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Cross-Temporal Analysis of Genetic Diversity in the Endangered Medium Tree Finch (Camarhynchus pauper) and Closely Related Darwin’s Finches

Student’s name: Colleen M. Metzger

This work and its defense approved by:

Committee chair: Kenneth Petren, PhD
Committee member: Joshua Gross, PhD
Committee member: Theresa Culley, PhD
Cross-Temporal Analysis of Genetic Diversity in the Endangered Medium Tree Finch (*Camarhynchus pauper*) and Closely Related Darwin’s Finches

By Colleen Metzger
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Abstract

Natural history collections can provide a direct view of past genotypes, which allows greater insight into evolutionary processes that are relevant for conservation and management. However, few studies have used broad surveys of multilocus genotypes from the past to address the wide range of processes that can affect conservation planning of a species today. Therefore, we assessed the history and status of the critically endangered medium tree finch, *Camarhynchus pauper*, an endemic finch of the Galápagos Islands. Using ancient DNA techniques, we quantified cross-temporal genetic change for 16 microsatellite loci in this species and its relatives. We tested the hypothesis that *C. pauper* has undergone a recent reduction in population size and loss of genetic diversity, and evaluated the hypothesis that *C. pauper* is genetically distinct from its two closest relatives, *C. parvulus* and *C. psittacula*. We assessed whether decline in *C. pauper* has led to increased hybridization with other species and evaluated a long-standing hypothesis of its origin from *C. psittacula* on another island using genetic distances, assignment tests, and migration analyses. Genetic diversity declined significantly in *C. pauper* over time, and several other tree finch populations showed similar losses of genetic diversity. Genetic distances and assignment tests supported the notion that *C. pauper* has remained genetically distinct from other species over time, and is actually becoming more distinct, and has experienced substantial genetic change, over the past century. Hybridization between *C. pauper* and other tree finch species was detected, but has declined to low levels over the last century. However, we could not detect a single origin of this species as hypothesized by Lack (1947). We conclude that *C. pauper* should be treated as a genetically distinct conservation unit. Management actions to restore habitat quality and mitigate the negative effects of
introduced parasites and predators should be continued, to ensure the persistence of this unique and iconic, endangered species.
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Introduction

Understanding the genetic and evolutionary history of threatened or endangered species is critical for effective management and conservation (Höglund et al. 2011; Haig 1998; Moritz 1994). The value of a potential conservation unit, and the most effective strategy for managing it, will depend on many factors including its evolutionary distinctness (Redding and Mooers 2006; Vane-Wright et al. 1991), effective population size (Waples 2010), recent population growth or decline (Antao et al. 2011; Luikart et al. 2010; Wang 2005), past population bottlenecks (Hundertmark and Daele 2009; Luikart et al. 1998), recent hybridization with relatives (Cureton et al. 2011; Nourisson et al. 2011; Caballero and Toro 2002; Legge et al. 1996), the degree of connectivity or isolation from other populations (Farrington and Petren 2011; Petren et al. 2005), habitat fragmentation within the conservation unit (Reed 2004; Harrison and Bruna 1999), and mating patterns that lead to increased inbreeding (Chaves et al. 2011; Frankham 2005; Keller and Waller 2002).

Genetic information for conservation is typically obtained from a “snapshot” of genetic variation collected during a single point in time (usually the present), from which a past history is inferred or a future trajectory is predicted (Wilson et al. 2003; Beaumont 1999). However, different historical scenarios may produce similar genetic patterns. For example, low levels of genetic diversity may be due to recent population decline or due to a consistently small population size maintained over time (Matocq & Villablanca 2001). Methods now exist to distinguish among different historical scenarios using genetic information from a single time point, but these methods rely on many assumptions (Wilson et al. 2003; Beaumont 1999).

Fortunately, specimens housed in natural history collections represent a major source of viable genetic data (“ancient DNA”) from the past (Wandeler et al. 2007; Austin & Melville
which can provide direct evidence of historic genetic composition. When coupled with modern genetic data, data from these collections can reveal more accurate, detailed, and complex genetic and demographic histories than single time-point estimates (Ramakrishnan & Hadly 2009; Storz and Beaumont 2002; Bouzat 2001). Such “cross-temporal” genetic studies allow for the direct assessment of genetic and demographic change over time, providing an appropriate temporal and genetic context for the evaluation and management of modern populations (Bouzat 2001; Matocq & Villablanca 2001).

Cross-temporal genetic analyses can be especially useful for endangered species that exist as island endemics or otherwise as single confined populations. Island populations are often small in size, isolated from immigration, and subject to strong environmental perturbations from both natural and anthropogenic causes (Frankham 1997, 1998). Such perturbations can cause a cascade of events: dramatic reductions in population size, inbreeding, loss of genetic variation and even extinction (Grant et al. 2005a; Frankham 1998; Keller 1998). Genetic monitoring can be useful for predicting and managing the threat of extinction due to such perturbations (Schwartz et al. 2007).

The Galápagos Islands, Ecuador, are home to an iconic avifauna currently facing pressure from anthropogenic habitat degradation, introduced species, and stochastic environmental fluctuations caused by El Niño events (Grant et al. 2000). This is especially true of Darwin’s finches, which comprise 14 endemic passerine species that exist in various island subpopulations throughout the archipelago. These species can be broadly categorized into three main groups based on their morphology and ecological niche: ground finches, tree finches, and cactus/warbler finches. In contrast, phylogenetic studies based on nuclear and mitochondrial DNA place Darwin’s finches into six distinct clades and suggest that all taxa originated from a single
ancestral species 2-3 mya (Petren et al. 1999; Sato et al. 1999). As a recently diverged group, Darwin’s finches are closely related genetically, but exhibit notable interspecific variation both ecologically and morphologically.

Two of the main groups, the tree finches (*Camarhynchus*) and the ground finches (*Geospiza*), differ noticeably in their ecology, morphology, and behavior (Lack 1947), and also, surprisingly, in their overall levels of genetic diversity (Petren et al. 2005; Grant et al. 2005b). Attempts to delineate species using mtDNA yielded low resolution of species in ground and tree finches (Petren et al. 2005; Sato et al. 1999). However, microsatellite loci can be used to successfully discriminate species (Petren et al. 1999, 2005), which reflects the observed biological and ecological differences among species. Related species often co-occur on the same island, yet remain distinct morphologically and behaviorally (i.e. in terms of song; Grant and Grant 2010a; Ratcliffe and Grant 1985). Some species occasionally exchange genes through hybridization and limited backcrossing (Grant and Grant 2010b; Grant 1999; Grant 1993). Overall levels of genetic diversity (observed heterozygosity) appear to be lower in the tree finches (45%) compared to the ground finches (67%; Petren et al. 2005), perhaps because tree finches tend to favor moist habitat that occurs in limited upland areas of the islands.

The small (*Camarhynchus parvulus*), medium (*Camarhynchus pauper*), and large (*Camarhynchus psittacula*) tree finches co-occur only on Floreana Island, which hosts the archipelago’s singular medium tree finch population. Lack (1947) posited that the medium tree finch originated as a subpopulation of the large tree finch. He hypothesized that large tree finches from Santa Cruz Island to the north migrated to Floreana Island. Some time later, another group from Santa Cruz also migrated to Floreana and these two taxa interbred with each other instead of the resident large tree finches on the same island. These two populations then
diverged to form both a medium and a large tree finch species. Such “double colonization” appears to account for endemic forms in other island communities (beetles: Jordal et al. 2006, sticklebacks: McPhail 1993; Schluter and McPhail 1992), and the initial stages of this process have recently been observed in Darwin’s finches (Grant and Grant 2009).

Most Darwin’s finch populations currently face a suite of environmental and anthropogenic threats, including habitat destruction, invasive species, and a growing resident human population (Watkins and Cruz 2007). Although no species of Darwin’s finch has gone extinct to date to the best of our knowledge, more than a dozen populations appear to have been recently extirpated from disturbed islands (Grant et al. 2005a; Grant 1999). Consequently, the mangrove finch (Cactospiza heliobates) and the medium tree finch (Camarhynchus pauper) are both listed as “critically endangered” by the IUCN (Brumm et al. 2010; Fessl et al. 2010; IUCN 2010; Dvorak et al. 2004). Most Darwin’s finches exist as a series of metapopulations dispersed across various islands, with occasional interisland migration (gene flow) linking different island populations (Farrington and Petren 2011; Petren et al. 2005; Grant 1999). However, the medium tree finch exists as a single population on Floreana Island, and is now critically endangered due to habitat degradation and introduced parasites and predators (IUCN 2010). Floreana Island has the longest history of human disturbance in the Galápagos archipelago (Lack 1947), and continues to experience habitat destruction and degradation, especially in the limited uplands where farming is possible, and where tree finches reside (Watson et al. 2009). As of 2010, only 22.5 km² of suitable tree finch habitat remained in the Floreana highlands (O’Connor et al. 2010b). To date, several finch populations have already disappeared from Floreana, including, most recently, the warbler finch (Grant et al. 2005a).
The finches on Floreana Island are also heavily impacted by the parasitic fly *Philornis downsi* (Diptera, Muscidae), a recent invader of the archipelago that infests the nests of land birds and severely reduces offspring survival, particularly in Darwin’s tree finches (O’Connor et al. 2010a). After hatching, the developing *P. downsi* larvae feed on the blood and tissues of the hatching birds, both externally and through the nasal cavities, causing considerable blood and tissue loss (Fessl et al. 2006). Medium tree finch nests are one of the most intensely parasitized of all Darwin’s finches, and nestlings experience a 41% mortality rate (O’Connor et al. 2010a; O’Connor et al. 2010b; Kleindorfer and Dudaniec 2009).

A recent census estimated the current *C. pauper* population to be 1,620 individuals (maximum), which is considerably smaller than the estimates for many other avian species on Floreana Islands (N_{small tree finch}=3,700 and N_{small ground finch}=4,680; O’Connor et al. 2010b). The effective, or breeding population size, however, is often substantially less than the census size (Ovenden et al. 2007; Turner et al. 2002; Frankham 1995). Fortunately, genetic analysis can provide independent estimates of effective population size (Beaumont 1999, Beerli 1991, 2001, Waples 1991).

We used cross-temporal genetic comparisons to evaluate the genetic identity of *C. pauper* and assess its current status. While our focus rested on the medium tree finch (*C. pauper*) of Floreana Island, we also examined its two closest relatives, the small tree finch (*C. parvulus*) and the large tree finch (*C. psittacula*), in order to place the recent history of *C. pauper* in context. We tested the following hypotheses: (1) Population size and genetic diversity have recently declined, as would be expected for a “critically endangered” species on a small, recently disturbed island. We predicted that modern measures of genetic diversity would be significantly lower than past (1906) levels, as population decline should increase levels of inbreeding and
genetic drift, and ultimately reduce genetic diversity. (2) *C. pauper* is genetically distinct from its two closest relatives, *C. parvulus*, and *C. psittacula*, and genetic differences among these species have increased over time. Even though these species are closely related, we predicted *C. pauper* would be distinct and therefore worthy of efforts to conserve it. (3) Hybridization has recently occurred among *C. pauper* and relatives, which may be predicted based on patterns of hybridization among other closely related Darwin’s finches (Grant and Grant 2010b; Grant and Grant 2008; Grant 1993). We expected hybridization to increase as numbers of *C. pauper* decline.

**Materials and Methods**

**Samples**

We genotyped a total of 78 modern and 142 historic specimens from six tree finch populations (representative of the three species), spanning three islands in the south of the Galápagos archipelago (Table 1, Figure 1). DNA from modern specimens was isolated from whole blood samples collected in the field by venipuncture and dried on EDTA-treated filter paper during expeditions to the Galápagos during the years 1988-2006 (Petren et al. 1999, 2005). The majority of samples were collected in 1997. Ancient DNA from historic specimens was extracted from tissue samples (~ 2 x 3mm) taken from the toe pad of specimens, originally collected in the field during the years 1897-1906, and housed at the California Academy of Science, the British Museum of Natural History, and the American Museum of Natural History. The majority of historic specimens were collected during the 1905-1906 California Academy of Science (CAS) Galápagos expedition.
**Laboratory Methods**

DNA extraction from modern blood samples followed previously published protocols (Petren 1998). Historic specimens were stored and processed in an isolated lab dedicated to ancient DNA work to reduce the risk of contamination from modern DNA. All tools and surfaces used in the extraction and processing of ancient DNA were UV irradiated prior to and after each use; work surfaces were frequently wiped down with 50% bleach, and access to the room was closely restricted. Researchers wore protective clothing, including sterile gloves and foot coverings. DNA was extracted from museum tissue samples using MP Biomedicals’ GENE CLEAN® for Ancient DNA kits; extracted DNA was eluted to a total volume of approximately 65 µL. Blank extractions, processed without tissue, were performed with each batch to ensure reagents remained uncontaminated.

Extracted DNA for both modern and historic specimens was amplified via multiplex PCR of sixteen polymorphic microsatellite loci (14 autosomal, 2 sex-linked) previously developed for Darwin’s finches (Petren 1998). Original PCR primers were redesigned to produce smaller PCR products (Petren et al. 2010) to increase amplification success, which decreases with increased fragment size in degraded DNA (Sefc et al. 2003). Multiplex PCR reactions included four loci, each labeled with a different color fluorescent dye, per reaction. To reduce genotyping errors in historic specimens, each sample was subjected to PCR amplification in triplicate to prevent errors due to allelic dropout, which is estimated to be around 20% for similar samples (Farrington & Petren 2011; Petren et al. 2010). Blank PCR amplifications, without DNA, were also performed with each batch to test for reagent contamination.

PCR amplifications were performed with a total volume of 15 µL, containing 7.5 µL QIAGEN multiplex PCR master mix, 0.30 µM of primers, and 1 µL of extracted DNA under the
following conditions: an initial denaturation step at 95°C for 15 minutes, followed by 33 cycles for modern DNA (40 cycles for historic DNA) of 30 seconds at 94°C, 1 minute 30 seconds at 52°C, and 1 minute 30 seconds at 72°C, and a final extension step at 72°C for 10 minutes. PCR products were then processed at an external facility (Cornell University Life Sciences Core Laboratories Center) with a LIZ-labeled size standard on an Applied Biosystems 3730xl DNA Analyzer. Genotypes were scored using the software Gene Mapper® (Applied Biosystems). Historic individuals with less than 50% genotype recovery across all loci were excluded from further analyses. Profiles for previously genotyped modern specimens from the populations of interest (78 total samples) were easily obtained for cross-temporal comparisons (Petren et al. 2005).

**Genetic and Statistical Analyses**

We calculated basic measures of genetic diversity across all 14 autosomal loci for each population and the two time points. Allelic richness (Aₑ), which accounts for variation in sample size among populations, was computed in FSTAT (Goudet 1995), a program which employs a rarefaction method using the smallest number of samples across all groups to reduce bias from uneven sample sizes among groups; for our data, we calculated the effective number of alleles using a minimum sample size of four diploid individuals. Expected heterozygosity (Hₑ) and observed heterozygosity (Hₒ) were calculated using GenAlEx (Peakall & Smouse 2006). Observed heterozygosity was used to reveal deviations in HWE that might indicate inbreeding or population substructure, but was not used as a main diversity indicator. Wilcoxon paired-sample tests (paired by locus) were performed in JMP (Pro v. 9; SAS) to test for significant cross-temporal differences in the diversity measures Aₑ and Hₑ. Utilizing all 16 microsatellite loci, a principal coordinates analysis (PCA) was performed for the historic and modern Floreana Island
specimens (5 populations total – modern *C. psittacula* specimens were not available for analysis) in GenAlEx (Peakall & Smouse 2006), and graphically compiled in JMP, to qualitatively measure the amount of genetic overlap among species and across time.

Genetic relationships among tree finch populations, both within a single time period and across the two time periods, were investigated in several ways. We explored patterns of genetic differentiation between population pairs by calculating Weir and Cockerham’s θ (1984), an analogue of FST that takes into account variation in size among populations, in Genetic Data Analysis (GDA, version 1.0; Lewis and Zaykin 2001); 95% confidence intervals based on bootstrapping across loci were also calculated to determine if populations were significantly differentiated from one another. To assess genetic change on the landscape scale, a Mantel matrix correlation was performed in GenAlEx (Peakall and Smouse 2006) to determine if pairwise θ values were correlated across time periods; a significant correlation between past and present populations would suggest that the genetic composition of populations did not change over time on the landscape scale.

The entire adaptive radiation of Darwin’s finches has occurred in less than 3 million years, and species comprising highly structured populations are not expected to attain reciprocal monophyly at commonly used marker loci over this time. We therefore need a different measure of genetic distinctness to evaluate biological differences among species. We conducted population assignment tests with the program STRUCTURE (v. 2.3.3; Pritchard et al. 2000), which uses a Bayesian approach to determine genetic distinctness of populations based on multilocus allelic information. We determined the number of genetic clusters (K) represented in our data by conducting 10 replicate runs per K of the admixture model, with correlated allele frequencies, inferred alpha values, and with location (in our case, this was temporal or
geographic population) as a prior to assist in membership assignment. We ran multiple K levels, from K=1 to K=n+1, where n is the number of sampling locations (or in our case, temporal and geographic populations) and selected the K with the highest likelihood value averaged from 10 independent simulations (Pritchard et al. 2000). We ran all historic and modern populations together to discern broad genetic differences by species and time period (a total of 13 temporal and/or geographic populations); simulations included a burn in of 500,000 steps followed by a sampling interval of 300,000 Markov chain Monte Carlo (MCMC) iterations. We then analyzed all populations within a given time period (past, present) to investigate genetic differences due to species and/or geographic location (historic: 7 populations; modern: 6 populations); temporal simulations included a burn-in of 400,000 steps, followed by 300,000 MCMC iterations. We ran the historic and modern populations occurring on Floreana Island only (5 populations) to explore relatedness and gene flow between C. pauper, C. parvulus, and C. psittacula. Simulations included a burn-in of 500,000 steps, followed by a sampling interval of 500,000 MCMC iterations each. For direct assessment of ancestry, Floreana populations were assessed by time point using the “gen back” option within the “USEPOPINFO” model, which uses sampling locations (or species in this case) to test whether individuals have recent immigrant ancestry from among the other sampling localities (which would indicate hybridization in our scenario). We explored two generations back, and simulations included a burn-in of 500,000 steps, followed by a sampling interval of 500,000 iterations.

Finally, because interisland gene flow is known to occur on a regular basis in other Darwin’s finches (Petren et al. 2005), and interisland movement is hypothesized in the origin of C. pauper (Lack 1947), we employed a coalescent-based method to estimate past and present population sizes and interisland migration rates using MIGRATE (v. 3.2.17; Beerli and
We analyzed our autosomal microsatellite data under the maximum likelihood framework, using a Brownian motion model, which has been shown to provide results very similar to those generated via the stepwise model with our data (Farrington and Petren 2011). We used randomly generated starting trees, excluded missing data, and performed searches with 10 short chains and three long chains; after a burn-in of 10,000 steps, samples were taken every 20 steps. We focused our MIGRATE analyses on migration between C. pauper and the other two tree finches of interest, C. parvulus and C. psittacula. We estimated \( \theta \) (assuming a mutation rate of \( 1 \times 10^{-4} \)), a measure of population size and \( M \) (migration rate) for every population and population pair.

**Results**

**Genetic Diversity Over Time**

Cross-temporal comparisons of genetic diversity in C. pauper revealed decreases in two diversity measures over the past century, a time span equal to roughly 25 finch generations. Allelic richness in C. pauper declined significantly by approximately 9%, from 3.42 to 3.19 alleles per diploid individual \( (F_{1, 13} = 32.5; P < 0.05; \text{Figure 2}) \); expected heterozygosity declined approximately 7%, from 0.541 to 0.505 \( (F_{1, 13} = 21.0; P = 0.20; \text{Table 2}) \). In contrast, observed heterozygosity, which was not our primary measure of genetic diversity, experienced a large, although non-significant, increase of nearly 28\% \( (F_{1, 13}; P = 0.08; \text{Figure 3}) \). Observed heterozygosity, however, is highly biased by the presence of subpopulation structure, sampling localities, and any deviations from Hardy-Weinberg equilibrium (HWE) due to factors such as inbreeding, genetic drift and gene flow, which is known to occur in the finches (Grant 1999).
On Floreana Island, *C. pauper* coexists with *C. parvulus* and *C. psittacula*; however, cross-temporal comparisons were only possible for *C. parvulus*, as modern *C. psittacula* specimens were unavailable. Contrary to the genetic declines observed in *C. pauper*, *C. parvulus* populations experienced increases in all measures of genetic diversity over time. Allelic richness and expected heterozygosity increased by approximately 4% and 2%, respectively (\(A_E: F_{1,13}; P = 0.50; H_E: F_{1,13}; P = 0.67\); Table 2; Figures 2, 3). Additionally, levels of observed heterozygosity in *C. parvulus* on Floreana increased significantly, by 48% (\(F_{1,13}; P = 0.01\); Table 2; Figure 3), perhaps indicating substantial gene flow from another source.

Although our focus was on genetic change in *C. pauper* over time, when we expanded direct cross-temporal comparisons to tree finch populations on the neighboring islands of Isabela and Santa Cruz, we encountered population decline in three additional populations (*C. psittacula* on Isabela and Santa Cruz, and *C. parvulus* on Santa Cruz), suggesting that genetic decline in Darwin’s tree finches may be occurring on a landscape-scale (Table 2; Figure 2). The *C. psittacula* population on Isabela Island experienced significant decreases of 15% and 18% in allelic richness and expected heterozygosity, respectively (\(A_E: F_{1,13}; P < 0.05; H_E: F_{1,13}; P < 0.01\); Figure 2). The *C. psittacula* population on Santa Cruz Island experienced a decline of 11% and 14% in allelic richness and expected heterozygosity, respectively (\(A_E: F_{1,13}; P = 0.12; H_E: F_{1,13}; P < 0.05\)). Lastly, we observed slight, but non-significant, declines in allelic richness and expected heterozygosity for the *C. parvulus* population on Santa Cruz (\(A_E: F_{1,13}; P = 0.36; H_E: F_{1,13}; P = 0.32\)). In contrast, *C. parvulus* populations on Floreana and Isabela Islands showed slight increases in their levels of genetic diversity (Table 2, Figure 2). In total, cross-temporal comparisons of genetic diversity revealed declines in four out of the six tree finch populations sampled (Figure 2).
**Genetic Structure Over Time**

Population structure analyses support the unique genetic identity of *C. pauper* compared to its congeners on the same and neighboring islands. When all species and populations from both time points are assessed together, three genetic clusters (K=3) are supported that mostly correspond to species (Figure 4b). Most individuals displayed an admixed genetic composition, which is to be expected in a group of recently diverged, closely related species like the tree finches. Individuals generally clustered according to species, with the exception of the modern Santa Cruz Island populations of *C. parvulus* and *C. psittacula*, which clustered together (Figure 4b). Historic *C. pauper* individuals clustered with modern *C. pauper* individuals, with assignment probabilities (*Q* in STRUCTURE) ranging from 0.704-0.737. Membership proportions for modern *C. pauper* individuals ranged from 0.775-0.872.

Similar patterns were observed when each time point was analyzed separately. In both the historic and modern time points, *C. pauper* remained distinct from the other tree finch species and *C. pauper* individuals segregated into their own distinct cluster. However, the amount of genetic information shared with other clusters varied temporally. Historically, *C. pauper* shared more genetic information with the *C. psittacula* cluster than with the *C. parvulus* cluster, whereas the modern *C. pauper* population shared a similar amount of genetic information with both species, but overall shared less information with congeners than it did historically.

**Genetic Differentiation Over Time**

Genetic distances (θ) reinforce the observation of significant genetic differentiation between *C. pauper* and individual populations of *C. parvulus* and *C. psittacula* from both time points (Table 3). The historic *C. pauper* population exhibited significant differentiation from all
historic *C. parvulus* and *C. psittacula* populations; the same was true for the modern *C. pauper* population (Table 3). Significant changes in genetic composition can be further substantiated by cross-temporal changes in allele frequency ($\theta$) over time. Of the six populations reviewed, three (*C. pauper* on Floreana, *C. parvulus* on Floreana, and *C. parvulus* on Santa Cruz) exhibited significant cross-temporal changes in allele frequency over time (Table 3). Two populations, *C. pauper* and *C. parvulus*, both on Floreana, also showed significant changes (the former decreasing and the latter increasing) in genetic diversity over time.

Comparing pairwise $\theta$ distances between time points with a Mantel matrix correlation revealed no significant correlation between historic and modern populations, which indicates that significant change in genetic composition has occurred over time across the landscape scale in Darwin’s tree finches ($P = 0.22; R_m = 0.04$). Similarly, a Principal Coordinates Analysis plot of genetic data from past and present tree finch populations on Floreana Island can visually illustrate the change in genetic composition over time (Figure 5). Genetic distances between the sympatric populations of *C. pauper* and *C. parvulus* on Floreana Island remained stable over time ($\theta$ values increased from 0.046 to 0.047). However, $\theta$ values between *C. pauper* and its allopatric congeners on Isabela and Santa Cruz Islands exhibited a general increase. Historic $\theta$ interspecies distances ranged from 0.032-0.042, whereas modern values spanned from 0.043-0.070; this increased differentiation may indicate a decline in interbreeding between *C. pauper* and its neighboring congeners.

**Hybridization and Structure on Floreana Island**

Levels of hybridization among these three species have never been directly assessed. Considering first only the tree finch populations (*C. pauper, C. parvulus,* and *C. psittacula*) on Floreana Island, our analyses revealed low levels of hybridization among the tree finches, both
historically and recently. In the historic Floreana simulations, three genetic demes were supported (K=3), corresponding to the three tree finch species. Similarly, the modern populations on Floreana grouped by species (K=2, since only two species were accessible for sampling). When looking two generations back, historic *C. pauper* individuals displayed a low (or zero) percent ancestry from other Floreana tree finch species. Individuals generally had less than 5% chance of belonging to, or having migrant history with, either *C. parvulus* or *C. psittacula*. Similar results ancestry results were obtained with modern Floreana populations.

When examining historic and modern Floreana tree finches together in the same simulations, we observed that historic *C. pauper* individuals displayed a slightly more admixed composition than modern *C. pauper* individuals (Figure 4a). Historic *C. pauper* shared genetic information with all other demes; however, most of modern *C. pauper’s* shared genetic information aligned with the *C. parvulus* cluster over the *C. psittacula* cluster. This change is consistent with decreasing levels of hybridization between the three tree finch species on Floreana Island.

**Migration and Population Size Over Time**

The levels of migration calculated via MIGRATE support the existence of only very low levels of interbreeding among the tree finch species on Floreana Island, and across nearby island populations (Table 4). Historic levels of migration between *C. pauper* and *C. parvulus* indicated low levels of hybridization, of approximately one immigrant per generation; similar levels were calculated across all other species and island populations. Thus, results were inconclusive regarding David Lack’s hypothesis of *C. pauper’s* origin. Overall, it appears that that low levels of gene flow among populations have been maintained over time in Darwin’s tree finches.
Effective population size estimates from MIGRATE did not indicate notable population declines over the past century, and calculated population sizes were similar across species, which does not agree with recent census data (O’Connor et al. 2010b; Table 5). MIGRATE calculated effective population sizes of between 2,000 and 3,000 individuals for each population; the historic effective population size for *C. pauper* was estimated at approximately 2,500 individuals, whereas the modern population was estimated at approximately 2,300 individuals.

**Discussion**

Utilizing both historic specimens housed in natural history collections and recently collected samples from the field, we quantified the genetic changes that have occurred over the past century in the critically endangered medium tree finch, *C. pauper*, and several related species and populations of Darwin’s tree finches. We found that the population size of *C. pauper* appears to be in decline, as reflected in the observed loss of genetic diversity over time. We found extensive evidence that *C. pauper* is genetically distinct from other species, and is therefore worthy of conservation efforts. Additionally, we found evidence for low levels of hybridization that are similar to rates found in other species of Darwin’s finches. Rates of introgression were similarly low to rates of interisland migration (approximately one immigrant per generation). Hybridization does not appear to be increasing over time as predicted, and if anything, appears to be declining over time. Many populations showed changes in genetic relatedness over time, presumably as a result of low levels of immigration and introgression.

**Genetic Diversity Over Time**

The levels of genetic diversity observed in this study are similar to those previously calculated, and show that tree finches maintain lower overall levels of genetic variation than
ground finches (Petren et al. 2005; Grant et al. 2005b). The significant decline in genetic diversity observed in *C. pauper* reflects a relatively recent (over the past 90 years) population decline in this species. The observed loss of genetic diversity in this small, isolated, and singular population may lead to increased inbreeding, followed by a decrease in fitness and adaptability, and thereby an increased probability of extinction. Our data suggest that population decline has been occurring over many decades (from the early 1900s to the 1990s), a time span that would not be greatly influenced by any particular episodic weather change such as an El Niño/La Niña event, which operates on a much smaller time scale of several years.

Habitat degradation and destruction have eliminated the majority of *C. pauper*’s restricted habitat (O’Connor et al. 2010b). This threat, in addition to increased mortality rates from introduced parasites and diseases (e.g. *P. downsi*: Dudaniec and Kleindorfer 2006, O’Connor et al. 2010a; avian poxvirus: Kleindorfer and Dudaniec 2006), has likely caused the reduction in population size, and hence in genetic diversity. Recent surveys of Floreana bird populations revealed a 61% decline from 2004 to 2008 in the population size of *C. pauper* (O’Connor et al. 2010b). Our modern sampling largely predates this period of possibly dramatic decline, and such a sharp decline may take some time to reveal itself genetically given the generation times of Darwin’s finches (~5 yrs.). Under these conditions, genetic diversity may provide an overestimate of actual population size. Current (2012) levels of genetic diversity may be much lower than our “modern” estimates from approximately 15 years ago (1997), given the recent observation of extreme population decline in the field (O’Connor et al. 2010b).

Our genetic assessment of the *C. parvulus* and *C. psittacula* populations on Floreana Island aligns with the recently collected census data on these species. The census observations of O’Connor et al. (2010b) indicated a healthy, stable *C. parvulus* population, composed of a
maximum of 4,680 individuals, whereas their estimates of the _C. psittacula_ population revealed a maximum of only 490 individuals. Our MIGRATE results indicated a slight increase in population size over time in _C. parvulus_, and supplement our findings that _C. parvulus_ has not experienced a decrease in genetic diversity over the past century. These results agree with the modern _C. parvulus_ census data, suggesting that perhaps _C. parvulus_ population sizes have increased over the past century (or at least remained constant over time) or received additional genetic input, via gene flow from neighboring populations (although this would be minimal since so few migrants are entering the population each generation).

Unfortunately, cross-temporal comparisons were not possible for _C. psittacula_ on Floreana. The reduction in _C. psittacula_ genetic diversity that we observed on neighboring islands is most likely occurring on Floreana Island also, especially in light of the extremely low census estimate (O’Connor et al. 2010b); modern genetic data from the Floreana population would be necessary to confirm this trend. Historic surveys during the past century were able to record and/or collect at most only a very few _C. psittacula_ individuals. Many avian surveys on Floreana Island have failed to observe any _C. psittacula_ individuals, which suggests that the population is endangered and at risk of local extinction (Grant et al. 2005a). However, O’Connor et al. (2010b) successfully observed several individuals during their recent Floreana survey.

Several populations other than the critically endangered _C. pauper_ showed substantial genetic change over time. It is unlikely that mutations could have had an impact in generating genetic change over the short time period examined in this study. Increases in levels of genetic diversity in the small tree finch ( _C. parvulus_ ) population on Floreana are most likely due either to hybridization with congeners or gene flow from neighboring _C. parvulus_ populations on Santa
Cruz, Isabela, or other nearby islands. However, MIGRATE analysis did not reveal a substantial amount of gene flow coming from either of the neighboring populations we included in our assessment. It is possible that migrants from other islands are entering the Floreana population; however, this remains unlikely. Modern hybridization of notable levels with *C. pauper* was not observed from our analysis. The genetic grouping of *C. parvulus* with two populations of *C. psittacula*, on Isabela and Santa Cruz Islands, could be indicative of more recent interbreeding among *C. psittacula* and *C. parvulus*. If nearby islands are experiencing a decrease in suitable habitat due to human activities, *C. parvulus* individuals may be migrating from nearby islands in search of new habitat. Lastly, it may be possible that *C. parvulus* population size is increasing due to open niches that resulted from the recent decline of potential competitors on Floreana (*e.g.* *C. psittacula*). A combination of recent hybridization with *C. psittacula* individuals, and an expanding population on Floreana Island, may be responsible for the observed increases in genetic diversity.

Similar findings were noted for *C. parvulus* and *C. psittacula* on Santa Cruz Island. The genetic changes observed in these two populations supplement the findings of a recent avian survey conducted on the island, where the *C. parvulus* population on Santa Cruz remained stable, but the *C. psittacula* population experienced a population decline (Dvorak et al. 2011). This demographic finding agrees with the significant reduction in heterozygosity that we observed in the Santa Cruz population of *C. psittacula*.

The overall maintenance of stable *C. parvulus* populations on the three islands examined here may be a reflection of the generalist foraging behavior of *C. parvulus* (Christensen and Kleindorfer 2009; Grant 1999). As habitat fragmentation continues on these islands, *C. parvulus* individuals are still able to forage successfully throughout the vegetation zones. In contrast, the
more specialized large tree finch is severely impacted by habitat fragmentation and degradation occurring within its preferred habitat (*Scalesia* zone, agricultural zone, and *Cinchona* zone; Dvorak et al. 2011; Grant 1999) to which it is constrained. Additionally, due to their larger body mass, the medium and large tree finches are more intensely parasitized than the small tree finch by *P. downsi* (O’Connor et al. 2010a).

**Hybridization Over Time**

Hybridization can compromise the genetic integrity of a species targeted for protection by artificially inflating levels of diversity and giving the false appearance of a robust population size (Grant et al. 2005b). We therefore wanted to investigate the possibility of hybridization between *C. pauper*, *C. parvulus* and *C. psittacula*, a prospect that was first suggested by David Lack in 1947. Intermediate morphological specimens housed in natural history collections gave rise to these ideas (Lack 1947), and recent morphological and genetic data also support the possibility of such interbreeding; for example, tree finch species are more genetically similar in sympatry than in allopatry (Grant et al. 2005b). Low, yet consistent levels of hybridization have been recorded in several of Darwin’s finches, usually among closely related species inhabiting the same island (Grant et al. 2005b, Grant 1999). For ground finches on Daphne Major, hybridization occurs in less than 2% of breeding pairs; overall, low-level hybridization is considered a persistent feature among Darwin finches (Grant and Grant 2008).

From our microsatellite data, it can be observed that, historically, a notable portion of *C. pauper*’s genetic information was shared with sympatric congeners *C. parvulus* and *C. psittacula*, supporting the notion that hybridization between these species occurred in the past. Over the past century, the amount of shared genetic information decreased in the tree finches. This may be due to increased habitat fragmentation (Watson et al. 2009) and/or decreased
population sizes of *C. psittacula* and *C. pauper*, which could lead to a reduced range overlap between species, and hence a reduction in interbreeding.

**Migration Over Time**

Extremely low levels of migration have been maintained over the past century among tree finch species on Floreana. There was no notable difference between the numbers of immigrants contributed to *C. pauper* by *C. parvulus* on Floreana or by *C. psittacula*. Approximately one individual from each sympatric tree finch species hybridized with *C. pauper* each generation. Conspecific tree finch migration among the islands of Floreana, Isabela, and Santa Cruz however, is notably lower than previously calculated levels of migration in Darwin’s finches. For example, warbler finches had an average of three to four migrants per generation (Farrington and Petren 2011), and other species show higher levels of interisland gene exchange (Petren et al. 2005). The lower migration in tree finches may be due to their more specialized diet and habitat requirements, which may limit their locations for migration, or it could be a recent phenomenon caused by lower reproductive rates that were in turn caused by recent increases in habitat deterioration and/or parasite burden.

**Implications for *C. pauper* Conservation**

*C. pauper* has remained a genetically distinct species over the past 100 years. Cross-temporal genetic comparisons support the assumption that *C. pauper* is a distinct species, not only morphologically, ecologically, and behaviorally, but also genetically. Significant θ values between *C. pauper* and its closely related congeners, in addition to *C. pauper* individuals clustering into a distinct genetic deme, illustrate the unique genetic identity of this species. Additionally, no evidence of genetic substructure was observed within the *C. pauper* population;
this knowledge can help in the development of future management strategies for the medium tree finch.

Genetic relationships between *C. pauper* and its sympatric congeners have changed very slightly over the past century, with *C. pauper* appearing more genetically distinct over time. In the early 1900s, the allelic composition of *C. pauper* and its congeners was more similar than in the present day. Between the two congeners, *C. pauper* once shared more genetic material with *C. psittacula* than *C. parvulus*; however, this trend reversed near the end of the century, and modern *C. pauper* individuals are genetically more similar to *C. parvulus* than to *C. psittacula*; such a trend may be the result of overall population decline in the *C. pauper* population, and the extremely small population size (<490 individuals) of the Floreana *C. psittacula* population, especially compared to the *C. parvulus* population size (O’Connor et al. 2010b). Census data estimated that the modern *C. pauper* population comprises a maximum of 1,620 individuals, compared to a maximum of 4,680 individuals estimated for the modern *C. parvulus* Floreana population (O’Connor et al. 2010b).

Our analyses reveal significant genetic decline in the critically endangered *C. pauper* over the past century. This decline is most likely a reflection of recent population reduction due to the expanding degradation of tree finch habitat, to nest predation, and to the recent invasion of the parasitic fly *Philornis downsi* (O’Connor et al. 2010a). If these threats to the *C. pauper* population persist, and the population continues on its current course, it will become more challenging for the species/population to respond to further environmental change. As genetic variation and population size declines, the extinction probability of *C. pauper* thus increases. Because this species exists as a single population (and as far as we know, always has), any benefit from immigration and the influx of new genetic information other Darwin’s finches
experience is not available for *C. pauper*. Levels of interbreeding among *C. pauper* and its congeners, and thus the influx of new, possibly beneficial alleles to the endangered population through introgression have also decreased over the past century, further reducing the prospect of increased viability via genetic rescue.

An immediate action plan should be developed that addresses the above-mentioned threats, and endeavors to increase the *C. pauper* (and hopefully also the *C. psittacula*) population of Floreana Island. Recent efforts to reduce rat predation on Mangrove finch nests on Isabela Island have been successful in reducing finch mortality, but the placement and monitoring of poison bait stations is time-consuming (Fessl et al. 2010). Efforts to target *Philornis downsi* parasitism, which accounts for 41% fledgling mortality in *C. pauper* nests, may have the largest impact on population restoration. In addition, guidelines for anthropogenic development in the Floreana highlands that prevent destruction or fragmentation of primary tree finch habitat may prevent further decline. Immediate conservation action and continued population monitoring of *C. pauper* on Floreana will be critical for the preservation of this unique endemic species.
References


Frankham, R. 1997. Do island populations have less genetic variation than mainland populations? Heredity 78: 311–327.


Tables and Figures

Table 1: Number of successfully genotyped samples available for each species by island and time period. *C. pauper* exists only on Floreana Island. The majority of historic samples were collected in 1905-1906; modern samples collected c. 1997.

Table 2: Genetic diversity measures from 6 cross-temporal population comparisons (historic versus modern). FL=Floreana Island; ISA=Isabela Island; SC=Santa Cruz Island. $A_E$: Allelic Richness; $H_E$: Expected Heterozygosity; $H_O$: Observed Heterozygosity. Sample sizes are indicated below population name as: (N historic, N modern). Bold values indicate the direction of significant change, and asterisks (*) indicate significant change over time.

Table 3: Pairwise $\theta$ (analogue of $F_{ST}$) values across 13 tree finch populations spanning two time periods. Numbers along top correspond to population numbers at left. H = historic (1905-1906) population; M = modern (c.1997) population (horizontal line between populations 7 & 8 marks temporal division). Bold values with asterisks (*) are deemed significant via bootstrapping over 10,000 intervals.

Table 4: Levels of migration among tree finch populations on three central islands in the Galápagos archipelago, both in historic and modern populations. Species/populations along the top of the table represent the receiving population, whereas species along the left of table indicate the source of the migrant individuals. Migration is reported among populations in the past (H) and among modern populations (M).
Table 5: Estimates of effective population size ($N_e$) for several island populations of three species of Darwin’s tree finches. Historic (c. 1906) and modern (c. 1997) estimates are provided. $N_e$ calculated from $\theta (\theta = N_e*4*\mu)$ values obtained using the software MIGRATE.

Figure 1: A map of the Galápagos archipelago with islands of interest labeled in capital letters.

Figure 2: Changes in three summary statistics for genetic diversity. Means ± standard error of allelic richness (A), expected heterozygosity (B), and observed heterozygosity (C) are graphed for each population over the past century. Gray bars represent historic populations (c. 1906); black bars represent modern populations (c. 1997). Asterisks (*) above bars indicate a significant change over time (p<0.05).

Figure 3: Changes in genetic diversity over the past century (historic c. 1906, modern c. 1997) represented as a percent increase or decrease in: A) allelic richness and B) Expected heterozygosity for three species of Darwin’s tree finches across three islands (FL = Floreana Island; ISA=Isabela Island; SC=Santa Cruz Island). * Indicate significance (P <0.05)

Figure 4: Results of Bayesian clustering analysis in STRUCTURE. A) Modern and historic tree finch populations only on Floreana Island analyzed together. Four genetic clusters were identified (K=4), corresponding to species, with the exception of modern C. pauper and historic C. pauper clustered independently. B) Modern and historic populations analyzed together. Three genetic clusters were identified (K=3; indicated by color), mostly corresponding to species (except modern Santa Cruz populations). Each column represents a pre-defined population, and
is labeled by species along the bottom: *C. pp.* = *C. pauper*; *C. pv.* = *C. parvulus*; *C. ps.* = *C. psittacula*. Island of origin is indicated in white.

**Figure 5:** Principle coordinates analysis (PCA) of historic (dashed lines and hollow symbols) and modern (solid lines and solid symbols) populations from Floreana Island. H = historic population (c. 1906); M = modern population (c. 1997). Circles are 50% ellipses. The first two axes account for 13.3% and 6.8% of the variation of the data.
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Figure 1:
Figure 2:
Figure 3:
Figure 4:

A

MODERN FLOREANA

HISTORIC FLOREANA

MODERN FLOREANA

B

HISTORIC POPULATIONS

MODERN POPULATIONS

FLOREANA ISLAND

ISABELA ISLAND

SANTA CRUZ ISLAND

FLOREANA

ISABELA

SANTA CRUZ
Figure 5:

- C. parvulus – M
- C. psittacula – H
- C. pauper – M
- C. pauper – H