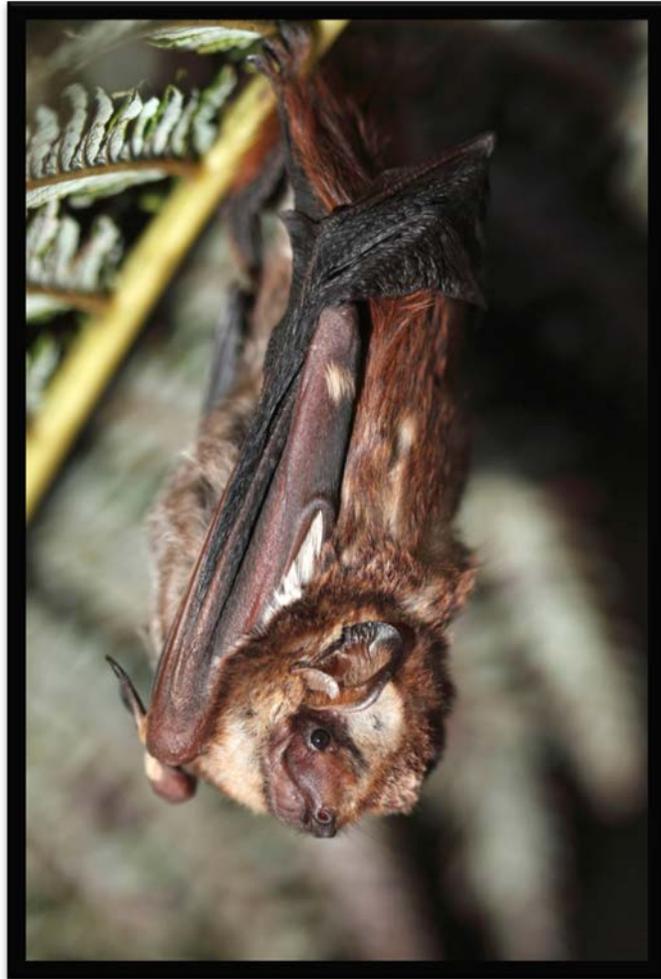


HAWAIIAN HOARY BAT CONSERVATION GENETICS



A Proposal Prepared for the State of Hawaii Endangered Species Recovery Committee

Submitted: July 15th, 2016

From: U. S. Geological Survey, Pacific Island Ecosystems Research Center

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SUMMARY

Basic information on population structure and status of Hawaiian hoary bats is unknown including the levels of genetic variability within populations, levels of migration and gene flow between islands and degree of population structure existing throughout the Hawaiian Islands (Hawaii DNL 2015, USFWS 1998). In light of this current lack of knowledge, gathering population genetic information on this bat will be invaluable for managing the recovery of this species and protecting it from current and future threats. Genetic variability and population structure of Hawaiian bats may affect resilience to sustained mortality from threats such as wind turbines, and capacity for long term adaptation to climate change. Recent work has shown that genetic clades of Hawaiian hoary bats are distinct with one “ancient clade” distinct from North American populations, and other clades bearing strong similarity to North America, indicating that multiple colonization events have occurred in the Hawaiian Islands and suggesting that more than one distinct management unit may exist for the Hawaiian hoary bat (Baird et al. 2015, Russell et al. 2015). Unfortunately, no practical techniques exist for the direct census of populations for tree bats (Schorr et al. 2014), and thus, an alternative course employing molecular genetics including the use of genomics techniques is required to estimate effective population size and structure. The main goals of this proposal are to quantify the genetic variation and population structure throughout the state and determine whether there are distinct population boundaries in order to identify if multiple management units for conservation will be needed. We will also determine the existence and location of source and sink populations for this species.

The factors contributing to bat collisions with wind turbines are still not well understood (Arnett & Baerwald 2013). Effective mitigation and minimization strategies may benefit from knowing if there is a sex-specific vulnerability or seasonal pattern in wind turbine associated deaths and if turbine mortality is impacting bats from a broader region. Genetic information from carcasses from fatality searches can be of assistance to identify sex (Cryan & Barclay 2009) and conduct population studies (Sovik et al. 2016). Unless a bat carcass discovered under a turbine is quite fresh (1-2 days old), more often than not, the sex of decayed or scavenged carcasses are difficult or nearly impossible to identify from external observation alone, leading to a male biased dataset because females are often identified as unknown (Korstian et al. 2013). A simple and reliable genetic method now exists to accurately determine the sex of bat carcasses from *Myotis* bats found under wind turbines, and has been validated for the North American hoary bat in a recent study by Korstian et al. 2013. We will genotype and identify the sex of all Hawaiian hoary bat carcasses collected from state wind facilities, as well as those samples in our current tissue collection and gathered from any concurrent bat capture projects. This new genetic information will be used to enhance available bat demographic information across the State of Hawaii; through determining differences between male and female bats in their mortality at wind facilities, genetic variability and gene flow rates, and dietary needs.

The research proposed herein will substantially advance our understanding of Hawaiian hoary bat (*Lasiurus cinereus semotus*) demographics and populations across the Hawaiian Islands and assist in studies related to diet composition and prey selection. Specifically, we will address the goals of 1) Basic research that gathers information for guidance of conservation efforts, and 2) Identifying limiting factors in food availability that impact survival and reproductive success. This project will utilize the latest molecular genetic techniques and incorporate both currently available bat biological samples as well as analyze samples from bat ecology projects running concurrently throughout the state. We aim to gather genetic information and uncover the biological associations and patterns with respect to bat population and diet from several hundred individuals on islands where guidance is needed to implement effective conservation measures that result in a net recovery benefit for this endangered species.

Our conservation genetics study of Hawaiian hoary bats will document the following points identified as Priority 1 Objectives by the ESRC

- Document demographic information
- Document genetic variability

Contributions of this research will aid with the following objectives by the ESRC

- Identify diet (1)
- Document prey selection (1)
- Conduct population modeling (2)

USGS has already made a substantial commitment to conservation genetics research of the Hawaiian hoary bat. Work to date includes the first ever molecular publication exploring the colonization history and dispersal of hoary bats to Hawaii from North America (Russell et al. 2015) using mitochondrial (mtDNA) and nuclear DNA markers; a master's thesis research in progress by C. Pinzari has genotyped mtDNA from over 100 bats across the islands of Hawaii, Maui, and Oahu with analysis to include population structure by island, and geographic variation in morphology; and finally a collaborative effort with D. Price and P. Michalak has sequenced whole genome data with next generation sequencing techniques and is currently analyzing the patterns of population structure, migration rates, and historic bottlenecks with thousands of single nucleotide polymorphisms (SNPs) for 23 bats across four islands. Strengths to perform this type of research lie in our extensive collection of Hawaiian hoary bat biological samples, our local group's field expertise with the proper permitted capture and study of this challenging bat species, and the technical knowledge our government and academic collaborators. Started in 2004, and containing tissue samples from over 200+ individuals, our extensive biological collection includes samples from live captured bats, downed bats from wind facilities, and even historical museum specimens.

This conservation genetics plan represents the largest and most comprehensive sampling effort ever attempted to characterize Hawaiian hoary bat population structure using next generation sequencing technology and bioinformatics analysis. Critically important research questions that require study for effective management of this bat and appear recommended in the most recent five-year U.S. Fish & Wildlife Service review (2011) include; 1) what are the effective population sizes of Oahu, Maui, and Hawaii Islands? 2) do one or more distinct populations of hoary bats exist in Hawaii, 3) what level of connectivity through gene flow occurs across the Hawaiian Islands? The management and conservation of suitable habitats for the Hawaiian hoary bat depends on our knowledge and understanding of their ecology, life history processes, and resource requirements. These needs can vary greatly between the sexes, resulting in sex-specific foraging and habitat use, movements and seasonal distributions, and survival.

Information forthcoming from this conservation genetics study will provide wildlife managers key data for planning recovery of the Hawaiian hoary bat, as well as information that will better guide planning and implementation of future mitigation areas. Examples of new critical information expected as outcomes include first estimates of the effective population sizes and structure across the state of Hawaii, demographic information on sex of bats for application to management objective such as REA and PVA modeling, correct sexual identification of historical bat take at Hawaiian wind facilities, and food habits of both male and female bats of varying ages and habitats.

Total Budget: \$393,355

Year 1- \$313,089

Year 2- \$32,709

Year 3- \$47,558

Budget Match Provided by USGS: \$15,680

GOALS

The first goal within this proposal is to perform basic research that provides information on the Hawaiian hoary bat documenting demographic information and genetic variability. The second goal will be to contribute to research which recognizes the factors limiting food availability for the bat through the identification of diet and documentation of prey selection. The expected results from this molecular dataset will consist of measures of genetic variation, population structure, effective population size estimates, migration rates, and food habits. The patterns in population dynamics and dietary composition, direct outcomes of this genetic research, will help managers make effective conservation decisions and enable assessment of wind energy impact on both local and cumulative bat populations. Understanding population boundaries and diet needs, contributes directly to a net recovery for the Hawaiian hoary bat because it allows managers to balance future threats to island wide bat populations while informing the selection and design of bat habitats within mitigation reserves.

OBJECTIVES

The tactical objectives in this proposed study of Hawaiian hoary bats will document:

- demographic information
- genetic variability

All of the above objectives are listed as high priority (1) by the ESRC contained in this request for proposals. Each of the objectives when fulfilled will contribute to a more informed guidance toward mitigation strategies for the future selection, restoration and protection of natural reserve lands for the recovery of Hawaii hoary bats.

Contributions of this research will aid with the following Priority 1 and 2 objectives by the ESRC

- Identify diet (1)
- Document prey selection (1)
- Conduct population modeling (2)

TASKS AND ACTIVITIES

- **Collection of bat tissue samples for DNA analysis and sex genotyping**
 - Tissue samples will be obtained from existing USGS collection, carcasses from wind facilities, live bats captured during this project, and any other concurrent projects that can donate tissue samples.
 - Information on the individual (age, sex, location and habitat, morphology, date, etc.) will document demographic status, and the DNA sample will document genetic variability.
 - The ability to obtain a high number of samples to perform rigorous and statistically sound population genetics analyses can be difficult, expensive, and time consuming for a cryptic, endangered species. Our group has already obtained a large number of samples and has extensive experience in successful capture of live bats across the state.
- **Increase geographic coverage of bats from Kauai, Maui, and lesser sampled islands such as Molokai and Lanai**
 - Historic tissue samples will be obtained from museum collections for lesser sampled islands, and the current population of bats on Kauai will be sampled during one collection trip. Museum tissues contain degraded DNA and a special process of using

multiple, overlapping primers to amplify and reconstruct the mtDNA CO1 region would be used to recover useable data from these specimens.

- Completion of this task would boost our temporal and spatial coverage significantly for population and demographic analyses. The statistical power to infer inter-island population structure, migration rates between islands, and estimate historical population sizes and past bottleneck events would improve for all islands.
- **Determine the sexual identity of all bats to be included in genetic studies**
 - Genotyping methods will be used to identify the sex of all bats, including carcasses from wind facilities.
 - Information on the individual (genetic sex) will assist in documenting demographic status, allow comparisons of data generated for population genetics, diet, home range, habitat use between the sexes, and provides reproductive information on bats taken at wind facilities to the agencies for management purposes.
 - Currently, the sexes of many individuals in our collection and from wind facilities are unknown and can't be confirmed on visual inspection due to decomposition. Using genetic methods to obtain the sex of bats overcomes the biases associated with identifying young bats captured live, fills missing gaps in sex data for previously collected individuals, and provides the sex of decomposed and desiccated carcasses.
- **Complete sequencing efforts for CO1 mitochondrial DNA marker and analyze mtDNA in Hawaiian bats**
 - Over 100 bats have been sequenced for this marker region as part of C. Pinzari's graduate research, additional samples for all islands will be added to this dataset
 - Completion of this task allows statistical analysis of genetic variability, maternal population structure, and estimates of effective population sizes.
- **Conduct nuclear micro-satellite analysis of 19 markers on all bat samples collected from Hawaii, Maui, and Oahu**
 - All markers chosen have been tested directly on hoary bats and published in scientific literature. USGS and collaborators have already used a subset of these markers and there is an existing microsatellite dataset for 30 individuals from Hawaii Island.
 - Completion of this task allows statistical analysis of intra-island population structure on three islands where bats face threats from wind energy (Hawaii, Maui, and Oahu) and estimates of effective population sizes.
- **Conduct genomic sequencing and analysis of single nucleotide polymorphisms (SNPs) in Hawaiian bats**
 - We will sequence up to 24 new bats to use in population genomics analyses using next generation techniques at the Virginia Biodiversity Institute.
 - Using a previous dataset of 23 bats across 4 islands, plus up to an additional 24 bats, a reference genome will be assembled and library of SNP loci generated.
 - SNPs will be examined for over 40 Hawaiian bats with bioinformatics software by P. Michalak and D. Price for differences between populations to detect genomic regions of divergence and conduct population-level analyses including identifying bottleneck events, allelic diversity, population structure measures, and effective population sizes. Completion of this task will greatly expand the genomic sequence analysis to detect important regions of the genome that could be especially important in the divergence of the different populations and under adaptive, positive selection.

OUTPUTS

Data outputs will include population metric and parameter measurements from all three sources of genetic marker data; 1 mitochondrial region of CO1 subunit, 19 nuclear microsatellites, and thousands of single nucleotide polymorphisms. Population metrics and parameters include measures of genetic variability, intra-island divergence, directional selection tests, number and structure of existing genetically distinct populations and their distances, estimates of effective population and recent bottleneck events for the islands of Hawaii, Maui, and Oahu. Maps which feature haplotype distributions and genetic distances will illustrate population boundaries and migratory connections. Genetic sequence data from this study will be deposited into a publically available database after publication. The data produced here, including the construction of a reference genome, will provide a valuable baseline for possible future genetic monitoring of Hawaiian hoary bat populations.

Data outputs related to the demographic nature of bats will include the genetic determination of sex for all available tissue samples from wind fatality carcasses obtained during the study period, maps of bat demographic information and tissue collection locations, and sex assignment data for bats to be used in the analyses of population structure metrics, dietary composition, and prey selection studies.

OUTCOMES

While the USGS is a research agency, its project staff will be available to advise state, federal and private organizations about the applicability of data outputs in relation to bat conservation genetics. We will do this through providing technical assistance by phone, conference meetings, management workshops, technical reports, and publication of peer reviewed scientific publications. We will frequently present preliminary data at appropriate conferences such as Hawaii Conservation Conference and the North American Symposium for Bat Research or at such local conferences as are hosted in the State of Hawaii.

MATERIALS AND METHODS

We propose a conservation genetics study that will run over a three year period, incorporate both historical and current individual bats, and extend geographic bat population information across the state, with a specific emphasis on the islands with wind energy facilities (Hawaii, Oahu, and Maui).

DNA Extraction and PCR Amplification

Tissue samples from our existing collection, new live captures, and museum specimens will have DNA extracted and sequencing performed at the University of Hawaii at Hilo. Total genomic DNA will be extracted from wing or muscle tissue using a Qiagen DNeasy Blood and Tissue kit and normalized for either genetic or genomic PCR. A suite of up to 19 microsatellite loci as well as one mitochondrial locus (cytochrome oxidase 1, CO1) will be amplified using PCR. Genotyping of microsatellite PCR reactions will be done at the UH Hilo Core Genetics Facility on a Beckman Coulter CEQ 8000. Resulting microsatellite fragments will be sized and scored by eye. Mitochondrial PCR reactions will be cleaned and bi-directionally Sanger sequenced on an Applied Biosystems 3500 Genetic Analyzer, and aligned using MEGA software. Tissue collection and laboratory work will be performed by C. Pinzari and F. Bonaccorso.

Population Genetic Measures and Parameters

Analysis of molecular variance (AMOVA) to test for geographic structure in mitochondrial CO1 sequence diversity will be performed using Arlequin software. DnaSP software will calculate mitochondrial DNA diversity from the number of haplotypes, haplotype diversity, Watterson's theta, per site nucleotide diversity, and Tajima's D. For the microsatellite data, analysis of population structure will use Bayesian clustering analysis to test for the presence of more than one genetic subpopulation among islands using the program Structure. Ima2 will be used to estimate effective population and historical sizes for Hawaii, Maui, and Oahu. This analysis will be done by C. Pinzari, D. Price, and F. Bonaccorso.

Sex Determination of Bats

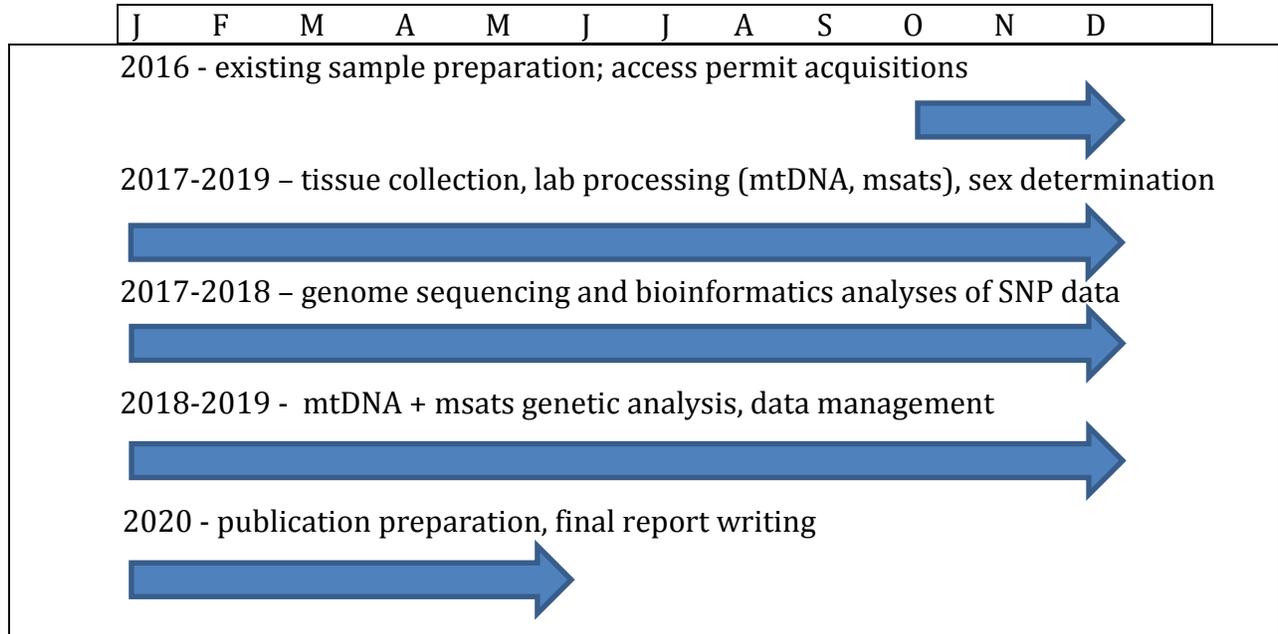
Genotypes will be determined from all individuals in our study, both known and unknown, and of all carcasses found on wind facilities in Hawaii available for sampling, following molecular methods in Korstian et al 2013. PCR will be used to amplify specific hoary bat markers on the X and Y chromosomes of bats, and gel electrophoresis with staining techniques will result in product bands which can be visualized with UV light for genotyping. Sex determination testing will be conducted at UH Hilo genetics laboratory by C. Pinzari and F. Bonaccorso.

Genome Sequencing and Population Genomic Analyses

We will select individuals (up to 24) with high quality extraction levels of DNA, balanced geographic distribution per island, and unique haplotypes from CO1 analysis to undergo whole-genome sequencing and bioinformatics analyses at Virginia Biodiversity Institute by P. Michalak and at University of Nevada by D. Price. A Hi-Seq 2500 Illumina platform will generate a TruSeq paired end library of 150 base pair length reads at approximately 71x coverage. A reference genome for the bat will be created from the mate pair library using de-novo assembly in the program Velvet. The reference genome will be used to discover tens of thousands of single nucleotide polymorphisms from the newly sequenced bats, as well as make genetic comparisons to publically available sequence data for a single hoary bat from North America. This variant discovery will employ bioinformatics programs such as Bowtie2, BWA, SamTools, and GATK. The unfolded site frequency spectra (SFS) will be estimated for each island, to determine the distribution of allele frequencies, and PCA clustering will quantify patterns of population structure. Population genomics summary statistics such as mutation rates, Tajima's D, fixation index (F_{st}), mean nucleotide diversity, average number of nucleotide substitutions, and estimates of effective population sizes will be generated from SFS data and custom scripts. The program Structure will also be used with genomic data to build phylogenetic trees based on nucleotide distances, and infer population admixtures. Between island gene flow estimates will be analyzed with Mcmctree based on a Bayesian Markov Chain Monte Carlo algorithm and an ABBA/BABA test within PAML software. Additional tests for gene flow will be conducted using Admixture and IMA2.

TIMETABLE AND MILESTONES

Hoary Bat Conservation Genetics Timeline



PERMITS AND AUTHORIZATIONS

U. S. Geological Survey holds current research/take permits to collect and store biological samples for endangered species from U. S. Fish and Wildlife Service (Permit TE 003483-29) and Hawaii Department of Lands and Natural Resources (Permit WL-16-04), and additionally has an active IACUC protocol approved by the University of Hawaii for vertebrate animal research. We maintain letters of transfer from the U.S. Fish and Wildlife local office for the shipment of biological samples to our partner labs and academic institutions. For historical DNA specimens, we are a partner on a vertebrate collections use permit with the Bernice Pauahi Bishop Museum. USGS has an excellent network of contacts with both private and public land stewards throughout the island of Hawaii that have frequently provided access to lands for bat research.

MONITORING AND EVALUATION

The USGS project manager will closely supervise all aspects of research. Staff will have periodic meetings (usually quarterly) with the project manager and with supervisory directors of USGS and HCSU. Genetic data will be reviewed on a bimonthly schedule as appropriate for specific analyses and cumulative data sets updated frequently as more bat samples are sequenced. Project managers will employ adaptive management to improve and refine data collection with major reviews of success or weakness each year as the project proceeds. Annual reports will be provided to key wildlife management contacts (ESRC, DOFAW, USFWS) as well as oral reports or posters at the annual Hawaii Conservation Conference. Research staff will be available for phone

consultations with wildlife managers when management issues arise in which new data inputs from the project may be helpful as updates.

ORGANIZATIONS AND KEY PERSONNEL

U. S. Geological Survey (USGS) is based at Kilauea Field Station inside Hawaii Volcanoes National Park and offers computer and research labs and a large multi-disciplinary staff of senior biologist researchers and technicians.

USGS Project Manager: Dr. Frank Bonaccorso, USGS Wildlife Biologist, earned his Ph.D. at the University of Florida in 1975. He has over 45 years of experience in working with bats from around the world including 11 years of intensive research on Hawaii Hoary Bats. He has published over 50 articles in peer reviewed scientific journals across the fields of community and population ecology, behavioral ecology, population genetics, and studies of bats on wind energy facilities.

Hawaii Cooperative Studies Unit (HCSU) is based at the University of Hawaii at Hilo and offers research lab facilities and opportunities to collaborate with senior staff, technicians and students in the biological sciences.

HCSU Hoary Bat Conservation Biologist: Corinna Pinzari, is a Master's degree candidate in the Tropical Conservation Biology and Environmental Sciences program at University of Hawaii at Hilo. Her thesis research focuses on determining the potential geographic structure, genetic and morphological variation in Hawaiian hoary bats across the state using conservation genetics techniques. She received her bachelor's degree of science from University of Washington in 2006, majoring in Ecology and Evolution, with minors in Aquatic Sciences and Restoration. She has 8 years of experience working directly with the Hawaiian Hoary bat, assisting USGS research and publications on the biology, ecology, and behavior of bats; with emphasis on developing techniques and deploying equipment for acoustic surveys.

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