across the Hawaiian Islands, and estimate both historic and contemporary effective population sizes for Hawaiian hoary bats. I also utilized genetic techniques to determine the sex of unknown individuals, which in turn assisted in improving morphological analyses and testing for sex biased dispersal. This approach has recognized that Hawaiian hoary bats may consist of multiple subpopulations, affirmed the theory of multiple founding events to the islands, and provided preliminary estimates of genetic diveristy and effective population sizes.

Geographic population structure and genetic diversity

Genetic structure develops within a species once it departs from panmixia conditions and forms subpopulations which become restricted from exchanging genetic material via dispersal and mating opportunities (Waples & Gaggiotti 2006). Genetic structure is expected to be present for island bat species that leave continental land masses and in instances where dispersal becomes limited by oceanic barriers between islands. Genetic isolation in island bat populations descending from a wide-ranging continental population has been demonstrated for molossid and vespertillonid species, with open water crossings between islands serving as a barrier to gene flow over time; *Myotis punicus* in the Mediterranean (Biollaz *et al.* 2010), *Nyctalus azoreum* in the Azores (Salguerio *et al.* 2004, 2007), *Tadarida brasiliensis* in The

Bahamas (Speer *et al.* 2017), and two species of *Miniopterus* bats in Madagascar (Weyeneth *et al.* 2011).

Individuals used in this study covered a broad geographic range across four islands and spanned an average time period of seven years. This study maintains the largest sampling of individual bats (140) for genetic analyses, thus doubling the sample size of Russell *et al.* (2015) and Baird *et al.* (2017), including over 50 bats of which neither study had access. My results demonstrate that more than one distinct population of hoary bat exists in Hawai`i, that these populations have high heterozygosity and thus remain genetically diverse. F_{st} estimates from mitochondrial and nuclear microsatellite analyses support population differentiation by island, with variation in mitochondrial DNA higher than nuclear microsatellites, suggests existence of island subpopulations in Hawai`i. The existence of unique haplotypes on each island also support a geographic pattern of island driven population structure.

Star-shaped haplotype networks are reported in many genetic studies of tree bats in North America, suggesting large populations with little to no levels of genetic differentiation due to high gene flow and the absence of geographic barriers. Star-shaped networks are composed of one large common ancestral haplotype at the center, while small numbers of unique haplotypes radiate out from the center with short branches. The haplotype network structure for COI in Hawaiian hoary bats is different from common star-shaped networks and seems to be more aligned with geographic island structure for Clade 1, with Maui hosting a connecting haplotype between Kaua`i/O`ahu and Hawai`i Islands. Although at least one fairly abundant haplotype was present per island, a few abundant haplotypes from two different lineages (Clade 1 & 2) were present on Maui and O`ahu, making these two islands a source of high genetic and nucleotide diversity. A few unique haplotypes radiate outwards from the central ancestral haplotype on Hawai`i Island, suggesting recent expansion of the bat population into the larger geographic space available on the largest island. Haplotype network results, which share a

majority of data taken from the same set of individuals, are fairly identical to networks presented in Russell *et al.* (2015) and Baird *et al.* (2017), furthering the reproducibility of Hawaiian maternal DNA population structure. However, I found evidence of 5 additional haplotypes not previously reported; both Russell *et al.* (2015) and Baird *et al.* (2017) reported only 10 haplotypes. Only one North American hoary bat study reports mitochondrial DNA for the same COI marker as used here. Korstian *et al* (2015) identified 32 haplotypes across 217 individuals for a regional area around the state of Texas, while in contrast, the Hawaiian hoary bat has about half as many, 15 for 140 individuals (this study). This lower haplotype number could be due to differences in sample size, founder effect, or past population bottleneck events. Increased sampling on all islands, including Lanai and Molokai, may uncover more haplotype diversity.

The six microsatellite markers I used are highly diverse in hoary and red bats (Korstian 2012, Korstian *et al.* 2014, Korstian *et al.* 2016), however I found CotoG12, LcM, and LbG to have consistent linkage disequilibrium and an excess of null alleles. The number of alleles present at these loci for Hawaiian hoary bats was far less than those reported for the loci in North American hoary bats. Microsatellite pairs developed in one species can sometimes be used in closely related species because primer sites are generally highly conserved. There may be some downward bias of genetic diversity estimates from microsatellite primers transferred across species when a small number of loci are selected based on high polymorphism in the focal species; in congeners these may be fixed or have relatively low polymorphism (Allendorf *et al.* 2013). A decrease in the number of alleles is expected due to the founder effect and subsequent genetic drift but might also indicate ascertainment bias or high mutation load in the primer region itself or the population from which the markers were described. Unequal sample sizes and locus-specific effects can create artifacts in heterozygosity calculation, and overestimate null alleles (Dharmarajan *et al.* 2013). Null alleles, which occur because of mutation in the primer regions and result in amplification failure, reduce gene diversity and can inflate population

differences when comparisons are made to populations without null alleles (Chapuis and Estoup 2007). Taking a conservative approach, I did not use microsatellites with high levels of null alleles, employed a correction factor, and performed analyses using an "unbiased-estimator." Eleven individuals failed to amplify at all 6 markers, so mutations may be present or it could be simply a result of using degraded DNA from carcass material which contains less nuclear DNA. Yet, the presence of unknown subpopulation structuring common under the Wahlund effect, might also cause excesses in homozygosity in some loci which could be misinterpreted as an effect of null alleles (Dharmarajan *et al.* 2013). There was consistent evidence across subpopulations (islands) for loci CotoG12 and LbG to have null alleles, and for LcM on O`ahu and Maui but not for Hawai`i Island. With the high potential for mutations and microsatellite polymorphism differences between North American and Hawaiian species, it is not clear if the Wahlund effect is also influences genetic diversity results. Subpopulations within islands may be present, even with this bat's flight ability and seasonal movements, but I was not able to collect enough samples to test for fine-scale population differentiation at the intra-island level. Additional species-specific microsatellites and larger sample sizes could possibly discriminate between the Wahlund effect and intra-island sub-structuring.

Estimates of genetic differentiation for nuclear microsatellites maintained significance in population structure between islands, but were much subtler than mitochondrial results. This is expected because the effective population size for mitochondria is lower than nuclear DNA (Allendorf *et al.* 2013). Mitochondrial DNA and nuclear microsatellites can display population genetic structure at different rates, and it is common to see geographically structured mtDNA trees without strong validation from nuclear genes, especially in populations with shorter periods of isolation (Zink & Barrowclough 2008). Moreover, microsatellites may underestimate F_{st} if high mutation rates cause size homoplasy, even when migration rates are low (Balloux *et al.* 2000). Analysis using the program Structure, based only on three microsatellites, did not display visual evidence of genetic structure by specific island, but suggested four genetically

distinct clusters, showing bats on each island with admixed ancestry. This admixture could be due to past gene flow between islands occurring through migration, or could be an effect of lower power of a small number of molecular markers. All bats were included in the Structure analysis; results may be different if bats were separated by clade. Meirmans (2012) reports that Structure results can also be muddled if isolation by distance exists in the dataset.

Estimation of gene flow

Migration rates estimated from microsatellites using BayesAss suggested little to no movement between islands, with all but one rate having confidence intervals including zero. The overall pattern shows directional movement to be consistent with the stepping-stone pattern, from older island (O`ahu) to younger island (Hawai`i), which supports bat founding events to older islands first, then population expansion to new islands. The largest differences in Fst estimates for mitochondrial DNA were found at distant ends of the island chain, Hawai`i and Kaua`i. Considering the estimates for each clade, greater separation exists between islands for mitochondrial Clade 1 than Clade 2. This is concordant with the scenario described by Baird *et al.* (2017) under which colonization events began with founding of populations first on Maui/O`ahu, then spreading out in each direction, northwest to Kaua`i, and southwest to Hawai`i Island. The most significant, and thus most contemporary, estimate shows greater levels of movement from Maui to Hawai`i Island than vice versa. The short distance (~30 miles) of the ʻAlenuihāhā Channel may facilitate bat flight between these two islands when winds are light. On the other hand, strong winds may decrease the crossing and keep gene flow lower than it would be for such a narrow channel because bats usually avoid flying in strong winds. BayesAss measures contemporary gene flow by detecting the percentage of migrant genotypes directly, one to a few generations after they occur and performs better at higher levels of differentitation (Wilson & Rannala 2003). Here, migration rates may be underestimated due to the minimal number of microsatellites used. Gene flow estimates using model-based clustering

approaches like program Migrate (for mitochondrial DNA) would be valuable for modeling historical migration patterns, which were not explored in this study.

Isolation by distance (IBD) was not evident for mitchondrial or nuclear DNA, despite Fst indicating significant differences in population structure between Hawai`i and Maui Islands, and Hawai`i and O`ahu Islands. Two factors drive this result: lack of population differentiation between O`ahu and Maui, and Hawai`i Island's show of closer genetic affinity to O`ahu than Maui, opposite of IBD expectations. I combined all bats and segregated data by island, which resulted in only six distance comparisons for mitochondrial DNA. The three microsatellite data points are also likely not sufficient. If individual distances between exact locations of bat samples were utilized, then more data points would be available for the Mantel test. Unfortunately, fine scale population sampling to achieve this was constrained by sample availability. We would expect to see significant isolation by distance if the oceanic channels between island were completely impassable to bats, this result proposes that bats did in fact cross this barrier at some point but that genetic variation is due to island population rather than spatial distance.

If populations are at or near migration-drift equlibirum, genetic differentation at a lower heirachial level (within islands) cannot exceed the genetic differentation at a higher heirachial levels (among islands). Mitochondrial DNA AMOVA results supported significant population structure due to dispersal for Clade 1 bats, and moderately when both clades are combined. Alternatively, if genetic differentation at lower hierarchical levels (within islands) exceeds genetic differentation at higher hierarchical levels, this possibility suggests that genetic structure is more determined by genetic drift than by dispersal. Mitochondrial DNA variance within islands exceeds variance among islands for Clade 2 bats, but was not significant. For nuclear microsatellites, subtle yet significant results were found for population structure caused by genetic drift. Drift is stronger in small populations (island scale) than in large populations

(archipelago scale) and deviates from migration-drift equlibrium, reflecting a future loss of genetic diversity.

Diversity differences found between mitchondrial and microsatellite markers may sometimes indicate sex-biased dispersal (Petit *et al.* 2001). If female bats are philopatric to the same maternity roost areas, this may result in mitochondrial DNA showing a stronger population differentiation signal than microsatellite loci. Mitochondrial DNA in this study showed greater levels of population differentiation than microsatellites, indicating potential female philopatry to island. High levels of male dispersal mediate gene flow and prevent declines in nuclear microsatellite heterozygosity and this seemed congruent with the lower differentitation results for microsatellites. However, I did not find bias in dispersal for either sex when I pooled bats across islands. It is likely that both sexes make relatively large scale and seasonal movements, however the sex-biased dispersal analyses should be re-analyzed by island group for a more correct interpretation.

Historical and recent estimates of effective population size

Effective population sizes should be considered in evaluating conservation programs for endangered species (Waples 2002). For measures of effective population size, rates of extinction and colonization, patterns of migration and variability in the size and productivity of subpopulations are important. Changes to local and global effective population sizes should be treated separately because each responds differently than the other to the same condition. Population sub-structure has a greater effect on variation at mitochondrial DNA than nuclear DNA because of the smaller effective population size of mitochondrial DNA. Effective population estimates are not reflective of the true census size of a natural population, and all estimates should be interpreted with caution. Estimates will also likely change with when newer genetic data becomes available. Methods that infer effective population size (Ne) assume simple

population models, but migration, spatial structure, and reproduction which are often found in meta-population dynamics can confound results (Waples 2011).

My hypothesis that estimated effective population sizes (Ne) are relative to island size was somewhat supported by contemporary effective population size results, based on microsatellite linkage disequilibrium. Negative values of Ne in this program are usually consistent with very large populations and confidence intervals that include infinity. Maui Ne was ~800, but the confidence interval also included infinity, making the estimate unreliable. My limited number of loci and samples is probably not sufficient to distinguish moderate and large population sizes (500 < Ne < 5000) (Luikart *et al.* 2010). O`ahu, the smallest island, had an Ne of 17 bats, and was the only estimate with a legitimate confidence interval, upwards of ~50 bats. It's possible that for Maui and O`ahu, the estimates were confounded by the sample sizes. I relied heavily on carcasses from wind facilities on O`ahu and Maui; these estimates may reflect the local population around those areas.

Contemporary long-term effective population estimates for Hawaiian hoary bats from this study are 91 - 17,834 bats, while North American hoary bat estimates (for a small region of Texas and Minnesota) using COI sequence data and the same mutation rate models reported 2,203 – 220,329 bats. I reported both high and low mutation rates for both SMM and IAM models because evolutionary mutation rates can vary across and within loci, but most loci behavior can be described to fall between the IAM and SMM models (Piry *et al.* 1999). Homoplasy is also allowed within the SMM model, and is expected to occur in microsatellites due to their small size and naturally high mutation rates. (Estoup *et al.* 2002). The IAM and SMM models produced somewhat similar estimates for each island, and suggest each island may need between a hundred to ten thousand bats to maintain an effectively reproducing population.

Historic female effective population size estimates were different depending on the mutation rate used and grouping or splitting by clade. Historic sizes on each island may have

been in the thousands, tens of thousands, and potentially hundreds of thousands, but the precision of this estimate is unknown because confidence intervals cannot be calculated. Historic female effective population estimates for Hawaiian hoary bats from this study are: 5,971 – 26,501 (Clade 1 +2 high mutation rate), 81,094 – 359,932 (Clade 1+2 low mutation rate), 2,434 – 7,349 (Clade 1 high mutation rate), 33,601 – 99,808 (Clade 1 low mutation rate) and 5,511 – 10,104 (Clade 2 high mutation rate), 74,856 – 137, 236 (Clade 2 low mutation rate). For comparison, North American hoary bat estimates (for a small region of Texas and Minnesota) using COI sequence data and the same mutation rate models reported 16,723 - 225,781 bats. Molecular estimates of effective population size between historic and contemporary estimates overlap when confidence intervals of the latter are taken into consideration.

Population bottlenecks are distinguished by relatively low allelic richness caused by loss of rare alleles, which has little effect on heterozygoisty. When the number of individuals is reduced, this leads to an excess of common microsatellite alleles compared to rare alleles than would be expected under equilibrium conditions (Cornuet and Luikart 1996). The M-ratio test gave evidence of population bottlnecks in the overall bat population, particually for populations on Hawai`i and O`ahu. The measures I used may not be able to detect recent decline or bottlenecks in Hawaiian hoary bats, since not all genetic diversity measures have power to detect changes over typical time managment frames for species with large effective population sizes, those with high pre-bottleneck diversity, and high connectivity (Peery et al 2012).

Population expansion or growth can be signaled by significantly negative Tajima's D values, high haplotype diversity and low sequence diversity. Tajima's D was significantly negative for Hawai`i Island, potentially reflecting recent population expansion which is congruent with gene flow and haplotype diversity results. Tajima's D values for O`ahu and Maui islands changed from positive to negative when bats were separated by mitochondrial clade. For clade 1, all islands showed negative Tajima's D values, with Hawai`i and O`ahu being

significant. For clade 2, only O`ahu had a significantly negative D value, and it was the highest of all values across clades. Bats were not known to occur on O`ahu in any number until the last decade, and this may reflect an actual expansion of a small population on O`ahu. However, Tajima's D must be interpreted carefully, in populations with no demographic changes negative results can indicate positive selection. Bottleneck testing would be greatly improved with demographic coalescent methods, which would put estimated dates on expansions, since these results have no time context.

Morphological distinctions and potential hybridization

Sex determination was succesful in a high percentage (94%) of individuals tested for this study. Because I was able to confirm sex for many unknown samples, sample sizes for statistical population and morphological tests was greatly improved, especially for the islands of O`ahu and Maui. The sex determination technique initially testing during this study was recently employed identify the sex of carcasses and investigate the sex ratios of Hawaiian hoary bats recovered at wind facilities on Maui and O`ahu Islands (Pinzari & Bonaccorso 2018). The methodology improved the demographic data collection at local wind facilities and showed that currently, both male and female bats are being taken equally by collisions with wind turbines.

Female bats were found to be larger than male bats regardless of maternal lineage, and this sexual dimorphism trend which Jacobs (1996) demonstrated for Hawai`i Island bats holds true for bats measured across all islands in this study, which supports my hypothesis of morphological differences between the sexes. Bat body and head size did not vary significantly between island or clade. Compared to North American hoary bats, Hawaiian hoary bats have undergone a reduction in body size and are 45% smaller (Jacobs 1996). I found forearm length ranges for females and males to be almost identical to those reported by Jacobs (1996) and significantly smaller than North American hoary bats measured in the same study.

The occurrence of two maternal lineages or clades in Hawaiian hoary bats was substantiated with additional evidence in this study, however my results do not support the presence of two distinct species of Hawaiian hoary bats within the islands. A divergence estimate higher than 4% has been used for intraspecific divergence in bats (Ziegler *et al.* 2016), but this represents matriarchal lineage only. I found divergence rates for COI of 2.95% between Clade1/Hawaiian and Clade2 hoary bats. Other rates reported are 3.04% for COI (Russell *et al.* 2015) and 4% for cytochrome b (cytb) (Baird *et al.* 2017). Analyzing divergence with a greater number of nuclear genetic markers would provide for more precise estimates of population structure or admixture, as is necessary to resolve this question.

I did not uncover highly significant differences in body size, when comparing head and wing size between mitochondrial clades, and this could be due to limited sampling or potential hybridization between clades. Baird *et al.* (2017) proposed hybridization, due to finding a single bat on Hawai`i Island with 'mismatched' genotypes, North American (Clade 2) mitochondrial DNA and Hawai`i (Clade 1) nuclear DNA. Unfortunately, data from this individual was not accessible for this study, however I examined its preserved skin at the Bishop Museum, and it appears to look physically similar to other Hawai`i Island bats (Clade 1) in size and fur coloration. Hybridization is likely the best explanation for the lack of significant difference in body size between Clade 1 bats (colonization more than 100, 000 years ago) and Clade 2 bats (colonization less than 1, 000 years ago). It is unlikely that changes in Clade 2 bat body size would happen in such a short evolutionary time period.

Potential hybridization between maternal lineages may explain why measures of population differentiation by island (F_{ST}) are subtle for nuclear microsatellite loci, contribute to the mixed ancestry patterns seen on each island in the Structure analysis, and dampen measures of inter-island gene flow. I also found no evidence that microsatellite alleles segregated by clade when I analyzed the topology of a neighbor-joining tree of Nei's standard genetic distance. The

neighbor-joining tree of nuclear genotypes for three microsatellite loci shows mixture between bats with mitochondrial clades 1 and 2, as well as mixture of alleles across islands.

Limitations and Future Studies

Population genetics studies of non-model organisms are often limited by factors related to sampling (numbers of individuals and loci), as well as monetary costs of chosen laboratory methods. In this study, an adequate number of individuals was sequenced for the mitochondrial marker COI to explore population structure across three islands and two clades; however, only three of six loci provided useful information for microsatellite analyses in a reduced subset of individuals from only three islands. With only three loci available for many genetic tests I was unable to further divide data into clade without facing serious issues with low sample sizes. The three available loci had generally low allelic variability and thus may not be fully representative of differences between island populations, for instance LcO was monomorphic in Hawai`i and O`ahu populations. I encountered problems that are frequently associated with model-based methods using microsatellite data: weak structure, confounding effect of incomplete lineage sorting, and the need for more variable loci to more accurately detect structure, admixture, and hybridization (Putman & Carbone 2014). This study would have benefited from more samples from Kaua`i Island to quantify if additional haplotypes are present. Additional genetic markers, such as cytochrome b, or a larger set (>12) of microsatellite loci would allow for tests of potential substructure across all islands.

This study also would be improved by the addition of demographic coalescent approaches to better understand Hawaiian bat population founding events and population trajectories; more accurate dating of bottlenecks and expansions would complement genetic diversity and population estimation information. Based on disagreement among proposed colonization dates in the literature, this topic is still under active scientific debate.

Statistical estimation techniques of effective population sizes are prone to uncertainty, and different tests often produce different results depending on which mutation rate is used. I also was limited to one of two methods for contemporary effective population size estimation; the temporal method available in NeEstimator required a much larger number of bats from each island and each year sampled. Again, incorporating new data from post-2017 years might improve the estimates, and use of the secondary temporal method.

Although I found some evidence of morphological differences between mitochondrial clades in skull length, more samples, an even sampling distribution across islands, and the addition of an allometric trait analyses is likely necessary to uncover informative phenotypic characters that could be used to address potential subspecies within Hawaii. I was limited in the number of available bats with both genetic and phenotypic information. This issue could be resolved with the addition of tissue samples from museum skulls already measured and the addition of skulls from newly collected carcasses. Fur coloration would be a useful character to investigate within Hawaiian hoary bats. The extent of white frost on the tips of guard hairs could be measured and compared between the sexes and maternal lineages. Bat species are also distinguished frequently by their echolocation calls; future studies would benefit from recording voucher calls of live bats when captured for genetic sampling. Subsequent comparisons could focus on characterizing any potential differences found in vocalizations between sexes and clades. One could also compare ultrasonic acoustic survey records between islands where clades co-occur such as O`ahu and Maui, to islands suspected to have mainly singular clade bat ancestry, such as Hawai`i and Kaua`i.

As a next logical step in conservation genetics research for Hawaiian hoary bats, I am collaborating with a team of population geneticists to employ genomic-level sequencing techniques; which by comparing hundreds of thousands of single nucleotide polymorphisms (SNPs). This will provide a higher resolution assessment of taxonomic divergence from the North American hoary bat, and could be used to refine estimates of population structure and

migration patterns, and produce more precise estimates of historic and contemporary island effective population sizes. The costs of genomic sequencing methods have been decreasing, and acquisition of fresh tissues from both capture and release studies and local bat carcasses obtained from wind turbine facilities is an attainable conservation goal. Data produced from genome-level studies could also aid in the construction of a reference genome for this endangered species and be used to create a suite of island specific microsatellite markers to monitor changes in local genetic diversity and population trajectories on islands where bats experience the greatest threats.

Management implications for Hawaiian hoary bats

For island bats, the influences of harvesting, intrinsic population factors and natural disasters have the largest negative effects on their survival (Jones *et al.* 2009). Hawaiian hoary bat populations may be negatively affected by increased fatal collisions from wind turbines, and would be most concerning on O`ahu. The true abundance of bat on each Hawaiian island is currently unknown. If long-term acoustic recordings reflect density levels, then the O`ahu population would be the lowest based on recent acoustic study findings (Gorresen *et al.* 2015, Gorresen *et al.* 2018, Bonaccorso *et al.* 2019, Starcevich *et al.* 2019) compared to the high volume of recordings from acoustic studies on the islands of Maui (Todd *et al.* 2016, Pinzari *et al.* 2019), and Hawai`i (Gorresen *et al.* 2013). Estimates of contemporary effective population sizes from this study also indicate that the O`ahu and Maui populations may be smaller than Hawai`i. The extent of available habitat for roosting and foraging may also constrain breeding populations of bats on O`ahu and Maui. Threats from habitat loss and collisions from wind turbines placed within bat habitats or commuting zones could certainly wipe out an important part of the population of bats on O`ahu. Overall, a decline in bat numbers on O`ahu and Maui has the potential to reduce the genetic diversity of current Hawaiian bat populations across the state. Unfortunately, captive breeding and translocation of bats from one island to another would not be an economically feasible or realistic option for this species.

It is also important to note that threat of fatal collisions wind turbines affects all bats, regardless of sex, mitochondrial clade, or potentially described subspecies. During my analyses of this tissue collection, I found that both sexes and both clades were represented in the bat fatalities I sampled from wind farm facilities on O`ahu and Maui (Pinzari *et al.* 2018). A recent population viability analysis by Frick *et al.* (2017), demonstrated the mortality from wind turbines may drastically reduce population size and increase the risk of extinction for the North American hoary bat. According to their model, a population of 2.5 million hoary bats with an assumed "normal" population growth rate ($\lambda = 1.01$), under current wind turbine mortality rates, could experience a 90% population decline in 50 years. It is extremely unlikely that Hawaiian bat population census sizes and genetic population structure mirror that of the continental United States; local island populations are minuscule in comparison. The North American hoary bat is a long distance migrant, with a genetically diverse, large, panmictic population, whereas Hawaiian bat populations have shown substructure and potentially limited migration between islands as demonstrated in this study. The Frick study used a conservative fatality estimate based on 75,570 Megawatts (MW) of installed wind generated energy. Currently, the Hawai`i Clean Energy Initiative aims to make the state completely fossil fuel free by 2045, with a goal of achieving 100% energy from clean, renewable resources such as wind power. As of 2019, Hawai`i had 206 MW of installed capacity, at least two upcoming projects will increase that number to 230 MW. According to a 2010 report by the National Renewable Energy Laboratory, Hawai`i has the potential capacity to install up to 3,000 MW of wind power. Although at full capacity, this is only about 4% of installed megawatts compared to North America, the future fatality risks to Hawaiian hoary bats will undoubtedly increase on an isolated population of bats already restricted in number and available habitat. And without mitigation or deterrent solutions that lessen these impacts or positively affect local bat population growth, wind fatalities could still lead to individual island population declines and possible extinctions in an even shorter time frame.

The IUCN Red List includes global assessments for subspecies, and defines geographically separate subpopulations of a species as "those populations that are so isolated from others of the same species that it is considered extremely unlikely that there is any genetic interchange…listings of such subpopulations should be restricted to those that have been isolated for a long period of time" (IUCN, 2012). The Hawaiian hoary bat (*L. cinereus semotus*) is scientifically regarded as a sub-species of the Hoary Bat, and the ICUN considers it a "range extension" from the North American population. Current scientific data supports colonization events in Hawai`i as occurring at a minimum of 800 years before present, and an even older colonization events at 30,000 years before present (Baird *et al.* 2015, Russell et al 2015, Baird *et al.* 2017). There is no evidence from genetic studies that Hawaiian hoary bats maintain a contemporary genetic "interchange" to North American populations: they remain physically separated by the Pacific Ocean, and Hawaiian genetic haplotypes do not appear in North American population studies of *L. cinereus* (Baird et.al 2017). *L.c. semotus* also appears phenotypically different to Hoary bats in size (Jacobs 1996), and potentially in coat color (personal observation). With further genome-level study and a thorough morphological comparison of Hawaiian bats to North American museum specimens, it is possible that strong biological evidence would support elevation of *L. c. semotus* to full species level. I believe the genetic evidence gathered from this study combined with information from Russell *et al.* (2015) and Baird *et al.* (2017) could aid the IUCN in updating scientific information on the Hawaiian hoary bat and I suggest a review of its current listed status of Least Concern. However, the ICUN will only accept new assessments of subspecies and subpopulations if a global assessment of the species as a whole is provided. This would require a global assessment of the North American hoary bat. Recent population decline and extinction risk estimates from a study of population viability under wind energy mortality in the North American hoary bat also warrants re-evaluation of the status of hoary bats from least concern to a threatened category on the IUCN Red List (Frick *et al.* 2017). Fortunately, Conenna *et al.* (2017) found a positive association

between research effort (publications) and improvement in Red List category for island endemic bats, even though extinction risk did not trigger more scientific attention.

Based on these findings, I recommend the following to enhance the conservation management of Hawaiian hoary bats: 1) bats have distinct island populations and bat habitat conservation plans should mitigate for bats on the same island where threats negatively impact bats from that population; 2) cumulative effects of bat fatalities should be considered for each island as well as the entire population across all islands; 3) federal and state management plans for the species should consider subpopulation structure, persistence of subpopulations, and support further research to refine island management units and asses genetic diversity and population estimates on islands without wind turbines 4) conservation strategies for Hawaiian bats will benefit from further genetic research on a temporal scale, thus efforts should continue to gather and characterize biological samples from post-construction monitoring (carcasses) and museum collections.

In conclusion, these results demonstrate the that Hawaiian hoary bats are genetically diverse, and population estimates based on the low mutation rate indicate evidence of fairly large historic population sizes, but considerably smaller population estimates when the higher mutation rate is considered. Mitochondrial DNA collected and examined in this study supports the theory of multiple founding events by North American hoary bats to the Hawaiian Islands and subsequent subpopulation genetic structure by island. Most importantly, the application and use of carcasses and museum specimens to study this species has led to increased sampling opportunities, an ability to gather demographic information in areas where fieldwork is difficult to conduct, and contributes valuable sex ratio information when the sex of a bat cannot be determined through physical examination. Furthermore, information provided through conservation genetics approaches will aid local wildlife management agencies and contribute to Hawai`i's future goals of balancing natural resource conservation with necessary sustainable agricultural and energy practices.

TABLES

Table 1. Number of mitochondrial sequences (mtDNA) and microsatellite genotypes (nDNA) obtained from male and female Hawaiian hoary bats collected on four islands from 2005 – 2017. Female (F), male (M), unknown (U).

		mtDNA			nDNA			
Island	Year	F	M	Total	F	M	U	Total
Hawai`i	2005	1	3	4	0	0	0	0
	2006	6	1	7	0	0	0	0
	2007	0	1	1	0	0	0	0
	2008	1	1	2	0	0	0	0
	2009	8	7	15	0	1	0	1
	2010	10	5	15	2	4	0	6
	2011	1	0	1	2	0	0	2
	2012	1	1	2	1	0	1	2
	2013	3	2	5	3	1	0	4
	2014	0	1	1	0	1	0	1
	2015	2	2	4	2	1	1	4
	2017	0	0	0	1	0	0	1
	ALL	**33**	**24**	**57**	**11**	**8**	**2**	**21**
Maui	2009	2	0	2	2	0	0	2
	2010	3	1	4	3	1	0	4
	2011	2	1	3	2	1	0	3
	2012	0	2	2	0	2	0	2
	2013	3	7	10	3	4	0	7
	2014	4	3	7	4	1	0	5
	2015	0	1	1	0	1	0	1
	2016	5	8	13	4	5	1	10
	ALL	**19**	**23**	**42**	**18**	**15**	**1**	**34**
O`ahu	2011	2	0	2	2	0	0	2
	2012	2	1	3	2	1	0	3
	2013	3	10	13	3	10	0	13
	2014	4	6	10	4	6	0	10
	2015	1	6	7	1	6	0	7
	2016	2	1	3	2	1	0	3
	ALL	**14**	**24**	**38**	**14**	**24**	**0**	**38**
Kaua`i	2008	0	1	1	0	0	0	0
	2009	0	1	1	0	0	0	0
	2015	1	0	1	0	0	0	0
	ALL	**1**	**2**	**3**	**0**	**0**	**0**	**0**
	TOTALS	**67**	**73**	**140**	**43**	**47**	**3**	**93**

Table 2. Table of 23 individuals used in skull morphology analyses, including the Bernice Pauahi Bishop Museum (BPBM) accession number, collection date (year), locality (island) of collection, sex, and data used (length or width).

BPBM	Date (Year)	Island	Sex	Data (L, W)
146222	1985	Kaua`i	M	L, W
161228	1984	Hawai`i	M	L
175758	1985	Kaua`i	M	L, W
175759	1986	Kaua`i	M	L, W
177883	1988	Hawai`i	M	L, W
178452	1989	Hawai`i	M	L, W
178453	na	Hawai`i	F	L, W
178911	1993	Maui	F	L
183973	1996	Maui	M	L, W
184308	1999	Maui	M	L
184834	2002	Hawai`i	F	L, W
185003	2000	Maui	M	L, W
185241	2007	Hawai`i	F	L, W
185283	2008	Maui	F	W
185478	2009	Kaua`i	M	W
185479	2009	Maui	F	L, W
185519	2010	Maui	M	W
185520	2010	Maui	F	L, W
185538	2009	Hawai`i	F	L, W
185539	2010	Maui	F	L, W
185540	2010	Maui	M	L, W
185541	2010	Maui	F	L, W
185710	2012	Maui	M	L, W

Table 3. Primer details of six microsatellite candidate loci for genotyping in the Hawaiian hoary bat. Repeats, size range, and number of alleles represent those found with the loci in North American hoary bats according to Korstian *et al.* 2014.

Group	Locus Name	Forward Primer	Reverse Primer	Repeat	Size Range (bp)	Alleles
E	CotoG12	TGCAAGTCTTAACTCACCTCATT	CCACTCCCCTAGTTTTCATCTAC	(AC) 23	220-256	17
E	LcM	CTGAGCTGGTTGTGCAGAAG	CTGCATCCCCAGCGCT	(AG) 13	188-272	29
E	LcO	GAGGTCCTGTTTGTGCCAAG	CAGGTCCGCGGTTAATTACG	(AC) 10	195-211	9
F	LcP	ATCTCACATGCTGGGTTAATTT	GCAGGAAAGGGGAGAATCTC	(GTTT) 7	244-292	5
F	LcU	GAAACAGCCTGCATTTACACA	AAATGTCTCCCCTGTCCTCA	(ATCT) 7	234-294	15
F	LbG	CTGGGATCACATGGGGAACT	ATGTGGACTCAGCTCACACA	(AC) 11	209-239	16

Table 4. Average pairwise genetic distances and percent sequence divergence for COI within and between clades; and for each clade, within and between islands.

	Clade 1	Clade 2/ North America	Clade 1/ Hawai`i	Clade 1/ Maui	Clade 1/ O`ahu	Clade 1/ Kaua`i	Clade 2/ O`ahu	Clade 2/ Maui
Clade 1	1.67 (0.25%)	-	-	-	-	-	-	-
Clade 2/North America	19.36 (2.95%)	2.05 (0.31%)	-	-	-	-	-	-
Clade 1/Hawai`i	-	-	0.44 (0.07%)	-	-	-	-	-
Clade 1/Maui	-	-	1.20 (0.18%)	0.67 (0.10%)	-	-	-	-
Clade 1/O`ahu	-	-	2.34 (0.36%)	1.45 (0.22%)	0.23 (0.04%)	-	-	-
Clade 1/Kaua`i	-	-	2.23 (0.34%)	1.33 (0.20%)	0.12 (0.02%)	0.00 (0%)	-	-
Clade 2/O`ahu	-	-	-	-	-	-	0.00 (0%)	-
Clade 2/Maui	-	-	-	-	-	-	2.11 (0.32%)	2.54 (0.39%)

Table 5. Mitchondrial haplotype data characteristics for each island using all bats; including number of sequences obtained (N), number of haplotypes (H), number of polymorphic sites, gene diversity (H) with standard deviation (SD), nucleotide diversity (π) with standard deviation, Tajima's D, and Watterson's estimator (θ). *P < 0.05, **P<0.002, ***P<0.0001

Island	N	H	Number of Polymorphic sites	Haplotype diversity (h) ± SD	Nucleotide diversity (π) ± SD	Tajima's D	θ
Hawai`i	57	7	6	0.39 ± 0.00	0.0006 ± 0.00	-1.64*	1.3
Maui	42	6	25	0.68 ± 0.05	0.0104 ± 0.00	0.64	5.77
O`ahu	38	4	22	0.45 ± 0.08	0.0120 ± 0.00	1.61	5.38
Kaua`i	3	1	0	-	-	-	-
All Islands	140	15	31	0.80 ± 0.01	0.0148 ± 0.00	0.15	3.11

Table 6. Mitchondrial haplotype data characteristics for each clade as inferred from COI; including number of sequences obtained (N), number of haplotypes (H), number of polymorphic sites, gene diversity (H) with standard deviation (SD) and nucleotide diversity (π) with standard deviation, Tajima's D, and Watterson's estimator (θ). *P < 0.05, **P<0.002, ***P<0.0001

Clade 1							
Island	N	H	Number of Polymorphic sites	Haplotype diversity (h) ± SD	Nucleotide diversity (π) ± SD	Tajima's D	θ
Hawai`i	57	7	6	0.39 ± 0.08	0.0006 ± 0.00	-1.64**	1.3
Maui	7	3	4	0.52 ± 0.20	0.0017 ± 0.00	-1.43	1.6
O`ahu	25	2	2	0.08 ± 0.07	0.0002 ± 0.00	-1.51*	0.53
Kaua`i	3	1	0	-	-	-	-
All Islands	92	11	11	0.68 ± 0.03	0.0019 ± 0.00	-1.15	0.86

Clade 2							
Island	N	H	Number of Polymorphic sites	Haplotype diversity (h) ± SD	Nucleotide diversity (π) ± SD	Tajima's D	θ
Maui	36	3	5	0.55 ± 0.05	0.0037 ± 0.00	2.7	1.2
O`ahu	9	2	6	0.22 ± 0.16	0.0020 ± 0.00	-1.72*	2.2
All Islands	45	4	6	0.52 ± 0.06	0.0035 ± 0.000	0.49	1.7

Table 7. Microsatellite data characteristics for six tested loci by island, including the number of alleles observed, observed and expected heterozygosity (Ho/He), significance of Hardy-Weinberg (H-W) tests, and null alelle frequencies. Bolded Hardy-Weinberg p-values are significant. Bolded loci contain null alleles; frequency of null alleles is based on Van Oosterhout *et al.* (2004).

Hawai`i						
Loci	Alleles	Genotypes	Ho	He	H-W test p-value	Frequency of null alleles
CotoG12	10	21	0.5714	0.8281	**< 0.001**	**0.1504**
LcM	12	21	0.6190	0.6701	0.3043	0.0231
LcO	1	21	mono	-	-	0.000
LcP	2	21	0.0476	0.0476	1.000	-0.0241
LcU	5	21	0.8571	0.7537	0.3698	-0.0804
LbG	7	21	0.4285	0.7363	**< 0.001**	**0.1997**
Maui						
Loci	Alleles	Genotypes	Ho	He	HWE p-value	Frequency of null alleles
CotoG12	8	32	0.5000	0.8070	**< 0.001**	**0.1861**
LcM	14	32	0.6875	0.8462	**< 0.001**	**0.0830**
LcO	3	32	0.1250	0.1205	1.000	-0.0638
LcP	3	33	0.3939	0.3268	0.6402	-0.2175
LcU	6	34	0.6764	0.6268	0.5327	-0.0459
LbG	6	34	0.3529	0.6150	**< 0.001**	**0.1997**
O`ahu						
Loci	Alleles	Genotypes	Ho	He	HWE p-value	Frequency of null alleles
CotoG12	7	34	0.6176	0.7699	**< 0.001**	**0.0506**
LcM	8	37	0.5675	0.7356	**0.008**	**0.0863**
LcO	1	38	mono	-	-	0.000
LcP	3	36	0.2777	0.2461	1.000	-0.1480
LcU	5	38	0.7368	0.7035	0.7112	-0.0390
LbG	7	38	0.6052	0.6928	**0.002**	**0.0510**

Table 8. Diversity measures for three microsatellite loci using the "nearly unbiased" estimation in SMOGD (Crawford 2010). Harmonic mean of population size (Ñ), Nei's within-subpopulation heterozygosity (H_{s_est}), total-subpopulation heterozygosity (H_{T_est}), relative differentiation (G_{ST_est}), genetic differentiation (G'_{ST_est}), and Jost's actual differentiation (D_{est}).

Loci	Ñ	H_{S_est}	H_{T_est}	G_{ST_est}	G'_{ST_est}	D_{est}
LcO	28.5212	0.0403	0.0412	0.0228	0.0243	0.0015
LcP	28.3823	0.2073	0.2144	0.0329	0.0459	0.0134
LcU	29.0285	0.6945	0.7046	0.0142	0.0627	0.0492

Table 9. Pairwise F_{st} comparisons calculated using mitchondrial data among four islands for all bats. Pairwise Fst values are located below the diagonal and relative p-values are above the diagonal. Bolded values indicate statistically significant α = 0.05.

	Hawai`i	**Kaua`i**	**Maui**	**O`ahu**
Hawai`i	-	**0.00**	**0.00**	**0.00**
Kaua`i	0.656	-	**0.00**	0.621
Maui	0.469	0.459	-	**0.00**
O`ahu	0.576	-0.015	0.351	-

Table 10. Pairwise Fst comparisons using mitchondrial data for each clade as inferred by COI. Pairwise Fst values are located below the diagonal and relative p-values are above the diagonal. Bolded values indicate statistically significant α = 0.05.

Clade 1				
	Hawai`i	**Kaua`i**	**Maui**	**O`ahu**
Hawai`i	-	**0.00**	**0.00**	**0.00**
Kaua`i	0.656	-	**0.00**	0.99
Maui	0.565	0.634	-	**0.00**
O`ahu	0.714	-0.197	0.810	-

Clade 2		
	Maui	**O`ahu**
Maui	-	**0.05**
O`ahu	0.135	-

Table 11. Summary of analysis of molecular variance (AMOVA) for pairwise differences in genetic distance of mitochondrial COI in all bats, Clade 1, Clade 2, and 3 microsatellite loci. Bold p-values indicate a significant test result at $p < 0.01$. Islands included, Hawai`i (H), Maui (M), O`ahu (O), and Kaua`i (K).

Group	Variance among islands	Variance within islands	p-value
All Bats (H, M, O, K)	65.02%	34.98%	**0.000**
Clade 1 (H, M, O, K)	80.00%	20.00%	**0.000**
Clade 2 (O, M)	11.04%	88.96%	0.095
Microsatellites (H, M, O)	2.45%	-9.71% 107.25% (within individuals)	**0.005**

Table 12. Pairwise Fst comparisons using microsatellite data for all bats among islands. Pairwise Fst values from Arlequin are located below the diagonal and Fst values that have been adjusted for null alleles using the ENA method (Chapuis and Estoup 2007) in FreeNA are above the diagonal. Significant values are bolded.

	Hawai`i	**Maui**	**O`ahu**
Hawai`i	-	0.071	0.057
Maui	**0.056***	-	0.076
O`ahu	**0.034***	0.007	-

Table 13. Pairwise G''_{ST} and Jost's D for each island from GenAlEx. G''_{ST} values are below the diagonal and Jost's D is above the diagonal. Asterisks indicate differentiators that are significantly greater than expected by random (*p < 0.05, **p< 0.005).

	Hawai`i	**Maui**	**O`ahu**
Hawai`i	-	**0.026****	**0.013***
Maui	**0.077****	**-**	0.003
O`ahu	**0.044***	0.008	**-**

Table 14. Results of the sex-biased dispersal test; estimated Fis, Fst, gene diversity (Hs), relatedness (Relat), mean assignment index (mAIc) and variance of the assignment index (vAIc), and correspondent significance levels.

	N	mAIC	vAIC	Fis	Fst	Relat	Hs
Females	46	0.0964	1.0054	-0.1062	0.0103	0.0227	0.3163
Males	47	-0.0943	1.3418	-0.0720	0.0204	0.0430	0.3281
P one-sided test		0.1960	0.1328	0.3893	0.7037	0.6970	0.3058
P two-sided test		0.3942	0.3908	0.6872	0.7499	0.7621	0.6610

Table 15. Estimates of historic female effective population size (Ne) calculated from *Lasiurus cinereus semotus* mitochondrial sequences based on high and low mutation rates for cytochrome b in the family Vespertilionidae. Data was analyzed using groupings of clade and island, plus an average value per group.

Group	Island	High Rate (μ)	Low Rate (μ)
Clade 1+ 2	Hawai`i	5, 971	81, 094
	Maui	26, 501	359, 932
	O`ahu	24,710	335, 604
Clade 1	Hawai`i	5, 971	81, 094
	Maui	7, 349	99, 808
	O`ahu	2, 434	33, 061
Clade 2	Maui	5, 511	74, 856
	O`ahu	10, 104	137, 236

Table 16. Estimates of long-term effective population size (Ne) calculated from *Lasiurus cinereus semotus* microsatellite loci using the expected heterozygosity for two different mutation rates in both the stepwise mutation model (SMM) and infinite allele model (IAM), and an average taken from both models and rates.

Island	SMM High/Low	IAM High/Low	Average High/Low
Hawai`i	108 / 10,773	91 / 9,112	99 / 9,943
Maui	178 / 17,834	139 / 13,945	159 / 15,890
O`ahu	143 / 14,259	116 / 11,578	129 / 12,919

Table 17. Estimates of contemporary effective population size (Ne) based on microsatellite linkage disequilibrium, a pcrit of 0.01, and 95% confidence intervals from jackknifing for *Lasiurus cinereus semotus*.

Island	n	Ne	Lower 95% CI	Upper 95% CI
Hawai`i	21	-134.5	108.8	∞
Maui	34	794.7	61.2	∞
O`ahu	38	17.7	7.8	48.6

Table 18. PCA loadings along the first two principal components for wing, skull length, and skull width characters.

	Wing		**Skull Length**		**Skull Width**	
Character	PC 1	PC2	PC1	PC2	PC1	PC2
FA	-0.449	0.569				
D5	-0.495	-0.224				
D3	-0.160	-0.525				
P4.1	-0.352	0.516				
P4.2	-0.357	0.497				
WA	-0.525	-0.391				
B			0.193	-0.920		
CB			0.090	-0.327		
M			0.977	0.212		
MT					-0.711	-0.282
ZW					-0.702	0.312
PL					-0.020	-0.906

Table 19. Summary statistics (mean and standard deviation followed by range and sample size) of skull measurements taken of Hawaiian hoary bats from up to three islands. Islands are in order from northwest to southwest.

Sex	Island	B	CB	MT	M	ZW	PW
Female	Maui	14.44 ± 0.98 13.5 – 16.0 (5)	15.73 ± 0.46 15.2 – 16.2 (4)	12 ± 0.41 5.6 – 12.4 (4)	8.88 ± 0.13 8.7 – 9.0 (5)	11.5 ±0.32 11.2 – 11.9 (4)	4.12 ±0.33 3.8 – 4.5 (4)
	Hawai`i	13.7 ± 0.78 13.1 – 14.8 (4)	15.28 +0.22 15 – 15.5 (4)	10.23 ±3.09 11.6 – 12.1 (4)	8.88 ± 0.22 8.7 – 9.2 (4)	11.1 ± 0.22 10.8 – 11.3 (4)	4.7 ±1.44 3.5 – 6.8 (4)
Male	Kaua`i	13.23 ± 0.12 13.1 – 13.3 (3)	15.17 ±0.06 15.1 -15.2 (3)	8.4 ±0.35 5.2 – 5.8 (3)	8.82 ± 0.15 8.7 – 9.0 (4)	11.2 ±0.26 10.9 – 11.5 (4)	3.85 ±1.22 4.2 – 6.7 (4)
	Maui	14.05 ± 0.97 13.3 – 15.4 (4)	16.1 (1)	12.1 (1)	8.70 ± 0.35 8.4 – 9.2 (4)	10.65 ±0.92 10 – 11.3 (2)	6.02 ±0.50 3.5 -4.2 (2)
	Hawai`i	13.38 ± 1.11 12.5 – 15 (4)	14.95 ±0.45 14.6 - 15.6 (4)	6.58 ±3.28 4.9 -11.5 (4)	8.57 ± 0.32 8.2 – 8.8 (4)	10.36 ±0.26 10.1 – 10.6 (3)	5.63 ±1.76 3.6 – 6.7 (3)

Table 20. Summary statistics (mean and standard deviation followed by range and sample size) of wing measurements taken of Hawaiian hoary bats from three islands. Islands are in order from northwest to southwest.

	Island	FA	D5	D3	P4.1	P4.2
Female	O`ahu	49.73 ± 1.65 47.3 – 52.0 (7)	59.98 ±2.57 56.5 – 62.7 (4)	98.7 ±7.43 92.4 -109.3 (4)	12.3 ±0.25 12 -12.6 (4)	14.7 ±0.46 14.4 – 23.2 (4)
	Maui	49.79 ± 1.6 46.5 – 52 (16)	63.78 ±5.07 56.6 – 74.9 (12)	92.3 ±13.82 67 -104.8 (12)	13.6 ±3.14 10.6 -20.8 (12)	15.45 ±2.55 12.6 – 21.1 (12)
	Hawai`i	50.34 ± 1.33 45 – 52.9 (36)	62.9 ±0.99 62.2 – 63.6 (2)	95.3 ±2.55 93.5 – 97.1 (2)	12.6 ±0.21 12.5 -12.8 (2)	13.9 ±0.42 13.6 – 14.2 (2)
Male	O`ahu	48.16 ± 2.14 43 – 51.5 (15)	60.72 ±7.84 53 – 76 (10)	86.4 ± 17.11 43 – 99.9 (10)	12.6 ±3.29 8.6 -19.1 (10)	14.32 ±6.25 10.5 -23.2 (10)
	Maui	47.52 ± 1.76 43.5 – 50.5 (21)	57.9 ±5.87 49.1 -68.7 (14)	87.1 ±8.82 67 -95 (14)	11.7 ±2.55 9 – 19.5 (14)	12.63 ±3.07 7.8 - 20.4 (14)
	Hawai`i	48 ± 1.57 43 – 50.3 (28)	60.5 ±2.26 58.9 -62.1 (2)	95.3 ±0.85 93.5 – 93.1 (2)	11.1±0.17 11 – 11.25 (2)	12.1 ±1.13 11.3 - 12.9 (2)

FIGURES

Figure 1. General locations of bat tissue samples used in mitochondrial and microsatellite analysis, collected between 2005 and 2017 across four of the major seven Hawaiian Islands.

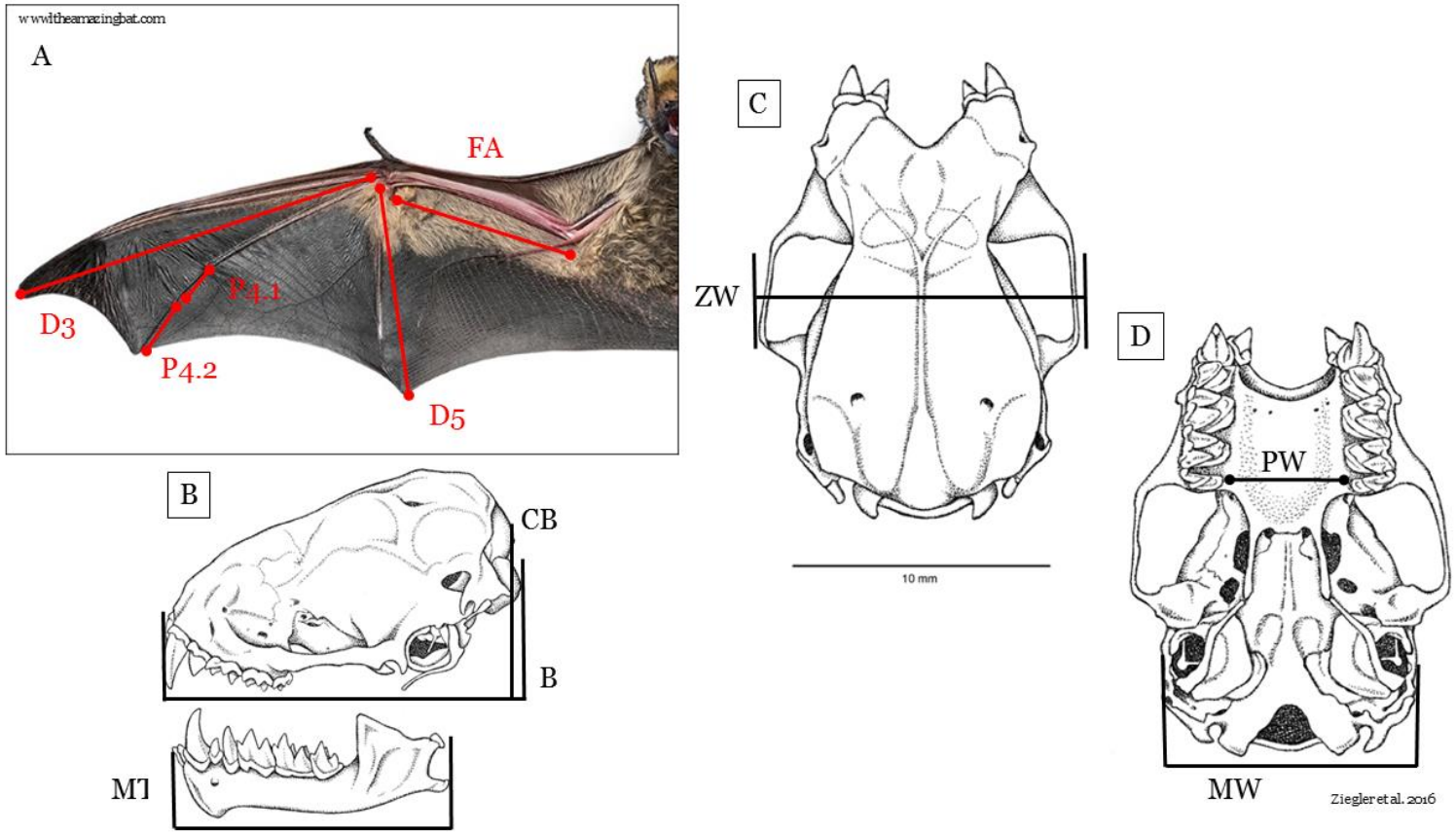


Figure 2. Morphological measurements collected from Hawaiian hoary bats. Extended bat wing illustrating five wing measurements (A), six measurements on the lateral (B), dorsal (C) and ventral (D) views of the skull of *Lasiurus cinereus semotus*. Skull images adapted from Ziegler *et al.* 2016.

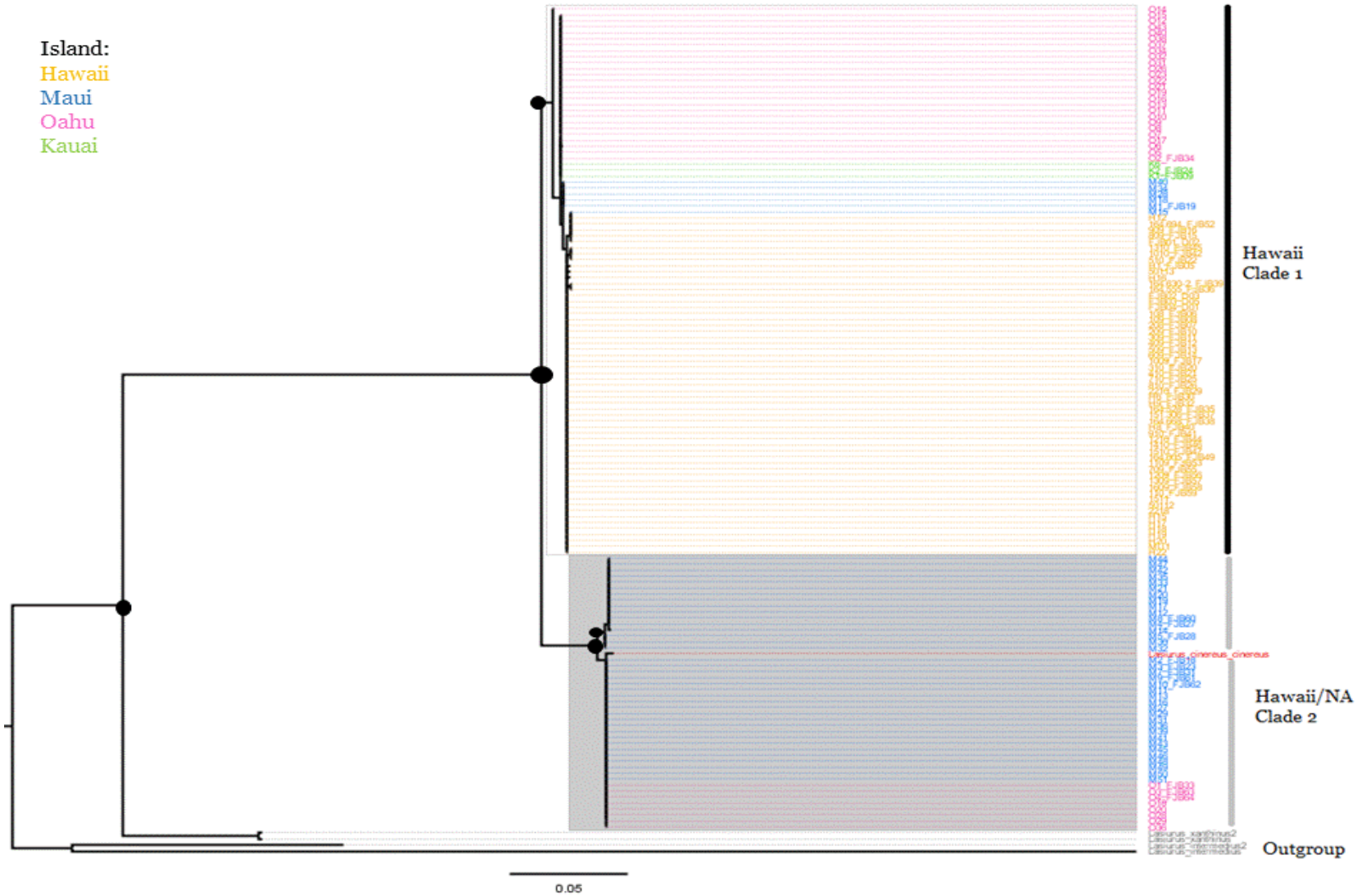


Figure 3. MEGA maximum likelihood mitochondrial phylogeny with tip labels corresponding to individuals of *Lasiurus cinereus semotus* from Hawai`i, Kaua`i, O`ahu, and Maui, including one specimen of *L. cinereus cinereus* from North America, and *L. xanthinus*, *L. intermedius* as outgroups. Nodes with greater than 90% bootstrap support are shown with black dots.

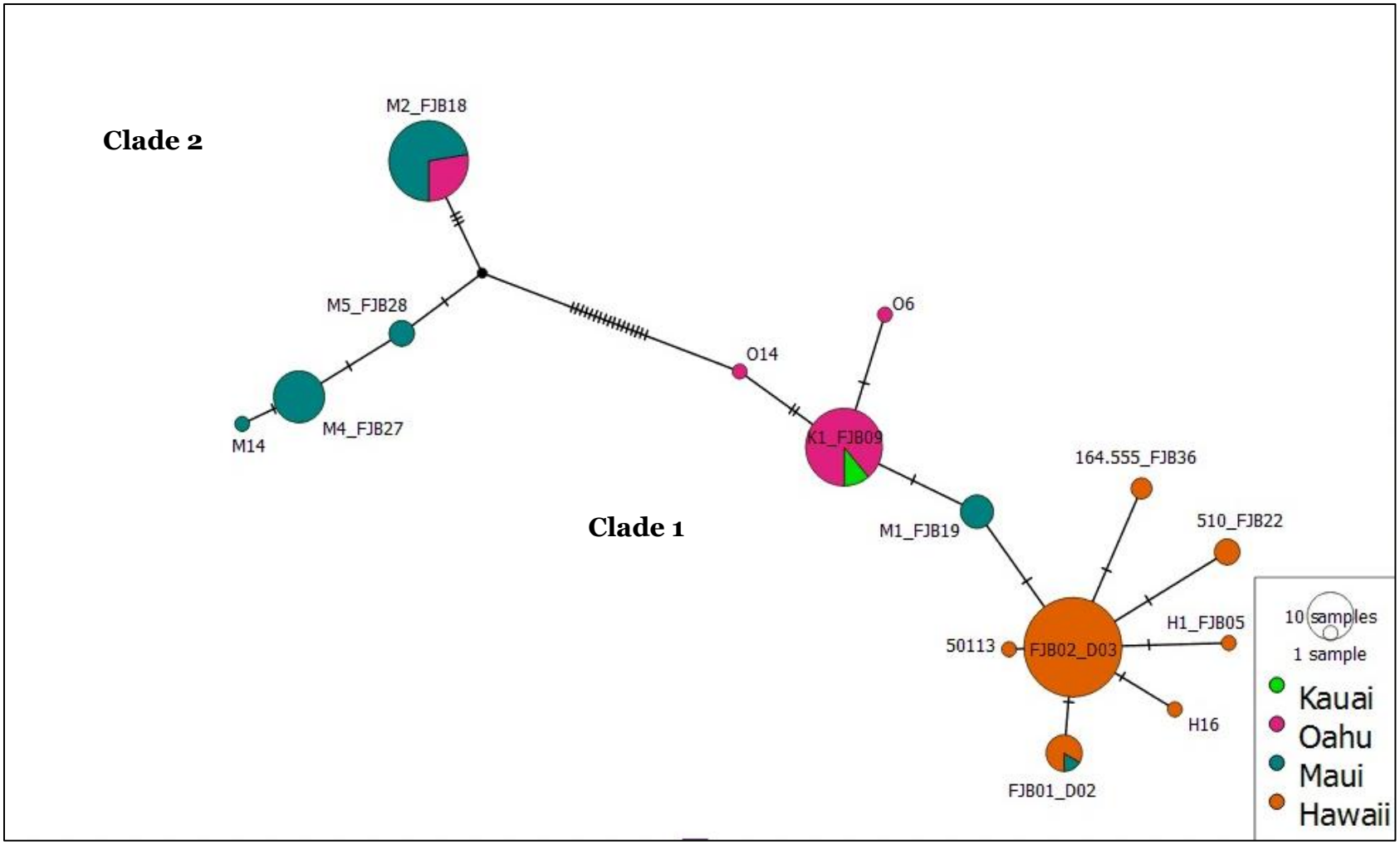


Figure 4. Haplotype parsimony network constructed from 15 unique mitochondrial haplotypes of the COI region of 140 Hawaiian hoary bats. Each colored circle represents a unique haplotype, and islands are defined by separate colors. Each small black circle represents a theoretical haplotype. Each line between circles represents a single base pair change between the two haplotypes. Size of circles is scaled to the approximate relative frequency of that haplotype in the population.

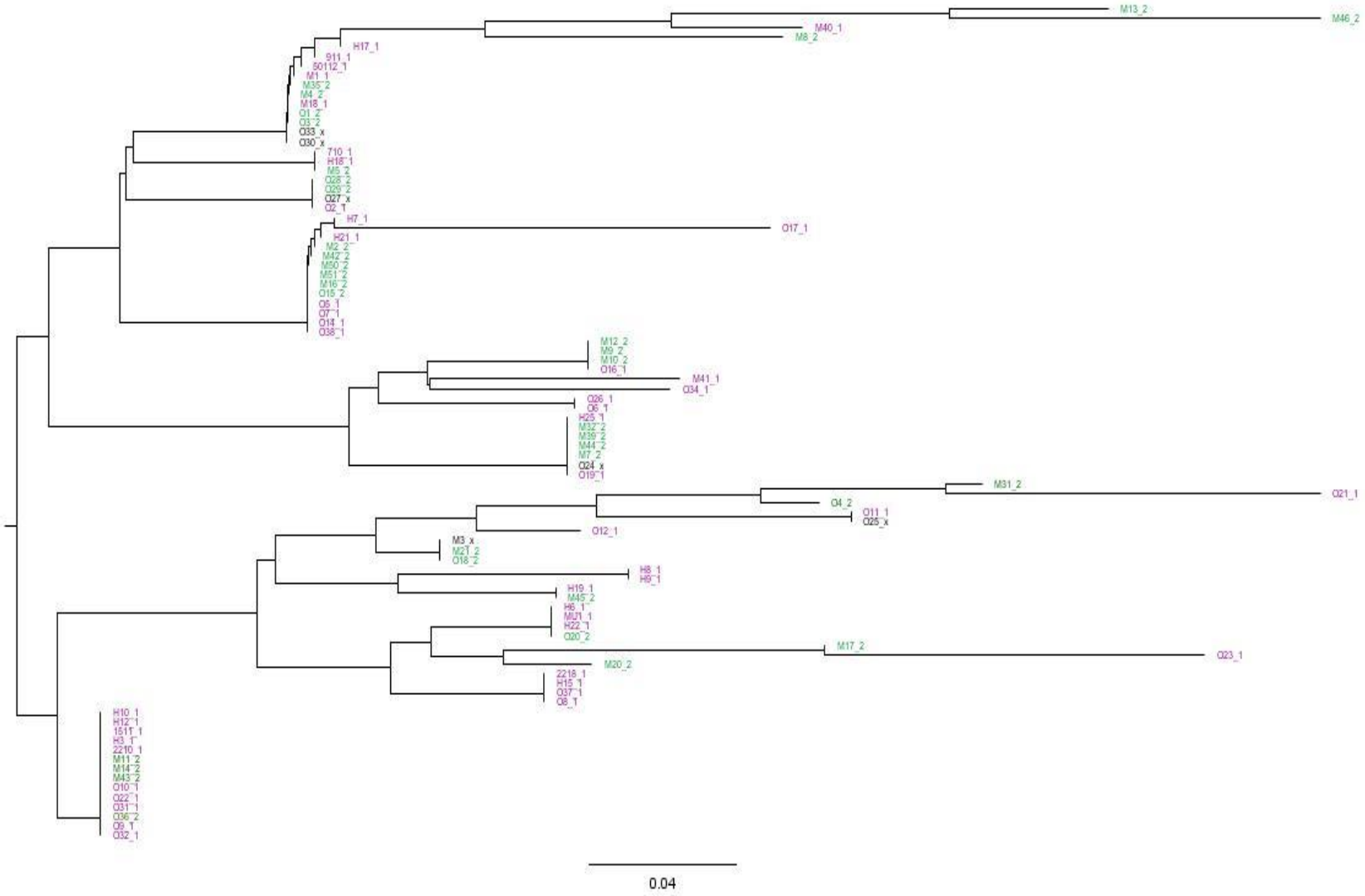


Figure 5. Neighbor-joining phylogeny based on Nei's standard genetic distances between 3 microsatellite loci alleles in 88 Hawaiian hoary bats across the islands of Hawai`i, Maui, and O`ahu. Purple denotes a sample with mitochondrial origin of Clade 1, green denotes a sample with mitochondrial origin of Clade 2.

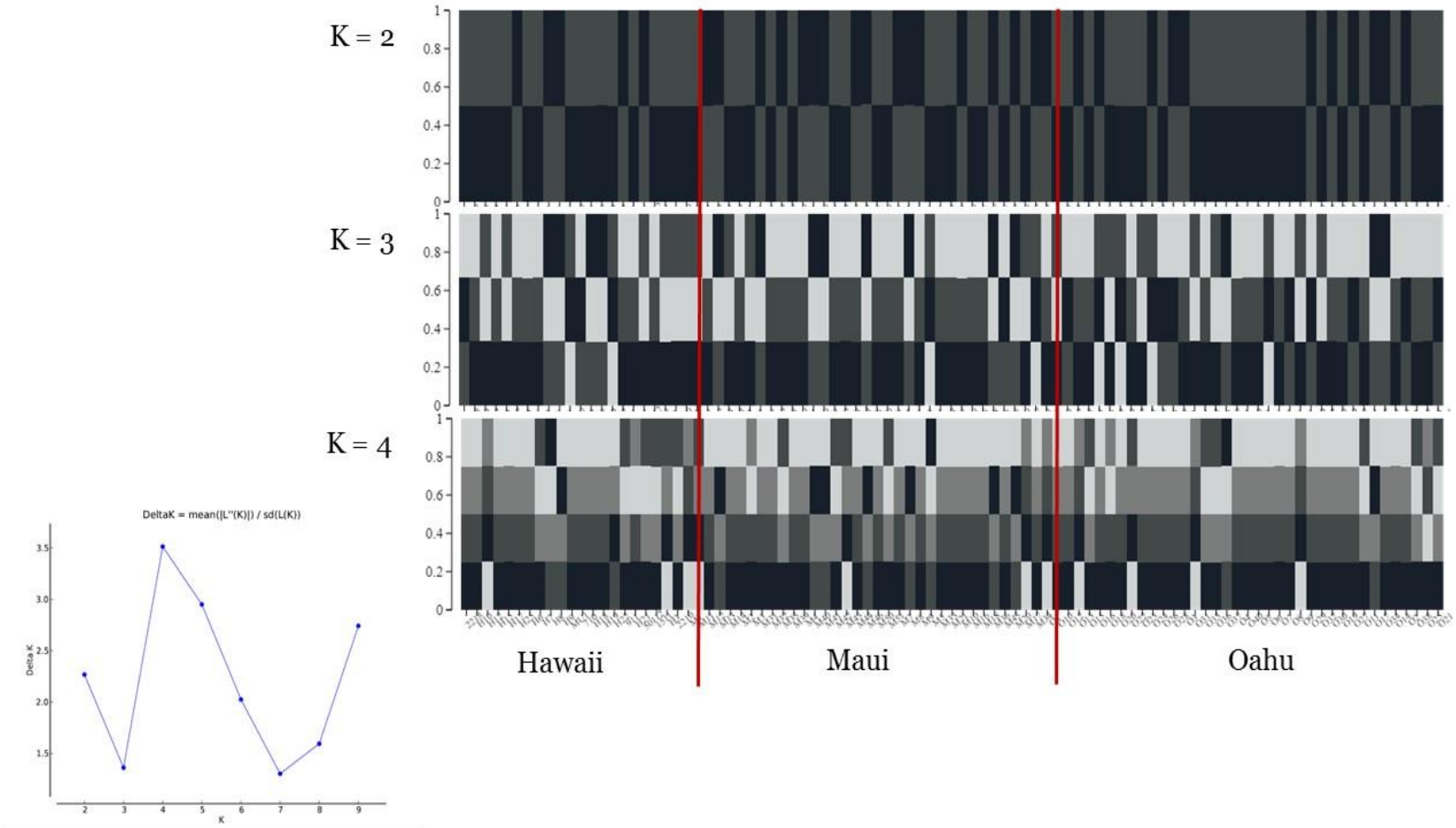


Figure 6. Structure results for 3 microsatellite loci in Hawaiian hoary bats of three islands. Bottom left graph: the most likely K number of genetically distinct clusters (denoted with the highest DeltaK) using the Evanno method. Top right: bar plots showing the probabilities of individual assignment to each genetic cluster of K=2 (top), K = 3 (middle), and K= 4 (bottom). Each vertical bar along the x-axis represents the genotype of an individual, grouped by their sampling island. Island groups are separated by red lines. The y-axis indicates the proportion of the individual's genotype that belongs to a given number of clusters (K). Each cluster is a different color.

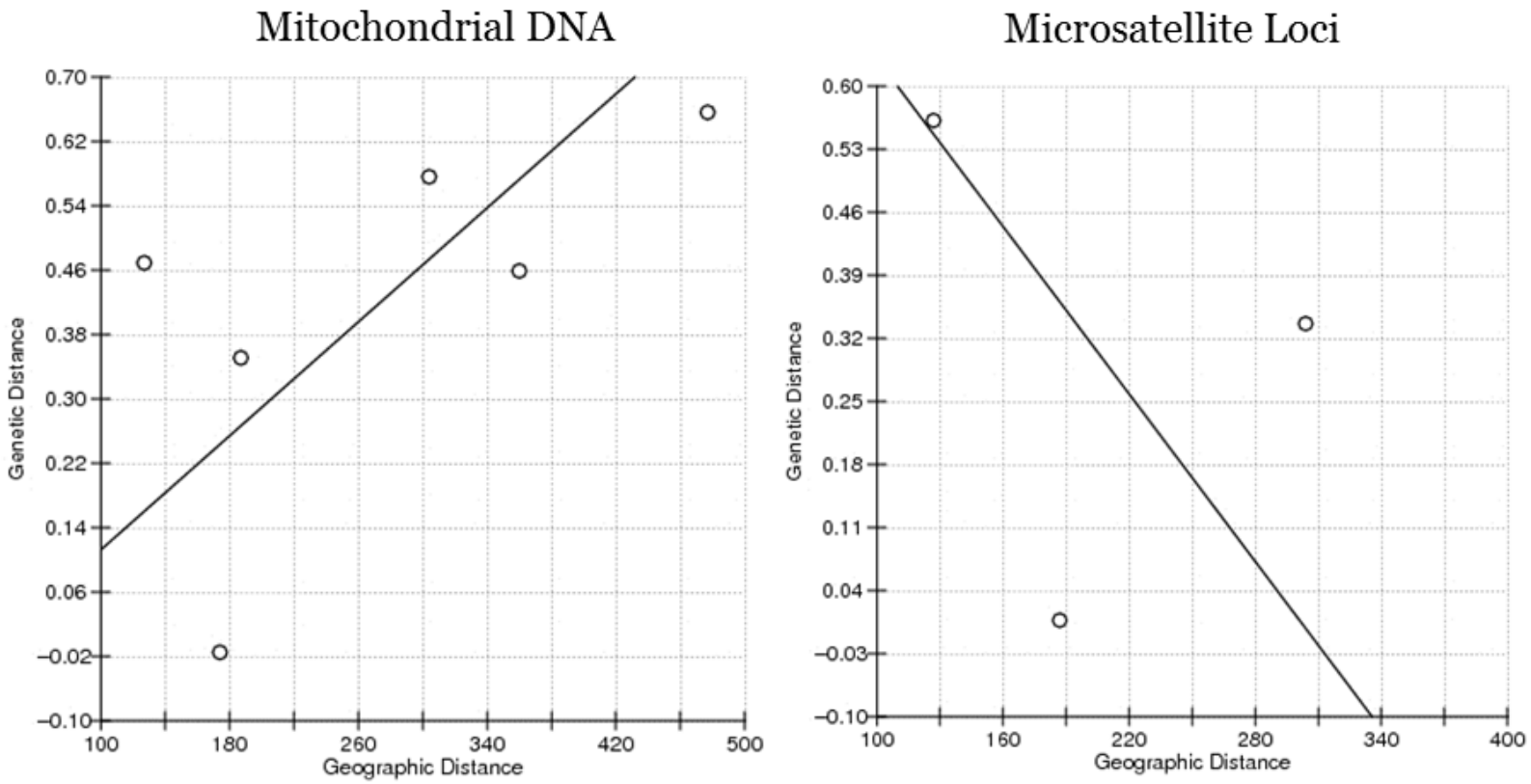


Figure 7. Correlation plots between pairwise F_{ST} and pairwise geographic distances. Pairwise values were calculated between islands using both mitochondrial DNA (left) and microsatellite loci (right).

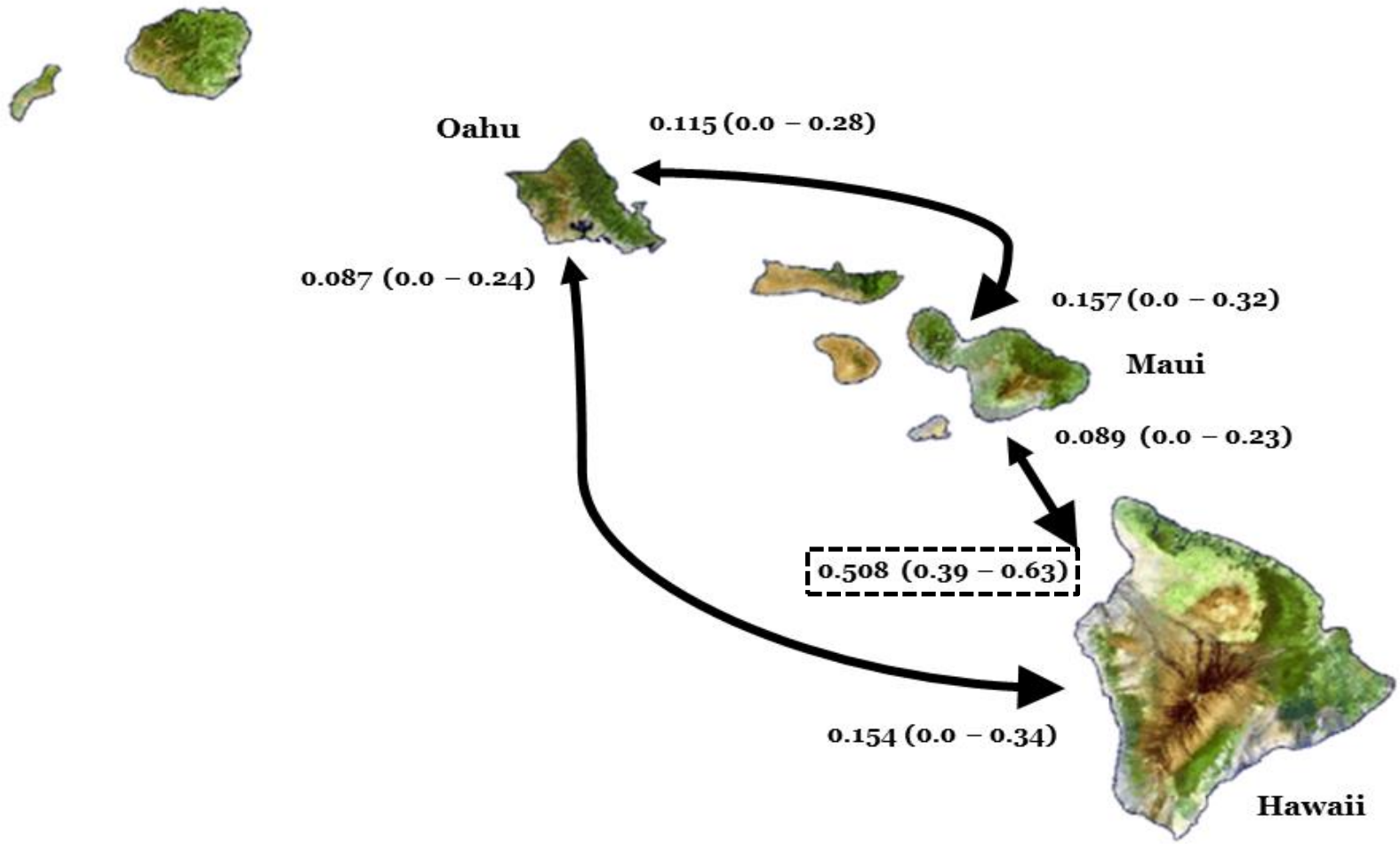


Figure 8. BayesAss estimates of migration rate and direction are illustrated across the main Hawaiian Islands. The estimated proportion of migrants is indicated next to the island, and black arrows indicate the pairs of islands for which gene flow was tested. Arrows with larger tip ends indicate a higher proportion of migrants in that direction. Number in parentheses equal the 95% confidence interval. The boxed estimate represents the only inter island result in which the data on proportion of migrants was informative, and the confidence interval did not contain zero.

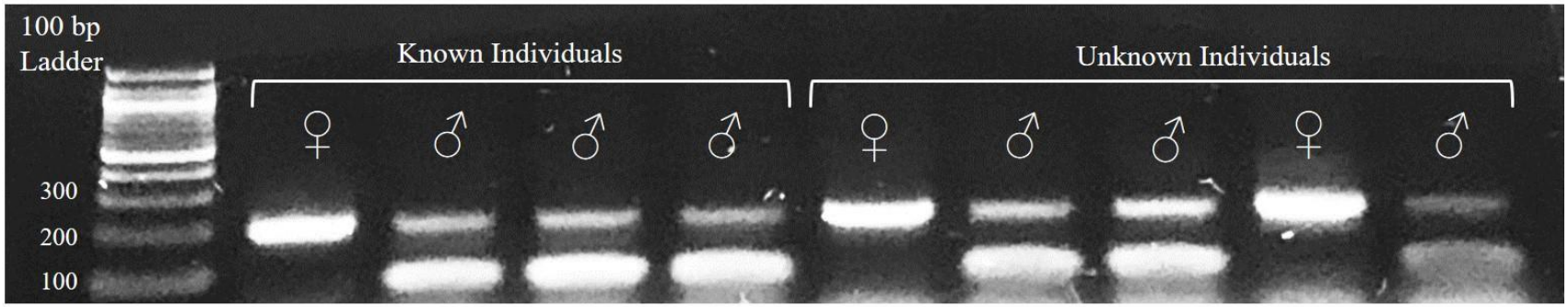


Figure 9. Image of agarose gel visualizing genotyping banding pattern present in male and female bats of known and unknown sex from live and carcasses for sex determination.

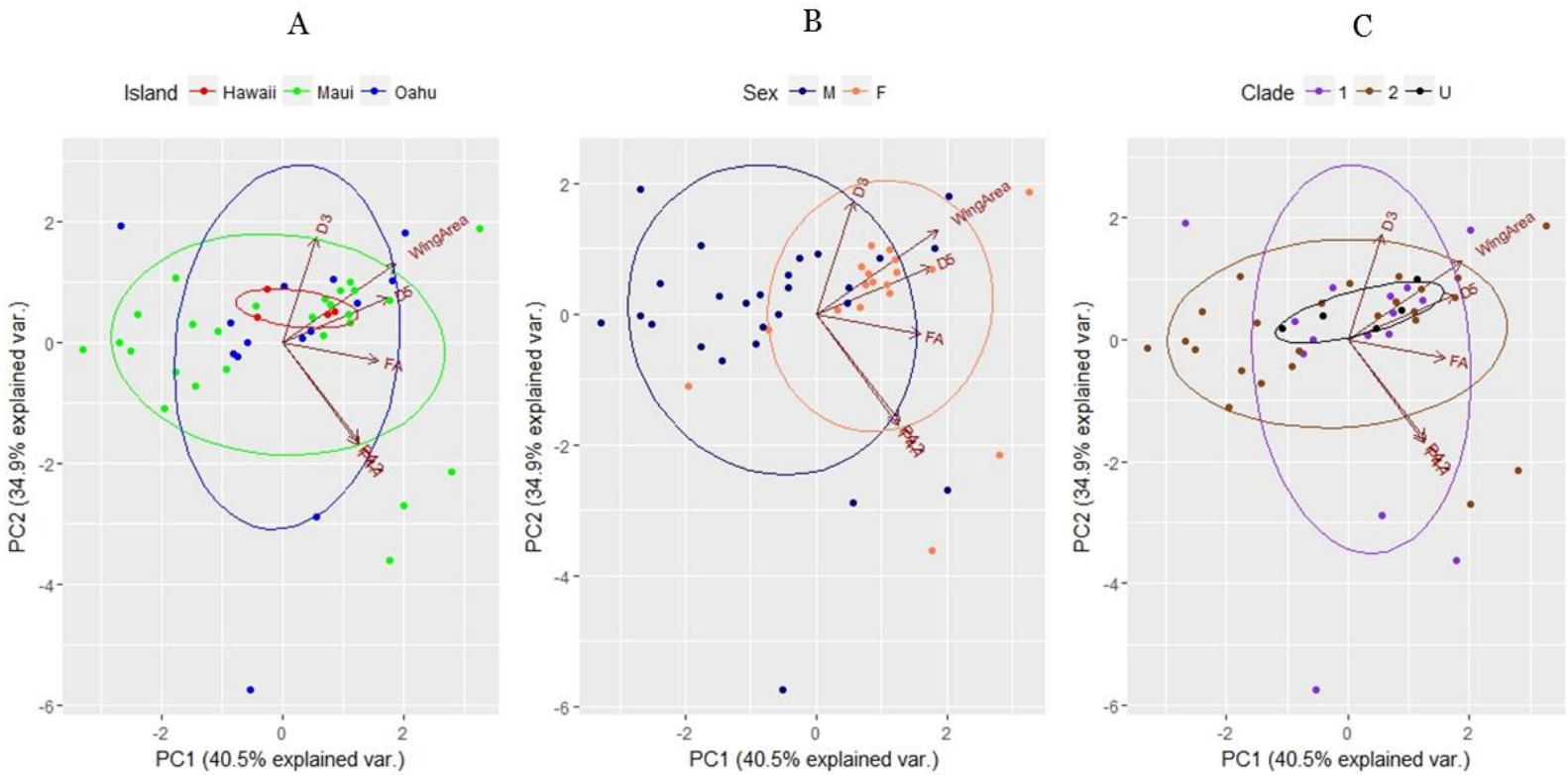


Figure 10. Principal component analysis of six morphological wing characteristics from 44 Hawaiian hoary bats across three islands. PC1 axis represents forearm length, and PC2 axis represents D5 length. Circles surround individuals from either island (A), sex (B), or clade (C). In graph C, "U" represents bats of unknown clade.

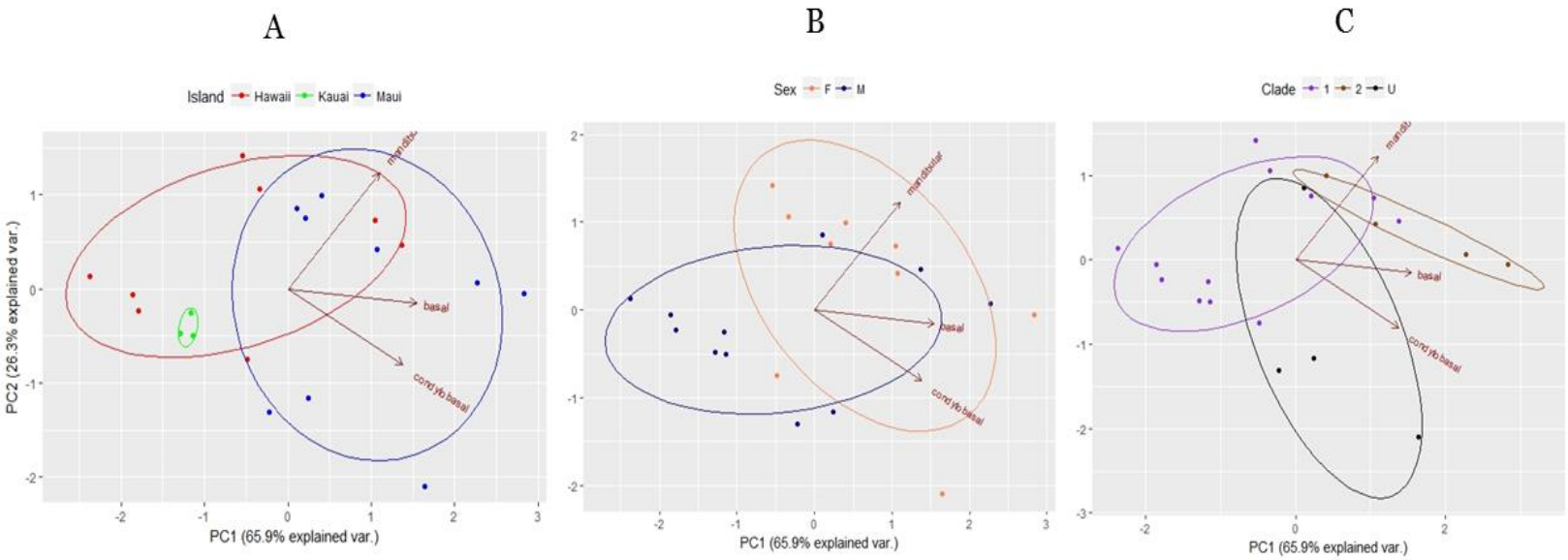


Figure 11. Principal component analysis of three morphological skull length characteristics in 20 Hawaiian hoary bats across three islands. PC1 axis represents basal length, and PC2 axis represents condylobasal length. Circles show the potential clusters of points by island (A), sex (B), and clade (C). In graph C, "U" represents bats of unknown clade.

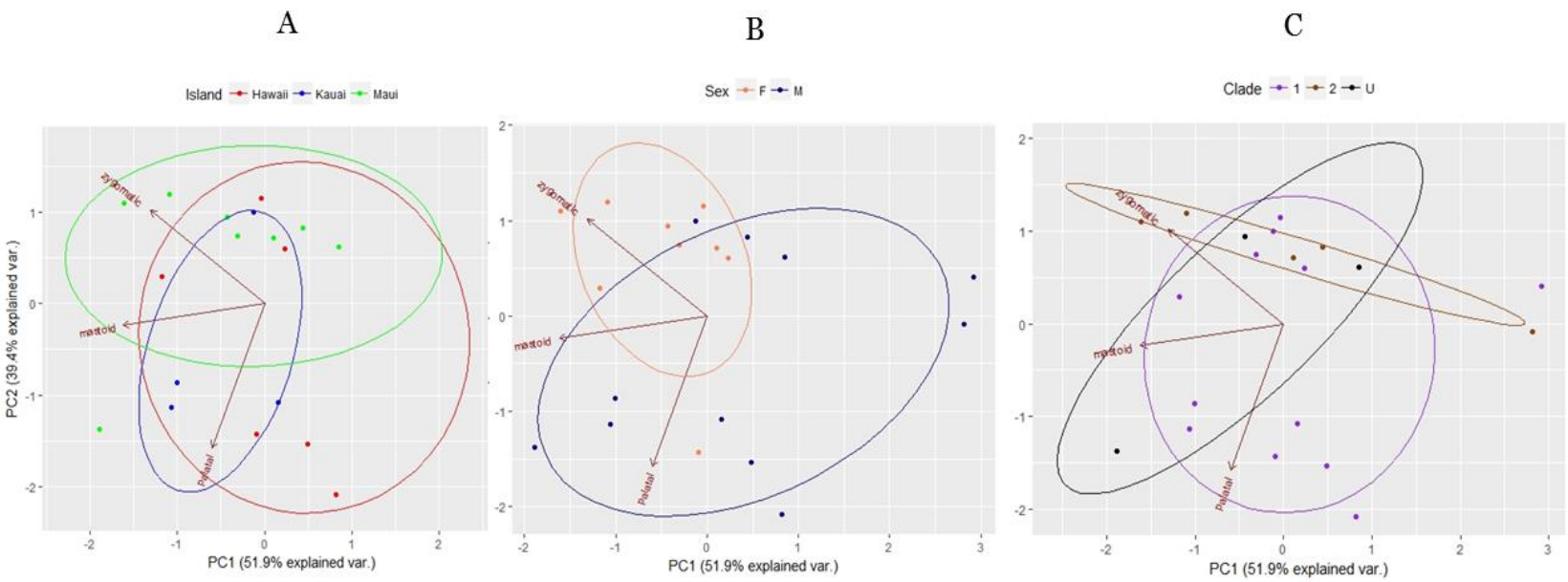


Figure 12. Principal component analysis of three morphological skull width characteristics in 20 Hawaiian hoary bats across three islands. PC1 axis represents mastoid width, and PC2 axis represents zygomatic width. Circles show the potential clusters of points by island (A), sex (B), and clade (C).

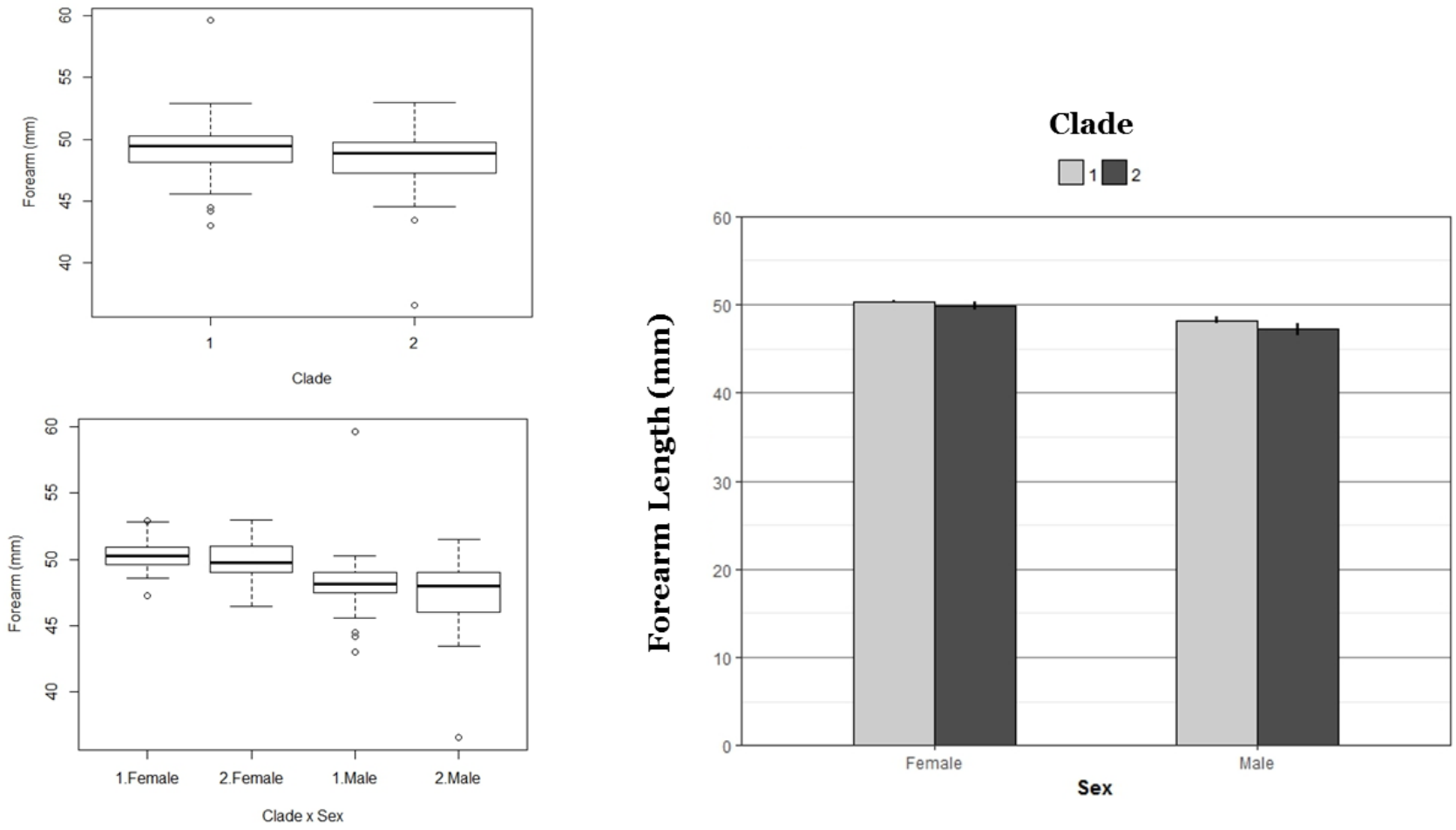


Figure 13. Variation in forearm length in 114 Hawaiian hoary bats between sex and mitochondrial COI clade.

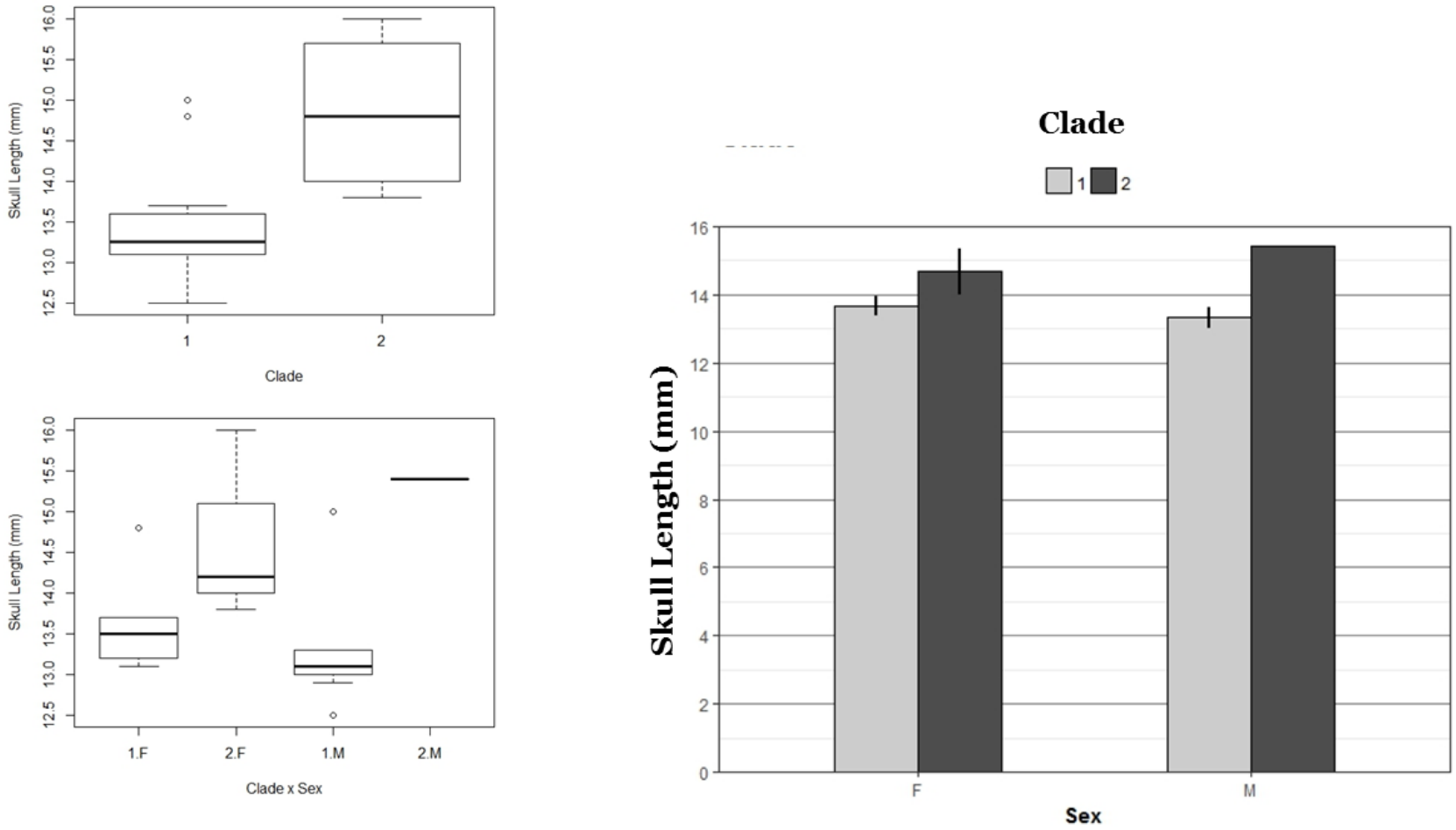
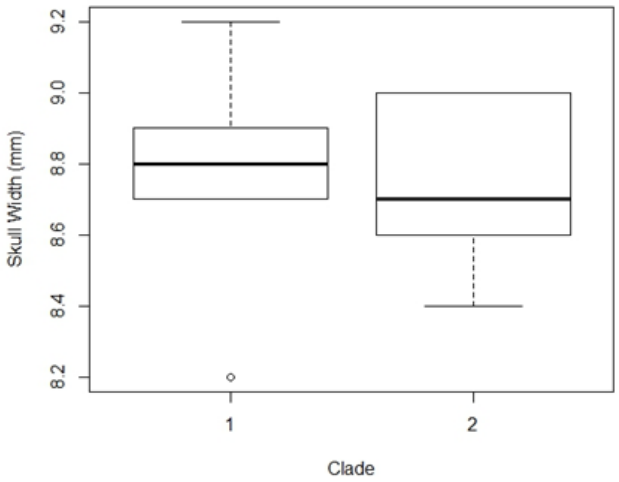
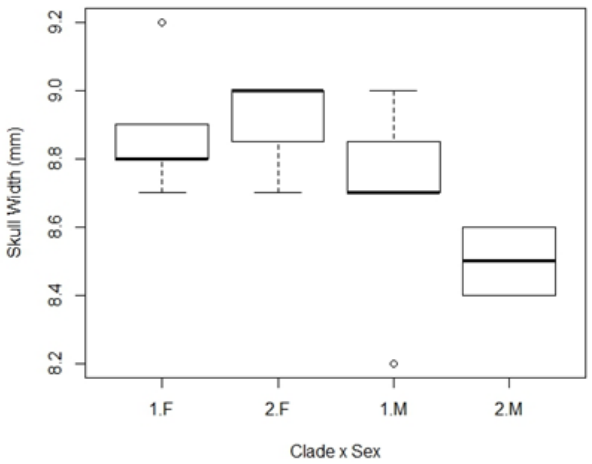


Figure 14. Variation in skull length in 17 Hawaiian hoary bats between sex and mitochondrial COI clade.





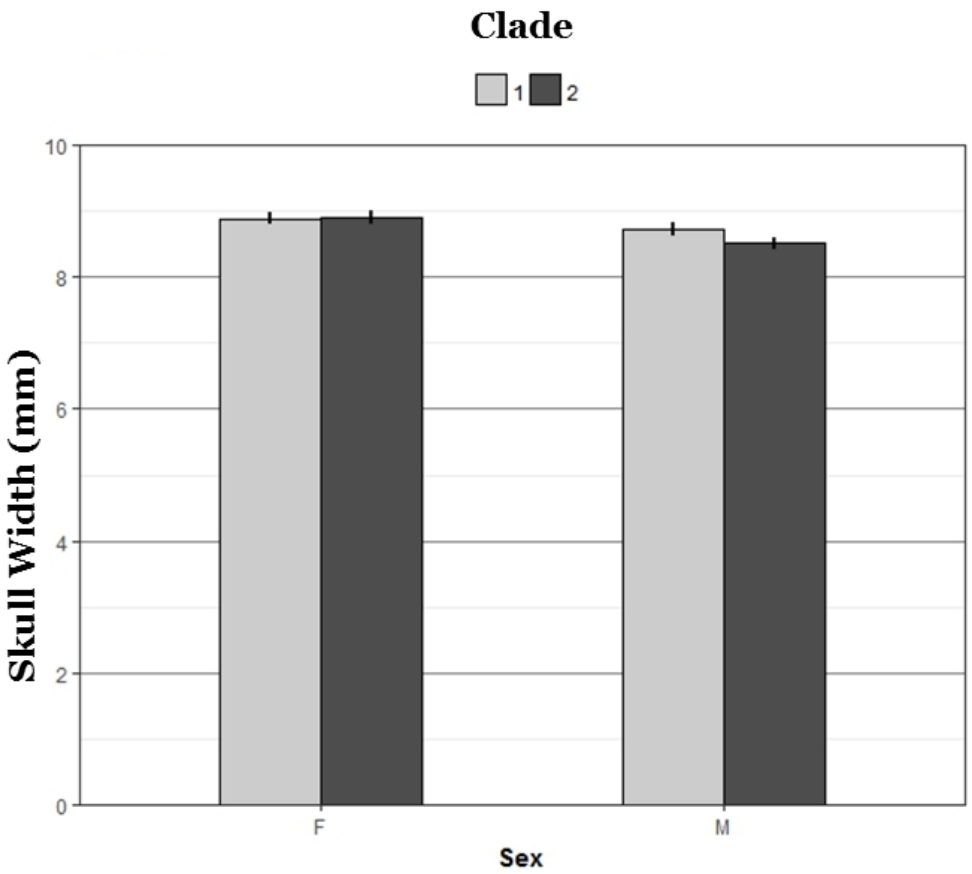


Figure 15. Variation in skull width of 16 Hawaiian hoary bats between sex and mitochondrial COI clade.

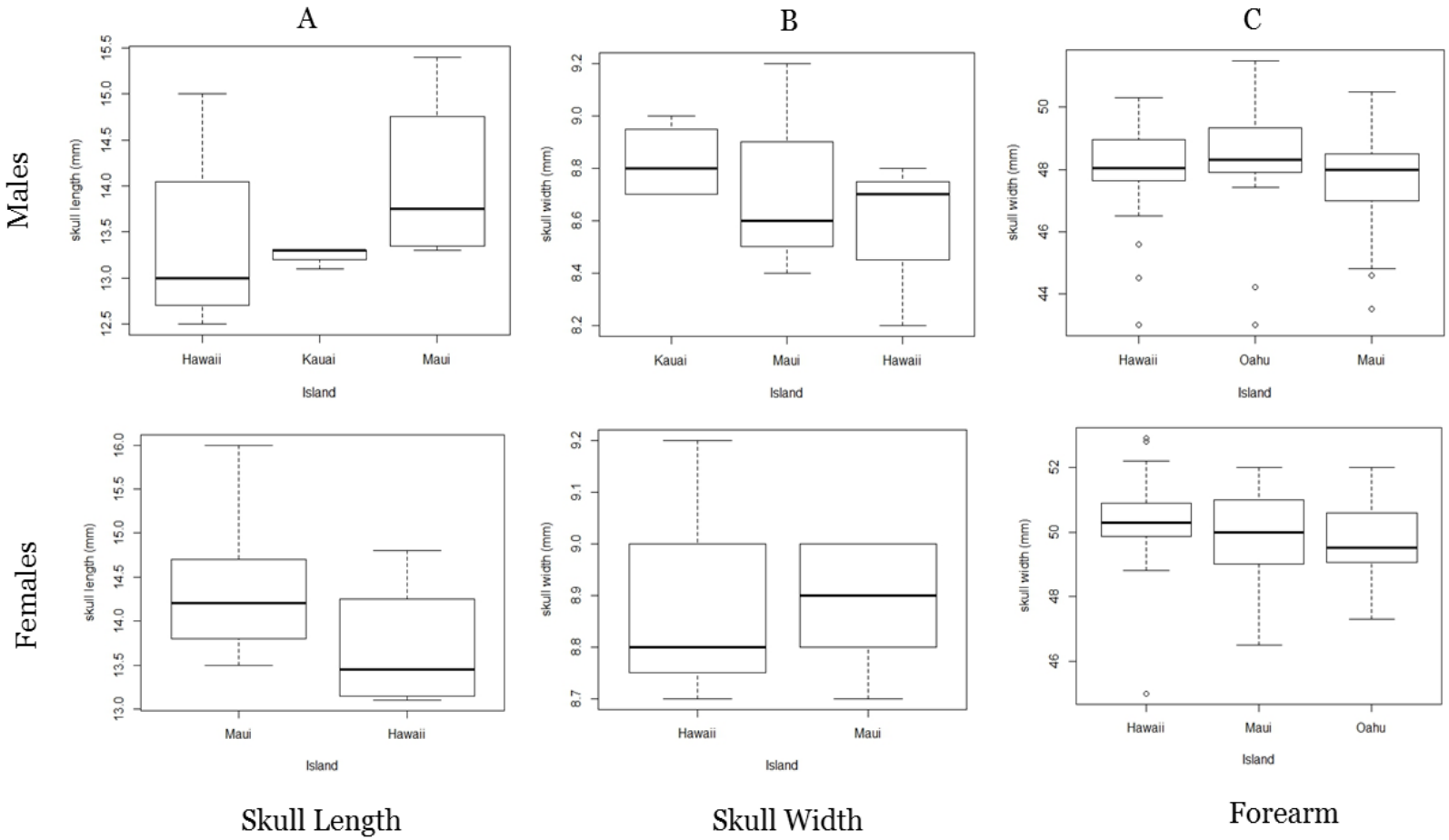


Figure 16. Variation in A) skull length, B) skull width, and C) forearm length for male and female Hawaiian hoary bats across Hawai`i, Maui, O`ahu, and Kaua`i.

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