ABSTRACT

Title of Dissertation: CONSERVATION GENETICS OF THE ENDANGERED HAWAIIAN PETREL (PTERODROMA SANDWICHENSIS) ACROSS SPACE AND TIME

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The endemic Hawaiian Petrel (*Pterodroma sandwichensis)* is a long-lived pelagic seabird. Although endangered, subfossil evidence indicates that it was formerly more abundant in the past. In addition to a wider distribution on islands where the petrel currently breeds, two colonies, one on Oahu and one on Molokai, have been extirpated since humans colonized the Islands. Despite this, little is known about this species. Here I use conservation genetic and ancient DNA techniques to investigate the taxonomic status and population dynamics of the Hawaiian petrel. Investigation of the timing and magnitude of divergence between the Hawaiian petrel and its sister species, the Galapagos petrel (*P. phaeopygia*), revealed that these taxa diverged approximately 550,000 years ago. In a phylogenetic tree constructed from mitochondrial data Galapagos

and Hawaiian petrels were reciprocally monophyletic, however, trees from the nuclear data set were unresolved. Low estimates of gene flow between taxa indicate that incomplete lineage sorting is causing the difference in resolution between data sets and that Galapagos and Hawaiian petrels are likely separate species. In addition to the mitochondrial and nuclear intron data sets, I developed a suite of 20 polymorphic microsatellite loci: I developed 10 specifically for the Hawaiian petrel, and characterized an 10 additional previously reported loci in this species. Using these three data sets I investigated the pattern of gene flow and divergence between modern, historical, and ancient populations of the Hawaiian petrel. The mitochondrial data set showed strong levels of differentiation between modern populations. The two nuclear data sets also revealed significant population structure, although it was weaker. Mitochondrial DNA sequences obtained from subfossil bones indicate that populations were significantly differentiated in the past, although there was low divergence between the extirpated Oahu and Molokai populations and modern birds from Lanai, suggesting that perhaps as colonies dwindled individuals dispersed to that island. Investigation of the effective population size indicates that no significant change has occurred on Hawaii or Maui. It appears that the long generation time of this species may have allowed it to escape a genetic bottleneck after the arrival of humans in the Hawaiian Islands.

CONSERVATION GENETICS OF THE ENDANGERED HAWAIIAN PETREL (*PTERODROMA SANDWICHENSIS*) ACROSS SPACE AND TIME

by

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INTRODUCTION

Humans have greatly impacted the Earth, leading to a biodiversity crisis (Pechmann and Wilbur 1994; Ceballos and Ehrlich 2002; Ehrlich and Wilson 1991; Myers and Knoll 2001; Pimm et al. 2006). Anthropogenic impacts include direct exploitation (Brashares et al. 2004; Milner-Gulland et al. 2003), habitat fragmentation or destruction (Debinski and Holt 2000; Brook et al. 2003), introduction of invasive species (Mooney and Cleland 2001), and introduction of exotic pathogens (Daszak et al. 2000; Kilpatrick et al. 2006). Thousands of species have likely gone extinct (Pimm et al. 2006), but even those that remain have been greatly impacted. The range of many extant species has been reduced, often due to population extirpations, and as a result, many are increasingly threatened by extinction (Hughes et al. 1997).

There are several reasons to conserve biodiversity including ecosystem services (Luck et al. 2003; Naeem et al. 1994) and economic benefits (Goodwin 1996; Kirkby et al. 2010), as well as both aesthetic and ethical reasons (Frankham et al. 2002). An understanding of genetics can aid in conservation efforts in many ways (Frankham 2005). For example, it can be used to identify species or populations of conservation concern, including identification of cryptic species, resolution of taxonomic uncertainty, and delineation of evolutionarily significant units (Yoder et al. 2000; Moritz 1994). Genetics can also be used to investigate population connectivity and isolation (Foote et al. 2011; Funk et al. 2005), as well as to investigate changes in genetic diversity and trends in effective population size (Lippé et al. 2006; Tessier et al. 1997)

At the species level, genetics can inform us about the process of evolution. If two populations become separated (either by extrinsic or intrinsic factors) they may diverge to the extent where they become reproductively isolated, and therefore be considered separate species (Coyne and Orr 2004). The genome of the organisms will reflect these changes, but the signal of divergence may accumulate at different rates (Hudson and Coyne 2002; Avise et al. 1983). For example, in most vertebrates, due to the haploid nature and the maternal inheritance mode of mitochondrial DNA, it will have a shorter coalescence time than that of nuclear DNA (Palumbi et al. 2001), and hence will often be a relatively 'leading indicator' of divergence (Zink and Barrowclough 2008). If not taken into account, this process of lineage sorting can make it difficult to discern taxonomic relationships (Weisrock et al. 2010). Still, genetic techniques have been successfully used to identify taxa that may warrant conservation efforts, but which might otherwise be overlooked (Hailer et al. 2011).

If two populations are not isolated, and instead they exchange migrants, then they may remain a single species. In this situation it can be useful to gain a better understanding of the patterns of gene flow and isolation between populations. This is particularly true for endangered species that may be isolated due to anthropogenic effects (Schwartz et al. 2005). Knowledge of population connectivity can also be important for reserve design (Palumbi 2003) and the selection of individuals for translocations (Schultz et al. 2011). Finally, investigations of gene flow can also reveal hybridization of endangered species with more common species, which can be a serious problem (Weigel et al. 2003).

Within populations, genetics can be used to investigate changes in population size. When species undergo a severe decline in number, or bottleneck, they may also exhibit a decrease in genetic diversity (Taylor et al. 1994) and this lower level of genetic diversity may increase the extinction risk (Frankham 2005). Molecular markers can also be used to investigate the effective population size of a species. The effective population size of a species is the size of an ideal population that would lose genetic diversity (i.e. through genetic drift) at the same rate as the population of interest (Frankham et al. 2002). This concept becomes important when individuals in a population do not contribute equally to the gene pool. This is often the case in real populations and can be caused, for example, by variation in reproductive success or unequal sex ratios (Allendorf and Luikart 2007). Changes in effective population size can also be informative for conservation, such as the impact of translocated individuals or supportive breeding programs (Tessier et al. 1997).

Combining genetic techniques with the use of historical, archeological, and paleontological specimens can lead to additional insights important for conservation (Leonard 2008). Through the use of ancient DNA, extinct species can be studied to determine phylogenetic relationships with extant species. For example, ancient DNA has been used to investigate a radiation of geese in the Hawaiian Islands. This radiation included a giant, flightless form which is not known from historical records (Paxinos et al. 2002b). From ancient samples it is also possible to learn about past levels of gene flow and changes in population size, for example, in response to anthropogenic impacts or climate change (Campos et al. 2010). While special care is required to work with ancient

DNA (Axelsson et al. 2008), it has the ability to give insights that cannot be obtained with modern DNA alone (Ramakrishnan et al. 2005; Leonard 2008).

The Hawaiian Islands have a rich subfossil history, including 39 species of land birds that are not known from historical records. Many of these went extinct after the colonization of the Islands by humans (Olson and James 1982b). Of the species that persisted, many have undergone a range contraction and a reduction in the number of populations, and many are endangered. The Hawaiian petrel (*Pterodroma sandwichensis*) is one such species.

The Hawaiian petrel is a long-lived pelagic seabird. It is endemic to the Hawaiian Islands and today it breeds on four of the main islands: Hawaii, Maui, Lanai, and Kauai (Simons and Hodges 1998). However, in the past, it was more widely distributed. Subfossil evidence indicates that it was formerly more wide spread on the islands where it is currently known to breed, with colonies in the past occurring just above sea level, whereas today colonies are found only at high elevation (Athens et al. 1991). A large colony was also present in the lowlands of Oahu, but there are no historical records documenting that Hawaiian petrels there (Olson and James 1982a). There was also a large colony on the island of Molokai, with birds so numerous that they were said to "darken the sky" (Munro 1955), however recent surveys have failed to locate a substantial number of birds breeding there (Simons and Hodges 1998).

It appears that humans have had great impacts on this species, both directly and indirectly. Historic records indicate that Hawaiian petrel chicks were considered a delicacy for the Hawaiian chiefs (Bryan 1908; Henshaw 1902). There is also evidence that the adults were consumed by Hawaiians on trips to acquire resources from high elevation sites (Athens et al. 1991; Hu et al. 2001). Humans also introduced exotic mammalian predators, such as rats, cats, and mongoose, which have been and continue to be a serious threat to their persistence (Carlile et al. 2003; Bryan 1908; Simons 1984). In addition to these factors, grazing by introduced ungulates and the introduction of invasive plants have led to habitat destruction (Simons and Hodges 1998). Today, at sea estimates indicate that there are between 11,000 and 34,000 Hawaiian petrels remaining, however given that the distribution of these birds was formerly much larger, in addition to high levels of predation by introduced mammals observed today, the number of Hawaiian petrels may have been much larger in the past.

Despite all of this, little is actually known about the Hawaiian petrel. As with most other procellariiform species, it spends the majority of its life at sea, but little is known about it's non-breeding distribution (Simons and Hodges 1998). When this species returns to land, it breeds in remote and inaccessible colonies, often nesting in burrows that are greater than 2 m long, making it difficult to study it directly (Simons 1985). And in addition to all of this, Hawaiian petrels return to land nocturnally (Simons and Hodges 1998). Even the taxonomic status of this species remains somewhat uncertain. For many years the Hawaiian petrel was considered a subspecies of the dark-rumped petrel, along with the Galapagos petrel. However, in 2002 these two were split based on behavioral,

biochemical, and subtle morphological differences. This split has not been investigated further.

The goal of this work is to use conservation genetics and ancient DNA techniques to investigate the taxonomic standing and population dynamics of the endangered Hawaiian petrel. In the first chapter I use mitochondrial and nuclear DNA sequences to investigate if Hawaiian and Galapagos petrels represent separate species. In the second chapter I describe the development of 10 microsatellite loci specifically for the Hawaiian petrel, as well as characterization of 10 previously reported loci in the Hawaiian petrel. In the third chapter, I combine these microsatellite loci with mitochondrial and nuclear intron sequences to investigate the patterns of gene flow and isolation in contemporary populations, as well as a population on Molokai that may have been extirpated in the last 100 years. Finally, in the fourth chapter I utilize ancient DNA from subfossil Hawaiian petrel bones to examine temporal trends in genetic diversity and gene flow as humans colonized the Hawaiian Islands.

CHAPTER I

Mitochondrial and nuclear DNA sequences reveal recent divergence in morphologically indistinguishable petrels*

Often during the process of divergence genetic markers will only gradually obtain the signal of isolation. Studies of recently diverged taxa utilizing both mitochondrial and nuclear data sets may therefore yield gene trees with differing levels of phylogenetic signal due to differences in coalescence times. However, several factors can lead to this same pattern, and it is important to distinguish between them in order to gain a better understanding of the process of divergence and the factors driving it. Here we employ three nuclear intron loci in addition to the mitochondrial *Cytochrome b* gene to investigate the magnitude and timing of divergence between two endangered and nearly indistinguishable petrel taxa: the Galapagos and Hawaiian petrels (*Pterodroma phaeopygia* and *P. sandwichensis*). Phylogenetic analyses indicated reciprocal monophyly between these two taxa for the mitochondrial data set, but trees derived from the nuclear introns were unresolved. Coalescent analyses revealed effectively no migration between Galapagos and Hawaiian petrels over the last 100,000 generations and that they diverged relatively recently, approximately 550,000 years ago, coincident with a time of intense ecological change in both the Galapagos and Hawaiian archipelagoes. This indicates that recent divergence and incomplete lineage sorting are causing the difference in the strength of the phylogenetic signal of each data set, instead of insufficient variability or ongoing male-biased dispersal. Further coalescent analyses show that gene flow is low even between islands within each archipelago suggesting that

divergence may be continuing at a local scale. Accurately identifying recently isolated taxa is becoming increasingly important as many clearly recognizable species are already threatened by extinction.

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INTRODUCTION

Genetic divergence can arise between populations very quickly, for example through chromosomal rearrangements, founder effects, or changes in ploidy (Navarro and Barton 2003; Soltis and Soltis 1999; Carson and Templeton 1984), but in many cases it can be a long process. Often when two taxa become isolated genetic markers will only gradually move to equilibrium - first giving a polyphyletic, then paraphyletic, and eventually a reciprocally monophyletic phylogenetic signal. The length of time required for lineages to sort out depends on the effective population size and coalescence time of the loci utilized (Avise 2004). For example, since mitochondrial loci have an effective population size one quarter that of nuclear loci, mitochondrial loci will have a shorter coalescence time (Palumbi *et al.* 2001). During the divergence process this can lead to gene trees with different levels of support for the same clade. For example, Hudson and Coyne (2002) point out that in the simplest theoretical case, where two populations diverge in the absence of gene flow and where population sizes are equal, it is expected that a single mitochondrial locus and a single nuclear locus will give different phylogenetic signals for approximately $6N_e$ generations (where N_e represents the effective population size), because of shared ancestral polymorphism alone.

Empirically, when gene trees with differing levels of phylogenetic support are obtained from multilocus, multigenomic data sets it can be difficult to determine the underlying causes (Maddison 1997; Maddison and Knowles 2006). The pattern of recent divergence and incomplete lineage sorting can be difficult to distinguish from ongoing male-biased gene flow or even from a lack of signal in nuclear gene sequences (Avise et al. 1983;

Degnan and Rosenberg 2009). Such situations may be encountered with increasing frequency as continual technological developments make it easier to obtain both mitochondrial and nuclear gene sequences for non-model organisms (Harismendy *et al.* 2009). In the case of gene trees where clades have different levels of phylogenetic support, methods that implement coalescent theory can be particularly helpful as they permit estimation of parameters such as gene flow and divergence time, and allow assessment of the uncertainty of these estimates (Kuhner 2009; Nielsen and Wakeley 2001). Disentangling the factors behind the observed difference in resolution can have important consequences for our understanding of the divergence process and the factors driving it, as well as for delimiting species boundaries in taxa threatened by extinction (Weisrock *et al.* 2010).

In this study we investigated evidence for recent and ongoing divergence between the endangered Galapagos and Hawaiian petrels (*Pterodroma phaeopygia* and *P. sandwichensis,* respectively). Traditionally these taxa have been considered a single species, likely as a result of their striking morphological similarity (Brooke 2004). Both taxa are medium sized (43 cm in length) and have black and white dichromatic plumage making them nearly indistinguishable, particularly at sea (Spear et al. 1995; Brooke 2004; Tomkins and Milne 1991; Simons 1985; Force et al. 2007). Seabirds, such as these petrels, are highly mobile and have few apparent physical barriers to dispersal. Yet many species exhibit strong philopatry and therefore have the potential to become genetically distinct over short geographical distances (Milot *et al.* 2008). A study of thirteen allozyme loci from blood samples found that eleven were fixed for the same allele in both

Hawaiian and Galapagos petrels, one locus was polymorphic in Galapagos petrels, and a single locus displayed a fixed difference between the two taxa (Browne *et al.* 1997). Based on these biochemical data as well as variation in breeding phenology and song, these taxa have recently been elevated to species status (Banks *et al.* 2002), but this split has not been investigated further. This is especially important as both of these taxa are increasingly threatened by extinction (Brooke 2004; Simons and Hodges 1998).

To gain a better understanding of the extent of genetic differentiation between the endangered and morphologically similar Galapagos and Hawaiian petrels, we sequenced the entire mitochondrial *Cytochrome b* gene and three nuclear intron loci. We employed both phylogenetic and coalescent-based approaches to examine evidence for recent divergence versus ongoing male-biased gene flow. Finally, we investigated migration between populations within each species to explore the potential for divergence at a local scale.

MATERIALS AND METHODS

Samples

We sampled a total of twenty-eight Hawaiian petrels, which included seven individuals from each of the four main Hawaiian Islands where they are currently known to breed (Figure 1, Table 1). Historically there was also a large colony on the island of Molokai, but it is now feared that this colony has been extirpated (BirdLife International 2009).

Samples included blood taken from grounded fledgling birds that were subsequently rehabilitated and released, as well as tissue, feather, and bone from depredated carcasses collected opportunistically during the breeding season from 1990 to 2008. Hawaiian petrels nest in burrows and are particularly vulnerable to exotic mammalian predators during this time. Non-breeding birds are known to fly over the colonies, but rarely come to ground and depart early in the breeding season (Simons and Hodges 1998), therefore samples are most likely to come from breeders or their offspring. Voucher information for Hawaiian petrel specimens can be found in Appendix A. A subset of the Galapagos petrel samples previously collected by Friesen *et al.* (2006) were utilized in this study. We obtained a total of thirty-five samples, which included seven individuals from each of the five Galapagos Islands that make up the entire breeding range of this bird (Figure 1, Table 1). For outgroup taxa, six blood samples each were obtained from the Atlantic petrel (*Pterodroma incerta*) and Soft-plumaged petrel (*P. mollis*) on Gough Island. Additionally, we obtained a tissue sample from the Bernice P. Bishop Museum (accession number BPBM184828) of one Murphy's petrel (*P. ultima*), which was originally collected at sea (Appendix A). These outgroup taxa represented both closely (*P. ultima*) and more distantly related (*P. incerta* and *P. mollis*) *Pterodroma* petrels (Nunn and Stanley 1998; Lawrence et al. 2009).

Molecular characterization

Total genomic DNA was extracted from Hawaiian petrel and outgroup samples using either the DNeasy tissue kit (Qiagen) for tissue and blood or a phenol/chloroform

method, developed for use with ancient and degraded DNA, for bone and feather samples (Horváth et al. 2005; Fleischer et al. 2000). DNA was previously extracted from Galapagos petrel samples (Friesen *et al.* 2006). The entire mitochondrial *Cytochrome b* (*Cytb*) gene was amplified using overlapping primers (Appendix B) designed in Primer3 (Rozen and Skaletsky 2000). A simulation study by McCormack *et al.* (2009) demonstrated a plateau in accuracy for sampling efforts beyond three loci and nine gene copies when utilizing coalescent-based approaches to investigate the relationships of recently diverged taxa. Therefore, three nuclear introns were amplified: α-enolase intron 8 (Enol), lamin A intron 3 (Lam), and ribosomal protein 40 intron 5 (RP40) (Friesen *et al.* 1999; Friesen *et al.* 1997). For samples with degraded DNA, shorter overlapping primer sets were developed (Appendix B). Polymerase chain reactions were carried out in a total volume of 15 uL, utilizing 1x PCR Gold Buffer (Applied Biosystems), 2.5 to 4 mM $MgCl_2$, 0.2 mM each dNTP, 1.2 mg/mL BSA, 0.5 μM of each primer, 1 unit AmpliTaqGold Polymerase (Applied Biosystems) and 1 to 2 uL of DNA extract. Amplifications took place under the following thermocycle profile: an initial denaturation step at 94^0C for 8 minutes, and then 35 cycles of 92^0C for 30 sec, a primer specific annealing temperature between 50^0C and 60^0C for 30 sec, and an extension step at 72^0C ranging from 30 sec to 1 min proportional to the length of the PCR product, followed by a final 7 minute extension at 72^0C.

Excess primers in the PCR product were degraded using a 1/10 dilution of ExoSAP-IT (USB) before cycle sequencing in both directions using the Big Dye Terminator v. 3.1 Cycle-Sequencing Kit (Applied Biosystems). Sequencing reactions were purified in

Sephadex (G-50 fine) columns and electrophoresed in a 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled in Sequencher v. 4.8 (Gene Codes Corporation) and visually inspected. Since some analyses conducted in this study (e.g. the Isolation with Migration program) required known phase for nuclear sequences, gametic phase was determined probabilistically using the program PHASE (Stephens and Donnelly 2003; Stephens et al. 2001). For individuals with resulting phase probabilities < 80% (Harrigan *et al.* 2008), haplotypes were determined via cloning using the TOPO TA cloning kit for sequencing (Invitrogen) and direct sequencing of more than five colonies. Phased haplotypic data were used for all nuclear analyses (i.e. each individual was represented by two sequences). Sequences for most loci were aligned in Sequencher, however the RP40 intron locus contained three short gaps and was aligned using ClustalX v2.0.12 (Larkin *et al.* 2007; Thompson *et al.* 1997). Gaps were retained in the data set and handled according to the default settings for each program. For example, gaps were treated as missing data in phylogenetic analyses, and discarded by the Isolation with Migration program.

Phylogenetic analyses

To investigate the magnitude of divergence between Galapagos and Hawaiian petrels, gene trees were constructed. First, however, *Cytb* sequences were translated in DAMBE (Xia and Xie 2001) and examined for the presence of stop codons and other indicators that they were nuclear copies (Sorenson and Fleischer 1996; Sorenson and Quinn 1998). Once the mitochondrial origin of the sequences was confirmed, the best-fit model of

nucleotide substitution for mitochondrial and nuclear DNA sequences was examined using jModelTest 0.1.1 (Posada 2008; Guindon and Gascuel 2003) with the Akaike information criterion.

Phylogenetic reconstruction was conducted with both maximum likelihood and Bayesian inference using Garli 0.96 (Zwickl 2006) and MrBayes 3.1.2 (Altekar et al. 2004; Ronquist and Huelsenbeck 2003), respectively. The HKY + I substitution model was utilized for both mitochondrial and nuclear data sets and analyses were conducted for each locus individually. Three additional partitioned Bayesian analyses were also carried out with all parameters except topology and branch lengths unlinked. First, the *Cytb* sequences were partitioned by codon position using the HKY, HKY + I, and HKY + G models for first, second, and third codon positions, respectively. Bayes factors were estimated in Tracer 1.4.1 to assess support for the codon-partitioned model over the unpartitioned model (Drummond and Rambaut 2007; Suchard et al. 2001; Kass and Raftery 1995). Second, a partitioned analysis was conducted on a data set that included all nuclear intron loci. Third, a partitioned analysis of the combined mitochondrial and nuclear data sets was performed. Finally, a maximum likelihood analysis was carried out for the three nuclear intron loci concatenated into a single data set. For maximum likelihood analyses, multiple runs were performed with random and stepwise addition, and support was assessed via 1000 nonparametric bootstrap replicates. Bayesian runs were conducted for between 10×10^6 and 50×10^6 generations each with four Markov chains and the default heating scheme. Output was recorded so that 10,000 data points were saved for each run and the first 10% were discarded as burn-in. Stationarity was

investigated by plotting –lnL across generations in Tracer 1.4.1 (Drummond and Rambaut 2007) and examining the effective sample size (ESS), which was greater than 500 for all estimated parameters. Convergence was assessed through comparison of multiple independent runs.

Genealogical sorting index

A measure known as the genealogical sorting index (gsi, Cummings *et al.* 2008) has recently been developed to quantify lineage divergence in cases where taxa do not form monophyletic groups during phylogenetic reconstruction. It measures divergence on a continuous scale from 0 to 1, where 1 represents the qualitative state of monophyly. To account for the uncertainty in the relationship between Galapagos and Hawaiian petrels in each nuclear intron phylogeny, the ensemble gsi statistic for each locus was calculated on the ~7,100 trees that represent the 95% credible set found in MrBayes. Significance of this statistic was determined through 10,000 permutations. For comparison, gsi was also calculated for an additional, previously published seabird mitochondrial DNA sequence data set investigating speciation in populations of band-rumped storm petrels breeding sympatrically in the Galapagos Islands during different seasons (Smith and Friesen 2007).

Inter-specific gene flow and divergence time

To investigate the timing and magnitude of divergence between Galapagos and Hawaiian petrels in a coalescent framework, the Isolation with Migration, (IMa; Hey & Nielsen 2007; Nielsen & Wakeley 2001) was utilized. IMa assumes no recombination within loci, and this assumption was tested using the difference of sums of squares method (DSS), a sliding window technique, implemented in TOPALi v 2.5 (McGuire and Wright 2000; Milne et al. 2009). Window size was set to 70 bp with a 10 bp increment, and statistical significance was determined through 100 parametric bootstraps. Initial IMa runs were conducted using extremely wide priors to establish the appropriate values for further analyses (Won and Hey 2005). Multiple independent final runs were conducted for between 5×10^6 and 2×10^8 generations both with and without heating. The first 10% of the genealogies were discarded as burn-in. Runs were considered to have reached stationarity after examination of plots of marginal posterior probability for each parameter and when the ESS for each run was > 200 (Won and Hey 2005).

Parameter estimates from IMa are compounded by the mutation rate, μ, and generation time. Therefore, to translate parameter estimates into values that are more easily interpretable, a mutation rate and generation time were applied. For the *Cytb* gene the mutation rate was determined by multiplying the sequence length (Appendix A) by an estimate of mutation rate for procellariiform seabirds of 1.89×10^{-8} substitutions/site/year (Weir and Schluter 2008). For the nuclear intron data set, sequence length for each locus was multiplied by a mutation rate of 3.6×10^{-9} substitutions/site/year (Axelsson et al. 2004; Carling and Brumfield 2008) and then the geometric mean of these three individual mutation rates was determined. The substitution rate of Axelsson *et al.* (2004) was

utilized since it was derived from birds and estimated from a broad sampling of 33 autosomal loci. To translate parameter estimates into demographic terms, generation time was also required. Unfortunately, little is known about generation time for these long-lived taxa. The average age at first breeding for procellariiform seabirds is roughly correlated with body size, and birds of similar size to Galapagos and Hawaiian petrels reach sexual maturity by approximately 5 or 6 years of age (Simons 1984; Warham 1990). Some albatross species are known to begin breeding regularly within 3 years after sexual maturity with the latest beginning to breed by about 12 years of age. While age at first breeding does not necessarily equal generation time, it seems reasonable that generation time for petrels of this size is between 6 and 12 years, therefore we calculated demographic estimates using generation times of both 6 and 12 years. Interpretation of results was the same independent of which estimate was utilized, therefore results for a generation time of 6 years are presented.

The IMa model also assumes that in the past there was one ancestral population that split into two daughter populations. This generally corresponds to the divergence between Galapagos and Hawaiian petrels, however, based on the literature (Friesen *et al.* 2006) and topology of the inferred mitochondrial gene tree, there is some evidence for population genetic differentiation within each taxon. Given these departures from the assumptions, we also estimated divergence time in BEAST v 1.5.2 (Drummond and Rambaut 2007). Outgroups were used to root trees constructed in BEAST, therefore analyses were conducted with a Yule tree prior. For *Cytb* data, a single haplotype from each island was utilized for the Galapagos and Hawaiian petrels, in addition to a

sequence from *Pterodroma ultima,* and sequences from *P. inexpectata,* and *P. externa* downloaded from the GenBank database (Accession numbers U74346 and U74339, respectively). However, for the intron data set only sequences for *P. ultima* in addition to Galapagos and Hawaiian petrels were available. Divergence dates were estimated using both a strict and relaxed clock model (Drummond *et al.* 2006) with the substitution rate fixed as indicated above. Multiple independent runs were completed for 10×10^6 generations, with the first 10% discarded as burn-in. Stationarity and convergence were assessed as described for phylogenetic analyses. Bayes factors were estimated to assess support for the relaxed clock model.

Intra-specific gene flow

To investigate the potential for divergence within each taxon at a local scale, gene flow between islands in each archipelago was examined using a coalescent approach as employed in the program MIGRATE v3.0.3 (Beerli 2006; Beerli and Felsenstein 1999). Coalescent based approaches for estimating intra-specific gene flow have higher statistical power than summary statistics, are robust, and allow investigation of confidence in estimates (Kuhner 2009). Also, they have been found to be accurate with small sample sizes. A simulation study by Felsenstein (2006) found the optimal number of haplotypes for some coalescent analyses to be eight, or possibly even fewer, per locus.

Migration rates were estimated from the data set of three nuclear introns. As mentioned above, gametic phase was determined for nuclear sequences and so each individual was

represented by two sequences in these analyses (i.e. N = 14 gene sequences per locus per population, for a total of 64 and 56 gene sequences for Galapagos and Hawaiian petrels, respectively). The Bayesian mode was employed with the transition/transversion ratio = 4.0 (as estimated in Garli), uniform priors, and a single long chain with three replicates. A static heating scheme with parameters set to 1.0, 1.5, 3.0, and 10.0 was utilized to increase the effectiveness of the search. The chain was run for 20 $x10^6$ generations with samples recorded every 1000^{th} generation, after discarding the first 10% as burn-in. Stationarity was examined and convergence was assessed from multiple independent runs initiated with different random seeds. In MIGRATE estimates are compounded by the mutation rate, μ, (i.e. $M = m/\mu$), and as such, they were scaled by the appropriate mutation rate from above.

RESULTS

We sequenced 73 individuals for all 1143 base pairs of the mitochondrial *Cytochrome b* gene and for 741 total base pairs from three nuclear introns. Three Galapagos petrel samples (two from Santa Cruz and one from Santiago) were discarded from all analyses due to missing data. All sequences were deposited in the GenBank database under accession numbers HQ420313-HQ420813. The *Cytb* data were examined to verify mitochondrial origin of sequences. No insertions or deletions were detected, and after translation no nonsense or stop codons were found. The ratio of transitions to transversions was 19:1 in Galapagos petrels and 12:1 in Hawaiian petrels, and the

majority of substitutions occurred in the third codon position. This evidence indicates that a mitochondrial origin of the *Cytb* sequences is likely.

Analyses conducted in Arlequin v.3.1 (Excoffier *et al.* 2005) on the *Cytb* and nuclear data sets revealed that they contained approximately the same number of variable sites. Fifty-one polymorphic sites were found for the mitochondrial data set while the combined data set of all three nuclear intron loci exhibited 56 total variable sites (10 in Enol, 24 in Lam, and 22 in RP40). Within Galapagos and Hawaiian petrels, 14 and 16 haplotypes were found for the *Cytb* gene, respectively, with no haplotypes shared. Additionally, there were 16 fixed differences found between the two taxa (Appendix C, Supplementary Table 1). For nuclear introns, 8, 21, and 17 haplotypes were discovered for Enol, Lam, and RP40, respectively, with 2, 1, and 8 haplotypes shared between taxa (Appendix C, Supplementary Tables 2 - 4). There were zero fixed differences found for nuclear sequences, but haplotype frequencies differed substantially (Appendix C, Supplementary Tables 2 - 4).

Phylogenetic Reconstructions

The gene tree produced from the mitochondrial data set showed strong support for the reciprocally monophyletic relationship of Galapagos and Hawaiian petrels, however gene trees constructed from the nuclear intron loci were unresolved. The total evidence data set yielded the same topology as the mitochondrial tree, but this could be biased by the relatively stronger signal in the mtDNA data. For analysis of the *Cytb* sequences Bayes

factors showed strong support for the codon-partitioned model over the unpartitioned model (2ln Bayes Factors = 298.688). The topologies of the Bayesian and maximum likelihood *Cytb* trees were congruent, and indicated that Galapagos and Hawaiian petrels were each monophyletic with moderate to high support (Figure 2). Within the Galapagos petrels, individuals from the same island did not group together. Within Hawaiian petrels, however, there were two clades with high support that mostly contained individuals from Lanai and Kauai, respectively, while individuals from Hawaii and Maui failed to form distinct clades.

The gene trees constructed for each nuclear intron showed a polyphyletic relationship between Galapagos and Hawaiian petrels. In two of the three intron trees (Enol and RP40) the outgroups, even the most distantly related species from the mitochondrial tree, were polyphyletic with Hawaiian and Galapagos petrels. However, in the tree reconstructed from the Lam intron sequences the distantly related Atlantic and soft-plumaged petrels formed a separate clade with high support, while Murphy's petrel remained polyphyletic with the ingroups (Figure 3). The tree reconstructed from the partitioned analysis of all intron loci, which included comparatively the same total number of variable sites as the *Cytb* locus (56 versus 51, respectively), nearly twice the number of variable sites in Galapagos petrels (25 versus 13), and approximately the same number of variable sites in Hawaiian petrels (17 versus 20) yielded the same topology as that obtained for the Lam intron, with high support for a clade containing Galapagos, Hawaiian, and Murphy's petrels (Figure 3). For nuclear loci the genealogical sorting index for Galapagos and Hawaiian petrels ranged from 0.1785 to 0.2783 and was highly

significant in all cases ($p < 0.0004$, Table 2). These values are also similar to those determined for the mitochondrial control region of band-rumped storm petrels (gsi = 0.2630 and 0.3527, $p < 0.0004$) that may be undergoing sympatric speciation (Friesen *et al.* 2007b). Despite the unresolved topology of the nuclear gene trees, the gsi analysis indicated the presence of weak, but statistically significant phylogenetic signal in the nuclear intron data set. Overall, phylogenetic signal was strong in the mitochondrial data set, but apparently weak in the nuclear intron data set.

Inter-specific gene flow and divergence time

To determine if the weak phylogenetic signal in the nuclear intron data set was due to incomplete lineage sorting or ongoing gene flow, as well as to investigate the timing of divergence of these two taxa, additional analyses were carried out utilizing the IMa program (Hey and Nielsen 2004). For both the *Cytb* and nuclear intron loci, plots of the marginal posterior probability for the migration parameters between the Galapagos and Hawaiian petrels were unambiguous and very narrow (Figure 4A, C). For three of the four migration parameters the peak in the posterior probability occurred in the smallest interval measured in the analysis (from 0 to 5.67×10^{-10} migrants per generation). While it is theoretically possible that given a finer resolution a nonzero peak may be found in a smaller interval, these estimates were considered effectively zero (Won and Hey 2005). The remaining migration parameter, the number of Hawaiian petrels (HAPE) per generation that migrated into the Galapagos petrel (GAPE) population estimated from the

intron data, had a wider HPD (highest posterior density), but was still effectively zero ($M_{HAPE \rightarrow GAPE}$ =2.86x10^{-6} migrants per generation, 90% HPD: 4.99x10^{-7} to 1.34x10^{-5}).

The divergence time estimated by IMa from the nuclear intron data indicated that these two taxa split approximately 570,700 years ago (90% HPD: 159,800 to 45,658,200 years ago; Figure 4B and Figure 5). A distinct peak occurred in the posterior distribution, however the tail hovered near zero for divergence times beyond 2,000,000 years resulting in the broad HPD interval (Figure 4). Hence, obtaining smaller HPD intervals may require sequencing a larger number of loci (Hey 2005). The mitochondrial data specified nearly the same time frame for the split (545,300 years ago, Figure 5), however the posterior distribution failed to converge to zero after a peak in the beginning of the distribution (Figure 4D). When taxa have reached the state of reciprocal monophyly at a locus, as the Galapagos and Hawaiian petrels have for the *Cytb* gene, there is little or no information remaining regarding the divergence process and therefore estimating the timing of divergence can be especially difficult (Edwards and Beerli 2000).

Since the potential presence of population structure within the Hawaiian petrels, as identified by the mitochondrial DNA phylogeny, may violate assumptions of the IMa model, divergence time was also estimated in the program BEAST. Divergence time estimates from BEAST were similar to those found in IMa but with narrower HPD intervals (Figure 5). Investigation of Bayes factors indicated little support for the relaxed clock over the strict clock model (2ln Bayes Factors = -1.116 and -2.736 for *Cytb* and intron data, respectively). For the strict molecular clock model and the *Cytb* data set and

the split between Galapagos and Hawaiian petrels was estimated to have occurred 588,600 years ago (95% HPD: 382,300 to 810,800 years ago) and for the intron data set, the split was estimated to have occurred 1,376,000 years ago (95% HPD: 683,900 to 2,170,200). Despite somewhat different point estimates obtained from each data set, the 95% HPD intervals overlap, indicating that the estimated dates do not differ significantly. Additionally, point estimates were similar between the BEAST and IMa analyses. This strengthens confidence in the divergence time estimates and indicates that IMa analyses were robust to slight model misspecifications, as demonstrated by Strasburg and Rieseberg (2010).

Intra-specific analyses

To investigate the potential for divergence of each taxon on a local scale, migration rates were estimated for Galapagos and Hawaiian petrels within their respective archipelagoes. Posterior distributions for demographic estimates from MIGRATE were narrow and unimodal for 31 of 32 parameters investigated. The only exception was for migration of Galapagos petrels from the island of Isabela to Floreana, for which the posterior distribution was narrow yet bimodal. Therefore this estimate should be interpreted cautiously. However the effective sample size for all estimates was greater than 100,000, which is one rough indication that the analyses were run for a sufficient length. Results indicated low levels of gene flow among islands within each archipelago (Tables 3 and 4). Estimates were roughly symmetric between islands and ranged from 0.007 to 0.848 and 0.092 to 0.802 migrants per generation for Galapagos and Hawaiian petrels,

respectively. Additionally, the 95% confidence intervals for more than half of the migration estimates for each taxon overlapped with zero.

DISCUSSION

Phylogenetic analysis of the *Cytb* gene demonstrated a reciprocally monophyletic relationship for Galapagos and Hawaiian petrels, indicating that these two taxa have diverged to a great extent (Figure 2). However, gene trees constructed from three nuclear intron loci were unresolved, depicting a polyphyletic relationship for these taxa, as well as the clearly distinct Murphy's petrel (Figure 3). This observed lack of resolution could result from insufficient variability in the nuclear data, ongoing male-biased gene flow, or a recent divergence with incomplete lineage sorting.

A comparison of the mitochondrial and nuclear data sets suggests that low variability or small sample size is not the culprit: the mitochondrial data set contained 51 variable sites, and the combined data set for three nuclear introns contained 56 variable sites. Further, if the observed polyphyly was due to insufficient variability in the data set then further population based analyses, such as estimates of migration obtained from IMa, should also be inconclusive, and this was not the case (Figure 4). Simulation studies of sampling design have suggested that when utilizing coalescent based approaches a data set consisting of three loci with three to nine gene copies per taxon recovers the relationships between recently diverged species with high accuracy (McCormack et al. 2009; Maddison and Knowles 2006). Here we sampled three nuclear loci with 14 gene copies

each. Additionally, a population level signal of divergence between taxa was apparent from haplotype networks constructed for the nuclear intron data sets (Appendix C, Supplementary Figures 1 - 3). Finally, the genealogical sorting index indicated the presence of weak, but statistically significant phylogenetic signal. Therefore, even though there was weak phylogenetic signal in the nuclear intron data set, there was still a strong signal of divergence at the population level.

The differing levels of support between gene trees could be caused by ongoing male-biased gene flow (Degnan and Rosenberg 2009). There are few obvious physical barriers to dispersal in seabirds (Friesen *et al.* 2007a) and in some species dispersal is common (Inchausti and Weimerskirch 2002). In some cases sex-biased dispersal has been documented (Young et al. 2009; Young 2010). However, for Galapagos and Hawaiian petrels, Isolation with Migration analyses indicate there is effectively no gene flow between taxa (Figure 4). Therefore, it is most likely that recent divergence and incomplete lineage sorting is causing the differences in the strength of the phylogenetic signal between the nuclear intron and mitochondrial gene trees. The mitochondrial gene sequences have acquired the signal of divergence between Galapagos and Hawaiian petrels, but the nuclear intron sequences are comparatively lagging as phylogenetic indicators of this divergence. Even when more than thirty nuclear loci were utilized to investigate recently diverged taxa, none demonstrated reciprocal monophyly (Lee and Edwards 2008).

Using the simplified scenario outlined by Hudson and Coyne (2002), along with an estimate of effective population size, the time required to reach the state of reciprocal monophyly at nuclear and mitochondrial loci can be estimated. Unfortunately, population size is difficult to estimate in procellariid seabirds such as the Galapagos and Hawaiian petrels. Current census population estimates for both taxa range between 10,000 and 30,000 individuals (Spear et al. 1995; Day and Cooper 1995). Census size often does not equal effective population size due to factors such as variance in reproductive success. However, life history characteristics of petrels, such as overlapping generations and a monogamous mating system, should act to decrease variance among individuals (Nunney 1995). Given the difficulties of estimating population size in these taxa, and taking into account that populations sizes were much larger in the recent past before humans colonized the islands (Athens 2002), we optimistically assume an effective population size of 20,000 individuals (equal to the average estimated census size). With this estimate the state of reciprocal monophyly would be achieved at a single mitochondrial locus in just 264,000 years, while it would take three nuclear intron loci between 1,044,000 and 1,416,000 years to reach the same state. Divergence time estimates for Galapagos and Hawaiian petrels from both BEAST and IMa pointed to a split occurring approximately 550,000 years ago. This is longer than the time required for reciprocal monophyly to be obtained at a mitochondrial locus, but well within the time frame where incomplete lineage sorting is predicted for nuclear loci.

The estimated divergence time of 550,000 years ago roughly corresponds to a period of intense ecological change in the Hawaiian and Galapagos archipelagos. Several of the

main islands in both archipelagoes formed during this period. For instance, Hawaii first appeared around 0.5 million years ago and the oldest rocks from Maui-Nui (which includes the Hawaiian islands of Maui, Lanai, Molokai and Kahoolawe) date to between 1.6 to 1.9 million years ago (Carson and Clague 1995). In the Galapagos, the islands of Isabela, Santiago, Santa Cruz, and Floreana all appeared between approximately 0.5 and 2.3 million years ago (Bailey 1976; Parent *et al.* 2008). This timing also coincides with the Pleistocene and petrels may have been impacted by changes in sea levels and productivity (Lea *et al.* 2000; Nunn 1998; Smith *et al.* 2007). Therefore periods of intense volcanism or climate change may have acted as a catalyst in initiating the divergence of these taxa. Regardless of the causes, reciprocal monophyly in the mitochondrial gene tree and evidence for a lack of gene flow from the nuclear intron data set indicate that Galapagos and Hawaiian petrels have progressed substantially in the process of divergence.

It is not only difficult to detect recently diverged taxa, it can also be difficult to determine when these taxa have diverged to the extent that they should be considered distinct species (Harrison 1998; de Queiroz 2007; Sites and Marshall 2004). In the past, taxonomic decisions have relied strongly on morphological evidence. In 1942, Ernst Mayr described the biological species concept stating that distinct species should not interbreed, or if they do they should not produce viable, fertile offspring (Mayr 1942). The phylogenetic species concept, on the other hand, emphasizes fixed differences and/or the formation monophyletic groups on gene trees (Coyne and Orr 2004). These species concepts are often difficult to apply to taxa early in the process of divergence. Such taxa

are often morphologically very similar, and depending on the extent of divergence, fixed genetic differences and phylogenetic resolution may also be lacking. Finally, for many species it may be impossible to directly test for reproductive isolation. In these cases, perhaps the evolutionary species concept is most appropriate. Under this definition, lineages that are independently evolving and that have their own evolutionary fate are considered distinct species (Wiley 1976).

Galapagos and Hawaiian petrels are just one example of the difficulty of identifying species boundaries for taxa that are in the process of divergence. These birds are difficult to distinguish, particularly at sea where they spend the majority of their lives (Spear et al. 1995; Brooke 2004; Tomkins and Milne 1991; Simons 1985). Their complicated life history makes directly testing for reproductive isolation nearly impossible. However, evidence of effectively no gene flow between Galapagos and Hawaiian petrels over the last 100,000 generations, despite their striking mobility, may give some limited, indirect evidence for reproductive isolation. The presence of sixteen fixed differences in mitochondrial DNA sequences, and reciprocal monophyly in the *Cytb* gene tree give some support for species status under the phylogenetic species concept, but the nuclear data set lacked resolution. While this is likely due to recent divergence and incomplete lineage sorting, the phylogenetic species concept does not take such factors into account. Given all of the evidence mentioned above it does appear that Galapagos and Hawaiian petrels are evolving independently and therefore could be considered separate species under the evolutionary species concept. This supports the recent taxonomic revision elevating Galapagos and Hawaiian petrels to the level of distinct species.

Investigations of migration within each species indicated that divergence may be occurring at the local geographic scale of islands within each archipelago. While sample sizes for population analyses were limited, there was sufficient signal to detect population differentiation. Additionally, low estimates of migration between populations of Galapagos and Hawaiian petrels demonstrated here are concordant with the literature. Using microsatellite markers Friesen *et al.* (2006) found evidence of strong population structure among Galapagos petrels breeding on different islands. Also, banding studies of both Galapagos and Hawaiian petrels found high levels of philopatry over multiple years, and even between multiple colonies on the same island (Simons 1985; Simons and Hodges 1998; Tomkins and Milne 1991). Further analyses with additional samples from each island will strengthen these conclusions.

Accurately identifying cryptic and recently diverged taxa can have important conservation implications (Bickford *et al.* 2007). In the case of seabirds, spuriously grouping taxa could be especially tragic as they are increasingly threatened, both at land and at sea. At the breeding grounds, for instance, seabirds must contend with predation by introduced mammals such as rats and cats, construction of obstacles such as power lines and wind turbines, and light pollution that leads to high juvenile mortality during fledging (Brooke 2004; Rayner et al. 2007; Cruz-Delgado et al. 2010). At sea, petrel mortality on fishing gear can be high (Tuck *et al.* 2001). For white-chinned petrels (*Procellaria aequinoctialis*) alone, up to 80,000 individuals are killed each year as a result of by-catch (Techow *et al.* 2009). It is important to gain a better understanding of

the distribution of cryptic diversity as taxa that are already threatened by extinction may in fact represent multiple species that have even smaller population sizes, even more restricted ranges (Simons 1985), and that may require even more rigorous intervention to ensure their continued existence (Bickford *et al.* 2007).

TABLES

Table 1. Summary of taxa sampled, collecting locality, and sample size (N)

Taxon	Location	N
Pterodroma phaeopygia	**Galapagos Islands**	**35**
	Floreana	7
	Isabela	7
	San Cristóbal	7
	Sana Cruz	7
	Santiago	7
Pterodroma sandwichensis	**Hawaiian Islands**	**28**
	Hawaii	7
	Maui	7
	Lanai	7
	Kauai	7
Pterodroma incerta	**Gough Island**	**6**
Pterodroma mollis	**Gough Island**	**6**
Pterodroma ultima	**At sea**	**1**

Table 2. Genealogical sorting index and associated p-values by locus for Galapagos and Hawaiian petrels.

	Galapagos Petrels		Hawaiian Petrels	
Locus	**gsi_T**	**p**	**gsi_T**	**p**
Cytb	1.0000	0.00001	1.0000	0.00001
Enol	0.2297	0.00001	0.2573	0.00001
Lam	0.2783	0.00001	0.2353	0.00001
RP40	0.1785	0.00040	0.1832	0.00001

Table 3. Migration estimates for Hawaiian petrels from the program Migrate. Values in bold are the estimated number of migrants per generation from the population on the left into the population at the top of the table. 95% confidences intervals are in parentheses below.

	Hawaii	**Maui**	**Lanai**	**Kauai**
Hawaii	-	**0.802** (0.631-0.946)	**0.171** (0.026-0.289)	**0.145** (0.000-0.263)
Maui	**0.145** (0.000-0.263)	-	**0.145** (0.000-0.263)	**0.118** (0.000-0.263)
Lanai	**0.197** (0.000-0.368)	**0.302** (0.105-0.447)	-	**0.171** (0.000-0.342)
Kauai	**0.118** (0.000-0.237)	**0.092** (0.000-0.210)	**0.171** (0.026-0.342)	-

Table 4. Migration estimates for Galapagos petrels from the program Migrate. Values in bold are the estimated number of migrants per generation from the population on the left into the population at the top of the table. 95% confidences intervals are in parentheses below.

	Santiago	**Isabela**	**Santa Cruz**	**Floreana**	**San Cristóbal**
Santiago	-	**0.243** (0.105-0.355)	**0.007** (0.000-0.171)	**0.072** (0.000-0.210)	**0.099** (0.000-0.197)
Isabela	**0.204** (0.039-0.381)	-	**0.848** (0.657-1.025)	**0.099** (0.000-0.210)	**0.151** (0.013-0.276)
Santa Cruz	**0.112** (0.000-0.223)	**0.112** (0.000-0.223)	-	**0.230** (0.000-0.342)	**0.112** (0.000-0.210)
Floreana	**0.059** (0.000-0.171)	**0.177** (0.053-0.302)	**0.007** (0.000-0.223)	-	**0.204** (0.079-0.315)
San Cristóbal	**0.059** (0.000-0.171)	**0.269** (0.131-0.407)	**0.112** (0.000-0.250)	**0.151** (0.013-0.276)	-

FIGURES

Figure 1 Map of the Pacific Ocean (a) depicting Hawaiian (light gray) and Galapagos (medium gray) petrel breeding distribution and foraging range (Brooke 2004). Maps of the Hawaiian (b) and Galapagos archipelagoes (c) showing islands where Hawaiian and Galapagos petrels are known to breed and where samples were obtained (light gray and medium gray, respectively).

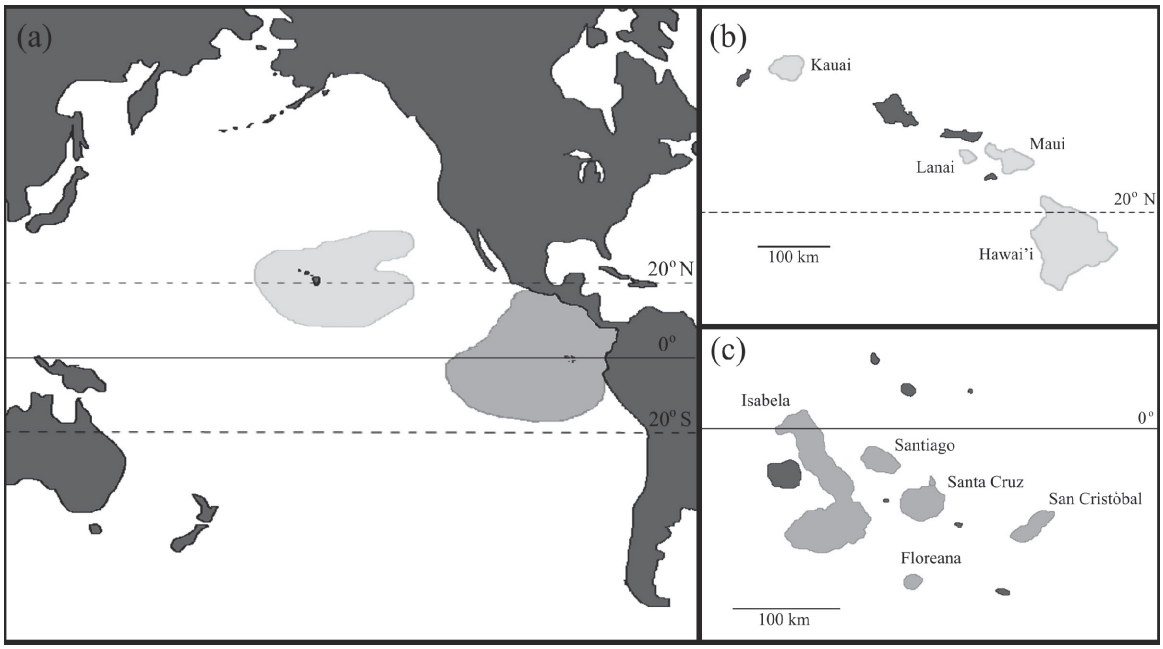


Figure 2. Bayesian majority rule consensus tree for the mitochondrial *Cytb* gene. A total evidence data set comprised of the *Cytb* gene and three nuclear introns resulted in a congruent topology, but lacked taxa for which no intron data was available (dashed branches). Numbers above the branch are posterior probabilities and maximum likelihood bootstrap support for the *Cytb* tree while those below the branch are posterior probabilities for the total evidence tree. Dashes indicate no substantial support. GAPE = Galapagos petrel, HAPE = Hawaiian petrel, MRPH = Murphy's petrel, SPP = soft-plumaged petrel, ATP = Atlantic petrel. Letters in parenthesis indicate island of origin for a haplotype: S = Santiago, Sc=Santa Cruz, F=Floreana, I=Isabela, C=San Cristobal, L=Lanai, K=Kauai, M=Maui, and H=Hawaii.

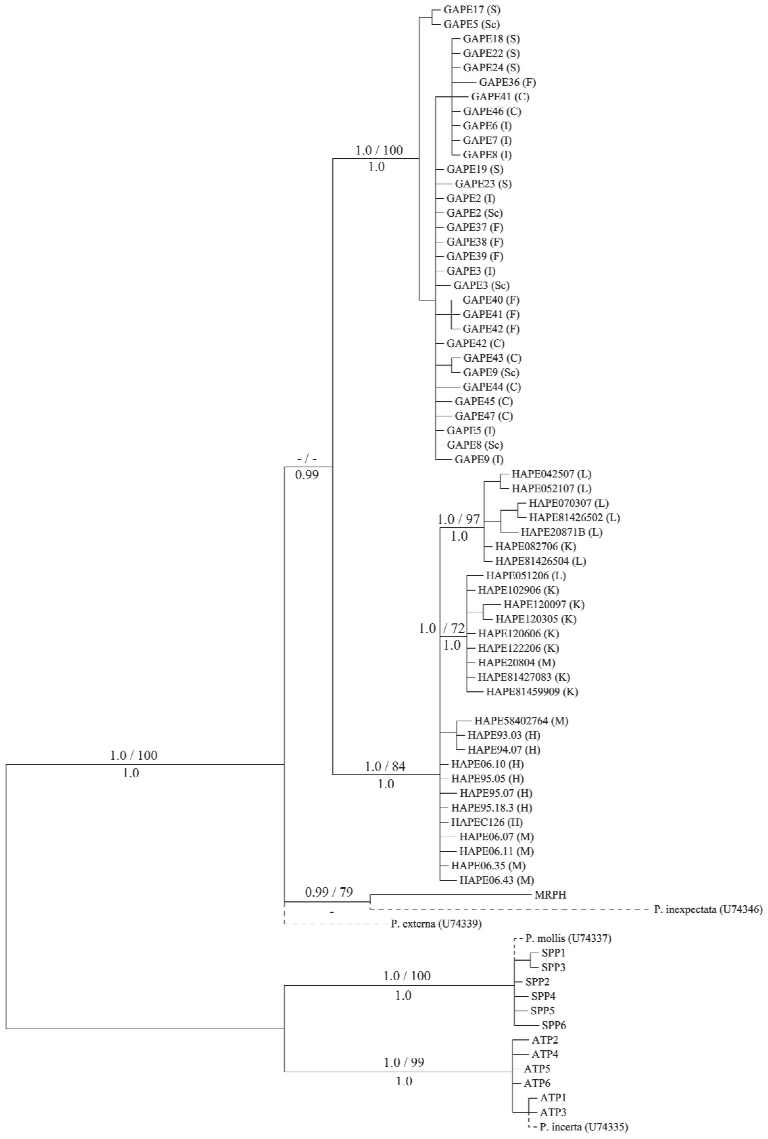


Figure 3. Bayesian majority rule consensus tree obtained for the Lam intron as well as the partitioned analysis of all three nuclear introns. Numbers above the branch are posterior probability and maximum likelihood bootstrap support for the Lam locus, below the branch is the posterior probability for the combined intron data set. Brackets and shading indicate location of taxa on the tree, with the darker, overlapping blocks where multiple species occur within a single clade. MRPH = Murphy's petrel, GAPE = Galapagos petrel, HAPE = Hawaiian petrel, SPP = soft-plumaged petrel, ATP = Atlantic petrel.

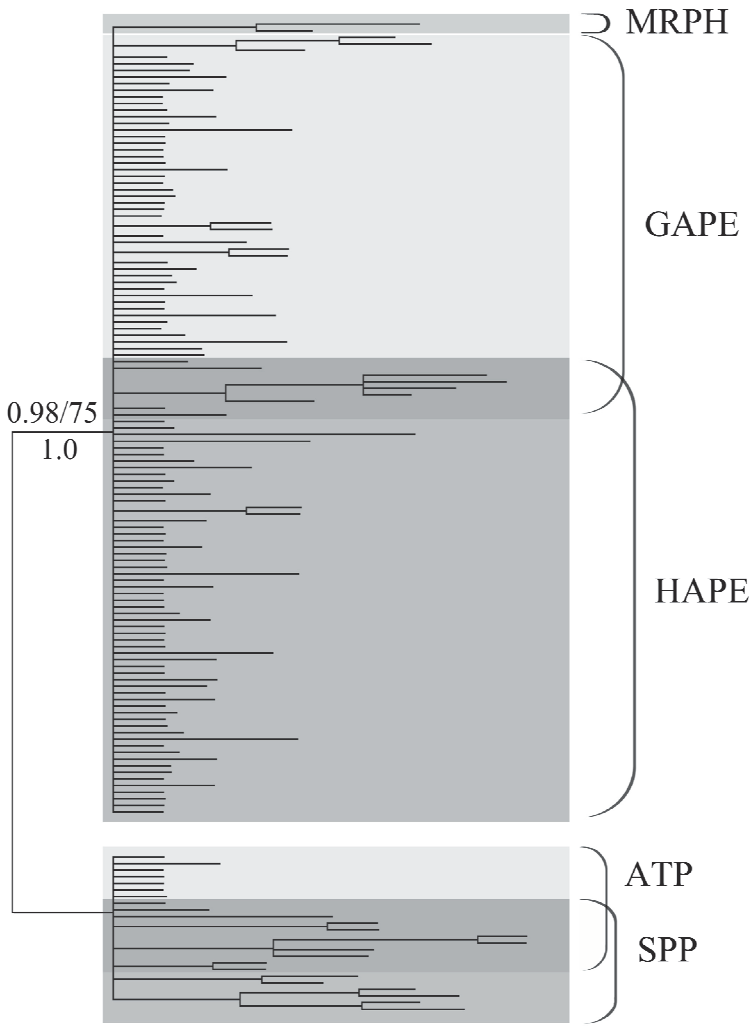


Figure 4. Marginal posterior distributions from the Isolation with Migration program (IMa) for migration rates and divergence time (years before present). (a) and (b) show results for a data set composed of three nuclear intron loci; (c) and (d) display results for the mitochondrial *Cytb* gene.

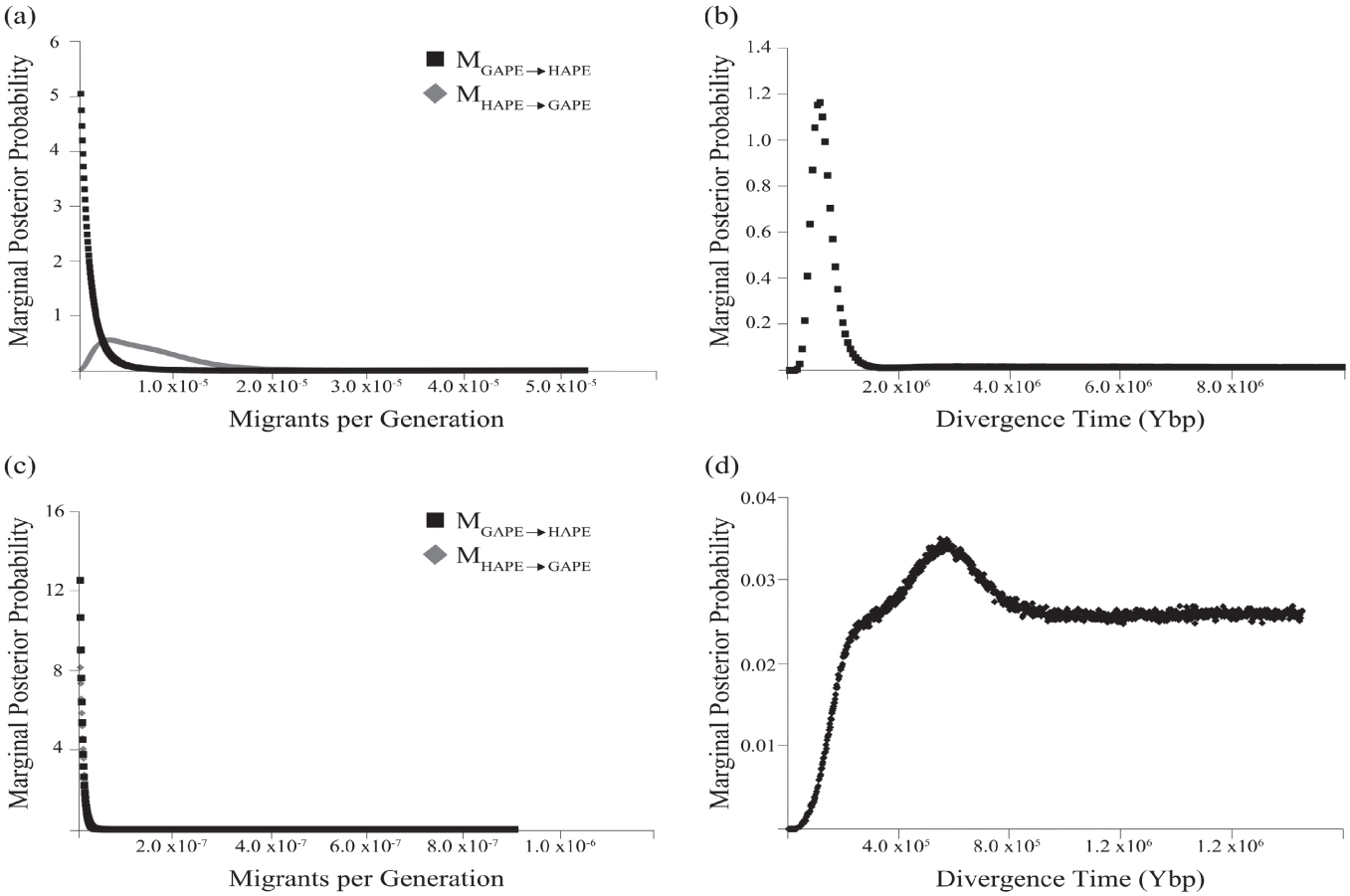
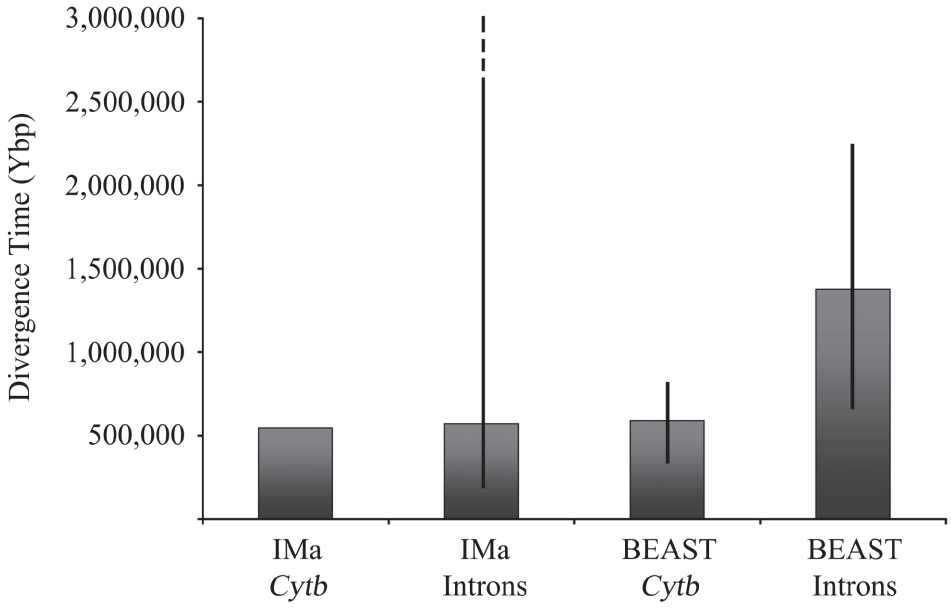


Figure 5. Comparison of divergence time estimates, in years before present, from IMa and BEAST for the mitochondrial and nuclear intron data sets. Black bars indicate the 90 and 95% highest posterior density (HPD) for estimates from IMa and BEAST, respectively. The posterior distribution for the divergence time parameter estimated in IMa from the *Cytb* data set failed to converge. Therefore confidence in this estimate cannot be assessed.



CHAPTER II

Polymorphic microsatellite markers for the endangered Hawaiian petrel (*Pterodroma sandwichensis)**

The endemic and endangered Hawaiian petrel (*Pterodroma sandwichensis*) breeds on four of the six main Hawaiian Islands, however the levels of gene flow between these islands remain unclear. We describe 10 novel polymorphic microsatellite loci isolated from this species, and characterize 10 additional previously published primer sets in 40 individuals from the island of Maui. Loci exhibited between 3 and 14 alleles (mean 6.85), and observed heterozygosity ranged from 0.150 to 0.825 (mean 0.540). Four loci showed evidence for null alleles, although only one locus was found to have significant deviation from Hardy-Weinberg equilibrium after Bonferonni correction. Two primer pairs showed significant gametic disequilibrium. The loci described here should provide a useful toolset for investigations of population connectivity and parentage.

*A. J. Welch and R. C. Fleischer. 2011. Conservation Genetics Resources, In press.

Seabirds face a multitude of threats, both on land and at sea. As a result many species are in danger of extinction (Brooke 2004). The endemic Hawaiian petrel, *Pterodroma sandwichensis*, was apparently abundant prior to human colonization of the Hawaiian Islands (Athens et al. 1991; Olson and James 1982a), but direct exploitation by humans, predation by introduced mammals, and habitat destruction have led to population declines and this species is now considered endangered (Simons and Hodges 1998). Several Hawaiian petrel colonies have been or are currently in danger of being extirpated: a colony on the island of Oahu was eliminated prehistorically, a formerly large colony on Molokai has not recently been relocated, and colonies on the Big Island and western Maui appear to be in peril (Simons and Hodges 1998; Birdlife-International 2010). A mitochondrial DNA sequence dataset suggests that gene flow may be low between petrel colonies on different islands (Welch *et al.* 2011), however additional information from biparentally-inherited markers is necessary to gain a better understanding of the patterns of gene flow and the impacts of population extirpation. Here were describe development of 10 novel polymorphic microsatellite markers and the characterization of 10 additional loci originally isolated in other species.

Microsatellite enriched genetic libraries were constructed following Hamilton *et al.* (1999). Briefly, genomic DNA was extracted from tissue samples of one male and one female Hawaiian petrel obtained from the University of Washington's Burke Museum (accession numbers 55605 and 55576) using the Qiagen DNEasy tissue kit. DNA was digested using the restriction enzymes *Hae*III, *Xmn*I, and *Nhe*I (New England Bioloabs) and fragments approximately 200 – 1000 bp in size were ligated to SNX linkers and

amplified using SNX primers. These fragments were then hybridized to $(CA)_{10}$, $(AAAG)_5$, $(AAAC)_5$, or $(AGAGG)_5$ biotinylated oligos and any remaining unhybridized fragments were removed using Dynabeads M-280 Streptavidin magnetic beads (Invitrogen). Fragments were amplified again, ligated into pBluescript II SK + plasmids, and then transformed into ultracompetent XL-10 Gold *E. coli* (Stratagene).

Positive clones were PCR screened using a combination of three primers: T7 (5'-GTAATACGACTCACTATAGGGC-3'), T3 (5'-AATTAAC- CCTCACTAAAGGG-3'), and the appropriate oligo from the enrichment step above. Clones that appeared as smears instead of distinct bands on agarose gels were then amplified using only the T7 and T3 primers, cycle sequenced using Big Dye Terminator v. 3.1, and electrophoresed on a 3130 Genetic Analyzer (Applied Biosystems). The resulting sequences were examined in Sequencher v. 4.9 (Gene Codes). Approximately 1400 colonies were screened. A total of 65 unique clone sequences contained sufficient flanking region for primer design and had ≥ 7 dinucleotide repeats or ≥ 5 tetra- or pentanucleotide repeats. However, 11 of these (17%) apparently contained an artifact consisting of the oligo used for hybridization ligated to a 9 bp remnant of the SNX linker.

Primers were designed for the remaining 54 clones using Primer3 (Rozen and Skaletsky 2000) with forward primers including an M13 tail (5'-TGTAAAACGACGGCCAGT-3'). One sequence, *Ptero06* (Table 1), contained an $(AAGG)_{13}$ motif as well as an upstream, interrupted $(TG)_{18}$ motif. Primers were designed for each region independently, and both produced multiple alleles, but results for only the (AAGG) motif are shown below. In

addition, we screened 37 previously published primer sets for cross amplification in the Hawaiian petrel, including all loci from Brown and Jordan (2009), Bried *et al.* (2008), Gonzalez *et al.* (2009), and Techow and O'Ryan (2004), as well as the individual loci RBG18, RBG29, Dpμ01, and DC16, which were found to be polymorphic in the closely related Galapagos petrel, *Pterodroma phaeopygia* (Given et al. 2002; Dawson et al. 1997; Burg 1999; Friesen et al. 2006). Some previously published primer sets were redesigned (indicated by M following primer name in Table 2) to amplify shorter fragments. Primer sets were initially screened for polymorphism using a total of 15 individuals from the islands of Hawaii, Maui, Lanai, and Kauai.

Amplifications were carried out following the three-primer protocol of Schuelke (2000), in which a fluorescently labeled M13 (5'-FAM-M13-3') primer was utilized in addition to the reverse and the M13-tailed forward primers. Amplifications were conducted in 15 μL reactions with 1x colorless GoTaq Flexi buffer, a primer-specific concentration of $MgCl_2$ (Tables 1 and 2), 0.2 mM each dNTP, 1.2 mg/mL BSA, 0.14 μM M13-tailed forward primer, 0.4 μM reverse and fluorescently labeled M13 primers, 1 unit GoTaq Flexi DNA polymerase (Promega) and 1.5 μL DNA extract. The thermocycle profile began with a denaturation step for 2 min at 95^{o}C, followed by 35 cycles of 95^{o}C for 30 s, a primer-specific annealing temperature (Tables 1 and 2) for 30 s, 72^{o}C for 30 s, and a final step at 72^{o}C for 30 min. Products were electrophoresed on a 3130xl Genetic Analyzer (Applied Biosystems) and alleles were called manually in GeneMapper v. 4.1 (Applied Biosystems). For loci that were found to be polymorphic, fluorescently labeled forward primers (without the M13 tail) were obtained and 40 individuals from the island

of Maui were amplified. Previously published loci that were found to be polymorphic in the Hawaiian petrel were sequenced, as described above, to examine homology to the original loci. The program MICRO-CHECKER v. 2.2.3 (van Oosterhout *et al.* 2004) was used to test for the presence of large-allele drop-out and null alleles. The program GENEPOP v. 4.0 (Rousset 2008) was used to test for departures from Hardy-Weinberg expectations and for linkage disequilibrium.

Of the 54 Hawaiian petrel (HAPE) specific primer pairs, 15 were monomorphic, 29 did not amplify or produced irresolvable stutter, and 10 produced two or more alleles in the initial screen. Of the 37 previously published primer pairs, 18 produced two or fewer alleles, 9 did not amplify, and 10 were polymorphic in initial screens. Therefore, 20 loci were genotyped in the larger set of 40 individuals (Tables 1 and 2). Two HAPE-specific primer pairs exhibited allele sizes inconsistent with the repeat motif. Sequencing of these alleles revealed a 38 bp deletion in the flanking region of *Ptero10,* and 2 bp and 5 bp deletions in *Ptero07*. None of the loci appear to be sex-linked and there was no indications of large-allele dropout. Four loci (*Ptero06*, *Ptero10*, *Parm05*, and *PuffPM2*) exhibited an apparent excess of homozygotes across all allele classes (van Oosterhout null allele frequencies were 0.215, 0.143, 0.150, and 0.154, respectively). The underlying causes of the observed homozygote excess remain unclear, but could potentially include the presence of additional undetected insertion/deletions or inbreeding within the Maui population. Despite the potential presence of null alleles, only *Ptero06* showed significant deviations from Hardy-Weinberg equilibrium after Bonferroni correction for multiple tests (Tables 1 and 2). Two pairs of loci were found to be in linkage

disequilibrium after Bonferroni correction: *Parm01* and *RBG29*, and *Parm01* and *Ptero03* ($p < 0.0001$ for both). Overall, the loci developed and characterized here should provide a useful toolset for investigations of population connectivity and parentage in the endangered Hawaiian petrel.

TABLES

Table 1. Characterization of 10 novel microsatellite loci isolated from the Hawaiian petrel (*Pterodroma sandwichensis*)

Locus GenBank #	Clone	Primer sequences (5' to 3')	Repeat motif	Dye	Size (bp)	$MgCl_2$ (mM)	T_a (^oC)	N_A	H_O H_E	HW (p)
Ptero01	27.28	**F:** GAAAACAACTCCCCCACAAC	$(CA)_7$	Hex	163-167	2.5	53	3	0.200	1.000
HQ918218		**R:** TCCGTCAGACCTGCTGTATG							0.187	
Ptero02	27.36	**F:** AAGCGCTTCACTGGAGGA	$(CA)_9$	Fam	131-141	3.0	53	5	0.550	0.425
HQ918219		**R:** TGACCTGTGTGCCTTCATTC							0.547	
Ptero03	27.72	**F:** TGTGTACAGCATGTGCTTGAG	$(CA)_9$	Fam	130-142	3.0	53	7	0.825	0.892
HQ918220		**R:** GCTGAATGGCAGTTTCTTCC							0.775	
Ptero04	27.96	**F:** TGCATTGTTTCTGTCCAAACTC	$(CA)_{13}$	Hex	146-160	3.0	53	8	0.775	0.752
HQ918221		**R:** GGCTGGAATGCATAGTACCAAC							0.734	
Ptero05	25.153	**F:** TGCTGTCGGCTGGGTTAC	$(AAG)_2AGG(AAG)_3$	Hex	206-215	2.5	53	3	0.150	1.000
HQ918222		**R:** CTGCCTGCCTTCCTGAAAC							0.142	
Ptero06	27.134	**F:** GTTCCATGGCGATGAAGG	$(AAGG)_{13}$	Hex	145-177	3.0	53	9	0.425	<0.0001*
HQ918223		**R:** CTGAAAATGGCATCCAAACG							0.801	
Ptero07	28.42	**F:** TTAAAAATCGGTCCAATAGTCG	…$(AAAG)_8$	Fam	253-289	2.5	53	8	0.825	0.690
HQ918224		**R:** GCACAGAGTTGACCGTGTTG							0.818	
Ptero08	28.50	**F:** GCACCTGCTGGTGATGAGTC	…$(AAGG)_8$	Hex	162-226	2.5	53	10	0.70	0.005
HQ918225		**R:** AGGGAAAAGGAACCATCCAG							0.823	
Ptero09	28.85	**F:** GCAAATACCAGTCTTCCAAAGG	$(AAGG)_8$…	Hex	212-236	2.5	53	7	0.775	0.902
HQ918226		**R:** TTTAAGATAAAGATGTTTGAGAACCAC							0.671	
Ptero10	25.06	**F:** AGCTGTGTGGGCTTAATTGC	$(TAGGA)_9$…	Fam	205-290	2.0	56	8	0.575	0.013
HQ918227		**R:** ACCCTACTCTGTGGCACCTC	$(TAGGA)_7$						0.810	
Mean								6.8	0.580	

Table 1 (Continued)
$MgCl_2$: Final magnesium chloride concentration (mM), T_a: Annealing temperature (^oC), N_A: Number of alleles in a sample of 40 Hawaiian petrels from Maui, H_O: Observed heterozygosity, H_E: Expected heterozygosity, HW: p value for test of departure from Hardy-Weinberg expectations, * Significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction. Mean number of alleles and observed heterozygosity shown on the last line.

Table 2. Characterization of 10 previously developed microsatellite loci in Hawaiian petrel (*Pterodroma sandwichensis*)

Locus / GenBank #	Primer sequences (5' to 3')	Original repeat motif / Repeat motif in HAPE	Dye	Size (bp)	$MgCl_2$ (mM)	T_a (^oC)	N_A	H_O / H_E	HW (p)	Ref
Parm01M	**F:** AGGGCAGAAGCTCGTCTATC	$(CA)_{13}$	Fam	189-219	3.0	53	13	0.675	0.773	a
HQ918214	**R:** AGTGCCCTCTTCCCCATAAC	$(CA)_{13}$						0.760		
Parm02M	**F:** TGTTATGTCCTGCGGATGAG	$(CA)_6TA(CA)_9$	Hex	168-180	3.0	53	6	0.225	1.000	a
HQ918215	**R:** TGAATCCAGGGTGAGTCAGG	$(CA)_7TA(CA)_8$						0.210		
Parm03M	**F:** ATTGCTCAGGGAGGTGCTG	$(CA)_5TA(CA)_{11}$	Fam	149-167	2.5	53	9	0.600	0.068	a
HQ918216	**R:** TTGGTTGAAGGTCTGTGTGG	$(CA)_{11}$						0.637		
Parm05M	**F:** CTCAGTGCCAATTCGTACCC	$(CA)_{11}$	Hex	93-99	3.0	53	4	0.300	0.003	a
HQ918217	**R:** AATGCAAGGCTGATGCTAATG	$(CA)_{10}$						0.464		
Cd2	**F:** CAACAACTCACCCTGCCTTT	$(TG)_{11}(CG)_2(TG)_2$	Fam	189-193	3.0	55	3	0.154	0.317	b
HQ918211	**R:** GCTCCTTTCTGAAGCTGTGG	$(TG)_6CG(TG)_2$						0.189		
Cd10	**F:** GGTGTGTGGGGAAAGAAACA	$(TG)_9$	Fam	187-201	2.0	54	7	0.650	0.359	b
HQ918212	**R:** TCTGTTCAGGGGCTGTCCT	$(TG)_{10}$						0.627		
PuffPM2	**F:** AAAGATGCTTAAGGATACATAACTGC	$(GT)_{16}$	Fam	183-189	3.0	55	3	0.375	0.004	c
HQ918228	**R:** TTGTTGGCAAAAGTGATAAGGTT	$(GT)_{13}$						0.589		
Paequ3	**F:** TGTGGGTGCAGTAGAGCA	$(GA)_{19}$	Hex	212-220	3.0	53	5	0.425	0.138	d
HQ918213	**R:** CAATAAGAAGATCAGCAGAACAGAC	$(GA)_{10}$						0.474		
RBG18M	**F:** TTGTTCCTTGGCTTCTATCTC	$(GT)_{11}$	Fam	98-108	3.0	53	5	0.825	0.073	e
HQ918229	**R:** CGCTGAAGATGTAGAGGGAAA	$(GT)_9$						0.617		
RBG29	**F:** CCTAGCTTTTTGGACTCAGT	$(GT)_{13}$	Fam	121-171	3.0	53	14	0.775	0.954	e
HQ918230	**R:** GTGGTGTTGCAATAGGATTC	$(GT)_{14}$						0.783		
Mean							6.9	0.500		

Table 2 (Continued)
$MgCl_2$: Final magnesium chloride concentration (mM), T_a: Annealing temperature (^oC), N_A: Number of alleles in a sample of 40 Hawaiian petrels from Maui, H_O: Observed heterozygosity, H_E: Expected heterozygosity, HW: p value for test of departure from Hardy-Weinberg expectations, * Significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction. [a] Brown and Jordan (2009),[b] Bried et al. (2008), [c] Gonzalez et al. (2009), [d] Techow and O'Ryan (2004), [e] Given et al. (2002) Mean number of alleles and observed heterozygosity shown on the last line.

CHAPTER III

Population divergence and gene flow in an endangered and highly mobile seabird*

The endemic Hawaiian petrel (*Pterodroma sandwichensis*) is a highly vagile seabird that was formerly distributed more widely across the Hawaiian Islands. Today it is considered endangered, and known to breed only on the islands of Hawaii, Maui, Lanai, and Kauai. Historical records indicate that a large population formerly bred on Molokai, but it is feared that this population has recently been extirpated. Given the great dispersal potential of petrels it remains unclear if populations are isolated from one another or if there is substantial gene flow between them. We sampled petrels from across their current range, as well as individuals collected on Molokai in the early 1900's. We sequenced 524 bp of mitochondrial DNA, 741 bp from three nuclear introns, and genotyped 18 microsatellite loci in order to investigate patterns of divergence and gene flow in the Hawaiian petrel. Mitochondrial DNA demonstrates significant, strong population genetic structure among all modern populations. Low differentiation was found between historic samples from Molokai and modern birds from Lanai. Weak, but significant structure was also detected in both nuclear data sets. Migration estimates were low, and a Bayesian clustering analysis revealed at least two genetic populations, one of which primarily consisted of birds from Hawaii and Lanai and the other consisted primarily of birds from Maui, with Kauai individuals representing a mixture. Evidence for significant population structure, in addition to ecological differentiation, indicates that distinct management

units may exist in this species. Conservation efforts should be considered on an island-to-island basis to preserve genetic diversity in Hawaiian petrels.

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INTRODUCTION

Populations are a fundamental unit in evolutionary biology and exist in a delicate balance between isolation and gene flow (Allendorf and Luikart 2007). Gene flow can decrease inbreeding (Madsen *et al.* 1999; Westemeier *et al.* 1998) and prevent losses of genetic diversity (Pinsky *et al.* 2010), but it can also lead to population genetic homogenization and outbreeding depression (Frankham 1995a). Populations are also of central importance for the persistence of species (IUCN 2001; Redford et al. 2011) and population loss may be an indicator increased threat of extinction (Ceballos and Ehrlich 2002). Indeed, it has been argued that changes in the size or number of populations may be a more immediate indicator of the long-term impacts of anthropogenic influences on species persistence than estimates of broad-scale extinction rates (Balmford *et al.* 2003).

Populations of many seabird species have declined in number or disappeared as a result of anthropogenic influences including habitat destruction, the introduction of exotic mammalian predators, and industrial fishing (Brooke 2004). Declines of seabirds could potentially have broad ecological consequences as they are top marine predators and play an important role in transferring marine nutrients to terrestrial ecosystems on oceanic islands (Fukami et al. 2006; Croll et al. 2005). However, information regarding seabird population size and dispersal, which is important for conservation management decisions, can be difficult to obtain for some species (Shirihai et al. 2009). Factors such as remote and inaccessible breeding locations, delayed maturity, high dispersal potential, and a pelagic lifestyle, can make direct observation difficult (Brooke 2004). Fortunately,

genetic methods have great potential power to accurately assess patterns of differentiation and dispersal without direct observation or manipulation of live birds (Friesen et al. 2007a).

The Hawaiian petrel (*Pterodroma sandwichensis*) is an endangered pelagic seabird that breeds exclusively in the Hawaiian Islands (Simons and Hodges 1998). It is known to forage long distances away from the colony during the breeding season, ranging from the equator to the Aleutian Islands (Spear et al. 1995; Adams et al. 2006; Simons and Hodges 1998). Today, the Hawaiian petrel is currently known to breed on the islands of Hawaii, Maui, Lanai, and Kauai, however colonies on Hawaii appear to be dwindling (Birdlife-International 2010). Based on subfossil evidence, it appears that this species was previously much more wide spread, both on islands where it currently breeds (e.g. at lower elevations than where modern colonies are located) as well as on two additional islands where it has apparently been extirpated (Simons and Hodges 1998; Olson and James 1982a). A large colony was formerly present in the lowlands of Oahu, but this species has not been recorded as breeding there in historical times (i.e. since the discovery of the Islands by Captain Cook in 1778; Olson and James 1998). Additionally, historical records (Munro 1955) indicate that there was a breeding population on the island of Molokai with petrels so abundant that they darkened the sky. However recent surveys have been unsuccessful in locating a substantial colony on that island (Simons and Hodges 1998). Fortunately, study skin specimens from this colony do survive in museum collections.

Little information is available about migration among Hawaiian petrel populations. These birds are difficult to study as they spend most of their life at sea and return to land nocturnally to breed. Additionally, they currently nest in burrows in high elevation colonies which are often located in remote and very rugged terrain (Simons and Hodges 1998). Banding studies in a colony on Maui have shown that adults are highly philopatric, returning to the same burrow to breed for multiple years (Simons 1985). However, Hawaiian petrels exhibit delayed maturity and don't begin breeding until approximately six years of age (Simons 1984), so it remains unclear if they demonstrate natal philopatry. Genetic information on dispersal is also limited. Browne et al. (1997) utilized allozymes to study genetic variation, but only in a single colony on Maui. Recently Welch et al. (2011) demonstrated that population structure may be present within this species, but a more extensive analysis is needed.

We have conducted a comprehensive, range wide population genetic study of the Hawaiian petrel. We have collected samples from all islands where these birds are currently, or were historically, known to breed and developed three genetic datasets, including both nuclear intron and mitochondrial DNA sequences as well as genotypes from 18 microsatellite loci, to investigate population divergence and gene flow among extant and potentially extirpated populations of this species.

METHODS

Samples

A total of 322 Hawaiian petrel samples were obtained from across the current and historical breeding range of this species (Table 1, Figure 1). Modern samples (N = 293) consisting of blood, tissue, bone, and feather were collected opportunistically from carcasses of birds depredated in breeding colonies on Hawaii, Maui, Lanai, and Kauai between 1990 and 2010, or from birds that were handled during conservation management procedures, such as rehabilitation after attraction to artificial lights and subsequent downing. Carcasses and downed birds were assumed to be breeding on that island (or the offspring of birds breeding on that island) since non-breeders depart early in the breeding season (Simons and Hodges 1998). Blood samples of chicks previously collected from Haleakala National Park, Maui, were also obtained (Browne *et al.* 1997). Toe pads were sampled from historical Hawaiian petrel museum specimens (N = 28) originally collected on Molokai between 1907 and 1914 and deposited at the Bernice P. Bishop Museum (BPBM) and the Los Angeles County Natural History Museum (Appendix A). It is possible that genetic differentiation could occur between different colonies on the same island (Friesen et al. 2007b), however sample sizes are not sufficient to address those questions here, and samples were grouped according to island for all analyses.

Molecular Techniques

Genomic DNA was extracted from blood and tissue samples using the DNEasy tissue kit (Qiagen). DNA was obtained from bone, feather, and toe pad samples via phenol/chloroform extraction and centrifugal dialysis (Fleischer *et al.* 2000). All extractions for historical samples were performed in a physically separate, dedicated ancient DNA laboratory, and a sample from a different species was extracted in between each Hawaiian petrel sample to detect cross-contamination. Multiple extraction and negative PCR controls were also used to detect contamination.

Three data sets were generated for this study. First, a 524 bp fragment of the mitochondrial *Cytochrome b* gene was amplified using the primers CytbL and CytbR9 according to Welch *et al.* (2011). Second, a set of three nuclear intron loci were sequenced, including α-Enolase intron 8, Lamin A intron 3, and Ribosomal Protein 40 intron 5 (Friesen *et al.* 1999; Friesen *et al.* 1997). For both sequence data sets Primer3 (Rozen and Skaletsky 2000) was used to design small, overlapping fragments for amplification with historic and degraded samples (Appendix B). Third, a set of eighteen polymorphic microsatellite loci were amplified (Welch and Fleischer 2011). For historic samples a minimum of two independent amplifications were conducted for each mitochondrial and nuclear intron sequence primer set. For microsatellites, loci were amplified and assayed between three and five times per individual (Taberlet *et al.* 1996).

Polymerase chain reactions were carried out in 15 μL (for modern) or 25 μL (for historical samples) total volumes. Reactions consisted of 1x colorless GoTaq Flexi buffer (Promega) or PCR Gold Buffer (Applied Biosystems, ABI), 2.0 – 4.0 mM $MgCl_2$, 0.2

mM each dNTP, 1.2 mg/mL BSA, 0.5 μM each primer, 1 unit of Promega GoTaq Flexi or AmpliTaq Gold DNA polymerase, and 1 – 3 μL DNA extract. Thermocycle profiles consisted of a denaturation step of either 95°C for 2 min (for GoTaq Flexi) or 94°C for 8 min (for AmpliTaq Gold), followed by 35 – 45 cycles of 95°C for 30 sec, a primer specific annealing temperature for 30 seconds, 72°C for 30 – 45 seconds, proportional to the length of the fragment, and a final 72°C extension step for either 7 minutes for sequences, or 30 min for microsatellites. For sequencing, PCR products were cleaned up using a 1:10 dilution of ExoSAP-IT (USB), cycle-sequenced in both directions using the Big Dye Terminator v3.1 Cycle-Sequencing kit (ABI), and then purified through Sephadex G-50 fine columns. All fragments were electrophoresed on an ABI 3130xL Genetic Analyzer. Sequences were assembled, aligned and visually inspected in SEQUENCHER v 4.9, and genotypes were called manually in GENEMAPPER v 4.1.

Data analysis

Prior to any analyses, the program GENECAP (Wilberg and Dreher 2004) was utilized to identify any individuals that may have inadvertently been sampled multiple times (e.g. individuals banded during rehabilitation and later depredated in the breeding colony). The probability of identity (Sib P[ID]) was calculated from modern microsatellite genotypes and individuals that were found to match with $p < 0.05$ were deemed to be duplicates. When duplicates were detected one of the pair was discarded from further analyses.

Sequences

Mitochondrial DNA sequences were investigated to determine whether they could represent a nuclear copy. Sequences were characterized using MACCLADE v 4.08 (Maddison and Maddison 2008) and translated in DAMBE v 5.1.2 (Xia and Xie 2001). To visualize relationships among haplotypes at the population level a statistical parsimony network for *Cytochrome b* sequences was constructed using TCS v. 1.21 (Clement et al. 2000) with a 95% connection limit. For both mitochondrial and nuclear sequence data sets, pairwise F_{ST} was calculated in ARLEQUIN v 3.1 (Excoffier et al. 2005) from a Kimura two parameter distance matrix. This substitution model was selected using JMODELTEST (Posada 2008) and the Akaike information criterion. Statistical significance of F_{ST} values was determined through 1000 permutations with the sequential Bonferroni correction (Rice 1989) applied for multiple tests. ARLEQUIN was also used to determine if there was a correlation between geographic and genetic distances. Mantel tests were conducted using Slatkin's linearized F_{ST} and a matrix of distances between each of the islands, with significance determined through 1000 permutations.

Although traditional frequency-based statistics are simple to calculate, they often rely on very simplistic assumptions such as equal population sizes and symmetrical gene flow (Beerli and Felsenstein 2001; Kuhner 2009). Coalescent-based analyses also have underlying assumptions (for example, some programs assume that population dynamics have been stable over many generations) and because of the stochastic nature of the coalescent they are best utilized with multiple loci. However, these analyses allow more realistic models to be utilized and can be more powerful (Kuhner 2009). Therefore, to

estimate migration of Hawaiian petrels between islands, we also utilized the coalescent-based program MIGRATE v 3.2.6 (Beerli 2006; Beerli and Felsenstein 2001). We used the Bayesian mode with uniform priors and substitution model parameters set to values estimated in JMODELTEST: the transition/transversion ratio = 14.0 for mitochondrial data and 8.0, 3.0, 8.0 for the nuclear introns Enol, Lam, and RP40, respectively, with rate heterogeneity for the mitochondrial locus modeled by a gamma distribution with alpha = 0.083. Three simultaneous replicate analyses were run with a single long chain of 20 million steps, of which the first 10% were discarded as burn-in. A static heating scheme with four chains was utilized to increase searching effectiveness, and heating parameters were set to 1.0, 1.2, 3.0, and 6.0. Convergence was assessed through examination of results from independent runs, and the effective sample sizes (ESS) for all parameters were greater than 1000. Effective population size (θ) and gene flow (m) estimates from MIGRATE are compounded by the mutation rate (i.e. $\theta = xNe\mu$ and $m = M/\mu$, where x is a scalar dependent on the ploidy and inheritance mode of the locus). Therefore, to avoid making an assumption about mutation rates (particularly for the microsatellite data set), the effective number of migrants per generation (NeM/x) was calculated.

Microsatellites

The microsatellite data were screened for the presence of large-allele dropout and null alleles using the program MICROCHECKER v. 2.2.3 (van Oosterhout et al. 2004). Null alleles arise from genotyping artifacts, leading to an excess of homozygotes. However inbreeding, which can be an important issue for insular populations of endemic species

(Frankham 1998), results in the same pattern. If null alleles are present in the data set in high frequencies they can bias estimates of population differentiation, such as F_{ST}. Therefore, we used the program INEST (Chybicki and Burczyk 2009) to simultaneously estimate the inbreeding coefficient and null allele frequency. The data were also checked for departure from Hardy-Weinberg expectations and the presence of gametic disequilibrium using the program GENEPOP v 4.0 (Rousset 2008). The program CONVERT (Glaubitz 2004) was utilized to create infiles for further population genetic analyses. Simulations were conducted in POWSIM (Ryman and Palm 2006) to determine if the microsatellite data set contained sufficient power to detect low levels of population genetic differentiation. In the simulated data sets the effective population size was set to 1000 and divergence time to 10, so that the overall F_{ST} of the simulated populations was 0.005. A total of 100 simulations were performed, with sample sizes from the simulated populations drawn corresponding to those utilized here, with significance determined through Fisher's exact test.

To investigate levels of differentiation between Hawaiian petrels breeding on different islands, we calculated an estimate of F_{ST}, G'_{ST}, and D for the microsatellite data set. F_{ST} was originally derived for biallelic data and depends on the variation of the loci utilized. In the case of highly polymorphic markers, such as microsatellites, F_{ST} may therefore underestimate genetic differentiation (Meirmans and Hedrick 2011). Several alternative measures have been suggested, including G'_{ST} and D. Both of these correct for maximum possible differentiation (Jost 2008; Hedrick 2005b) but differ in respect to the aspect of genetic diversity examined: G'_{ST} examines heterozygosity whereas D takes into account

the effective number of alleles (Meirmans and Hedrick 2011). An estimate of D was calculated using the package DEMETICS (Gerlach et al. 2010) implemented in R (R_Development_Core_Team 2009) and G'_{ST} was calculated using DEMETICS in conjunction with the program RECODEDATA (Meirmans 2006). The unbiased estimator was utilized for both statistics and significance p-values were estimated using 100 bootstrap replicates (Gerlach et al. 2010). As mentioned above, both of these statistics rely on assumptions like symmetric gene flow. Therefore, gene flow was also estimated using the program MIGRATE, as described above except that the Brownian motion approximation of the stepwise substitution model was utilized instead.

The number of genetic populations was investigated using the Bayesian clustering program STRUCTURE v. 2.3.3 (Pritchard et al. 2000; Falush et al. 2003). This program clusters individuals according to their multilocus genotype. We conducted analyses for 1 – 8 population clusters (K) using the admixture ancestry model and the correlated allele frequency model, both with and without sampling location information included as part of the prior (Hubisz et al. 2009). The model including sampling location information has been found to be sensitive to weak population structure, but yet unbiased when no structure is exists. Five independent replicates were performed, and runs were conducted for 3 million generations with the first 10% discarded as burn-in. Results from STRUCTURE were input into the program STRUCTURE HARVESTER (Earl 2009), to calculate the ad-hoc ΔK statistic suggested by Evanno (2005) that takes into account the change in the log probability of the data between increasing numbers of clusters.

RESULTS

Tests for duplicate modern samples from GENECAP revealed three individuals ($p < 0.05$) that were each sampled twice: first as a chick or downed fledgling, and then later as a carcass (e.g. a set of wings and tail or a clusters of feathers) collected on the same island. The initial sample was retained for further analyses and the other discarded from the data set. Therefore three samples, one from Maui and two from Kauai, were removed from the data set.

Mitochondrial sequences

Mitochondrial DNA sequences were obtained for a total of 322 modern and historical Hawaiian petrel samples (Table 1, Figure 2). The success rate for historical samples from Molokai was 100% with a mean combined sequence length of 479 bp. A total of 35 haplotypes were found with 11, 18, 7, and 9 haplotypes from Hawaii, Maui, Lanai, and Kauai respectively. There were no gaps present in the alignment, and after translation no nonsense or stop codons were detected. The majority (~72%) of substitutions occurred in the third codon position and 34 out of 35 were transitions. This evidence indicates that a mitochondrial, and not nuclear, origin of the sequences is likely.

Overall, mitochondrial DNA sequences revealed strong differentiation between petrels breeding on different islands (global $F_{ST} = 0.425$, $p < 0.001$). Pairwise F_{ST} ranged from

between 0.037 and 0.633 (Table 2) and was significant in 9 of 10 comparisons. The highest F_{ST} occurred between the islands of Lanai and Kauai ($F_{ST} = 0.633$), but differentiation was also high between Hawaii and Lanai ($F_{ST} = 0.405$), as well as between Maui and Kauai ($F_{ST} = 0.574$). F_{ST} was not significantly different from zero between Lanai and Molokai after sequential Bonferroni correction, and divergence was relatively low, but significant, between Hawaii and Maui. Mantel tests indicated there was no significant relationship between isolation and geographic distance ($p = 0.15$).

Results from MIGRATE concurred with estimates of F_{ST}, showing low migration between all pairs of populations. In analyses including only modern samples from the islands of Hawaii, Maui, Lanai, and Kauai, posterior distributions were unimodal and narrow. In analyses including modern samples as well as historic samples from Molokai, all posterior distributions were unimodal, however the migration parameters of from Hawaii into Maui, from Lanai into Molokai, and from Kauai into Molokai were relatively wide. However, all analyses showed that migration was very low. The highest migration estimate obtained was 2 migrants per 1000 generations (from Hawaii into Maui), but the average migration rate was approximately 1 individual per 1000 generations ($m = 0.00072$ migrants/generation). For 13 out of 20 migration parameters the 95% confidence interval included a migration rate of 0.

Nuclear intron sequences

Three nuclear introns were sequenced for each of 164 individuals, for a combined total of 741 bp (Table 1). Sufficient nuclear data could not be obtained for historical Molokai samples, and so they were not included in further analyses. Sixteen variable sites were discovered in the remaining four populations. There were 14 transitions and 2 transversions. A total of 25 haplotypes were found: 3 for the Enol locus, 8 for Lam, and 13 for RP40.

Nuclear sequences revealed a somewhat different pattern of differentiation than that obtained from mitochondrial sequences. Overall, significant differentiation was found (global $F_{ST} = 0.066$, $p < 0.001$), but it was lower than that for mitochondrial sequences, with F_{ST} estimated from the intron data set ranging from 0 – 0.145 (Table 2). The highest pairwise F_{ST} occurred between Maui and Lanai, however there was no significant differentiation between birds from Hawaii and Lanai or between Maui and Kauai. Mantel tests suggested that there was no relationship between isolation and distance ($p = 0.76$). Results from MIGRATE again concurred with estimates of F_{ST}. Posterior distributions were narrow and unimodal. Migration estimates were low, approximately 1 migrants per 1000 generations or less (e.g. $m = 0.0009$). The 95% confidence intervals for all parameters ranged from 0 to a maximum of 0.06 migrants/generation. The highest migration rate occurred from Maui into Kauai ($m = 0.0009$ migrants/generation), with slightly lower rates between Lanai and Hawaii ($m = 0.0006$ migrants/generation).

Microsatellite data set

A total of 232 individuals were genotyped for 18 microsatellite loci and there was an average of 6.75 alleles per locus. Expected heterozygosity ranged from 0.08 to 0.88, with an average expected heterozygosity for each population between 0.57 and 0.62. Results from MICROCHECKER indicated that no loci exhibited large-allele dropout, but two loci (*Ptero06* and *Ptero10*) did display evidence for the presence of null alleles. Simultaneous estimation of the inbreeding coefficient and the null allele frequency from INEST indicated that inbreeding was low. Null allele frequencies ranged from 0.115 to 0.247 for *Ptero06*, and from 0.125 to 0.252 for *Ptero10*. The *Ptero10* locus was previously found to contain a 38 base pair deletion in some individuals (Welch and Fleischer 2011), and additional undetected insertions or deletions could explain the relatively high incidence of null alleles. The cause of the observed excess of homozygotes for the *Ptero06* locus remains unclear. Regardless, both loci were discarded from further analyses. No other loci deviated from Hardy-Weinberg equilibrium after sequential Bonferroni correction. Two loci (*Parm01* and *RBG29*) were found to be in significant linkage disequilibrium after correction for multiple tests, therefore the *Parm01* locus was also discarded and a total of 15 loci were utilized in further analyses. Despite the removal of three loci, simulations demonstrated that the microsatellite data set still contained sufficient power to detect very weak population structure. Population structure was detected with 100% accuracy for simulated populations with an F_{ST} of 0.005. Even when F_{ST} was decreased to 0.0025, structure was correctly detected in 93% of simulations.

The microsatellite data set revealed patterns of differentiation similar to those of the nuclear intron data set. As in the nuclear intron data set, there was weak but significant

differentiation (global F_{ST} = 0.019, $p < 0.001$). The highest levels of F_{ST} occurred between Lanai and Maui and between Kauai and Lanai (Table 3). Estimates of G'_{ST} and D were very similar, although D was slightly higher, with G'_{ST} ranging from 0.015 to 0.057 and D ranging from 0.022 to 0.060. Both were higher than F_{ST}, as expected (Table 3). Estimates of G'_{ST} or D were marginally non-significant after sequential Bonferroni correction. Mantel tests also found no significant relationship between isolation and distance ($p = 0.94$). Migrate results demonstrated similar patterns. Point estimates of most migration rates were low, with 9 of 12 parameters showing a rate of about 1 or 2 migrants per 100 generations (e.g. m = 0.014 migrants/generation) or less. However migration rates from Hawaii into Kauai and from Lanai into Kauai were slightly higher (m = 0.71 migrants/generation), and rates from Maui into Kauai were higher yet, with an estimate of about 8 individuals per generation. Overall, 95% confidence intervals were much broader than for previous analyses and ranged from 0 to about 5 migrants per generation for most parameters.

Population structure was also investigated using a Bayesian clustering analysis. Under the model including sampling location information as part of the prior, runs with the number of clusters (K) set to 2 (Figure 3) grouped individuals from Hawaii and Lanai separately from individuals on Maui, similar to the nuclear intron data set. Kauai birds appeared to be an admixture of the two groups. With K = 3, Hawaii and Lanai were still grouped together, but Kauai was separated from that group as well as from Maui. Finally, with K = 4, birds from each island formed separate groups, however, individuals from Hawaii still contained some admixture with the Lanai population. Under the criteria of Evanno *et*

al. (2005), the grouping K = 2 received the highest support (Figure 4) as ΔK falls substantially and tends to level off for values beyond this. Additionally, even though it isn't possible to assess support for the situation where K = 1 using the criteria of Evanno *et al.*, the log probability of the data under this model is worse compared to models where K = 2 (e.g. -8780 vs. -8660) and above. In the analysis without sampling information, the grouping of K = 3 received the highest support under the Evanno criteria, however the maximum log probability of the data occurred for K = 1.

DISCUSSION

We conducted a population genetic study of the endangered Hawaiian petrel using three data sets: sequences of the mitochondrial *Cytochrome b* gene, sequences of three nuclear intron loci, and genotypes from 15 microsatellite loci. Analyses of the mitochondrial data set revealed strong population genetic structure between nearly all populations. No significant divergence was found between museum specimens from a potentially extirpated colony on Molokai and modern birds breeding on Lanai. Nuclear sequences and microsatellites revealed significant, but relatively lower signal of divergence among populations. Gene flow appeared to be highest between individuals from Hawaii and Lanai and between individuals from Maui and Kauai. The highest levels of differentiation were found between Lanai and Maui.

Mitochondrial DNA results show that contemporary populations of petrels on the islands of Hawaii, Maui, Lanai, and Kauai are significantly isolated from one another, with females rarely dispersing to breed. Pair formation in procellariiform birds requires multiple years and is thought to occur primarily on the breeding grounds (Warham 1990). Although it has not been directly observed, it is hypothesized that copulation of Hawaiian petrels takes place at the breeding grounds as well, likely within the burrow (Simons 1985). If birds spend the non-breeding season in population-specific areas then they may return together to the same breeding colony, which could in turn lead to population differentiation (Burg and Croxall 2001). This may be the case for Hawaiian petrels, as the stable isotope composition of flight feathers grown during the non-breeding season differs significantly between some islands (Wiley et al. 2011). Strong philopatry could also lead to differentiation between birds on different islands (Friesen et al. 2007a), and procellariiform seabirds in general are known to be highly philopatric (Brooke 2004; Friesen et al. 2006; Smith et al. 2007; Fisher 1976). Recent work also demonstrates that vocalizations of Hawaiian petrels are unique on each island (Judge 2011), suggesting they may be philopatric as well.

Significant differentiation was found between petrels sampled on Hawaii and Maui, although F_{ST} appeared to be lower than that found between other islands, suggesting that low levels of gene flow may be occurring. This could potentially be due to individuals dispersing from the dwindling population on Hawaii to Maui. Colonies on Maui and Hawaii both occur at high elevation with little precipitation and scrubby vegetation (Hu et al. 2001; Brandt et al. 1995), so dispersing birds would find more similar habitat on

these islands than on Lanai or Kauai, which differ strikingly in both elevation and rainfall (Ainley et al. 1997). Simons (1985) suggested that the eggs of birds breeding on Maui, which exhibit reduced shell thickness and functional pore area, may be specifically adapted to prevent water loss during incubation at high elevations. Such local adaptation could potentially limit gene flow between high and low elevation colonies but allow gene flow between high elevation sites. In addition to significant differences in vocalization, Judge (2011) found that adults from Maui were significantly larger than adults from Hawaii in both wing chord and tarsus length. While this could result from several factors, such as diet and distances to foraging locations, it may also indicate that contemporary gene flow is minimal.

Investigation of a potentially extirpated population on the island of Molokai revealed significant differentiation from all of the modern populations except for Lanai. By the early 1900's the number of Hawaiian petrels breeding on Molokai had apparently already begun declining, likely due to the introduction of mongoose to the island (Bryan 1908). During a 1907 collecting trip W. A. Bryan found a female mongoose with five young living in a former petrel burrow (Bryan 1908). The situation on Lanai around this time was probably also less than ideal for petrels. Foraging by introduced ungulates led to severe habitat degradation as early as 1870, and continued foraging impeded natural recovery until 1911 when goats were removed from the island (Munro 2007). There are few, if any, records of breeding Hawaiian petrels (on any island) between about 1914 and 1948, and this species was feared extinct (Baldwin and Hubbard 1948). Therefore, the recent discovery of a large colony on Lanai (Birdlife-International 2010), just a handful

of generations later, is somewhat unexpected given that these long-lived petrels lay a single egg each year and exhibit delayed maturity. Failure to relocate a formerly large colony on Molokai coupled with no significant divergence between birds from Molokai and Lanai, may indicate that petrels from Molokai dispersed to Lanai as the colony dwindled, or at least that there was a significant level of gene flow between the two islands prior to the decline of the Molokai population.

Dispersal can be substantial in seabirds. It has been hypothesized that adults may disperse if reproductive success becomes low (Friesen et al. 2007a). Social attraction is important for many seabirds and therefore individuals may disperse from dwindling colonies (Parker et al. 2007). Zador et al. (2009) describes dispersal of groups of up to 256 common murre (*Uria aalge*) up to 30 m away from other nesting sites, although dispersal of < 10 individuals was more common over the 11 year study. Laysan albatrosses, which have been found to be highly philopatric, have re-colonized several previously extirpated colonies, as well as expanded their range to new sites, within the last 35 years (Young 2010). This seeming inconsistency between strong philopatry and high dispersal ability has been termed the "seabird paradox" (Milot et al. 2008). There are several potential explanations. Dispersal may occur most frequently in young birds scouting potential burrow locations (including formerly extirpated colonies) who then remain philopatric to their chosen location in subsequent years (Brooke 2004). From the mitochondrial data examined here, it appears that contemporary female Hawaiian petrels predominantly exhibit strong natal philopatry, although in the past birds may have dispersed from the failing Molokai colony to Lanai.

Compared to the mitochondrial data, nuclear sequences and microsatellites revealed a slightly different pattern. Overall population genetic structure was weaker than that found from the mitochondrial data set. The Bayesian clustering analyses suggested the presence of at least two modern genetic populations. Recently the models for the Bayesian clustering method used in STRUCTURE were extended so that sampling locations could be included as prior information, resulting in increased power to detect weak structure (Hubisz et al. 2009). Despite this increased sensitivity, models were not found to be biased in cases where population structure was absent (Hubisz et al. 2009). Here the presence of population structure found in the STRUCTURE analysis is corroborated by other analyses of the microsatellite data set (e.g. F_{ST} and MIGRATE) as well as by analyses of the nuclear intron data set. Although estimates of G'_{ST} and D were higher than F_{ST}, they were marginally non-significant.

Both the nuclear intron and microsatellite data sets indicated that differentiation was low between individuals from Hawaii and Lanai and between individuals from Kauai and Maui. On the other hand, isolation appeared to be high between birds sampled on Maui and Lanai. These patterns of gene flow do not seem to be linked to distance, considering that colonies on Maui and Lanai are just ~75 km apart, whereas colonies on Hawaii and Lanai are separated by approximately 225 km, and colonies on Maui and Kauai are about 350 km apart. Additionally, as mentioned above, birds dispersing between Hawaii and Lanai or between Maui and Kauai would find very different habitat than in their natal colony (Brandt et al. 1995). Wind patterns are an important factor in the flight of seabirds

(Spear and Ainley 1997) and could affect dispersal, but it remains unclear if wind direction or speed differ substantially between islands in a manner consistent with these patterns. Interestingly, Wiley et al (In prep) conducted a study on the foraging ecology of the Hawaiian petrel using primary feathers (which are grown in the non-breeding season) and found that adults from Hawaii and Lanai had a very similar stable carbon and nitrogen isotope composition, whereas individuals from Maui and Kauai both differed significantly in nitrogen isotope values. Friesen et al. (2007a) have suggested that population specific foraging ranges or non-breeding distributions may act to reduce gene flow, and that could be one mechanism operating in the Hawaiian petrel.

While mitochondrial and nuclear data sets differed subtly in the pattern of differentiation between populations (i.e. four apparent contemporary genetic populations vs. two), the differences in magnitude of divergence were also conspicuous. This could be due to male-biased dispersal, which has been documented in some seabirds (Young 2010). However, evidence for low overall gene flow was present in both nuclear intron and microsatellite data sets, as shown by significant F_{ST} and by low levels of gene flow found in MIGRATE analyses. Sex-biased dispersal in birds can be difficult to study using molecular markers alone because in birds females are the heterogametic sex. In addition to sex-biased dispersal the differences in the strength of signal could be due to different levels of variability among the data sets. Mitochondrial sequences yielded 31 haplotypes from the 4 modern populations, whereas the nuclear intron data set contained a slightly lower number of 25 haplotypes, and this may account for some difference. However, power was high in the microsatellite data set, with simulations showing that even very

weak population genetic structure could be accurately detected. Microsatellite allele size homoplasy can also be problematic in some cases, particularly when population sizes are large and the mutation rate is high, but Estoup *et al.* (2002) point out that in general it does not pose a significant problem for most studies. Observations of differing levels of differentiation between mitochondrial and nuclear data sets can also be due to recent divergence. It appears that the Hawaiian petrel and its sister species, the Galapagos petrel (*P. phaeopygia*), diverged approximately 550,000 years ago (Welch *et al.* 2011) and that nuclear intron sequences for each species are still undergoing the process of lineage sorting. In such cases mitochondrial DNA, with its smaller effective population size, may act as a leading indicator of population divergence with nuclear DNA relatively lagging (Zink and Barrowclough 2008).

Genetic differentiation has implications for management of endangered populations. While the criteria for delineating evolutionarily significant units has been hotly debated (Fraser and Bernatchez 2001), it appears that distinct management units may exist within the Hawaiian petrel. Here we demonstrate that populations of Hawaiian petrels breeding on different islands are genetically isolated to some degree, although not to the extent where outbreeding depression would be a major issue (Frankham et al. 2011). In addition to significant levels of genetic differentiation, some populations demonstrate foraging segregation during the non-breeding season and could be ecologically distinct (Wiley et al. 2011). Finally, management concerns and challenges are unique on each island. For example, goats have been successfully removed from Lanai, but are still present on the islands of Kauai and Hawaii and may cause significant habitat degradation. Additionally,

the sources of predation vary on each island and can be substantial. On Kauai and Lanai predation by cats can be high, and on Maui and Hawaii introduced mongoose pose an additional threat (Hodges and Nagata 2001; Carlile et al. 2003). Therefore we propose that conservation measures for this species should be considered on an island-to-island basis.

Seabirds play an important role in both marine and terrestrial ecosystems (Croll *et al.* 2005), so it is important to gain a better understanding of their population dynamics and dispersal patterns. Here we demonstrate that birds breeding on one island may be genetically isolated from conspecifics breeding on other islands less than 100 km away. Many seabirds are increasingly threatened by extinction, and identifying isolated populations can be especially important for developing conservation management plans. Additionally, even in species that are relatively common, distinct evolutionary lineages may go unnoticed without further investigation (Hailer et al. 2011).

TABLES

Table 1. Sample sizes for Hawaiian petrels (*Pterodroma sandwichensis*) obtained from all islands where this species is currently, or was historically, known to breed.

Island	Time period	Sample Size		
		mtDNA	*nuDNA*	*Micros*
Hawaii	Modern	71	51	48
Maui	Modern	122	54	114
Lanai	Modern	38	25	28
Molokai	Historic	28	0	0
Kauai	Modern	63	34	42
Total		322	164	232

Table 2. Population differentiation of historic and modern Hawaiian petrels. Pairwise Fst values for the Cytb gene are below the diagonal, while those for a data set of sequences from three nuclear introns are above. *Indicates the estimate is significantly different from zero after sequential Bonferroni correction, ▲indicates significance before correction but not after, and † indicates p-values > 0.05. NA = not available.

	Hawaii	**Maui**	**Lanai**	**Molokai**	**Kauai**
Hawaii	-	0.092*	0.060†	NA	0.064▲
Maui	0.068*	-	0.095*	NA	-0.030†
Lanai	0.405*	0.543*	-	NA	0.145*
Molokai	0.226*	0.404*	0.037†	-	NA
Kauai	0.511*	0.574*	0.633*	0.424*	-

Table 3. Population differentiation for the microsatellite data set in modern Hawaiian petrels. Pairwise F_{ST} is shown below the diagonal and G'_{ST} is shown above. *Indicates the estimate is significantly different from zero after sequential Bonferroni correction, ▲indicates significance before correction but not after, and † indicates p-values > 0.05.

	Hawaii	**Maui**	**Lanai**	**Kauai**
Hawaii	-	0.028▲	0.030▲	0.021▲
Maui	0.016*	-	0.057▲	0.015▲
Lanai	0.021*	0.033*	-	0.046▲
Kauai	0.010*	0.012*	0.027*	-

FIGURES

Figure 1. Map of the main Hawaiian Islands with approximate locations of modern (Hawaii, Maui, Lanai, and Kauai) and historically known (Molokai) breeding colonies shaded. After Simons and Hodges (1998). Hawaiian petrels were formerly known to have a wider distribution on these islands, and there was an additional colony on the island of Oahu, however it was extirpated prior to the arrival of Captain Cook in 1778.

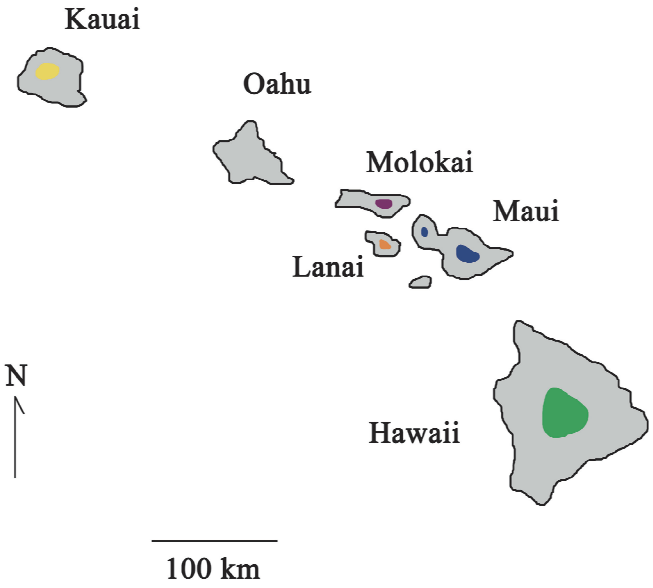


Figure 2. Haplotype network for 319 modern and historic Hawaiian petrel mitochondrial *Cytochrome b* sequences. The sizes of the circles are proportional to the haplotype frequency, and lines indicate the number of substitutions between haplotypes.

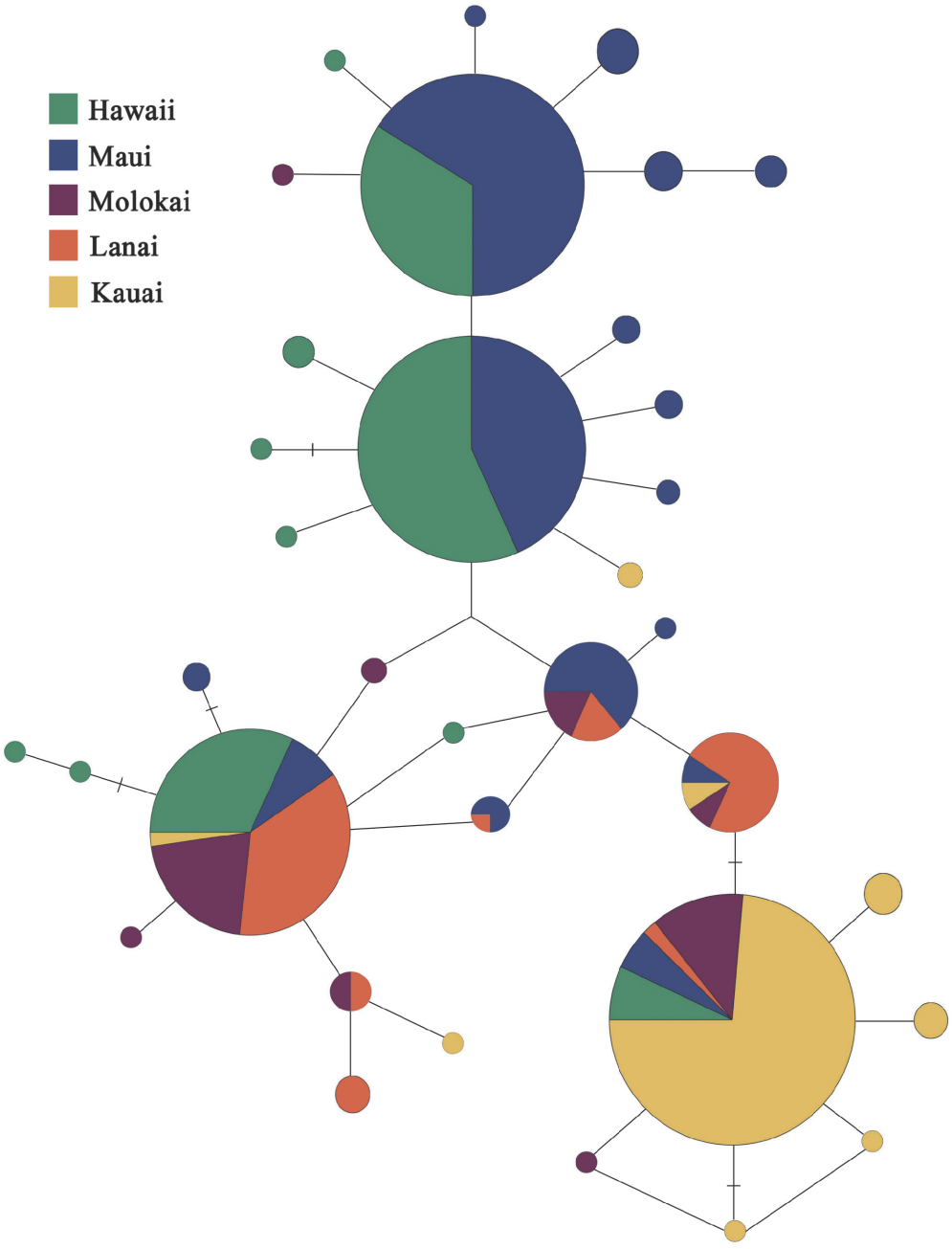


Figure 3. Genetic ancestry of Hawaiian petrels as estimated by the program STRUCTURE from the microsatellite data set using the admixed model with correlated allele frequencies and sampling location as a prior. Top K=2, Middle K=3, Bottom K=4.

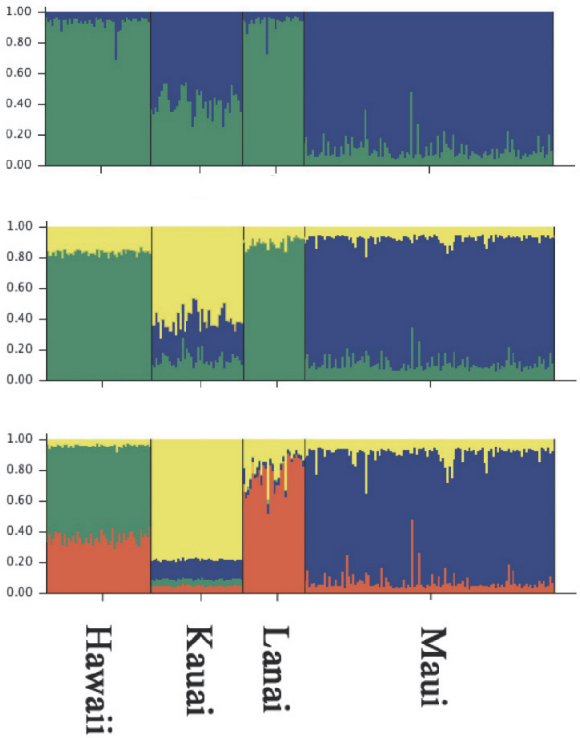
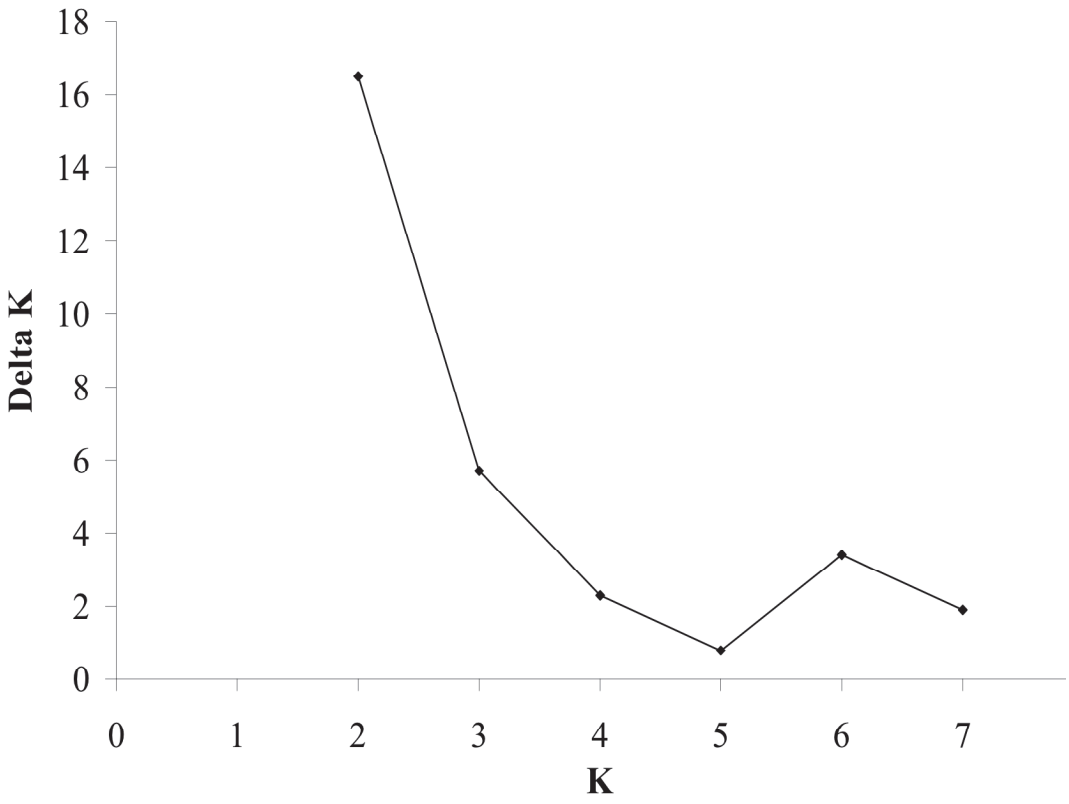


Figure 4. Graph of ΔK statistic (Evanno et al. 2005) for STRUCTURE runs with the number of clusters (K) set between 1 and 8. This statistic takes into account the change in the log probability of the data as K increases.



CHAPTER IV

Ancient DNA reveals resilience despite the threat of extinction: three thousand years of population genetic history in the endemic Hawaiian petrel*

Humans have had great impacts on Pacific Islands. In the Hawaiian Islands human colonization is associated with the extinction of nearly 50% of the terrestrial avifauna and a decline in the population size and range of many species, including several seabird species. The endemic Hawaiian petrel has escaped extinction, but colonies on two islands have been extirpated and populations on remaining islands have decreased. We obtained mitochondrial DNA sequences from 100 subfossil bones, 28 museum specimens, and 289 modern samples to investigate patterns of gene flow and temporal changes in the genetic diversity of this endangered species. Overall, differentiation was high between modern birds from Hawaii, Maui, Lanai, and Kauai, as well as between ancient samples from Hawaii, Maui, and Oahu. However, gene flow was substantial between the extirpated colonies on Oahu and Molokai and modern birds from the island of Lanai. No significant reductions in genetic diversity occurred over the three thousand year period, despite fears early in the last century that this species may have gone extinct. Modeling suggests that given current census size estimates, the decline was probably not as severe as previously thought. Simulations show that even a decline to a stable effective population size of 100 individuals would result in the loss of only 5% of the expected heterozygosity over a 150-year period. Simulations also show that in closed populations high levels of genetic diversity may be retained due to the long generation time of this species. Therefore, it appears that a pattern of dispersal from declining colonies, in addition to long generation

time, may have allowed the Hawaiian petrel to escape a severe genetic bottleneck after the arrival of humans in the Hawaiian Islands.

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INTRODUCTION

There is little debate that humans have had a great impact on global biodiversity (Ehrlich and Wilson 1991; Myers and Knoll 2001). Human colonization of the islands of the Pacific Ocean was no exception (Steadman 1995). The area known as Eastern Polynesia, which includes the Cook Islands, Society Islands, the Hawaiian Islands, as well as Easter Island and New Zealand (Steadman 2006), was one of the last habitable areas to be colonized by humans (Wilmshurst et al. 2011). Despite this relatively more recent arrival, there has been great debate about the exact chronology of colonization of Hawaii (Kirch and Kahn 2007). It appears most likely that the Hawaiian Islands were colonized some time between 1200 and 800 years ago (Wilmshurst et al. 2011; Kirch 2000). The earliest known settlements are located on Oahu, Molokai, and Hawaii in areas that would have had plentiful resources. Over time, however, humans colonized even the most marginal of habitats and population sizes may have exceeded 200,000 individuals prior to contact with Europeans (Kirch 1985). It appears, though, that even before human abundance reached maximum levels there may have been large ecological impacts (Athens *et al.* 2002).

The Hawaiian Islands are well known for their endemic avifauna (Carson and Clague 1995). The fossil record appears to indicate that the background extinction rate of birds in the Pacific was low prior to human colonization (Steadman 2006). In Hawaii, extinction rates increased dramatically coincident with the arrival of humans (Olson and James 1982b). These declines have often been associated with the introduction of exotic species, habitat destruction or modification, and direct exploitation (Blackburn et al. 2004;

Milberg and Tyrberg 1993; Athens et al. 1991; Athens et al. 2002). Extinctions primarily consisted of flightless species of geese and rails, although many other species also went extinct, including several raptors, crows, and more than a dozen honeycreepers (Olson and James 1982b). One seabird species, *Pterodroma jugabilis,* also disappeared during this time (Olson and James 1982a). Another wave of extinction occurred after European contact (Pratt 1994). In addition to extinction, populations of many species declined in number or experienced range contractions (Olson and James 1982a). Today many of the remaining native and endemic species of Hawaiian birds are threatened by extinction and have been listed under the US Endangered Species Act.

Severe population declines can have impacts on the genetic diversity of a species (Frankham *et al.* 2002). Declines in population size can lead to inbreeding, which can decrease survival and reduce reproduction. Additionally, decreased genetic diversity may limit evolutionary potential. All of these factors may in turn increase the risk of extinction (Frankham 2005). The amount of genetic diversity lost as a species passes through a decline depends on the length of time and effective population size during the decline: $H_t = H_0 (1 - 1/(2N_e))^t$, where H_0 and H_t represent heterozygosity in the past and the present, t represents time, and N_e represents the effective population size (i.e. the size of an ideal population that would lose genetic diversity at the same rate as the population of interest) (Allendorf and Luikart 2007). Ancient changes in population size can be difficult to detect because populations will have had an opportunity to recover and mutations may have accumulated in the intervening time. Fortunately, ancient DNA techniques allow utilization of temporally spaced samples, which increases power, and

can allow us to look back in time to gain a better understanding of the evolutionary history of a species (Ramakrishnan *et al.* 2005).

Several studies have demonstrated the power of this approach empirically. Using ancient DNA, Shapiro et al. (2004) investigated if temporal population dynamics of bison were associated with the presence of humans, and Campos (2010) investigated if changes in genetic diversity of musk ox resulted from climatic fluctuations. Another ancient DNA study, of penguins in New Zealand, showed that one endangered species actually underwent a range expansion after the extinction of a conspecific (Boessenkool et al. 2009). Ancient DNA has also been utilized to investigate temporal changes in genetic diversity of Hawaiian species. The endangered Hawaiian goose, or nene, was found to have undergone a severe prehistoric bottleneck (Paxinos et al. 2002a), coincident with human arrival in the Islands. It remains unclear how genetic diversity has changed over time for other bird species that have survived through this period.

The endemic Hawaiian petrel (*Pterodroma sandwichensis*) is a long-lived pelagic seabird. Subfossil bones of this species have been found on the islands of Hawaii, Maui, Lanai, Molokai, Oahu, and Kauai, but their range is considerably smaller today (Figure 1). The lowlands of Oahu were apparently the home of a large petrel colony, but historical records of it are lacking, indicating that it may have been extirpated prior to the arrival of Europeans. Historical accounts tell of a large colony on the island of Molokai with birds so plentiful that they "darkened the sky" (Munro 1955), however recent survey trips have failed to locate a substantial colony on this island (Simons and Hodges 1998).

Of the four remaining islands where the Hawaiian petrel currently breeds (Hawaii, Maui, Lanai, and Kauai) it appears that the birds have undergone a range contraction. Formerly on these islands bones were found over a range of elevations, from the coast up to the slopes of the highest volcanoes (Athens et al. 1991; Olson and James 1982a).

Many factors may have contributed to the decline of the Hawaiian petrel, including predation by both humans and introduced mammals such as rats, cats, and mongoose. In Hawaii several lines of evidence indicate direct exploitation of petrels by humans. Historical accounts indicate that Hawaiian petrel chicks were considered a delicacy and were reserved for the social elite (Henshaw 1902; Bryan 1908). At some sites on the island of Hawaii approximately 70% of bird bones found in an archeological context belonged to Hawaiian petrels (Athens et al. 1991), and in a modern breeding colony on Mauna Loa, 19 of 41 burrows discovered during a survey occurred in pits that were apparently modified by humans in prehistoric or early historic times (Hu et al. 2001). In addition to humans, cats, rats and mongoose have been significant sources of mortality for the Hawaiian petrel (Simons and Hodges 1998; Henshaw 1902; Bryan 1908; Hu et al. 2001) Due to these factors, and an apparent lack of sightings, ornithologists in the mid-1900's feared that this species was extinct (Baldwin and Hubbard 1948). Since that time the Hawaiian petrel has been rediscovered, and recent census population estimates now range from a total of 11,000 to 34,000 individuals with potentially 3,750 to 4,500 breeding pairs (Spear et al. 1995). Census sizes on each island are not precisely known, but it is likely that today there are several thousand birds each on Maui, Lanai, and Kauai.

On Hawaii populations appear to be declining and there may be fewer than 500 birds remaining (Birdlife-International 2011).

Here we investigate the temporal population dynamics of the endangered Hawaiian petrel. We obtained mitochondrial *Cytochrome b* sequences from 100 ancient, 28 historic, and 289 modern Hawaiian petrel samples collected from all islands where this bird has been known to breed, including a prehistorically extirpated colony on Oahu and a potentially historically extirpated colony on Molokai. We examined patterns of gene flow and divergence, as well as changes in population size over the last 3000 years as humans colonized the Hawaiian Islands and became increasingly abundant.

METHODS

Samples and dating

A total of 512 Hawaiian petrel samples were obtained for this study, representing approximately 3500 years of the population history of this species (Table 1). A total of 289 samples were obtained opportunistically from modern petrels on Hawaii, Maui, Lanai, and Kauai. Bone, feather, and tissue samples were collected from carcasses of birds that had been depredated in breeding colonies or that died as a result of grounding (i.e. after attraction of fledglings to artificial light sources). Blood was sampled from grounded birds that were rehabilitated and subsequently released. We also obtained blood

samples that were previously acquired from chicks captured in burrows on Maui (Browne et al. 1997). Since non-breeders depart early in the season (Simons and Hodges 1998), we assume that birds found on a given island represent breeders or their offspring. Because a large colony on Molokai may have been recently extirpated contemporary samples were unavailable; therefore we sampled toe pads of 28 Hawaiian petrel museum specimens that were collected on Molokai between 1907 and 1914 and deposited at the Bernice P. Bishop Museum and at the Los Angeles County Museum of Natural History (Appendix A). Finally, 195 ancient Hawaiian petrel bone samples were acquired from the Smithsonian's National Museum on Natural History, the Bernice P. Bishop Museum (Appendix A), or collected in the field at both archeological and paleontological sites (see for example Olson and James 1982a; Figure 1). In sites where skeletal remains were dissociated, a single element was sampled (e.g. a right humerus) to prevent duplication.

Radiocarbon dates of bones were obtained through accelerator mass spectrometry using a protocol modified from Stafford et al. (1988). As described by Wiley et al. (In prep), XAD-treated, gelatinized bone collagen samples were combusted to CO_2, graphitized, and dated at the W. M. Keck Carbon Cycle Accelerator Mass Spectrometry Lab, at University of California, Irvine. Since Hawaiian petrels forage at sea they obtain all of their carbon from the ocean. Mixing of carbon in the ocean and the atmosphere is not immediate, and upwelling can lead to mixing of ocean layers of differing ages, so carbon dates can be biased by several hundred years. We applied the global marine reservoir correction (Reimer et al. 2004) in addition to a regional correction of 54 years developed specifically for the Hawaiian petrel (James *et al.* In prep). Radiocarbon dates were

calibrated using the program CALIB v. 6.0 (Stuvier and Reimer 1993) and are presented as calendar years before present. Radiocarbon dates were obtained for all bones found in a paleontological context. For bones found in an archeological context, which generally accumulate over a shorter period of time, a series of dates was obtained and remaining samples were assigned the average age.

Molecular Techniques

Genomic DNA was extracted from blood and tissue samples using the DNEasy tissue kit (Qiagen) and from bone, feather, and toe pad samples via phenol/chloroform extraction and centrifugal dialysis (Fleischer et al. 2000). Stringent protocols were maintained to prevent contamination of ancient samples. All extractions for ancient and historical samples were performed in a physically separate, dedicated ancient DNA laboratory and work was conducted moving up a concentration gradient of PCR products. For 45% of the ancient and 100% of the historical samples, a sample from a different species was extracted in between each Hawaiian petrel sample to detect cross-contamination. For the remaining ancient samples Hawaiian petrel bones from different islands were alternated whenever possible. Multiple extraction and negative reagent controls were also used to detect contamination. Initial mitochondrial DNA sequencing results indicated the presence of cytosine deamination artifacts in ancient samples. Therefore 50 μL aliquots of all ancient DNA extracts from bone were incubated with 1X uracil-DNA-glycosylase buffer and 1 unit uracil-DNA glycosylase at 37^{o}C for 10 minutes, followed by incubation with 1 unit uracil glycosylase inhibitor (New England Biolabs) at 37^{o}C for 10 min, and a

final incubation for 10 min at 95°C. This treatment should destroy uracil residues that result in miscoding legions (Hofreiter *et al.* 2001).

We amplified a 524 bp portion of the 5' variable region of the mitochondrial *Cytochrome b* gene. In well-preserved modern samples this region was amplified using the primers CytbL and CytbR9 (Welch *et al.* 2011). Many population genetic studies utilize the mitochondrial control region instead, but this region has been duplicated in procellariiform seabirds (Abbott *et al.* 2005). *Cytochrome b* has been widely use for seabirds studies and has high levels of variation (Welch et al. In Prep; Nunn and Stanley 1998; Zino et al. 2008; Techow et al. 2009). For ancient, historical, and degraded modern samples we amplified seven short (< 150 bp) overlapping fragments (Welch et al. In Prep). For ancient and historical samples, each fragment was amplified at least twice. Polymerase chain reaction (PCR) and sequencing were carried out as in Welch et al. (In Prep). Briefly, PCR was conducted with 15 μL total reaction volumes, 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems), 1- 2 μL DNA template, and 35 cycles for modern samples and 25 μL total reaction volumes, 1 unit Amplitaq Gold DNA polymerase, 2 - 4 μL DNA template, and 45 cycles for ancient and historical samples. We also attempted to amplify nuclear intron and microsatellite loci (Welch et al. In Prep), but success was low, even for historical samples, and so those data were not utilized in further analyses. All fragments were electrophoresed in an ABI 3130 xL Genetic Analyzer (Applied Biosystems) and sequences were aligned and visually inspected in SEQUENCHER v 4.9 (GeneCodes).

Data analysis

Sequences were characterized in MACCLADE v. 4.08 (Maddison and Maddison 2008) and translated in DAMBE v. 1.5.2 (Xia and Xie 2001) to examine the potential for presence of nuclear copies in the mitochondrial data set (Sorenson and Fleischer 1996). The program JMODELTEST v. 0.1 (Posada 2008) was utilized to select the best fitting substitution model: the HKY model of nucleotide substitution with rate heterogeneity modeled by a gamma distribution. To depict the relationship between haplotypes a statistical parsimony network was created in TCS v. 1.21 with a 95% connection limit.

Gene flow and population divergence

Rates of gene flow and levels of differentiation were investigated between birds sampled on each island, including between modern samples and those from the extirpated colony on Oahu and the potentially extirpated colony on Molokai. It is also possible that the philopatric nature of procellariiform seabirds could lead to population differentiation on a local scale (Smith et al. 2007). To investigate this further we examined gene flow and differentiation between four groups of samples from the largest island of Hawaii: modern birds that breed on the slopes of Mauna Loa in Hawaii Volcanoes National Park (HAVO), ancient birds that were discovered in an archeological context at a site known as Fireplough Cave, ancient birds that were discovered in a paleontological context in the Pu'u Wa'a Wa'a region on the Kona side of the island, and finally a group that consisted

of birds from a mixture of the remaining sites that died during approximately the same time frame (100 – 400 years ago) (Figure 1).

We estimated pairwise F_{ST} values in ARELQUIN v. 3.1 (Excoffier et al. 2005) from a Kimura two-parameter distance matrix. Depaulis et al. (2009) pointed out that there could be bias in summary statistics, such as F_{ST}, calculated from heterochronous (i.e. ancient DNA) data sets. However, bias should be small if sampling occurs over a relatively short time as compared to evolutionary time. Here, corrected and uncorrected estimates of F_{ST} differed by at most 0.002, and so uncorrected estimates are shown. Significance of p-values was determined after sequential Bonferroni correction for multiple tests (Rice 1989). We also estimated migration rates using the program MIGRATE v. 3.2.7 (Beerli 2006; Beerli and Felsenstein 2001). We employed the Bayesian mode with uniform priors. The transition/transversion ratio was set to 14.0, and rate heterogeneity was modeled by a gamma distribution with alpha set to 0.083, as determined from JMODELTEST. Analyses were conducted both with and without the use of sampling dates, and both analyses produced very similar results with 95% confidence intervals overlapping. Three simultaneous replicates were performed, each for 20 million generations, with the first 10% discarded as burn-in. A four-chain heating scheme was used to increase the effectiveness of the search, with heating parameters set to 1.0, 1.2, 3.0 and 6.0. Convergence was assessed through multiple independent runs started from different random number seeds. Effective sample sizes for all runs were > 1000. Migration estimates from the program MIGRATE are compounded with the mutation rate

(i.e. $m = M/\mu$), so migration rates were scaled by an estimate of the mutation rate obtained from BEAST analyses (1.0×10^{-7}, see below).

Temporal changes in effective population size

We also investigated changes in effective population size (N_e) and genetic diversity over time to determine the impact of the decline in Hawaiian petrel population sizes. A coalescent Bayesian skyride analysis was conducted in BEAST v.1.6.1 (Drummond and Rambaut 2007; Minin et al. 2008). The Bayesian skyride is similar to a Bayesian skyline analysis but requires relatively weaker prior assumptions about population history. In the skyline analysis, the number of points where the effective population size is allowed to change, which is related to smoothness of the population size trajectory, must be set a priori. In the skyride analysis this is determined from the data by penalizing changes between coalescent intervals. This can be done in a 'time-aware' fashion by assuming that changes in successive intervals are smaller than changes in more distant intervals (Minin et al. 2008; Ho and Shapiro 2011).

A Bayesian skyride analysis was conducted separately for samples collected on Hawaii and Maui. A maximum likelihood tree was generated in the program GARLI v. 0.96b (Zwickl 2006) to provide a starting tree for subsequent analyses. The HKY substitution model was implemented with rate heterogeneity modeled by a gamma distribution as determined from JMODELTEST. The 95% confidence interval of the radiocarbon date was utilized as a prior on the sampling time for ancient samples. Analyses were performed for

1.5 x 10^8 generations sampling every 2,000 generations. Convergence was assessed through multiple independent runs, and effective sample sizes were examined in Tracer v. 1.5 (Drummond and Rambaut 2007). Since post-mortem damage of ancient DNA could potentially artificially inflate genetic diversity in the past (Axelsson et al. 2008), in addition to uracil-DNA-glycosylase treatment, we performed analyses in BEAST both with and without a model of DNA damage (Ho et al. 2007). Finally, we performed a BEAST analysis under the coalescent constant population size model. Support for various models was compared using Bayes factors, as estimated in Tracer (Suchard et al. 2001; Kass and Raftery 1995).

In addition to coalescent-based estimation of changes in effective population size, we utilized the 'temporal alleles' approach to estimate the variance effective population size. This method estimates the harmonic mean of N_e in the time period between the sampling points by assuming that changes in allele frequencies are due to genetic drift (Luikart et al. 2010). Samples from Hawaii were binned by age and any sequences with greater than 15% missing data were excluded from the analysis. We also excluded samples from the oldest time bin (i.e. older than 1000 years) because of the relatively low sample size. Estimates were obtained using the program TM3.1 (Berthier et al. 2002) assuming a maximum N_e of 100,000 with 20,000 iterations. For comparison, nucleotide diversity and gene diversity were calculated for the same samples in ARLEQUIN.

Modeling and Simulations

We utilized modeling and simulations to investigate the severity of the decline of the Hawaiian petrel as well as the effect of generation time on retention of genetic diversity. First, we used population models to investigate if, given the long generation time of the Hawaiian petrel, this species could have actually been on the brink of extinction in the early and mid-1900's, as previously suggested. We did this by modeling the minimum population growth rate necessary for Hawaiian petrels during this time and comparing it to estimates obtained from the literature. To our knowledge the last major record of the Hawaiian petrel from the early 1900's is the collection of 26 birds in breeding colonies on Molokai by W. A. Bryan in 1914 (Banko 1980; Bryan 1908). After this no breeding colonies were known until 1964 when a colony of between 150 and 300 birds was discovered in Haleakala National Park, Maui (Banko 1980). In 1995, Spear et al. estimated that there were 11,000 to 34,000 individuals based on at-sea sightings.

Since Hawaiian petrels have a long life span, delayed sexual maturity, and low annual fecundity, they have a very low population growth rate. To estimate the minimum growth rate necessary we used the simple exponential growth equation $N_t = N_0e^{rt}$ (where N represents population size, r represents growth rate, and t represents time). In this model individuals can begin reproducing in the first year. While this extreme scenario is unlikely to actually occur it represents the absolute minimum growth rate required during this time period. Therefore, if growth rates obtained from the literature (*i.e.* based on observations of Hawaiian petrels in the field) are far below this level, then there would be evidence that Hawaiian petrels may not have been as near extinction as previously thought. We tested various values for each parameter of the model (Appendix E,

Supplementary Table 1). We also used this equation, along with an estimate of the maximum population growth rate for Hawaiian petrels of between 0.005 and 0.008 (Simons 1984), to obtain a range of estimates of the number of individuals that may have actually been present in the past (Appendix E, Supplementary Table 2).

Next, we used simulations to investigate the severity of a decline needed to result in a decrease in genetic diversity of the Hawaiian petrel, given its life history characteristics. Assuming a life span of 36 years, with 100% overlapping generations, an age of first reproduction of six years, and an equal sex ratio (Simons 1984), we simulated various declines in the effective population size using the program BOTTLESIM (Kuo and Janzen 2003) and examined the resulting change in genetic diversity in terms of expected heterozygosity and allelic richness. One hundred simulations were run using haplotype frequencies observed in the ancient Hawaii data set.

A second set of simulations was used to explore the effect of life history (i.e. longevity and age at first reproduction) on the retention of genetic diversity. Assuming that a species starts with the level of genetic variation observed in the ancient Hawaiian petrel samples collected on Hawaii, and that there is no gene flow or selection, we used BOTTLESIM to simulate different life history strategies and examined the effect (in expected heterozygosity and allelic richness) of a decline 150 years ago to a stable effective population size of 250 individuals. The life history strategies investigated included that of the Hawaiian petrel (as described above), a range of theoretical values, and several life histories similar to those of other animals that live in Hawaii or for which

ancient DNA has been used to investigate changes in genetic diversity over time (Appendix E, Supplementary Table 3). These included life histories similar to elephants (*Loxodonta africana*, and, by proxy, mammoths), musk ox (*Ovibos moschatus*), bison (*Bison bison*), nene (*Branta sandvicensis*), and a Hawaiian honeycreeper, the Palila (*Loxioides bailleui*;) (Laursen and Bekoff 1978; Meagher 1986; Lent 1988; Banko et al. 1999; Banko et al. 2002; Debruyne et al. 2008).

Population contribution to overall genetic diversity

Finally, we investigated the contribution of each Hawaiian petrel population to overall genetic diversity. Total genetic diversity can be partitioned into a diversity component (i.e. the probability that two alleles of a gene drawn at random are different) and a richness component (i.e. the number of alleles) and compared across populations. Each of these components can be further broken down into two subcomponents: diversity within a population and the divergence between that population and others (Petit et al. 1998). We used the program CONTRIB v. 1.02 to calculate the contribution of modern and extirpated populations to overall genetic diversity. Since allelic richness can be sensitive to sample size, samples sizes were rarefied to 12, which represents the smallest samples size (Oahu) in the study.

RESULTS

Mitochondrial DNA sequences were obtained from a total of 417 Hawaiian petrel samples: 100 ancient, 28 historical, and 289 modern birds (Table 1). The success rate for ancient bones from Hawaii was about 76% (77/101), and ages ranged from 112 to 3228 years, although the mean age was 467 years (Figure 3; Appendix E, Supplementary Table 4). The success rate for ancient samples from Maui was 38% (9/24), with samples ranging from 555 to 3435 years old and a mean age of 1317 years (Figure 3). Success was 18% (12/66) for Oahu, with samples ranging from 615 to 1863 years old and an average age of 1310 years (Appendix E, Supplementary Figure 1). Relatively fewer bones were available from the islands of Lanai and Molokai and success was low: 1/5 for Lanai and 1/3 for Molokai. No bones were available from Kauai for destructive analyses. The success rate was 100% for historical museum specimens collected on Molokai (N = 28) approximately 100 years ago. The sequence length was 524 bp for modern samples, and the average combined sequence length was approximately 480 bp and 400 bp for historic and ancient samples, respectively. No instances of Hawaiian petrel contamination were found in either extraction, blank, or alternate species control samples. Additionally, Bayes factors indicate no support for BEAST analyses that included an age-dependent model of DNA damage over analyses without (2 ln Bayes factor = -4.70).

A total of 47 haplotypes were found from 51 variable sites. Of these, 21% occurred in the first codon position, 4% occurred in the second, and 75% occurred in the third. Ninety-one percent of the variable sites exhibited transitions. There were no gaps in the alignment and after translation no nonsense or stop codons were found. This evidence

indicates that a mitochondrial, and not nuclear, origin of the sequences is likely. A total of 18 haplotypes were found in birds from Hawaii, 20 from Maui, 8 from Lanai, 9 from Molokai, 6 from Oahu, and 10 from Kauai (Figure 2). A total of 34 haplotypes were found in modern and historical samples, and 21 were found in ancient samples with 8 shared between time periods.

Gene flow and population divergence

Using ancient DNA we investigated patterns of gene flow and divergence between extant and extirpated populations of the Hawaiian petrel. We also examined these patterns through time. Overall, differentiation was high (Table 2). In the analysis with modern Hawaiian petrels, as well as birds sampled from the two extirpated populations, 12 out of 15 pairwise F_{ST} values were significantly greater than zero. Of these twelve, 11 had an F_{ST} of about 0.20 or greater, with a maximum F_{ST} of 0.633 observed between Kauai and Lanai. There was no significant differentiation between modern individuals from Lanai and individuals from the extirpated populations on Oahu and Molokai. In the past, individuals from Hawaii, Maui, and Oahu were also significantly differentiated (Table 3). F_{ST} ranged between 0.229 and 0.437 with the highest level found between Hawaii and Oahu. Results from Migrate were consistent with those obtained from estimation of F_{ST} (data not shown).

We also investigated the potential for genetic differentiation on a local scale on the island of Hawaii. Samples found in a paleontological context in the Pu'u Wa'a Wa'a region

(ranging in age from 100-400 years) were significantly differentiated from all other groups of individuals, including modern birds sampled on the eastern side of the island at HAVO ($F_{ST} > 0.350$, Table 4). Birds found in an archeological context at Fireplough Cave (which was utilized by Hawaiians from about 400 to 500 years ago) were not significantly differentiated from any group but Pu'u Wa'a Wa'a. Geographically, Fireplough cave and the Pu'u Wa'a Wa'a site are relatively much closer to each other than to HAVO (Figure 1). Low, but significant, differentiation was found between the modern birds from HAVO and the combined group of samples from across the island ($F_{ST} > 0.100$, Table 4). Again, results from Migrate were consistent with those obtained from estimation of F_{ST} (data not shown).

Temporal changes in effective population size

The Bayesian skyride analysis for birds from Hawaii shows a population trend that has been relatively stable over time (Figure 3). Starting about 845 years ago and continuing toward the present a decrease can be seen. While the change appears small in Figure 3 because of the log scale for the y-axis, it represents a change in effective population size of 41% over the last 800 years. The 95% confidence interval, however, is wide. The lower interval includes a scenario in which Hawaiian petrel populations decline by 76%, but the upper interval indicates that a constant population size, with no change, is also possible. Bayes factors indicate some support for the constant population size model over the skyride model (2 ln Bayes factor = 27.37), suggesting that while there may have been a decline in effective population size it did not significantly change genetic diversity.

Samples were also combined into three different time bins (400 – 1000 years old, 100 – 400 years old, and modern) and changes in genetic diversity were investigated using haplotype and nucleotide diversity. Haplotype diversity was significantly higher in the oldest time bin (Table 5, $p < 0.05$), however there was no significant difference between the 100 - 400 and the modern time bins. There was also no significant difference in nucleotide diversities between any time bins. Finally, we investigated changes in effective population size on Hawaii using the 'temporal alleles' approach, which estimates the harmonic mean of the effective population size in the period between two samples. The effective population size between the middle and older time bins was 47,693 (95% Confidence interval (CI): 1415 to 97,397) and between the modern and middle time bin it was 954 (95% CI: 287 to 82,759). Confidence intervals for these estimates overlap, indicating no significant change over a period of about 1000 years.

The Bayesian skyride analysis for birds from Maui showed an increase in effective population size over time until about 1100 years ago, after which it stabilized and remained constant. This could be due to a relatively low ancient sample size (N = 9) for this island, which would result in few coalescent events in the past. Bayes factors indicate no support for the skyride model over the constant size model (2 ln Bayes factor = -8.80). Since the ancient sample size was lower for Maui as compared to Hawaii, samples were divided into two time bins, modern and ancient (> 500 years), and haplotype and nucleotide diversity were calculated. No significant changes were found (Table 6). Since

there were only two time bins, change in effective population size using the temporal alleles approach was not investigated.

Modeling and Simulations

We used the exponential growth model to investigate if Hawaiian petrels could have actually been near extinction in the middle of the last century given their life history and recent estimates of census population size. The smallest population growth rate found (to reach the minimum census population estimate of 11,000 individuals in 1995) was 0.03 (Appendix E, Supplementary Table 1), assuming that Hawaiian petrels declined to a minimum census size of 1000 individuals and had 80 years to recover. Growth rates based on larger declines (e.g. to 50 or 200 individuals), larger population sizes in 1995 (e.g. the average or maximum census size) or shorter times for recovery (e.g. 60 or 40 years), resulted in minimum necessary growth rates between about 0.04 and 0.12. These rates are much higher than those described in the literature. Simons (1984) modeled petrel population dynamics based on observations of a colony on Maui. His models included a breeding frequency of 89%, annual adult survival of 93%, and annual juvenile survival of 80%. The maximum reproductive success that he observed during a three-year field study of this species was 72%, which yielded a growth rate of 0.005. He also reported a maximum reproductive success of 75% for similar seabird species, which would translate to a maximum growth rate for Hawaiian petrels of 0.008. Using growth rates from the literature it appears that there would have been at least 5,800 Hawaiian

petrels in the 1900's and very likely many more than that (Appendix E, Supplementary Table 2).

We conducted simulations in order to determine the severity of a population decline that would be required to reduce genetic diversity in the Hawaiian petrel. Simulations were conducted in which a population declines from an effective population size of 50,000 at 150 years in the past to current effective population size from 10 – 1000 individuals. Simulations showed that declines to an effective population size of between 100 and 1000 resulted in approximately 5% or less loss in expected heterozygosity (Figure 4). More severe declines, down to an N_e of 50, 25, and 10 resulted in a loss of 11%, 23%, and 44% of the expected heterozygosity, respectively. Allelic richness showed a similar pattern but with more severe reductions in the percentage of alleles retained (Appendix E, Supplementary Figure 2)

To investigate what role longevity and generation time may play in maintaining genetic diversity, we simulated demographic changes in species with various life histories (i.e. longevity and age at first reproduction), ranging from a species that lives 50 years and begins breeding in the tenth year, to a species that lives 2 years and begins reproduction in the first year. Simulations, in which a population declines from 50,000 individuals 150 years in the past to a stable number of 250 individuals, demonstrated that species exhibiting long life spans and delayed reproduction lost less than 8% of the expected heterozygosity, while species with shorter life spans that began breeding in the first year lost between 21% and 45% of their expected heterozygosity (Figure 5A). For

comparison, we simulated the change in genetic diversity under life histories similar to those of species for which changes in genetic diversity have recently been investigated using ancient DNA or which also live in the Hawaiian islands and were also impacted by human colonization. Elephants (and by proxy mammoths), with the longest life span and latest age at first reproduction, demonstrated the least decline, losing about 3% of expected heterozygosity (Figure 5B). The palila, an endemic Hawaiian honeycreeper, experienced the largest loss of expected heterozygosity of about 16%. The bison and musk ox lost slightly less (13%), while the Hawaiian petrel and the Nene, the endemic Hawaiian goose, were intermediate loosing 6% and 8% respectively. Again, allelic richness showed a similar patter, but with more severe reductions (Appendix E, Supplementary Figure 3).

Population contribution to overall genetic diversity

An analysis of the contribution of each population to overall genetic diversity showed that birds from Maui and Kauai made the largest contribution to the genetic diversity component (0.17%) while birds from Maui and Lanai made the largest contribution to the allelic richness component (0.56% and 0.28%, respectively). Breaking each of these into subcomponents of diversity and differentiation allows for a more detailed investigation of these patterns (Figure 6). For the genetic diversity component of birds from Kauai, the diversity subcomponent showed levels below the average, but the differentiation subcomponent was very high, and the net difference between these two subcomponents lead to the comparatively high genetic diversity component overall. For the allelic

richness component, the diversity and differentiation subcomponents effectively canceled each other out, leading to an average level of contribution to the allelic richness component overall. Therefore, for birds from Kauai the largest contribution appears to come from differentiation while diversity is about average. This is in contrast to the extirpated populations on Molokai and Oahu. These populations both had slightly higher than average genetic diversity, but low differentiation from other populations swamped this out. Birds from Lanai had a high relative contribution, but only to allelic richness, whereas birds from Maui had high contributions to both diversity and richness. Birds from Hawaii had lower contributions in both of these components than average.

DISCUSSION

Here we examined patterns of gene flow and differentiation, as well as changes in genetic diversity and effective population size, in an endangered seabird over the last 3000 years. In general differentiation was high between petrels sampled across most islands, both currently and in the past. However, low levels of F_{ST} were found between modern birds from Lanai and extirpated populations from Molokai and Oahu. Examination of effective population size over time on Hawaii and Maui revealed a relatively stable trend since human colonization of the Hawaiian Islands, although reductions of about 40% may have occurred on Hawaii. Modeling indicates that Hawaiian petrel population sizes approximately 80 years ago may have been as high as 7000 individuals and simulations

demonstrate that long life span and generation time allow higher levels of genetic diversity to be retained over time.

Gene flow and population divergence

Investigation of differentiation between modern birds from Hawaii, Maui, Lanai, and Kauai indicates that isolation is strong between populations. This also appears to be the case in the past, as well. Significant F_{ST} was found between ancient individuals from Hawaii, Maui, and Oahu ($F_{ST} > 0.229$). Many seabirds, and especially procellariiform seabirds, are known to exhibit strong philopatry despite being highly mobile (Smith et al. 2007; Friesen et al. 2006; Zino et al. 2008). The Hawaiian petrel is known to make foraging trips of greater than 10,000 km to the Gulf of Alaska, however on average it appears to disperse less than 300 km to breed. Differentiation may also have been stronger in the past than it is today. An F_{ST} of 0.372 was found between ancient samples from Maui and Hawaii, whereas an F_{ST} of 0.068 was found between modern birds on the same island. Ancient samples from Oahu also exhibited higher F_{ST} when compared to ancient samples from Hawaii ($F_{ST} = 0.437$) than with modern samples ($F_{ST} = 0.192$), although the pattern is reversed for individuals from Maui, with higher F_{ST} in comparisons with modern birds ($F_{ST} = 0.349$). While mitochondrial DNA sequences only reflect the movements of females, weak but significant differentiation has been found between modern birds from each island using data sets consisting of multilocus microsatellite genotypes and nuclear intron sequences (Welch et al. In Prep).

It appears that population extirpation may cause Hawaiian petrels to deviate from their generally philopatric tendencies. While birds breeding today are significantly differentiated from one another, extirpated populations show low levels of divergence between them, as well as with modern birds from Lanai. Since the population on Oahu is known only from the fossil record it is likely that that population was extirpated first (the most recent radiocarbon dated petrel bone from Oahu in this study was 615 years old (95% CI: 546 – 662 years). Social attraction may be important for seabird species (Parker et al. 2007; Danchin et al. 1998), and individuals from Oahu may have dispersed to either Lanai or Molokai as numbers in their own colony dwindled. Then later, as the number of birds on Molokai declined birds may have dispersed to Lanai. The ancient samples used here were collected from various caves around each island at elevations lower than where petrels are currently known to breed, and may potentially represent extirpated breeding colonies.

Zador et al. (2009) noted that groups of common murre (*Uria aalge)* ranging in size from 2 - 256 individuals formed new subcolonies up to 30 m away from other nesting areas. In procellariiform seabirds dispersal can be high. Laysan albatross have often been found to breed within 20 km of their hatching site, however, this species has undergone a recent range expansion, which includes the re-colonization of formerly extirpated sites (Young 2010). A recent population genetic study demonstrated low differentiation among colonies (Young 2010; Young *et al.* 2009). Perhaps the lack of isolation could be a result of population contraction as birds dispersed away from declining populations. It also seems reasonable that given the long life span of these birds, which can be greater than

60 years (Kaufman 2011), individuals re-colonizing a former site may have actually hatched there themselves (Parker *et al.* 2007). A similar process of displacement from extirpated colonies may be occurring in Hawaiian petrels.

On a more local scale, colonies on the island of Hawaii may have been significantly differentiated from one another. Significant pairwise F_{ST} was found between individuals discovered in a paleontological context (i.e. without the presence of native Hawaiian artifacts) at the Pu'u Wa'a Wa'a site on western Hawaii and modern birds breeding on the eastern slopes of Mauna Loa. However, about 300 years separates these populations, and as such could have lead to changes in allele frequencies over time. It does provide limited evidence of the potential for fine-scale population differentiation, which is difficult to assess today as it appears that most islands now contain a single breeding colony. No significant differentiation was found between bones discovered in an archeological context at Fireplough cave and either bones from around the island (excluding the Pu'u Wa'a Wa'a site) or modern birds from Mauna Loa. This could indicate that Hawaiians were collecting birds from a large area and bringing them to Fireplough cave to process them, although it has been hypothesized that adult petrels were consumed opportunistically while parties hunted passerines for feathers or petrel chicks, which were a delicacy reserved for the social elite (Athens *et al.* 1991; Henshaw 1902). Another possibility is that birds breeding near Fireplough cave dispersed to the eastern Mauna Loa area as the colony declined. If additional caves with ancient Hawaiian petrel bones are discovered near where petrels currently breed, they may provide additional information about the contraction of populations on each island. Also,

discovery of additional Hawaiian petrel bones from Lanai and Molokai would be helpful in determining if gene flow occurred between islands in the past or if birds only dispersed as colonies dwindled.

Temporal changes in effective population size and genetic diversity

Investigation of changes in effective population size over time on the island of Hawaii suggests relative stasis over the past three thousand years, as Polynesians, and then Europeans colonized the islands. Humans have had broad ecological impacts on islands across the Pacific, including Hawaii. Humans have altered habitat, introduced exotic mammalian predators, and directly exploited plant and wildlife populations (Steadman 2006; Athens et al. 1991; Athens et al. 2002). Coincident with human arrival in the Islands many species went extinct, including one seabird species, *Pterodroma jugabilis* (Olson and James 1982a; Olson and James 1982b). Even for species that didn't go extinct, such as the nene or Hawaiian goose, declines in genetic diversity have been observed (Paxinos et al. 2002a). However, significant declines in the effective population size of the Hawaiian petrel have apparently not occurred, as demonstrated by both a Bayesian skyride analysis as well as the traditional temporal alleles approach. A similar pattern was found on Maui. While these findings are based only on mitochondrial DNA, it is unlikely that nuclear DNA would show a different pattern given that nuclear markers themselves have a higher effective population size than mitochondrial loci.

The median posterior probability of the Bayesian analysis for the island of Hawaii showed a trend in which the effective population size decreased by about 40%. Even though this does not represent a significant change in the genetic diversity of the Hawaiian petrel, it may indicate that the census size in the past was larger than today. Since the effective population size of mitochondrial DNA is about one quarter that of nuclear DNA, due to its haploid nature and maternal inheritance pattern, the total effective population size of petrels on Hawaii when they were most abundant (just eight hundred years ago) could have been 1.5 million. Given the ratio of effective to census population size (N_e/N_c), an estimate of the total number of birds at that time could be obtained. While N_e/N_c has been found to be about 0.10 on average (Frankham 1995b), overlapping generations and relatively low variance in reproductive success of the due to a monogamous breeding system, suggests that for the Hawaiian petrel N_e/N_c may be closer to 0.50 (Hedrick 2005a; Nunney 1995). If these assumptions are true then the total population size on Hawaii may have been as high 3 million individuals. A decline of 40% would represent the loss of about 1.2 million individuals, but given the current population size estimates, the decline was likely to be much greater than that. Since Hawaiian petrels are top marine predators, and transfer nutrients from the ocean to the terrestrial ecosystem on the islands, a severe decline in numbers could have broad ecological impacts.

The observed pattern of stasis in effective population size could be caused by low variability or small sample sizes in the data set. However, this does not appear to be the case. Mitochondrial DNA sequences contained 51 variable sites, from which 47 haplotypes were discovered. Also, on Hawaii ancient samples (N = 77) outnumbered the

modern samples (N = 71). However, due to the stochastic nature of the coalescent, additional loci may be beneficial (Kuhner 2009). This pattern of stasis could also be explained if gene flow brought genetic diversity from other islands. This also does not appear to be the case, either, as F_{ST} was found to be high between individuals from Hawaii, Maui and Oahu in the past and between modern individuals from all other islands, including the extirpated population on Molokai. Two additional explanations could lead to the observed pattern: The population decline of the Hawaiian petrel was not as severe as previously thought, and/or perhaps the long generation time of the bird is buffering it against loss of genetic diversity.

To investigate if Hawaiian petrels may have been on the brink of extinction, as previously feared (Munro 1955; Simons and Hodges 1998; Baldwin and Hubbard 1948), we used the exponential growth equation to model the minimum required population growth rate. Assuming that the Hawaiian petrel was near extinction (i.e. 50 birds) in 1915, the year after the last major museum collection, a growth rate of 0.067 would be required to reach the lower 95% confidence limit of the estimated census population size (Spear *et al.* 1995). This is an order of magnitude larger than highest measured growth rate (0.005) for a population of petrels studied on the island of Maui (where predator control programs were already in effect) and almost an order of magnitude larger than the growth rate (0.008) resulting from the best case scenario of 75% reproductive success proposed by Simons (1984). Therefore, it appears unlikely that the Hawaiian petrel was near extinction in the beginning of the last century. Using the growth rates of Simons (1984), and assuming exponential growth of Hawaiian populations, it is estimated that

between 5,800 and 7,374 individuals remained, or likely even more. It is understandable that this species may have been overlooked. The Hawaiian petrel spends the majority of its life at sea, only coming to land to breed. Even then it returns to breeding colonies nocturnally and nests in underground burrow that are often greater than 2 m long. In addition extant colonies occur in rugged terrain and can be difficult to access (Simons and Hodges 1998).

While Hawaiian petrel populations may not have declined to the brink of extinction, the distribution of subfossil bones suggest that they were more abundant in the past (Athens et al. 1991; Olson and James 1982a). Therefore it is possible that the long generation time of the Hawaiian petrel is buffering it against a significant loss of genetic diversity (Amos and Balmford 2001). We performed simulations to explore if, given the diversity observed in ancient Hawaiian petrels from Hawaii, different combinations of life span and age at first reproduction would result in different levels of genetic diversity being retained when held at an N_e of 100 for 150 years. Similar to other reports and consistent with population genetics theory (Amos and Balmford 2001; Hailer et al. 2006; Lippé et al. 2006; Kuo and Janzen 2004), we found that species with a longer life span retained higher levels of expected heterozygosity as well as a larger number of alleles. For species with a life span > 20 years a maximum of 8% loss was observed, whereas species with a life span < 10 lost between 20 and 45% heterozygosity. This may mean that a loss of an entire generation of offspring may not be as detrimental for long-lived species, such as the Hawaiian petrel, as it would be for other species with shorter generation times.

We compared the Hawaiian petrel with other species for which changes in genetic diversity have been investigated using ancient DNA (Paxinos et al. 2002a; Debruyne et al. 2008). Assuming that all of these species started with the same level of genetic diversity as the Hawaiian petrel, and assuming that they more or less fit the model equally well, elephants (and by proxy, mammoths, Debruyne *et al.* 2008) and Hawaiian petrels show the lowest losses of genetic diversity, whereas bison and musk ox show higher losses. Therefore, if all else were equal it might be expected that Hawaiian petrels and mammoths would show fewer changes in effective population size over time than either bison or musk ox. This is consistent with empirical findings (Campos et al. 2010). Nene should be intermediate between these groups, and changes in genetic diversity were found (Paxinos *et al.* 2002a). Also, for comparison we simulated the impact of a decline on the palila, an endemic Hawaiian honeycreeper. A large portion of honeycreeper species have gone extinct over the last 1500 years, since humans have arrived in the Hawaiian Islands (Olson and James 1982b), and many remaining species are endangered. The palila, with a relatively lower generation time, exhibited the largest decrease in simulations with a loss of 15% heterozygosity. Of course, in reality, many assumptions of these simulations may be violated. For example, even if generation time is very short, gene flow may prevent significant losses of genetic diversity. Also, life history is more complex than that modeled here, including biased sex ratios, non-random mating, multiple offspring per reproductive event, and so on. However, despite these violations simulations suggest that the generation time of an organism may mask temporal changes in genetic diversity, for example, in response to climate change or anthropogenic impacts, therefore careful interpretations of observed patterns are required.

Given the relatively constant level of effective population size observed over time, we also simulated population declines of varying severity to explore how severe a decline would need to be before genetic diversity in the Hawaiian petrel to decrease. Simulations were conducted of a decline in effective population size from 50,000 to a size of 1000 individuals or less. Results indicate that a very severe decline would be required to lose high levels of genetic diversity. Effective population size could decline to 50 individuals and remain there for 150 years and only 10% of the expected heterozygosity would be lost. Below this effects seemed to increase substantially, and a decline to an effective population size of 10 would result in a loss of 45% heterozygosity. However, in these drastic cases, long generation time would actually impede population growth, allowing the effects of the decline to perhaps persist longer than expected (Allendorf and Luikart 2007).

Conservation implications

Overall, genetic diversity appears to be relatively stable in the Hawaiian petrel. An analysis of the net contribution of birds from different islands to the total genetic diversity suggests that populations on Maui and Kauai contribute the most to genetic diversity, and that Maui and Lanai contribute most to allelic richness. However, closer investigation shows that every population makes a contribution to genetic diversity in some respect, even if the net contribution appears to be about the average. For example, extirpated populations on Molokai and Oahu had somewhat higher than average genetic

diversity but lower than average differentiation. So while loss of these populations would not reflect a large net loss, they could still be important, assuming that neutral genetic diversity is related to quantitative traits or fitness (Reed and Frankham 2001; Hansson and Westerberg 2002; Foerster et al. 2003; Reed and Frankham 2003). In addition to genetic diversity other aspects of populations are important to take into account when evaluating their contribution towards the continued existence of this species, including their ecology, census size, and distribution.

It may be best for conservation management actions to focus on stabilizing population trends and increasing the population growth rate on each island, for example through continued predator control programs. Since genetic diversity is relatively high, inbreeding depression will probably not be a major issue for birds on most islands. However, the same life history traits that have lead to retention of genetic diversity in this species also prevent quick population recovery after a decline. If a severe decline occurs, slow demographic recovery could result in an extended population bottleneck and a longer period of time in which genetic diversity is vulnerable to erosion (Allendorf and Luikart 2007). In these situations is may be beneficial to encourage gene flow, perhaps through translocation of individuals or the use social attraction (Parker et al. 2007). One such case may be occurring on the island of Hawaii where colonies appear to be dwindling. It appears that in the past Hawaiian petrels may have used dispersal as a mechanism to avoid extirpation. Habitat on Maui is similar to that on Hawaii, and individuals from Hawaii and Maui show relatively lower genetic differentiation in mitochondrial DNA. However, nuclear DNA indicates low differentiation with individuals from Lanai (Welch

et al. In Prep). Given the levels of divergence observed here, outbreeding depression is not likely to be a major issue (Frankham et al. 2011), so increasing movements between these islands could be beneficial.

Overall, it appears that a combination of long generation time and the ability to disperse in response to population extirpation have prevented declines in Hawaiian petrel population size and range from significantly decreasing genetic diversity. This could also account for the observation that while populations of many seabird species have been extirpated, relatively few species have gone extinct (Steadman 1995). The story of the Hawaiian petrel makes a case for some optimism despite the broad ecological and evolutionary impacts of humans on Pacific islands.

TABLES

Table 1. Number of Hawaiian petrel samples, by island and time period, for which DNA sequences were successfully obtained. NA indicates samples were not available due to colony extirpation or lack of bones for destructive analyses.

Island	Time Period		Total
	Ancient	Historical/Modern	
Hawaii	77	71	148
Maui	9	119	128
Lanai	1	38	39
Molokai	1	28	29
Oahu	12	NA	12
Kauai	NA	61	61
Total	100	317	

Table 2. Pairwise F_{ST} (below the diagonal) and associated p-values (above the diagonal) for *Cytochrome b* sequences obtained from ancient, historic, and modern Hawaiian petrels, representing samples from all islands where they have been known to breed. *Indicates significant and †indicates non-significant comparisons after sequential Bonferroni correction.

	Time	Hawaii	Maui	Lanai	Molokai	Oahu	Kauai
Hawaii	Modern	-	0.000	0.000	0.000	0.000	0.000
Maui	Modern	0.068*	-	0.000	0.000	0.000	0.000
Lanai	Modern	0.405*	0.543*	-	0.072	0.099	0.000
Molokai	Historic	0.226*	0.404*	0.037†	-	0.432	0.000
Oahu	Ancient	0.192*	0.349*	0.074†	-0.004†	-	0.000
Kauai	Modern	0.511*	0.574*	0.633*	0.424*	0.538*	-

Table 3. Pairwise F_{ST} (below the diagonal) and associated p-values (above the diagonal) for sequences from ancient Hawaiian petrels. *Indicates significant comparisons after sequential Bonferroni correction.

	Time	Hawaii	Maui	Oahu
Hawaii	Ancient	-	0.000	0.000
Maui	Ancient	0.372*	-	0.040
Oahu	Ancient	0.437*	0.229*	-

Table 4. Pairwise F_{ST} (below the diagonal) and associated p-values (above the diagonal) for sequences from petrels from different areas on the island of Hawaii. HAVO indicates individuals collected in Hawaii Volcanoes National Park on the eastern slope of Mauna Loa, PWW indicates bones of individuals from the Pu'u Wa'a Wa'a area, Fireplough indicates bones of individuals found in Fireplough Cave, and Other represents a grouping of individuals from all other sites from about the same time (see Figure 1) *Indicates significant and † indicates non-significant comparisons after sequential Bonferroni correction.

	Time (YBP)	HAVO	PWW	Other	Fireplough
HAVO	0 - 20	-	0.000	0.006	0.626
PWW[a]	100 - 400	0.364*	-	0.000	0.000
Other[b]	100 - 400	0.111*	0.431*	-	0.384
Fireplough[c]	400 - 500	-0.026†	0.373*	-0.003†	-

[a]Paleontological site
[b]Mix of archeological and paleontological sites
[c]Archeological site

Table 5. Haplotype and nucleotide diversity, and their 95% confidence intervals, by time bin for samples obtained on Hawaii.

Time period (YBP)	N	Haps	Haplotype Diversity	95% CI	% Nt Diversity	95% CI
400 - 1000	11	9	0.964	0.862 – 1.000	0.377	0.000 – 0.913
100 - 400	33	9	0.816	0.739 – 0.894	0.368	0.000 – 0.856
Modern	70	9	0.772	0.720 – 0.824	0.409	0.000 – 0.919

Table 6. Haplotype and nucleotide diversity, and their 95% confidence intervals, by time bin for samples obtained on Maui .

Time period (YBP)	N	Haps	Haplotype Diversity	95% CI	% Nt Diversity	95% CI
Ancient	9	7	0.917	0.733 – 1.000	0.554	0.000 – 1.289
Modern	15	8	0.791	0.581 – 1.000	0.384	0.000 – 0.897

FIGURES

Figure 1. Map of the main Hawaiian Islands with approximate locations of modern (Hawaii, Maui, Lanai, and Kauai) and historically known (Molokai) breeding colonies shaded, as well as collection localities of sub-fossil bones. Approximate locations of archeological sites are marked with a triangle, paleontological sites are marked by circles. Specific sites on Hawaii are indicated, as well, for investigation of local population differentiation.

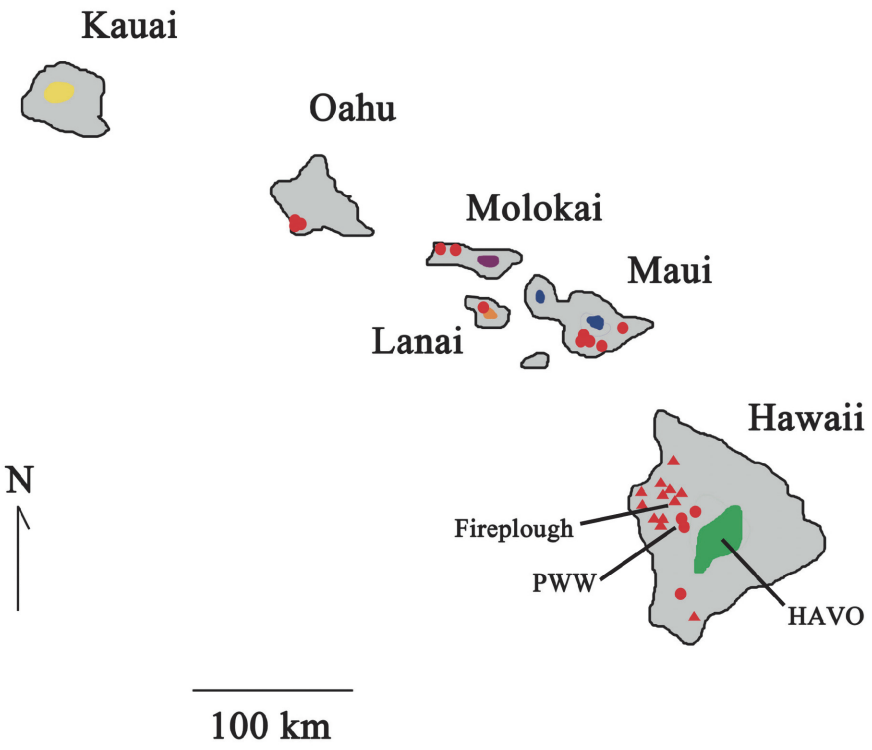


Figure 2. Haplotype network depicting relationships between *Cytochrome b* sequences obtained for 417 ancient, historical, and modern Hawaiian petrels from all islands where the birds are currently known, or have been known in the past, to breed. The size of the circle is proportional to the haplotype frequency, and dashes indicate the presence of multiple substitutions between haplotypes.

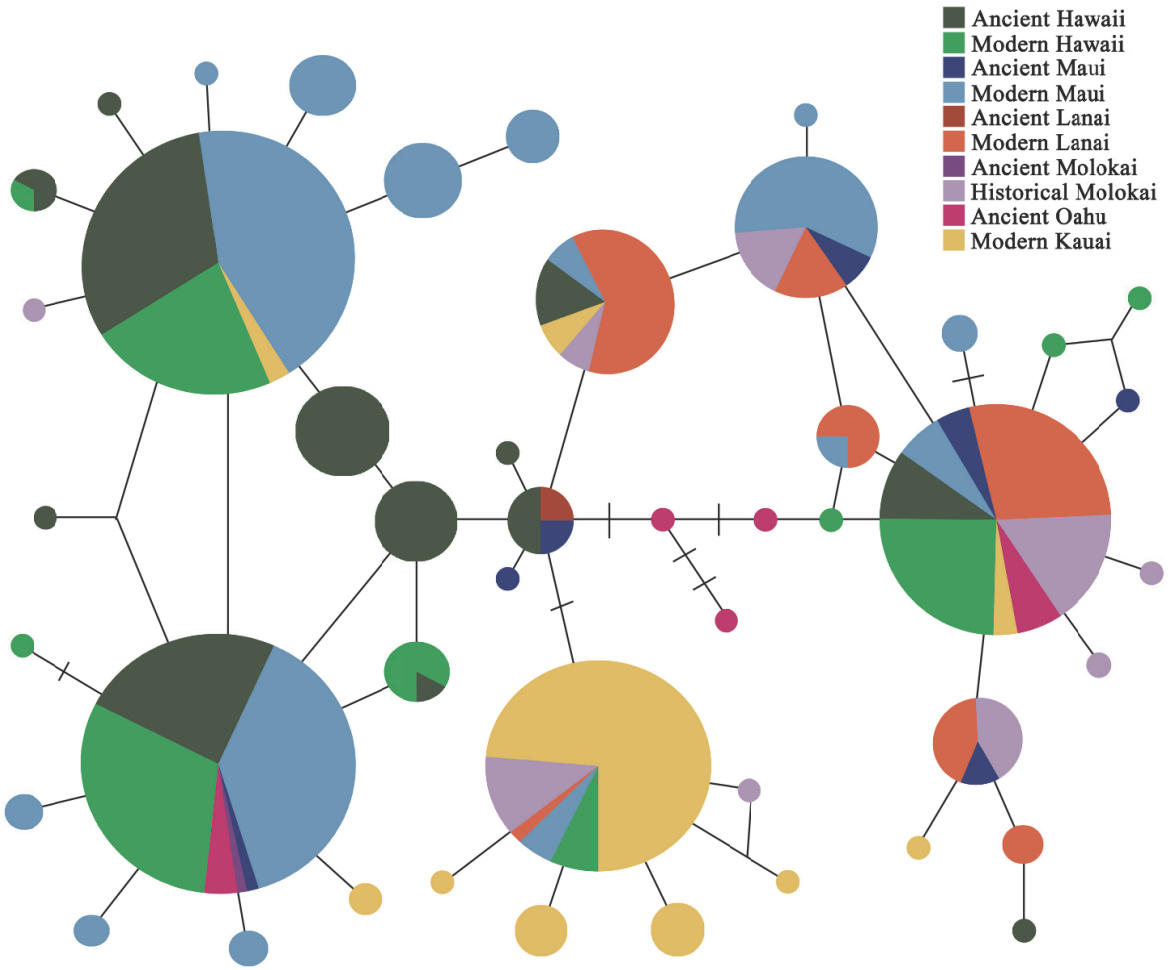


Figure 3. Temporal trend in the effective population size of the Hawaiian petrel. Upper plots show the Bayesian skyride of effective population sizes (thick black line is the median posterior probability, thin black lines are the upper and lower 95% confidence intervals) on Hawaii and Maui. Lower plots show number of samples and 95% confidence interval for their radiocarbon ages (ybp). Shaded areas coincide with periods of colonization and expansion by humans. White = pre-human arrival, Light grey = Polynesian colonization and early population growth, Medium grey = Polynesian population expansion, Dark grey = European arrival and expansion.

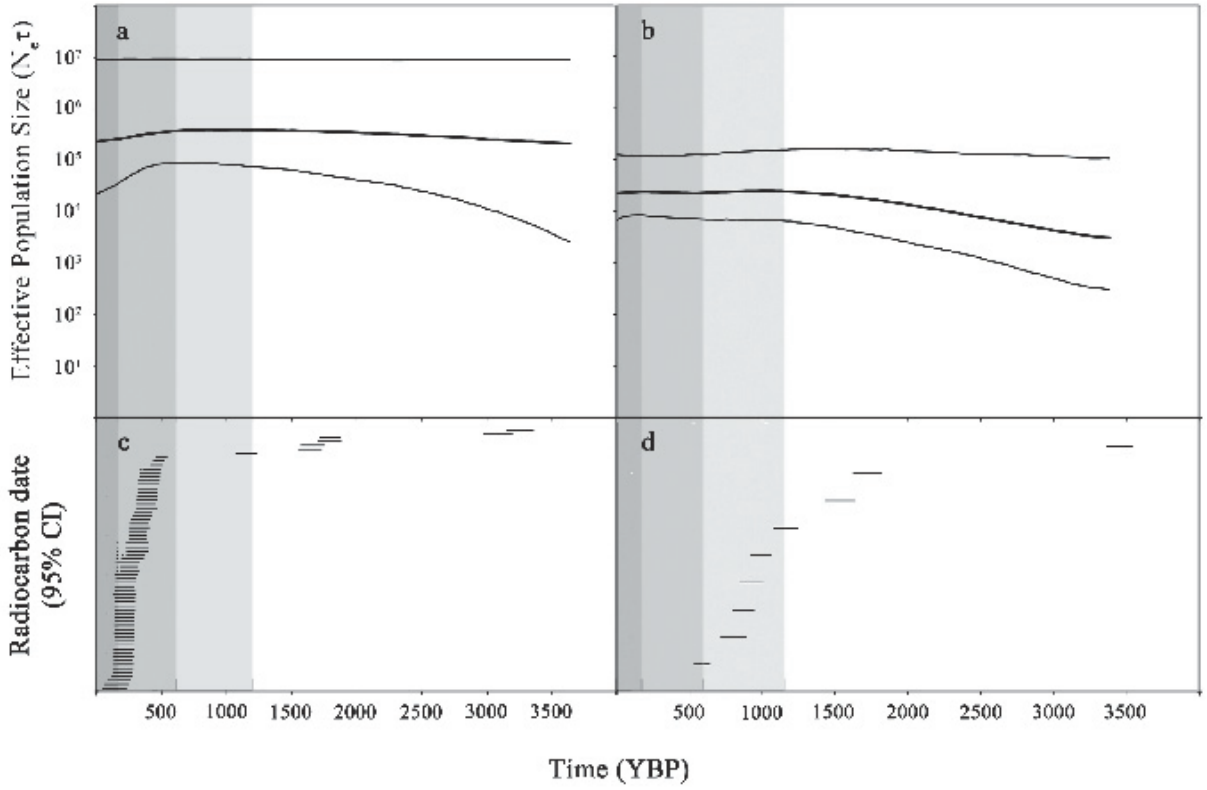


Figure 4. BOTTLESIM simulations of change in the expected heterozygosity (H_e) of the Hawaiian petrel as a result of a decline to a constant effective population size ranging from 10 to 1000 individuals at 150 years in the past.

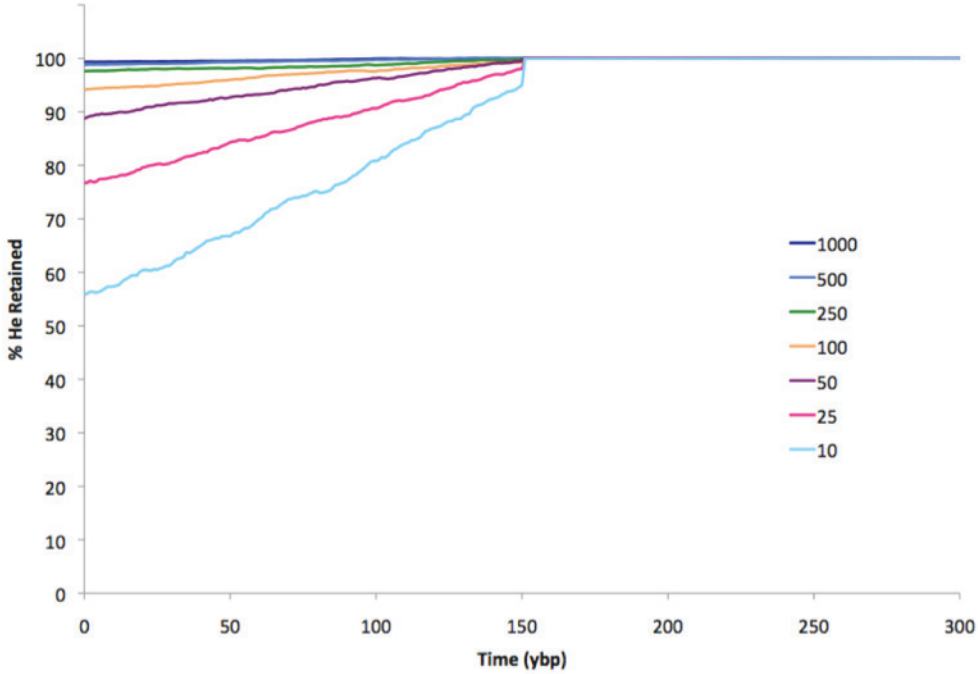
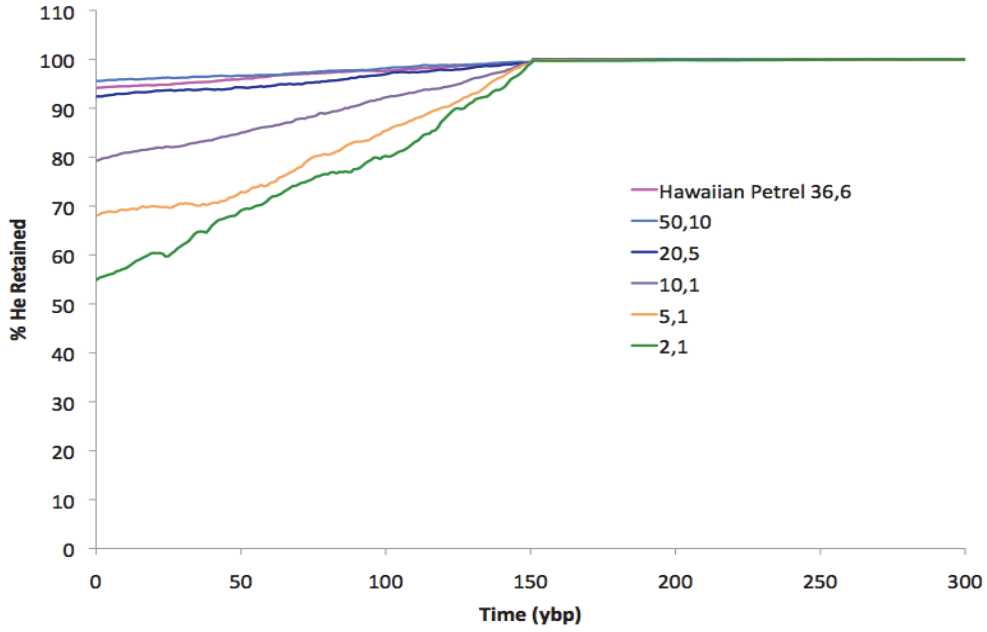


Figure 5. BOTTLESIM simulations of the impact of life history characteristics on the change in the expected heterozygosity (H_e) as a result of a decline to a constant effective population size of 100 individuals at 150 years in the past. (A) The Hawaiian petrel compared to species with theoretical life histories (longevity, age at first reproduction); (B) The Hawaiian petrel as compared to species for which ancient DNA has been used to investigate temporal changes in genetic diversity or for species that may have been impacted by arrival of humans on the Hawaiian Islands.

A.



B.

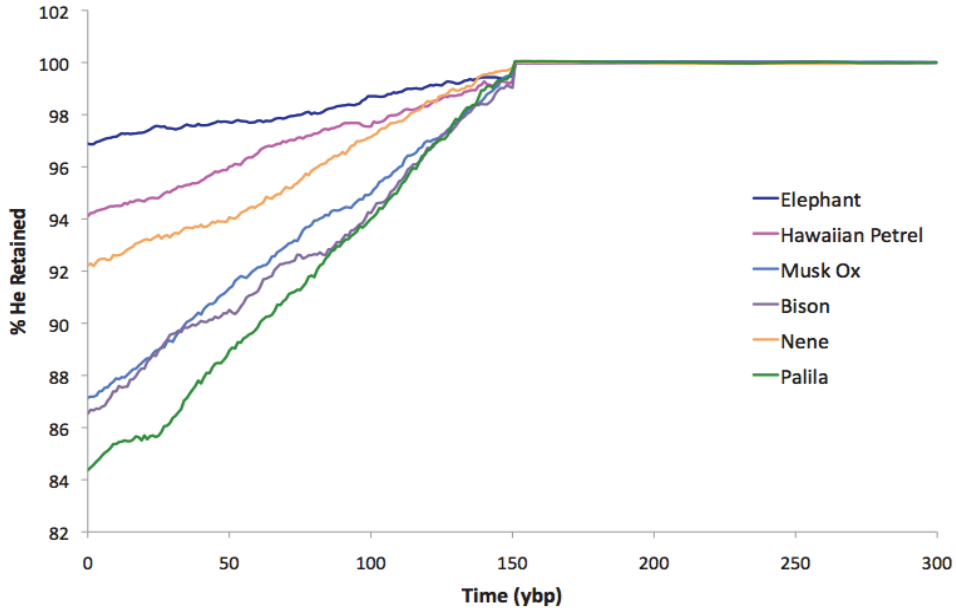
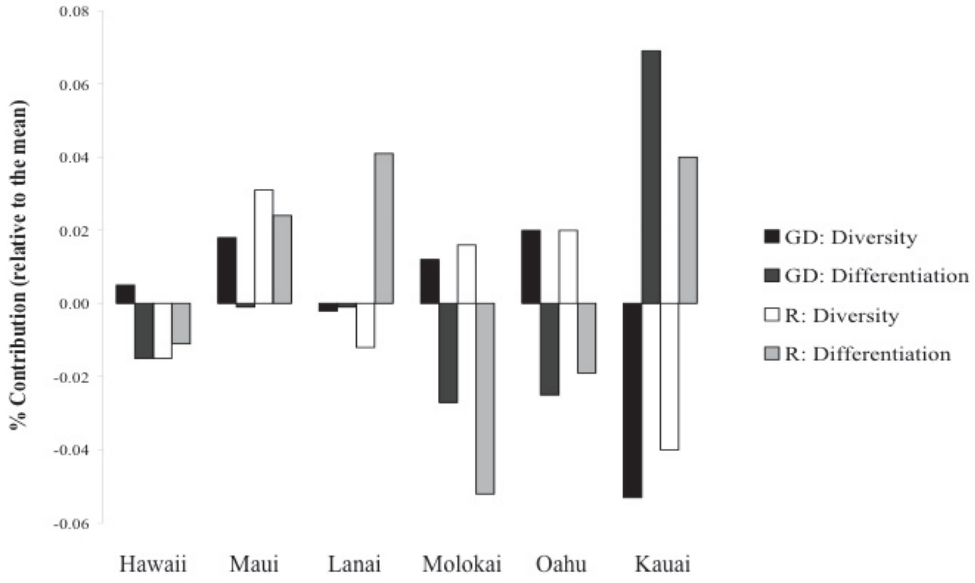


Figure 6. Relative contribution of modern and extirpated Hawaiian petrel populations to overall genetic diversity (GD) and allelic richness (R). Each of these has been further divided into diversity and differentiation subcomponents (see methods).



APPENDIX A

Museum specimen and voucher information

Unless otherwise indicated all specimens are Hawaiian petrels. In some cases, multiple subfossil bones were assigned the same catalogue number, but only one element (e.g. left humerus) was sampled from each group to prevent duplicate sampling. The remaining subfossil bones have not been catalogued. Catalogue indicates the institution's catalogue number, Year indicates year collected, and Type indicates type of specimen: S = study kin and B = bone/skeleton.

Museum	Catalogue	Year	Island	Type	Collector
BPBM[1]	X150206	1976	Maui	B	Howarth et al.
BPBM	X157438	1979	Hawaii	B	Unknown
BPBM	X157513	1980	Kauai	S	Unknown
BPBM	1992.166.1	1991	Hawaii	B	Giffen & Stone
BPBM	1992.166.2	1992	Hawaii	B	Giffen & Stone
BPBM	183557	1995	Kauai	S	Montgomery, S.
BPBM*	184828	2005	At Sea	S	Unknown
BPBM	4662	1907	Molokai	S	Bryan, W.A.
BPBM	4663	1907	Molokai	S	Bryan, W.A.
BPBM	4669	1907	Molokai	S	Bryan, W.A.
BPBM	4671	1907	Molokai	S	Bryan, W.A.
BPBM	4672	1907	Molokai	S	Bryan, W.A.
BPBM	4673	1907	Molokai	S	Bryan, W.A.
BPBM	4674	1907	Molokai	S	Bryan, W.A.
BPBM	4675	1907	Molokai	S	Bryan, W.A.
BPBM	4676	1907	Molokai	S	Bryan, W.A.
BPBM	4677	1907	Molokai	S	Bryan, W.A.
BPBM	4679	1907	Molokai	S	Bryan, W.A.
BPBM	4680	1907	Molokai	S	Bryan, W.A.
LACM[2]	20266	1914	Molokai	S	Bryan, W.A.
LACM	20267	1914	Molokai	S	Bryan, W.A.
LACM	20268	1914	Molokai	S	Bryan, W.A.
LACM	20269	1914	Molokai	S	Bryan, W.A.
LACM	20270	1914	Molokai	S	Bryan, W.A.
LACM	20271	1914	Molokai	S	Bryan, W.A.

LACM	20272	1914	Molokai	S	Bryan, W.A.
LACM	20273	1914	Molokai	S	Bryan, W.A.
LACM	20274	1914	Molokai	S	Bryan, W.A.
LACM	20275	1914	Molokai	S	Bryan, W.A.
LACM	20280	1914	Molokai	S	Bryan, W.A.
LACM	20281	1914	Molokai	S	Bryan, W.A.
LACM	20282	1914	Molokai	S	Bryan, W.A.
LACM	20283	1914	Molokai	S	Bryan, W.A.
LACM	20284	1914	Molokai	S	Bryan, W.A.
LACM	20285	1914	Molokai	S	Bryan, W.A.
USNM[3]	343310	1981	Oahu	B	Olson et al.
USNM	391137	1980	Oahu	B	ARCH
USNM	391317	1980	Oahu	B	Unknown
USNM	391422	1980	Oahu	B	Unknown
USNM	391726	1977	Oahu	B	Olson et al.
USNM	392735	1980	Oahu	B	Unknown
USNM	392780	1976	Oahu	B	Unspecified
USNM	434261	1981	Oahu	B	Olson et al.
USNM	434310	1981	Oahu	B	Olson et al.
USNM	434349	1977	Oahu	B	Olson et al.
USNM	434412	1977	Oahu	B	Olson et al.
USNM	434417	1981	Oahu	B	Olson et al.
USNM	434654	1981	Oahu	B	Olson et al.
USNM	442732	1981	Oahu	B	Olson et al.
USNM	442817	1981	Oahu	B	Olson et al.
USNM	442916	1977	Oahu	B	Olson et al.
USNM	442984	1977	Oahu	B	Olson et al.
USNM	443027	1977	Oahu	B	Olson et al.
USNM	443054	1977	Oahu	B	Olson et al.
USNM	443178	1977	Oahu	B	Olson et al.
USNM	447585	1981	Oahu	B	Olson et al.
USNM	447815	1977	Oahu	B	Olson et al.
USNM	447915	1977	Oahu	B	Olson et al.
USNM	494137	1964	Maui	S	Unknown
USNM	494138	1964	Maui	S	Unknown
USNM	639494	2005	Kauai	S	Imberski, M.

USNM	639495	2005	Kauai	S	Unknown
USNM	639496	2006	Maui	S	Nohara, T.
USNM	639497	2006	Kauai	S	Vercelli, J.
USNM	639498	2002	Maui	S	Duvall, F.
USNM	639500	2006	Kauai	S	Unknown
USNM	639501	2006	Kauai	S	Vercelli, J.
USNM	639502	1997	Kauai	S	Unknown
USNM	639503	2005	Maui	S	Alexander, C.
USNM	639504	2006	Maui	S	Medeiros, F.
UWBM[4]	55576	1991	At Sea	S	Unknown
UWBM	55605	1991	At Sea	S	Unknown

[*]Murphy's petrel (*Pterodroma ultima*)
[1]Bernice P. Bishop Museum, Honolulu, HI
[2]Los Angeles County Museum of Natural History, Los Angeles, CA
[3]National Museum of Natural History, Smithsonian Institution, Washington, DC
[4]University of Washington Burke Museum of Natural History and Culture, Seattle, WA

APPENDIX B

Mitochondrial and nuclear DNA amplification and sequencing primer information

Locus	Primer	Nucleotide sequence (5' – 3')	Size (bp)	Source
Cytb			**524**	
	CytbL	CCTCAAACATCTCTGCTTGAT	547	**a**
	CytbR9	CGATACCGAGAGGGTTGT		
	aCytb1.1F	CCCCTCAAACATCTCTGCTT	133	**a**
	aCytb1.1R	AGCGACGGATGAGAAAGCTA		
	aCytb2F	CGGCCTCCTACTAGCCATAC	121	**a**
	aCytb2R	GGCTCCATTTGCATGTAGGT		
	aCybt3F	CTCACACCTGCCGAAATGTA	108	**a**
	aCytb3R	AGAATCCTCGTCCGATGTGT		
	aCytb4.1F	CCTACACATCGGACGAGGAT	105	**a**
	aCytb4.1R	CCTACGAAGGCAGTTGCTATG		
	aCytb5.2F	CCCTCATAGCAACTGCCTTC	126	**a**
	aCytb5.2R	CCCATTCTACGAGGGTTTGG		
	aCytb6F	GAGGTGCTACAGTCATCACCAA	125	**a**
	aCytb6R	GTGTAGGGCGAAGAATCGAG		
	aCytb7F	CCCTACATTAACTCGATTCTTCG	121	**a**
	aCytb7R	TCGATACCGAGAGGGTTGTT		
α-Enolase			**212**	
Intron 8	EnolL731	TGGACTTCAAATCCCCCGATGATCCCAGC	270	**b**
	EnolH912	CCAGGCACCCCAGTCTACCTGGTCAAA		
	aEnol1L	GACTTCAAATCCCCCGATG	158	**a**
	aEnol1R	ACRGAGGGAATGCACCTATC		
	aEnol2L	AGGGAATCAGCACTGG	158	**a**
	aEnol2R	AGGCACCCCAGTCTACCTG		
Lamin A			**220**	
Intron 3	LamL724	CCAAGAAGCAGCTGCAGGATGAGATGC	274	**b**
	LamH892	CTGCCGCCCGTTGTCGATCTCCACCAG		
	aLam1L	CGTTGTCGATCTCCACCAG	181	**a**
	aLam1R	GAAGAACATTTACAGCGAGGTG		
	aLam2L	GGGATGGGGACACAACAC	158	**a**
	aLam2R	AGAAGCAGCTGCAGGATGAG		
Ribosomal Protein 40			**309**	
Intron 5	RP40L	GGGCCTGATGTGGTGGATGCTGGC	392	**c**
	RP40H	GCTTTCTCAGCAGCAGCCTGCTC		
	aRP401L	CTGGCTCGGGAGGTTCTG	180	**a**
	aRP401R	CTGTCCTCAACAGCAAGCAC		
	aRP402L	ATCTCCTGYTTCTGCTCCTG	221	**a**
	aRP402R	TGCTCCTCCTTTTCGATCTG		

[a]This study, [b]Friesen et al. 1997, [c]Friesen et al. 1999

APPENDIX C

Supplementary tables and figures for Chapter I:

Mitochondrial and nuclear DNA sequences reveal recent divergence in morphologically indistinguishable petrels

Supplementary Table 1. Haplotypes, their frequencies, and the number of variable sites for the mitochondrial *Cytochrome b* gene in Galapagos and Hawaiian petrels

Cytb **Haplotype**	**Galapagos petrels**	**Hawaiian petrels**
1	0.06	-
2	0.22	-
3	0.28	-
4	0.03	-
5	0.03	-
6	0.03	-
7	0.03	-
8	0.09	-
9	0.03	-
10	0.06	-
11	0.03	-
12	0.03	-
13	0.03	-
14	0.03	-
15	-	0.07
16	-	0.03
17	-	0.07
18	-	0.07
19	-	0.07
20	-	0.17
21	-	0.03
22	-	0.03
23	-	0.03
24	-	0.03
25	-	0.17
26	-	0.07
27	-	0.03
28	-	0.03
29	-	0.03
30	-	0.03
# of variable sites	13	20

Supplementary Table 2. Haplotypes, their frequencies, and the number of variable sites for the Enol nuclear intron locus in Galapagos and Hawaiian petrels

Enol Haplotype	Galapagos petrels	Hawaiian petrels
1	0.44	0.39
2	0.44	-
3	0.06	-
4	0.02	-
5	0.02	-
6	0.03	0.04
7	-	0.55
8	-	0.02
# of variable sites	6	3

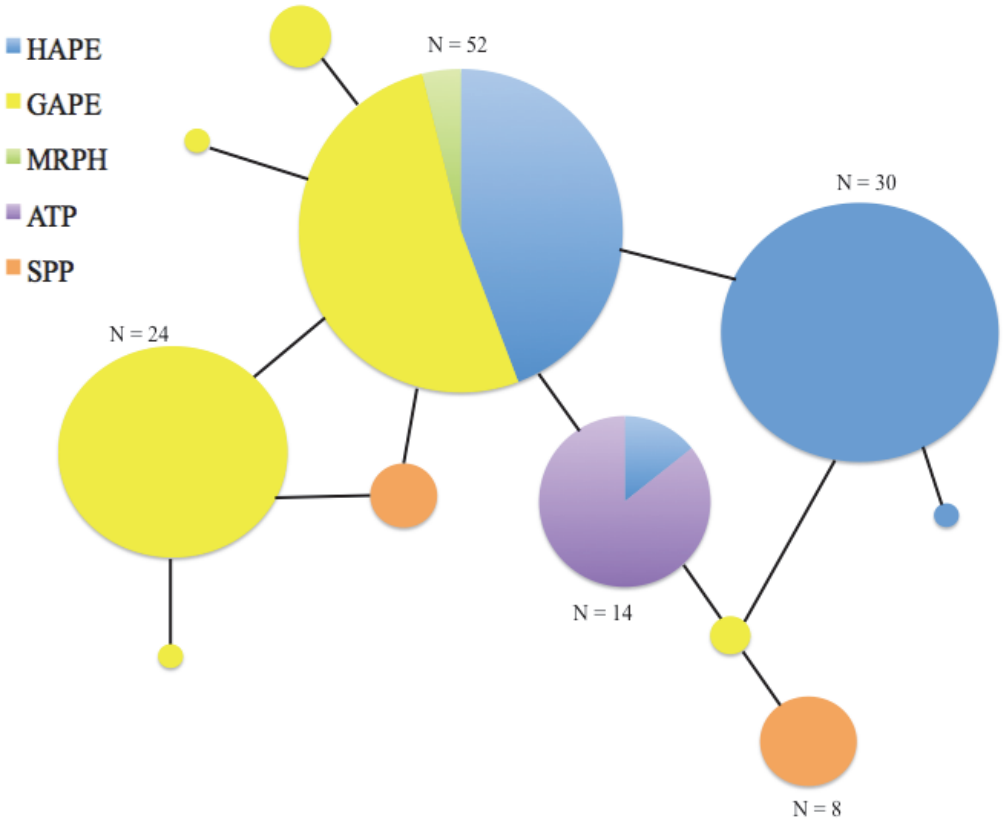
Supplementary Table 3. Haplotypes, their frequencies, and the number of variable sites for the Lam nuclear intron locus in Galapagos and Hawaiian petrels

Lam Haplotype	Galapagos petrels	Hawaiian petrels
1	0.06	-
2	0.73	-
3	0.02	-
4	0.03	-
5	0.03	-
6	0.03	-
7	0.02	-
8	0.02	-
9	0.02	-
10	0.02	-
11	0.03	0.11
12	-	0.55
13	-	0.02
14	-	0.05
15	-	0.09
16	-	0.07
17	-	0.02
18	-	0.04
19	-	0.02
20	-	0.02
21	-	0.02
# of variable sites	12	8

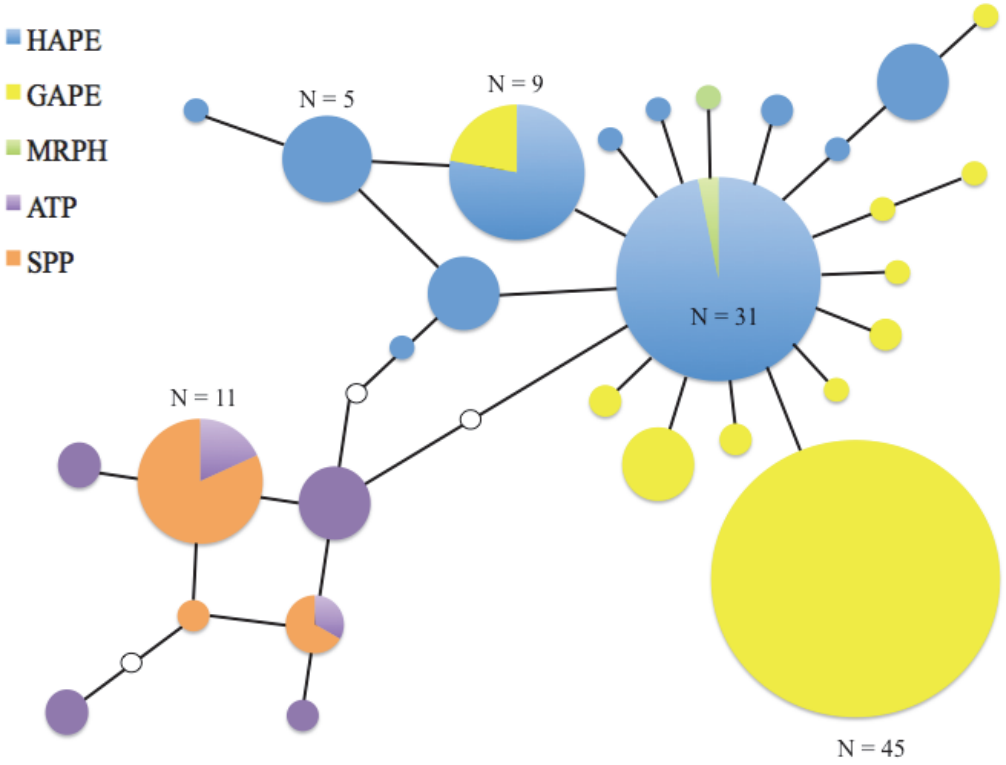
Supplementary Table 4. Haplotypes, their frequencies, and the number of variable sites for the RP40 nuclear intron locus in Galapagos and Hawaiian petrels

RP40 Haplotype	Galapagos petrels	Hawaiian petrels
1	0.12	-
2	0.04	-
3	0.05	0.07
4	0.12	-
5	0.05	-
6	0.10	-
7	0.19	0.45
8	0.04	-
9	0.06	-
10	0.05	-
11	0.03	0.04
12	-	0.04
13	0.03	0.33
14	0.02	0.02
15	0.03	0.02
16	0.02	0.02
17	0.03	0.02
# of variable sites	7	6

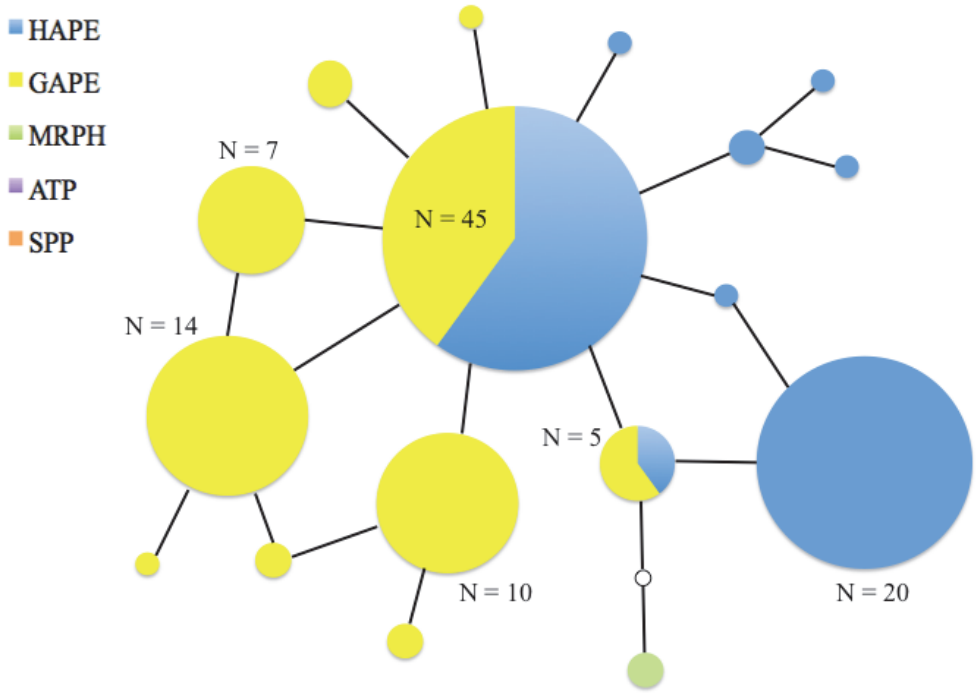
Supplementary Figure 1 Statistical parsimony haplotype network constructed in TCS (Clement *et al.* 2000) for the Enol nuclear intron data set. Circle size is proportional to haplotype frequency and color indicates the taxonomic origin for individuals possessing that sequence. The number of individuals is indicated for common haplotypes. Open circles indicate an inferred haplotype that was not sampled. HAPE = Hawaiian petrel, GAPE = Galapagos petrel, BPBM = Murphy's petrel, ATP = Atlantic petrel, and SPP = soft-plumaged petrel.



Supplementary Figure 2 Statistical parsimony haplotype network constructed in TCS (Clement *et al.* 2000) for the Lam nuclear intron data set. Circle size is proportional to haplotype frequency and color indicates the taxonomic origin for individuals possessing that sequence. The number of individuals is indicated for common haplotypes. Open circles indicate an inferred haplotype that was not sampled. HAPE = Hawaiian petrel, GAPE = Galapagos petrel, BPBM = Murphy's petrel, ATP = Atlantic petrel, and SPP = soft-plumaged petrel.



Supplementary Figure 3 Statistical parsimony haplotype network constructed in TCS (Clement *et al.* 2000) for the RP40 nuclear intron data set, with gaps treated as a fifth state. Circle size is proportional to haplotype frequency and color indicates the taxonomic origin for individuals possessing that sequence. The number of individuals is indicated for common haplotypes. HAPE = Hawaiian petrel, GAPE = Galapagos petrel, BPBM = Murphy's petrel, ATP = Atlantic petrel, and SPP = soft-plumaged petrel. With gaps treated as a fifth state, Atlantic and soft-plumaged petrel sequences did not join the network.



APPENDIX D

Supplementary tables and figures for Chapter III:

Population divergence and gene flow in an endangered and highly mobile seabird

Supplementary Table 1. Allele frequencies (%) for 15 microsatellite loci amplified in modern Hawaiian petrels. Continued on the next page.

Parm02M	Hawaii	Kauai	Lanai	Maui
168		2.44	1.79	0.88
170			1.79	
172	4.17	6.10	8.93	0.44
174	89.58	85.37	78.57	89.47
176	5.21	3.66		5.70
178	1.04	2.44	7.14	1.75
180			1.79	1.75

Parm03M	Hawaii	Kauai	Lanai	Maui
149	2.08	5.95		7.02
151	9.38	9.52	10.71	7.46
153		4.76	1.79	4.82
155	2.08		1.79	
157	48.96	35.71	26.79	54.39
159	8.33	13.10	8.93	4.82
161	11.46	15.48	17.86	7.02
163	12.50	4.76	25.00	9.65
165	4.17	4.76	5.36	3.51
167	1.04	1.19		0.88
169		1.19	1.79	
171		3.57		0.44

Parm05M	Hawaii	Kauai	Lanai	Maui
93	12.90	2.47	1.79	4.82
95	55.91	75.31	69.64	73.68
97	16.13	13.58	16.07	14.04
99	13.98	4.94	12.50	6.14
101	1.08	2.47		1.32
103		1.23		

Pacqu3	Hawaii	Kauai	Lanai	Maui
212		12.05	3.57	10.53
214	1.09	3.61		6.14
216	5.43	1.20	1.79	1.75
218	77.17	68.67	89.29	74.56
220	9.78	10.84	1.79	5.70
222	3.26	3.61		1.32
224	3.26		3.57	

RBG18M	Hawaii	Kauai	Lanai	Maui
96	1.06			
98		2.44	1.79	1.32
102	46.81	34.15	37.50	46.49
104	51.06	53.66	60.71	39.04
106	1.06	7.32		10.53
108		2.44		2.63

RBG29	Hawaii	Kauai	Lanai	Maui
121				0.44
129	2.08	1.19	7.14	3.07
131	19.79	29.76	21.43	25.88
133	46.88	28.57	44.64	30.26
135	10.42	5.95	5.36	7.02
137	12.50	4.76	1.79	7.89
139		1.19		0.44
143	2.08	4.76	3.57	0.44
145		3.57	1.79	1.32
147	1.04	5.95	7.14	4.82
149		1.19		2.63
151	5.21	3.57	3.57	0.44
153		4.76		7.46
155		1.19		
159		2.38		3.51
167				0.44
169		1.19		0.44
171				3.07
173			3.57	0.44

Cd10	Hawaii	Kauai	Lanai	Maui
183	2.60			
187	1.30			4.05
189	23.38	20.51	11.11	13.51
191	1.30	16.67	1.85	4.05
193	1.30		9.26	0.45
195	54.55	46.15	72.22	58.56
197	9.09	7.69	3.70	13.96
199	3.90	5.13		4.95
201	1.30	3.85	1.85	0.45
203	1.30			

Supplementary Table 1 (cont.)

Ptero01	Hawaii	Kauai	Lanai	Maui
163	83.33	91.67	73.21	93.42
165	16.67	8.33	26.79	4.82
167				1.75

Ptero02	Hawaii	Kauai	Lanai	Maui
37	1.05			
131	1.05			2.20
133	61.05	72.29	74.55	59.47
135	32.63	21.69	25.45	33.04
137	3.16	1.20		2.20
139	1.05	4.82		1.32
141				1.76

Ptero03	Hawaii	Kauai	Lanai	Maui
126		1.19	1.79	
128				0.44
130		1.19		0.88
132	9.38	7.14	5.36	8.33
134	29.17	26.19	28.57	26.32
136	3.13	2.38		1.75
138	40.63	36.90	48.21	42.98
140	16.67	14.29	16.07	11.84
142		4.76		6.58
144		4.76		
146	1.04	1.19		
152				0.88

Ptero04	Hawaii	Kauai	Lanai	Maui
146			1.79	4.39
148	14.58	2.38	8.93	4.39
150	52.08	46.43	64.29	48.68
152	26.04	33.33	14.29	15.79
154	2.08	4.76	1.79	14.47
156	2.08	10.71	8.93	8.33
158	3.13			1.32
160		2.38		2.19
162				0.44

Ptero05	Hawaii	Kauai	Lanai	Maui
206	6.67	1.25	7.14	0.88
209	1.11			
212	92.22	95.00	87.50	96.02
215		3.75	5.36	3.10

Ptero07	Hawaii	Kauai	Lanai	Maui
241		2.44		1.75
253	6.25	2.44	7.14	1.32
257	7.29	1.22		
261	16.67	12.20	14.29	23.25
265	13.54	25.61	17.86	26.75
269	18.75	12.20	25.00	14.47
273	9.38	12.20	19.64	16.23
277	10.42	13.41	14.29	10.09
281	14.58	12.20		4.82
285	3.13	4.88	1.79	0.44
289				0.88
293		1.22		

Ptero08	Hawaii	Kauai	Lanai	Maui
158	1.09		3.57	0.44
162		1.28		0.88
170		3.85		1.75
174	5.43	1.28	1.79	0.88
190		1.28		
198	4.35	5.13	1.79	1.75
202	20.65	8.97	8.93	12.28
206	8.70	17.95	12.50	19.74
210	23.91	23.08	14.29	28.95
214	27.17	20.51	50.00	12.72
218	8.70	10.26	5.36	11.84
222		6.41	1.79	4.82
226				3.51
298				0.44

Ptero09	Hawaii	Kauai	Lanai	Maui
212	10.71	2.50	10.71	13.16
216	3.57	3.75		3.51
220	7.14	15.00	3.57	3.07
224	34.52	38.75	57.14	54.82
228	16.67	15.00	21.43	12.72
232	21.43	11.25	3.57	5.26
236	4.76	12.50		6.14
240	1.19			1.32
244			3.57	
248		1.25		

Supplementary Table 2. Migration estimates from the mitochondrial data set obtained from the program MIGRATE. Values in bold are the estimated number of migrants per generation from the population on the left into the population at the top of the table. 95% confidences intervals are in parentheses below.

	Hawaii	**Maui**	**Lanai**	**Kauai**
Hawaii	-	**0.001** (0.000-0.260)	**0.001** (0.000-0.026)	**0.004** (0.000-0.093)
Maui	**0.003** (0.000-0.685)	-	**0.001** (0.000-0.027)	**0.004** (0.000-0.093)
Lanai	**0.003** (0.000-0.680)	**0.001** (0.000-0.255)	-	**0.004** (0.000-0.095)
Kauai	**0.003** (0.000-0.675)	**0.001** (0.000-0.253)	**0.001** (0.000-0.027)	-

Supplementary Table 3. Migration estimates from the nuclear intron data set obtained from the program MIGRATE. Values in bold are the estimated number of migrants per generation from the population on the left into the population at the top of the table. 95% confidences intervals are in parentheses below.

	Hawaii	**Maui**	**Lanai**	**Kauai**
Hawaii	-	**0.001** (0.000-0.002)	**0.004** (0.003-0.006)	**0.001** (0.000-0.002)
Maui	**0.001** (0.000-0.014)	-	**0.001** (0.000-0.002)	**0.001** (0.000-0.002)
Lanai	**0.001** (0.000-0.011)	**0.001** (0.000-0.002)	-	**0.001** (0.000-0.002)
Kauai	**0.001** (0.000-0.010)	**0.002** (0.001-0.003)	**0.001** (0.000-0.002)	-

Supplementary Table 4. Migration estimates from the microsatellite data set obtained from the program MIGRATE. Values in bold are the estimated number of migrants per generation from the population on the left into the population at the top of the table. 95% confidences intervals are in parentheses below.

	Hawaii	**Maui**	**Lanai**	**Kauai**
Hawaii	-	**0.013** (0.000-3.763)	**0.001** (0.000-0.011)	**0.715** (0.000-214.6)
Maui	**0.014** (0.000-4.504)	-	**0.001** (0.000-0.014)	**7.870** (0.000-327.7)
Lanai	**0.014** (0.000-4.056)	**0.013** (0.000-3.687)	-	**0.715** (0.000-280.5)
Kauai	**0.014** (0.000-4.868)	**0.013** (0.000-3.890)	**0.001** (0.000-0.016)	-

APPENDIX E

Supplementary tables and figures for Chapter IV:

Ancient DNA reveals resilience despite the threat of extinction: three thousand years of population genetic history in the endemic Hawaiian petrel

Supplementary Table 1. Minimum population growth rate required for Hawaiian petrels in the early 1900's using various parameter values in the exponential growth model $N_t = N_0e^{rt}$. N is population size, t is time, and r is the growth rate.

Parameter Varied	N_0	N_t	t	$r = (\ln (N_t/N_0))/t$
N_0	50	11000	80	0.067
	100	11000	80	0.059
	200	11000	80	0.050
	500	11000	80	0.039
	1000	11000	80	0.030
N_t	100	11000	80	0.059
	100	22500	80	0.068
	100	34000	80	0.073
	500	11000	80	0.039
	500	22500	80	0.048
	500	34000	80	0.053
t	100	11000	80	0.059
	100	11000	60	0.078
	100	11000	40	0.118
	500	11000	80	0.039
	500	11000	60	0.052
	500	11000	40	0.077

Supplementary Table 2. Minimum census population sizes of Hawaiian petrels in the early 1900's using various parameter values in the exponential growth model $N_t = N_0e^{rt}$. N is population size, t is time, and r is the growth rate. Estimates of r taken from Simons 1984.

Parameter Tested	$\mathbf{N_t}$	**t**	**r**	$\mathbf{N_0 = N_t/e^{rt}}$
r, $\mathbf{N_t}$	11000	80	0.008	5801
	11000	80	0.005	7374
	22500	80	0.008	11865
	22500	80	0.005	15083
	34000	80	0.008	17929
	34000	80	0.005	22792
r, t	11000	80	0.008	5801
	11000	80	0.005	7374
	11000	40	0.008	7988
	11000	40	0.005	9006
	34000	80	0.008	17929
	34000	80	0.005	22792
	34000	40	0.008	24690
	34000	40	0.005	27837

Supplementary Table 3. Life history parameter estimates used for simulation of the loss of genetic diversity after a decline to a constant effective population size of 100 individuals at 150 years in the past. Simulations were conducted assuming 100% overlapping generations, that random mating occurred within each species, and that sex ratios were equal. Longevity of some species is not well known, therefore the age of the oldest known individual were used instead.

Organism	Scientific Name	Longevity	Age at First Reproduction
African elephant	*Loxodonta africana*	70	10
Hawaiian petrel	*Pterodroma sandwichensis*	36	6
Musk ox	*Ovibos moschatus*	20	3
Bison	*Bison bison*	15	3
Nene	*Branta sandvicensis*	28	3
Palila	*Loxioides bailleui*	13	2

Supplementary Table 4. Information for all Hawaiian petrel bones that were radiocarbon dated for this study. Eleven bones from which DNA sequences were obtained were not radiocarbon dated: ten from Hawaii and one from Oahu. Location indicates cave where bone was discovered.

Sample Name	Island	Location	DNA Sequence	Median Age (YBP)	Lower 95% CI (YBP)	Upper 95% CI (YBP)
HFJ05.06	Hawaii	Above Kawaikae Bay	Y	112	41	230
NMNH08.20	Hawaii	Palisades Long Cave	Y	160	54	253
AM05	Hawaii	Ambigua Cave	Y	187	89	266
PC92	Hawaii	Petrel Cave	Y	203	115	281
PC94	Hawaii	Petrel Cave	Y	208	121	282
AM13	Hawaii	Ambigua Cave	Y	210	128	278
AM06	Hawaii	Ambigua Cave	Y	214	126	284
PC103	Hawaii	Petrel Cave	Y	214	126	284
PC97	Hawaii	Petrel Cave	Y	216	131	280
HFJ09.06	Hawaii	South Point	Y	216	131	280
PC86	Hawaii	Petrel Cave	Y	222	130	286
PC99	Hawaii	Petrel Cave	Y	225	134	282
PC96A	Hawaii	Petrel Cave	Y	230	133	289
SC05	Hawaii	Shangri-la Cave	Y	230	133	289
AM04	Hawaii	Ambigua Cave	Y	234	137	285
AM11	Hawaii	Ambigua Cave	N	234	137	285
PC108	Hawaii	Petrel Cave	Y	234	137	285
PC99B	Hawaii	Petrel Cave	Y	234	137	285
SC03	Hawaii	Shangri-la Cave	N	234	137	285
AM07	Hawaii	Ambigua Cave	Y	239	136	292
PC93	Hawaii	Petrel Cave	Y	239	136	292
PC100B	Hawaii	Petrel Cave	Y	242	140	288
PC87	Hawaii	Petrel Cave	Y	243	132	300
AM08	Hawaii	Ambigua Cave	Y	246	138	295
AM10	Hawaii	Ambigua Cave	Y	246	138	295
PC106	Hawaii	Petrel Cave	Y	246	138	295
PC91	Hawaii	Petrel Cave	Y	246	138	295
HFJ09.23	Hawaii	Puu Keanui Cave	Y	246	138	295
UMI08.01	Hawaii	Umii Manu	Y	248	142	292
AM12	Hawaii	Ambigua Cave	N	254	144	296
PC100A	Hawaii	Petrel Cave	Y	262	142	309
SC06	Hawaii	Shangri-la Cave	N	262	142	309
PC90	Hawaii	Petrel Cave	Y	263	145	168
NMNH08.19	Hawaii	Palisades Long Cave	Y	266	143	316
SC07	Hawaii	Shangri-la Cave	Y	270	144	326
PC107	Hawaii	Petrel Cave	Y	271	146	165
PC98	Hawaii	Petrel Cave	Y	271	146	165
HFJ09.22	Hawaii	Puu Keanui Cave	Y	274	146	165
PC101	Hawaii	Petrel Cave	Y	278	149	160
PC109	Hawaii	Petrel Cave	N	278	148	162
BBMX157438	Hawaii	Pu'uanahulu	N	278	148	162

Supplementary Table 4 (cont.)

Sample Name	Island	Location	DNA Sequence	Median Age (YBP)	Lower 95% CI (YBP)	Upper 95% CI (YBP)
HFJ09.15	Hawaii	Kiholo Bay Cave Along Shore	Y	282	149	159
PC105	Hawaii	Petrel Cave	Y	282	149	159
PC95	Hawaii	Petrel Cave	Y	282	149	159
AM03	Hawaii	Ambigua Cave	N	294	249	384
HFJ09.11	Hawaii	Cave N of Umi Heiau	N	294	249	384
HFJ09.20	Hawaii	Cave N of Umi Heiau	Y	294	249	384
RCF05.03	Hawaii	Kona Palisades Refuge Cave	Y	294	249	384
HFJ09.07	Hawaii	South Point	Y	294	249	384
HFJ09.21	Hawaii	Cave N of Umi Heiau	Y	300	252	396
HFJ05.03	Hawaii	Pohakuloa 10649	Y	302	248	404
HFJ09.14	Hawaii	Kiholo Bay Cave Along Shore	Y	323	266	407
AM09	Hawaii	Ambigua Cave	Y	348	280	422
SC04	Hawaii	Shangri-la Cave	Y	352	285	422
HFJ05.04	Hawaii	Pohakuloa 10649	Y	382	299	458
PC104	Hawaii	Petrel Cave	N	383	302	455
SC01	Hawaii	Shangri-la Cave	Y	383	304	452
HFJ09.19	Hawaii	Puuanahulu Cave	Y	394	307	462
HFJ09.25	Hawaii	Fireplough Cave	Y	400	310	467
HFJ09.16	Hawaii	Makalawena Cave System	Y	408	317	469
HFJ09.17	Hawaii	Makalawena Cave System	Y	408	317	469
RCF05.04	Hawaii	Fireplough Cave	Y	413	317	475
HFJ06.17	Hawaii	Fireplough Cave	Y	422	321	484
RCF05.01	Hawaii	Fireplough Cave	Y	428	325	488
PC96B	Hawaii	Petrel Cave	Y	431	333	345
HFJ06.21	Hawaii	Fireplough Cave	Y	437	333	345
HFJ09.18	Hawaii	Puuanahulu Cave	N	439	334	344
UMI08.02	Hawaii	Umii Manu	N	460	407	504
HFJ09.27	Hawaii	Fireplough Cave	Y	463	413	505
HFJ05.08	Hawaii	Waikulukulu Cave	Y	479	428	519
HFJ09.24	Hawaii	Fireplough Cave	Y	499	452	541
HFJ09.13	Hawaii	6000' Cave	Y	1144	1055	1228
HFJ09.11	Hawaii	KFU Old FWS camp cave	N	1515	1409	1600
SC08	Hawaii	Shangri-la Cave	Y	1632	1535	1714
PC88	Hawaii	Petrel Cave	Y	1654	1556	1743
PC102	Hawaii	Petrel Cave	N	1702	1608	1796
BBM19921662	Hawaii	Puu WaaWaa	Y	1777	1688	1867
HFJ09.10	Hawaii	KFU Old FWS camp cave	Y	1783	1701	1860
HFJ09.12	Hawaii	Kalahiki Cave System	N	2057	1963	2144
HFJ09.08	Hawaii	Palani Ranch Jefferys Cave	N	2660	2529	2726
UMI08.03	Hawaii	Umii Manu	Y	3058	2944	3170
BBM1992161	Hawaii	Puu WaaWaa	Y	3228	3117	3329
HFJ09.01	Lanai	Feather Cave	N	994	919	1070

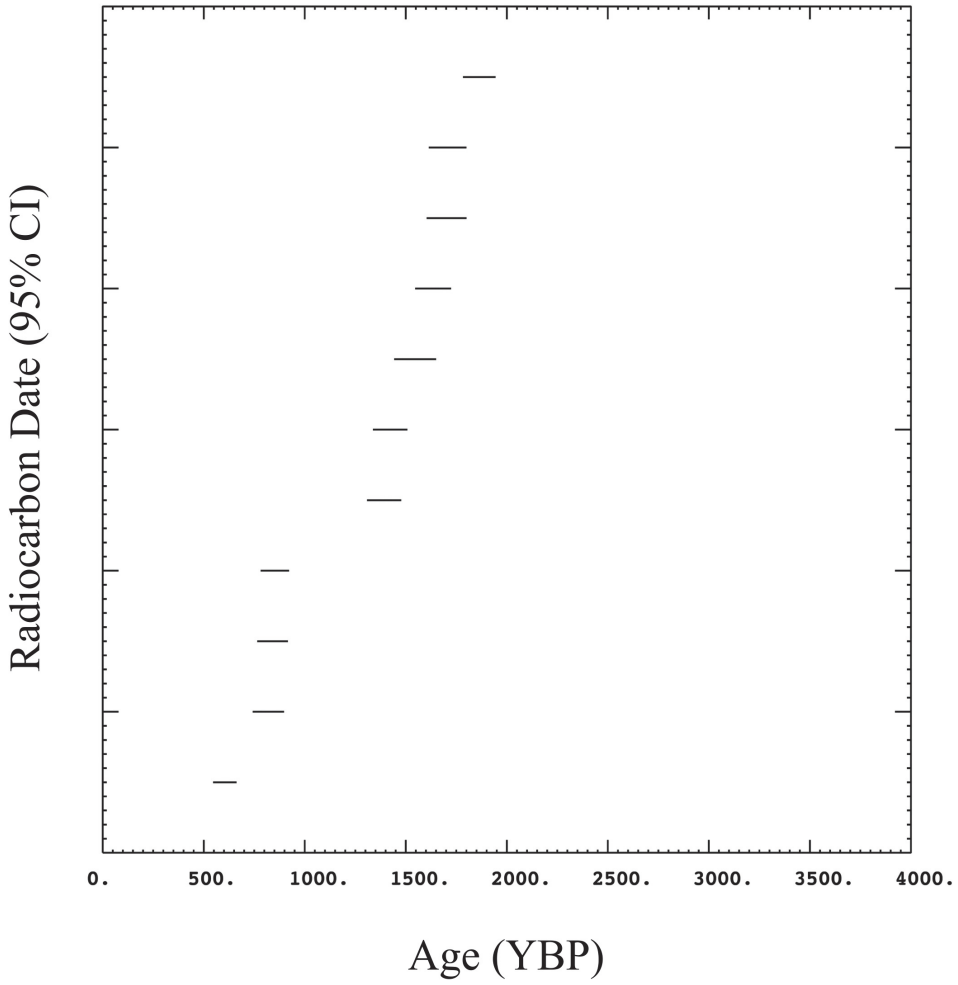
Supplementary Table 4 (cont.)

Sample Name	Island	Location	DNA Sequence	Median Age (YBP)	Lower 95% CI (YBP)	Upper 95% CI (YBP)
IIFJ09.02	Lanai	Feather Cave	Y	899	800	960
HFJ09.03	Lanai	Feather Cave	N	1088	990	1170
HFJ09.05	Lanai	Feather Cave	N	1014	931	1103
HFJ09.04	Lanai	Maunalei Gulch Feather Cave	N	823	738	899
LA06	Maui	Kanaio	N	243	132	300
NMNH08.18	Maui	Puu Naio	Y	555	505	620
BBMX150206	Maui	Haleakala Crater	N	651	569	576
HFJ06.35	Maui	Kiakeana Cave	Y	769	693	869
LA019	Maui	Kanaio	N	837	748	913
LA05	Maui	Kanaio	Y	855	773	922
LA021	Maui	Kanaio	Y	914	818	984
LA022	Maui	Kanaio	Y	955	899	1039
HFJ05.05	Maui	Kiakeana Cave	Y	1144	1055	1228
HFJ05.07	Maui	Puu Makua Cave	Y	1527	1409	1621
LA020	Maui	Kanaio	Y	1702	1604	1800
LA014	Maui	Kanaio	N	1789	1705	1865
NMNH08.14	Maui	Lua Lepo	N	2228	2139	2307
LA07	Maui	Kanaio	N	3095	2970	3207
LA01	Maui	Kanaio	N	3360	3274	3438
HFJ06.36	Maui	Kahawaihapapa Cave	Y	3435	3355	3539
NMNH08.12	Maui	Lua Lepo	N	4192	4080	4305
NMNH08.13	Maui	Lua Lepo	N	4395	4288	4496
NMNH08.11	Maui	Lua Lepo	N	4409	4310	4503
HFJ06.01	Oahu	9679-P1	Y	615	546	662
HFJ06.27	Oahu	50-80-12-2706-22B	N	644	565	585
HFJ06.26	Oahu	50-80-12-2706-22B	N	699	649	763
NMNH08.04	Oahu	50-Oa-B6-22	Y	823	743	898
NMNH08.01	Oahu	50-Oa-B6-22	Y	846	765	917
BSHP08.14	Oahu	9659	N	852	775	917
NMNH08.02	Oahu	50-Oa-B6-22	Y	861	782	922
BSHP08.04	Oahu	50-Oa-B6-100C	N	1049	960	1141
NMNH08.09	Oahu	50-Oa-B6-22	N	1076	987	1160
BSHP08.24	Oahu	50-Oa-B6-78	N	1144	1055	1228
BSHP08.07	Oahu	50-Oa-B6-100C	N	1150	1062	1228
BSHP08.11	Oahu	50-Oa-B6-100C	N	1150	1062	1228
BSIIP08.20	Oahu	BPBSPA III Site 9659	N	1213	1138	1276
BSHP08.15	Oahu	BPBSPA III Site 9659	N	1321	1270	1382
BSHP08.27	Oahu	50-Oa-B6-78	N	1342	1277	1412
NMNH2010.05	Oahu	50-Oa-B6-22	Y	1382	1308	1477
BSHP08.21	Oahu	BPBSPA III Site 9659	Y	1420	1338	1508
HFJ06.31	Oahu	9670-P1	Y	1546	1443	1650
BSHP08.10	Oahu	50-Oa-B6-100C	N	1596	1521	1683

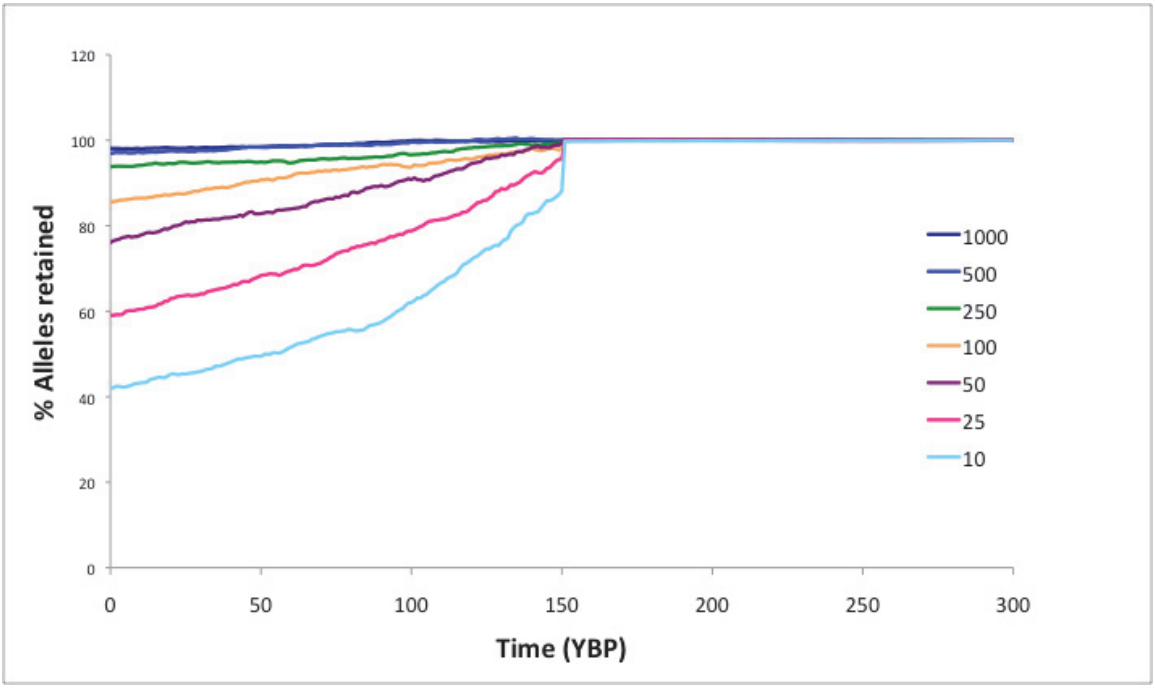
Supplementary Table 4 (cont.)

Sample Name	Island	Location	DNA Sequence	Median Age (YBP)	Lower 95% CI (YBP)	Upper 95% CI (YBP)
BSHP08.29	Oahu	50-Oa-B6-78	N	1596	1521	1683
NMNH08.03	Oahu	50-Oa-B6-22	Y	1643	1547	1724
HFJ06.33	Oahu	9670-P1	Y	1702	1604	1800
NMNH08.05	Oahu	50-Oa-B6-22	Y	1709	1614	1800
NMNH08.08	Oahu	50-Oa-B6-22	N	1756	1679	1844
NMNH2010.08	Oahu	50-Oa-B6-22	Y	1863	1783	1944
BSHP08.31	Oahu	50-Oa-B6-78	N	2022	1935	2110
BSHP08.08	Oahu	50-Oa-B6-100C	N	2040	1946	2128
BSHP08.28	Oahu	50-Oa-B6-78	N	2057	1963	2144
BSHP08.26	Oahu	50-Oa-B6-78	N	2584	2460	2693
BSHP08.22	Oahu	50-Oa-B6-78	N	2639	2505	2719

Supplementary Figure 1. Distribution of radiocarbon dates obtained for subfossil bones from the extirpated colony on Oahu that yielded ancient DNA sequences.

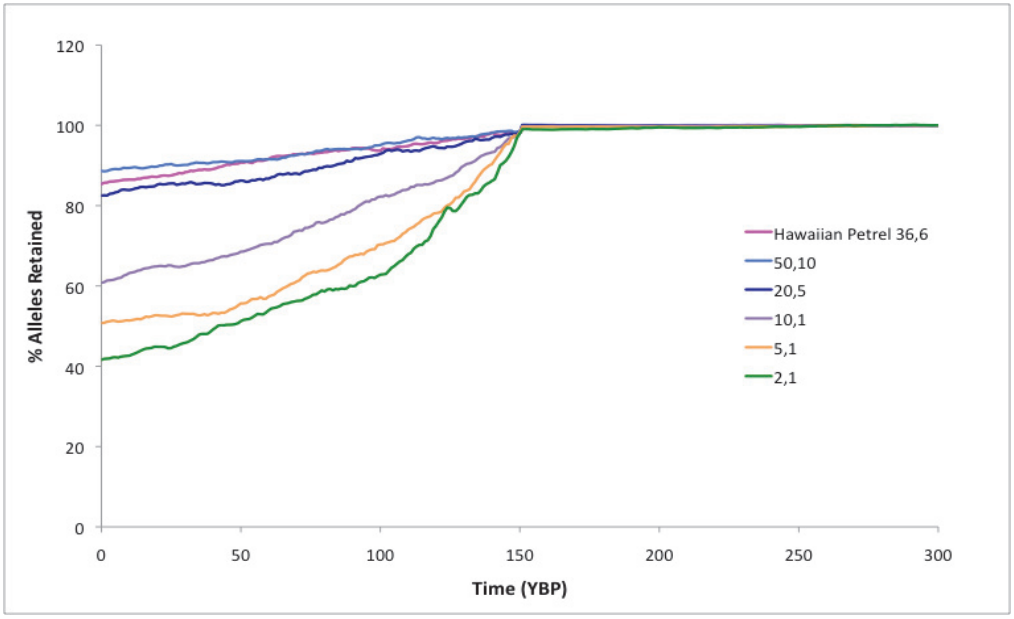


Supplementary Figure 2. BOTTLESIM simulations of the change in the number of alleles in the Hawaiian petrel as a result of a decline to a constant effective population size ranging from 10 to 1000 individuals at 150 years in the past.

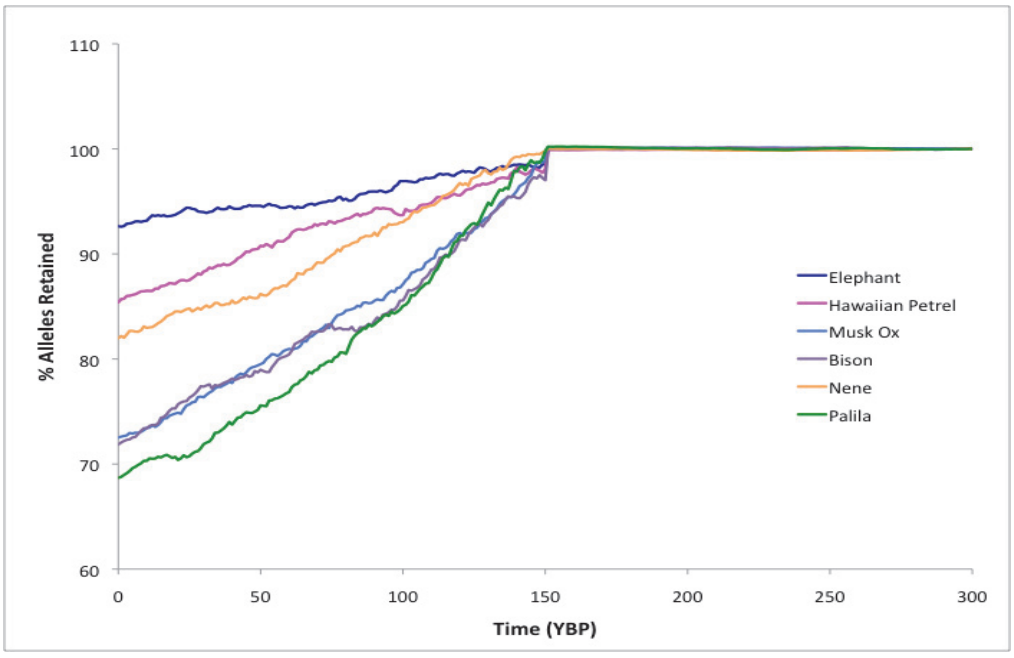


Supplementary Figure 3. BOTTLESIM simulations of the impact of life history characteristics on the change in the number of alleles retained after a decline to a constant effective population size of 100 individuals at 150 years in the past. (A) The Hawaiian petrel compared to species with theoretical life histories (longevity, age at first reproduction); (B) The Hawaiian petrel as compared to species for which ancient DNA has been used to investigate temporal changes in genetic diversity or for species that may have been impacted by arrival of humans on the Hawaiian Islands.

A.



B.



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