SPECIATION GENETICS IN TWO PAIRS OF HIGH-LATITUDE, MIGRATORY BIRD TAXA.

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# SPECIATION GENETICS IN TWO PAIRS OF HIGH-LATITUDE, MIGRATORY BIRD TAXA.

A

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## ABSTRACT

I investigated and characterized the divergence of two pairs of putatively young, high-latitude, migratory bird taxa with data from mitochondrial and nuclear DNA. I chose pairs exhibiting natural history attributes suggesting divergence scenarios that probably did not involve strict allopatry. First, I examined *Pluvialis dominica* and *P. fulva*, migratory plover species with a largely parapatric breeding range in Beringia. Secondly, I examined *Aegolius acadicus acadicus* and *A. a. brooksi*, a subspecies pair of owls where one subspecies (*brooksi*) is endemic to Haida Gwaii, Canada, a location where subspecies *acadicus* occurs during migration, resulting in cyclic sympatry (heteropatry) with *brooksi*. Using mtDNA sequence data and AFLPs I made inferences about population parameters, inferred the likely number of populations, and sought evidence of selection. Gene flow was very low in both pairs. The plovers are much older than was anticipated (1.8 Mybp), although hybridization does occur. Evidence for parapatric or speciation with gene flow scenarios was not found in the plovers, perhaps because the speciation event occurred far in the past. The owl's divergence date was relatively young (~16,000 ybp). Some evidence was found suggesting that heteropatric divergence contributed to the owl's differentiation, although the process could also have reinforced differences acquired largely in allopatry.

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## GENERAL INTRODUCTION

The processes that cause lineage divergence and ultimately speciation are fundamentally important to our understanding of Earth's biodiversity. Many of these processes (e.g., allopatric or sympatric divergence) are well understood theoretically, and some are widely held to be common in nature, with increasing data on their genomic signatures. However, we are still in the initial stages of testing proposed speciation models that differ from strict allopatry and obtaining genomic characterizations of them. As our knowledge of speciation processes has grown, the development of new models has increased, incorporating such common phenomena as migration and heteropatry, gene flow and parapatry, etc., important attributes of real organisms. These models need empirical examples if we are to understand how important they may be among all the divergence processes that result in speciation.

Birds are a well-known group of organisms and have been used extensively in the development of speciation theory. The highly mobile nature of migratory birds provides a unique geographic perspective on a process that has traditionally been defined using patterns of geographic distribution. Migratory bird taxa often exhibit multiple geographic distributional conditions throughout a migratory cycle (e.g., allopatry *and* sympatry). Examining these dynamics in relatively young taxa that are at or near the species threshold increases the likelihood of understanding the processes leading to their speciation, both because genomic markers are likely to display the patterns present during divergence and because processes acting now are likely to have been acting in the recent past, when divergences began. In this study I attempted to characterize and seek evidence for non-traditional speciation models in two cases of divergence, both putatively recent with complex geographic relationships (i.e., parapatric and heteropatric, respectively).

The plover species *Pluvialis dominica* and *P. fulva* were relatively recently elevated to species status based on field work conducted on the Seward Peninsula. The Seward Peninsula constitutes a small area of sympatry between these two species which are otherwise allopatric, *P. dominica* occuring across arctic North America, *P. fulva* occuring across much of arctic Russia. The current area of contact in Beringia would have been much larger in the past. I sought evidence of parapatric speciation (a type of

speciation with gene flow), which seemed likely given their biological attributes (i.e., long-distance migrants with a largely parapatric distribution). I used mitochondrial DNA, a well understood marker in avian genetics research, to make a number of inferences about population history, including gene flow, level of divergence, and divergence date. An anonymous nuclear marker technique (amplified fragment length polymorphisms, AFLPs) was also employed to obtain a genomic perspective, to infer the most likely number of populations given the sampling strategy, and to determine whether there was evidence of selection within the genome. Samples were drawn from sympatric and allopatric breeding populations of *P. dominica* and *P. fulva* in an attempt to assess whether sympatry had any effect on gene flow or population genetic distance parameters.

In contrast to the plovers, which are currently recognized as separate species, I also examined a case of subspecific divergence in owls that exhibit a similarly complex geographic pattern (heteropatry). Long recognized as a distinct subspecies of the Northern Saw-whet Owl, *Aegolius acadicus brooksi* is endemic to the Queen Charlotte Islands of British Columbia, Canada. Nominate *acadicus* passes through these islands during migration in low numbers. Previous genetic work had shown that *brooksi* was genetically distinct from the nominate mainland form. I expanded on this work and sought to determine how they diverged from their mainland counterpart. Molecular methods similar to those used in the plovers were employed to estimate a number of population history parameters, including gene flow, level of divergence, population sizes, divergence date, and the most likely number of populations and their genomic makeup, and to seek evidence of selection within the genome.

This approach, choosing young taxa exhibiting patterns of parapatry and heteropatry, was chosen to maximize my ability to infer aspects of their divergence/speciation histories and to characterize the genetic legacies of lineage divergence in two pairs of non-model organism likely diverging by non-traditional speciation processes (i.e., not in strict allopatry). Not only does this work increase our knowledge of how, why, and what speciation looks like in nature, but it is knowledge that can inform our understanding of how organisms might respond in a time of increasingly rapid climate change.

# Chapter 1: Speciation in a migratory shorebird lineage, the *Pluvialis dominica-fulva* complex.[1]

## 1.1 Abstract

Diversification through vicariant events during the Pleistocene is thought to have contributed significantly to taxonomic diversity at high latitudes. However, historic allopatry can be uncertain, and highly mobile migratory taxa have an increased likelihood of interpopulation gene flow, which leaves open the possibility of speciation with gene flow. High-latitude migratory taxa affected by Pleistocene glacial cycles therefore offer possible cases of speciation with gene flow, particularly when extreme phenotypic similarity suggests recent divergence. We sought evidence of speciation with gene flow in American and Pacific golden plovers (*Pluvialis dominica* and *P. fulva*), a high-latitude pair of obligate migrants recently recognized as full biological species. We used 1041 bp of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) from 20 *dominica* and 22 *fulva* and 242 amplified fragment length polymorphisms (AFLPs) from 29 individuals of each species, sampled from sympatric and allopatric breeding populations, to assess gene flow, levels of divergence, and divergence date. This taxon pair speciated longer ago than suspected (~1.8 Mybp). Very little gene flow was detected (0.0023 individuals per generation, effectively zero), significant genetic divergence was found between species (4.7% uncorrected sequence divergence in mtDNA; $F_{ST} = 0.21$ in AFLPs), and one backcrossed hybrid individual was found. We found no evidence for speciation with gene flow, despite its potential in this system, perhaps at least in part because the speciation event occurred so long ago. It is likely that ecological and possibly sexual selection acted to limit gene flow during the divergence of these cryptic species.

## 1.2 Introduction

Populations diverging in allopatry have long been thought to be the main route to speciation (Mayr 1963, Coyne & Orr 2004). However, speciation with gene flow is

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[1] Jack J. Withrow and Kevin Winker, Speciation in a migratory shorebird lineage, the *Pluvialis dominica-fulva* complex, in preparation for Molecular Ecology.

proving to be more common than previously thought (Nosil 2008a, Papadopulos *et al.* 2011, Cristescu *et al.* 2012, Galligan *et al.* 2012). The repeated cycles of glaciation at high latitudes during the Quaternary, causing cyclical changes in habitats over large areas, are thought to have caused diversification and/or speciation in many taxa (Hewitt 1996, 2000). The genetic effects of this history have been studied in diverse life forms using several different molecular markers (e.g., Taberlet *et al.* 1998, Shafer *et al.* 2010), with allopatric diversification often invoked. Many recent discussions of speciation, however, have moved past distributional conditions (e.g., allopatry, sympatry) to instead examine the multifaceted process in which geographic context is but one attribute of the divergence process (Fitzpatrick *et al.* 2009, Butlin *et al.* 2012). Increasingly, ecological, environmental, and behavioral factors are also being considered as important contributors to speciation (Schluter 2001, Gavrilets 2003, McKinnon *et al.* 2004, Verzijden *et al.* 2012). These factors may in some cases complement and act in conjunction with allopatry or parapatry to bring speciation to completion (Nagel & Schluter 1998, Howard & Berlocher 1998, Schluter 2000, Nosil 2008b).

Our ability to infer important drivers of the speciation process is complicated by several major factors, including crude reconstructions of past geographic ranges, uncertainty about the initiation and duration of divergence, and the discounting or ignoring of major phenotypic attributes. Recent work has attempted to mitigate some of these uncertainties (e.g., ecological niche modeling; Cicero & Koo 2012) but variation in mutation rates and the complexities of past demographic parameters continue to make correlation of organismal history with past events imprecise (Lovette 2004, Ho *et al.* 2005). Phenotypic attributes such as increased philopatry or diminished dispersal abilities are often implicitly invoked in order to fit an assumption of allopatry. However, it can be difficult to demonstrate historic allopatry in many widely distributed and migratory taxa. Thus, models of speciation with gene flow, often given the distributional label of 'parapatric speciation' in the theoretical literature (Gavrilets 2003), may be more appropriate in some taxa. Speciation with gene flow is well supported theoretically, and although its frequency in nature is uncertain, it is probably common (Coyne & Orr 2004, Price 2008).

Birds were instrumental in the development of much of speciation theory (Mayr 1963, Price 2008), including the widespread acceptance of allopatric speciation. Given the highly mobile nature of many birds this is perhaps surprising. Migratory birds in particular can cover large distances in their semiannual movements, making the realization of allopatry difficult and increasing the opportunities for gene flow. These cyclic movements of birds can reduce differentiation within species by increasing dispersal distances and promoting gene flow (Montgomery 1896, Mayr 1963), but these movements can also accompany divergence and thus have been proposed as sometimes being a driver of speciation (Winker 2010). Thus, migratory birds exhibiting divergence may help us better understand speciation with gene flow (e.g., Peters *et al.* 2012).

Here we examine the American and Pacific golden plovers *(Pluvialis dominica* and *P. fulva*), a recently recognized species pair with a Holarctic breeding distribution. Due to their extreme similarity in appearance and habits and their parapatric distribution, they would appear to be an excellent candidate system in which to study speciation with gene flow. These birds are arctic-breeding, obligate long-distance migrant shorebirds whose ancestor was also likely migratory (Sauer 1963, Baker *et al.* 2012). Long considered subspecies of the same biological species (e.g., Peters 1934, Gabrielson & Lincoln 1959, Mayr & Short 1970, AOU 1983), they were elevated to full species status in 1993 (Monroe *et al.* 1993). They breed across a large geographic area of arctic and subarctic tundra, with *fulva* occupying a largely Palearctic range from the Yamal Peninsula, Russia, to western Alaska (Vaurie 1965, AOU 1998), and *dominica* occupying a Nearctic breeding range from western Alaska to Baffin Island, Canada (AOU 1998; Figs. 1.1, 1.2). These birds migrate long distances to their wintering grounds in the south Pacific and Australia (*fulva*) and the pampas of South America (*dominica*), often covering thousands of kilometers nonstop over open ocean. The largely parapatric nature of this distribution includes areas of sympatry, which enabled researchers to determine that reproductive isolation appears to be complete between these two phenotypically very similar species (Connors *et al.* 1993; Fig. 1.3).

During the glacial-interglacial cycles of the Pleistocene, the breeding ranges of these two lineages would have fluctuated dramatically (Bartlein & Prentice 1989), with a

likelihood that they were often connected. At the height of many of these glacial cycles the current area of sympatry (Beringia) would have been much larger than it is today (Hopkins 1967, Kaufman & Manley 2004). The traditional speciation hypothesis (Larson 1957, Connors 1983) suggested that these plovers speciated during geographic isolation. Actual evidence for this scenario is lacking, however, and it was based primarily on the assumption that allopatry is required for speciation. The problem with the scenario of allopatric speciation in this pair is that it requires fundamental differences in historic breeding range occupancy and in migratory capacity for strict allopatry to have been realized.

This species pair thus represents a case of probable speciation with gene flow in a region that underwent rather dramatic cyclic changes during the Pleistocene. Further, it is an example of cryptic speciation (Bickford *et al.* 2007). Our first goals were to determine the level and nature of genetic divergence exhibited by this species pair and to estimate levels of gene flow. Secondly, we wished to estimate the timing of their divergence and how this may have been correlated with past geographic and climatic events. Finally, we explore the roles that migratory direction, pair bonding, and wintering ground adaptations may have had in the divergent selection likely required to produce these species, and how these compare to other high-latitude avian taxa at various stages in the speciation process.

### 1.3 Materials and Methods

We used two types of molecular data: DNA sequences from the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) and amplified fragment length polymorphisms (AFLPs; Vos *et al.* 1995). ND2 is a well-known mtDNA marker in birds and has been shown to be particularly informative and approximately neutrally evolving (Zink *et al.* 2005), hence providing confidence when used to estimate population parameters (Lovette 2004). AFLPs have several benefits, including sampling of the entire genome and inclusion of many unlinked loci. They have also been successfully used to study the genetics of closely related species (e.g., Parchman *et al.* 2006, Toews & Irwin 2008, Maley & Winker 2010, Brelsford *et al.* 2011).

### 1.3.1 Mitochondrial sequence data and sampling

Specimens from the University of Alaska Museums vouchered tissue collection were used. Approximately half of each species' samples came from the Seward Peninsula, were the species occurs in sympatry. In *fulva*, 12 individuals came from the Near Islands in the Aleutian Islands, where most migrant birds are from Asia (Gibson & Byrd 2007); nine more were from the Seward Peninsula, and one was from the Alaska Peninsula (Fig. 1.2; details in Appendix 1.A). In *dominica*, 13 birds came from the Seward Peninsula area, five came from the North Slope, and two were from Fairbanks (Fig. 1.2; Appendix 1.A).

We amplified 1041 bp of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) for 20 *dominica* and 22 *fulva.* DNA was extracted from frozen tissue using a Qiagen (Valencia, CA) DNeasy Tissue Kit following the manufacturer's protocol. PCR amplification was conducted using 2.5 μL each of 10 μM primers H6313 (Johnson & Sorenson 1998) and L5215 (Hackett 1996), 3 μL of a 10 μM solution of dNTPs, 0.2 μL (1 unit) *Taq* DNA polymerase, 6μL of 25 mM $MgCl_2$, 5 μL of 5X *Taq* buffer (Promega Corp., Madison, WI), 2.5 μL of 1 mg/mL BSA, and 23.3 μL water for a total reaction volume of 50 μL. PCR thermal regime began with 2 min. at 94° C followed by 29 cycles of 48° C for 2 min., 72° C for 2 min., with a final elongation step at 72° C for 5 min. PCR products were cleaned with either polyethylene glycol and cold ethanol or an ExoSAP process (USB, Cleveland, Ohio). Cycle sequencing was done with 2 μL Big Dye (Applied Biosystems Inc. [ABI], Foster City, CA), 1 μL of 5X *Taq* buffer (Promega Corp., Madison, WI), 4 μL water, and 2 μL of template with the same primers from the initial PCR reaction. This product was cleaned by passage through a sephadex column and run on an ABI 3100 automated sequencer (ABI). Sequences were aligned and edited using Sequencher 4.7 (Gene Codes, Ann Arbor, MI).

### 1.3.2 Amplified fragment length polymorphisms data and sampling

AFLP data were generated using a protocol modified from Vos *et al.* (1995) for 29 *dominica* and 29 *fulva*. To make comparisons between sympatric and allopatric population samples, and to broadly encompass any geographic genetic variation, we

choose roughly half the sampled birds of each species from the Seward Peninsula (where they occur in sympatry) and half from areas away from the Seward Peninsula (Fig. 1.2, details in Appendix 1.B). In *fulva*, 13 birds came from the Near Islands. Of the remaining 16 birds, 11 were from the Seward Peninsula and were grouped with five birds taken in migration south of there (Fig. 1.2). Sixteen of the *dominica* specimens came from the Seward Peninsula area and 13 came from areas away from breeding *fulva*.

Sample DNA concentration was quantified on a spectrophotometer prior to subsequent experiments; all had concentrations of 30-70 ng/μL. Restriction and ligation steps of adapter pairs were performed together. A master mix consisting of 0.28 μL water, 1.1 μL 10X T4 buffer (New England BioLabs, Inc. [NEB], Ipswich, MA), 0.55 μL 1M NaCl, and 0.55 μL 1 mg/mL BSA (NEB), 0.1 μL at 10,000 units/mL MseI (NEB), and 0.25 μl at 20,000 units/ml EcoRI (NEB) per sample was scaled up to accommodate the number of samples being run. The restriction enzymes MseI and EcoRI were added last, immediately before distributing the master mix into 6 μL of template DNA. The reaction mixture was vortexed and placed in a thermal cycler at 37° C for approximately 12 hr. This reaction mixture was subsequently diluted with 94.5 μL of 0.1M TE buffer and frozen until preselective amplification.

Preselective amplification was done following normal PCR protocols. A master mix containing 5.68 μL $dH_2O$, 1.0 μL 5X GoTaq (Promega Corp., Madison, WI), 0.6 μL 25mM MgCl, 0.1 μL 10 μM dNTPs, and 0.125 μL (0.625 units) *Taq* per sample was mixed on ice and added to 2.0 μL of diluted template. The thermal-cycler regime consisted of 1 min at 72° C followed by 19 cycles of 94° C for 20 sec, 56° C for 30 sec, and 72° C for 2 min followed by 30 min at 60° C. After preselective amplification, reactions were diluted with 80 μL of 0.1X TE buffer and frozen until selective amplification.

Selective amplifications were done using the same PCR mix as in the preselective amplification, but extended, dye-labeled MseI and EcoRI primers were used to selectively amplify a subset of DNA. All reagents were mixed on ice as quickly as possible. The thermal cycler regime consisted of 2 min at 94° C followed by 11 cycles of 94° C for 20 sec, 66° C for 30 sec, and 72° C for 2 min. During each cycle the annealing

temperature was dropped by one degree, and at 56° C 19 additional cycles were run at that annealing temperature followed by 30 min at 60° C. Samples were selectively amplified and 1.0 μL of each sample was loaded into a 96-well plate before running. This plate was vacuum-centrifuged to remove moisture and then frozen overnight. The next morning 8.5 μL deionized formamide and 0.5μL GeneScan 500 LIZ size standard (ABI), was added to each sample. The samples were then heated to 95° C for 2 min immediately before running on an ABI 3100 automated sequencer (ABI).

We used seven primer pair combinations for selective amplification (Table 1). GeneMapper ver. 3.7 (ABI) was used to score the chromatograms. Only unambiguous loci with clean, well-defined peaks were scored. We used a minimum peak width of 1.5 bp and a minimum peak height of 75 as a starting point, but then examined each peak individually to maximize the useful phylogenetic signal (Holland *et al.* 2008). We discarded two samples that did not amplify properly, likely due to tissue degradation related to specimen care in the field. Data were transformed into a binary state matrix using a Microsoft Excel® macro, which also transformed the matrix into nexus format (Rinehart 2004). All bands were considered independent, orthologous loci.

### 1.3.3 Genetic differentiation and population structure

A median-joining network illustrating haplotype frequencies was generated for mtDNA data using NETWORK 4.6.0.0 (Bandelt *et al.* 1999). We used Arlequin 3.11 (Excoffier *et al.* 1992) to calculate pairwise $\Phi_{ST}$ values between populations. Genotypes were permuted 1000x to obtain *P*–values determining whether $\Phi_{ST}$ values were significant.

For AFLP data we calculated $F_{ST}$ (and *P*-values) following Lynch & Milligan (1994) using AFLP-SURV 1.0 (Vekemans *et al.* 2002), using the Bayesian method with non-uniform priors, 10,000 random permutations, and 1,000 bootstraps for genetic distances. To assess intra- and inter-species divergence and divergence related to geography we made five $F_{ST}$ comparisons: between species, between Seward Peninsula (i.e., sympatric populations) and non-Seward Peninsula birds within a species, between

Seward Peninsula birds across species, and between non-Seward Peninsula birds across species.

We also analyzed AFLP data in STRUCTURE 2.2 (Pritchard *et al.* 2000, Falush *et al.* 2007) to determine the most likely number of populations and to identify any admixed individuals. STRUCTURE uses MCMC simulations in a Bayesian framework to assign individuals to populations based on Hardy-Weinberg/linkage equilibrium and can be used to determine the most likely number of populations (Pritchard *et al.* 2000). The program's model-based clustering method effectively deals with the genotypic ambiguity present in dominant markers such as AFLPs (Falush *et al.* 2007). Preliminary runs indicated that a burnin of 20,000 iterations was sufficient. We then ran three independent simulations under the admixture model for 100,000 iterations with the number of populations ($K$) varying from one to five, then calculated the likelihood of $K$ given the data as $P(K|X)$. To avoid biasing the inference of population structure, we did not use population origin information, although some individuals were phenotypically identifiable. We used the program Distruct (Rosenberg *et al.* 2002) to transform and apply information from STRUCTURE.

A mismatch between mtDNA and AFLP markers in one individual (a chick) resulted in follow up work in which the hybrid individual was re-extracted and re-sequenced for ND2, and all AFLP primer pairs were rerun to verify that the original data were correct. This second independently scored AFLP profile was nearly identical to the original profile, which was nearly identical to the putative male parent. Unfortunately, the putative female parent of the hybrid was not present and was therefore not sampled.

### 1.3.4 Divergence time, effective population size, and gene flow

The program IM (Nielson & Wakeley 2001) was used with mtDNA data to estimate divergence time ($t$) and population parameter ($\theta = 4N_e\mu$; where $N_e$ is effective female population size and $\mu$ is the mutation rate) and also to assess the likely number of migrants ($m = 2M/\theta$, where $M$ is the effective number of migrants moving into a population per year) between populations. Initial runs using a six parameter model where $\theta$ was estimated for both current populations and the ancestral population and two

migration rates showed poor convergence, possibly because mtDNA had sorted to reciprocal monophyly and therefore did not contain enough information to estimate a full set of parameters (Nielson & Wakeley 2001). A simpler model was used in which we constrained the analysis so that all three effective population sizes and both migration rates were constant. We ran three independent runs using the initial starting maxima of $\theta$ = 100, $m$ = 2, and $t$ = 50, with a burnin of 500,000 steps and a different random number seed for each run. An estimated generation time of 5 years was used based on Johnson and Connors (2010). The HKY model of molecular evolution (Hasegawa *et al.* 1985), which takes into account multiple nucleotide substitutions at the same position, nucleotide frequency differences, and any transition/transversion bias was used in all runs. We let each run proceed for more than 10 million updates to achieve a minimum effective sample size (ESS) of 100 for any given parameter estimate (Hey & Nielson 2004); most ESS values were several orders of magnitude higher than this. Results from the three runs were similar, and we report here only the parameters estimated in the longest run of 87,479,393 updates after burnin. The parameters estimated by IM are dependent on the mutation rate, which is an uncertain quantity (Lovette 2004, Ho *et al.* 2005). A mutation rate of 2.61% divergence per Myr (0.0000135 per year per 1041 bp of ND2) was used (following Weir & Schluter 2008), together with a range to incorporate uncertainties. We set this range at 0.48 – 4.31% divergence per Myr (0.00000249-0.00002243 per year per 1041 bp), the lower bound was based on Pacheco *et al.* (2011) for ND2 in Charadriiformes and the upper bound was based on Weir and Schluter (2008) for cytochrome *b* in Charadriiformes. Following calculations outlined in Hey (2005), we estimated the effective population sizes of *dominica* and *fulva* ($N$), the number of individuals coming into a population from the other population per year ($Nm$), and the time since divergence ($t$).

### 1.3.5 Genetic diversity and selection

We used DnaSP (Rozas *et al.* 2003) to calculate nucleotide diversity ($\pi$) and haplotype diversity ($H_d$) in ND2 following Nei and Chesser (1983). We conducted $\chi^2$ tests of genetic differentiation between populations based on haplotype frequencies and

nucleotide diversity indices. The $\chi^2$ tests with Yates continuity corrections were conducted in PopTools 2.6.9 (Hood 2005), an add-in for Microsoft Excel®.

We counted the number of AFLP loci that were fixed in one species but polymorphic in the other, as well as the number of loci that were present in one species but absent in the other. We used $\chi^2$ tests as implemented in PopTools 2.6.9 (Hood 2005) to test for significant population differences in these values.

To determine whether the genes sampled through AFLPs diverged via genetic drift or selection we compared $F_{ST}$ and heterozygosity estimates for each locus in our dataset with a simulated dataset acting under drift alone using an infinite-alleles model. To produce these simulated data we used the program fdist2 (Beaumont & Nichols 1996, Beaumont 2000). This program uses an average divergence of $F_{ST}$ and expected heterozygosity ($H_S$) calculated from the data to simulate the expected distribution of differentiation across loci (Campbell & Bernatchez 2004, Bonin *et al.* 2006). It uses an $F_{ST}$ calculated by the method of Nei (1977) as modified by Nei and Chesser (1983) and generates a uniform distribution of heterozygosities in place of a specified mutation rate. This distribution is then used to calculate quantiles of the median and upper and lower 99% confidence intervals of the distribution of loci for the population diverging under drift alone. It also calculates $F_{ST}$ and $H_S$ for all polymorphic loci in the dataset, which are then plotted against the confidence intervals. Loci falling outside this distribution in excess of expected false positives are assumed to be under selection or closely linked to loci under selection (Beaumont & Nichols 1996, Nosil *et al.* 2009). The data were also analyzed to obtain an estimate of the average $F_{ST}$ across all loci. The model was then fit to this $F_{ST}$ for simulation. We ran the simulation for 50,000 realizations, with two demes total, sampling the two populations of *Pluvialis* with an expected $F_{ST} = 0.11$ and an average sample size per population of 29 individuals (i.e., all of them).

## 1.4 Results

### 1.4.1 Genetic differentiation and population structure

Our data clearly showed that *dominica* and *fulva* are genetically distinct and have likely been so for a considerable period of time. There were 49 fixed differences (4.7%)

in ND2 between *dominica* and *fulva*. Mitochondrial DNA was highly structured between species ($\Phi_{ST}$ = 0.65, $P < 0.001$). The haplotype network showed that 18 of 22 *fulva* shared a common haplotype, while 16 of 20 *dominica* shared a common haplotype (Fig. 1.4). Other haplotypes were composed of single individuals, differing by one or two bases from the common haplotypes within a species (Fig. 1.4).

Genomic measures of differentiation mirrored those seen in mtDNA. The full interspecies comparison resulted in an $F_{ST}$ = 0.21 ($P < 0.001$; see Fig. 1.5). Both intraspecific AFLP comparisons showed low but significant levels of differentiation (*fulva* $F_{ST}$ = 0.038; $P$ = 0.033 and *dominica* $F_{ST}$ = 0.030; $P$ = 0.016). Comparisons between Seward Peninsula *dominica* and *fulva* ($F_{ST}$ = 0.24; $P < 0.001$) were similar to those between non-Seward Peninsula *dominica* and *fulva* ($F_{ST}$ = 0.22; $P < 0.001$).

Three independent 100,000-step Bayesian clustering algorithms run on AFLP markers gave similar results and clearly separated the two species (Fig. 1.6). These runs estimated that the most likely number of populations involved in the samples was two (ln Pr($K|X$) = -2323.8; P($X|K$) = ~1; Table 1.2). No individuals were misassigned to a population, although none were estimated to contain genomic material originating entirely from the source population, i.e., the species it phenotypically resembled. Most individuals (81%) were estimated to contain greater than 99% genomic material originating from their putative population of origin. The lowest estimate was 91.7% (Fig. 1.6).

One individual exhibited a mismatch between mitochondrial and nuclear DNA markers (Figs. 1.4, 1.6). This individual had the common *fulva* ND2 haplotype but exhibited a nuclear genome of *dominica*. STRUCTURE estimated that the hybrid individual had essentially all (99.6%) of its nuclear alleles originating from the *dominica* population (Fig. 1.6). Although previous reports of hybrids exist, we could find no convincing physical evidence (i.e., museum specimens) to support them (contra Gray 1958, McCarthy 2006, and references therein).

### 1.4.2 Divergence time, effective population size, and gene flow

Coalescent analysis of mtDNA estimated the high point and 95% confidence interval (in parentheses) of the following population parameters: $t$ = 23.98 (18.0-48.7), $\theta$ = 4.6 (2.2-11.7), and $m$ = 0.001 (0.001-0.12; Figs. 1.7a, b, c). Using our best estimate of $\mu$ this resulted in an estimated divergence time of 1.8 Mya and a long-term effective population size of 16,800 individuals (females). Ranges of these values based on different values of $\mu$ appear in Table 1.3. The estimated effective number of migrants per year was 0.0023, or two every 1,000 years, effectively zero (Table 1.3). IM's estimated long-term effective female population size of 16,800 (lineages constrained to be equal) is lower than current estimated census population levels in both *dominica* (134,000 - 200,000 breeding individuals) and *fulva* (90,000 – 250,000; Morrison *et al.* 2000, Delany & Scott 2006).

### 1.4.3 Genetic diversity and selection

Nucleotide diversity was lower in *fulva* ($\pi$ = 0.00040) than *dominica* ($\pi$ = 0.00048; $\chi^2$ = 1177.9, df = 1, $P < 0.001$), but haplotype diversity was similar between taxa (*fulva* $H_d$ = 0.35, *dominica* $H_d$ = 0.29; $\chi^2$ = 2.9, df = 8, $P$ = 0.089).

A total of 242 bands were produced by the seven AFLP primer pairs. Of these, 137 (58.9%) were polymorphic (Table 1.1) when both species were included. There were 23 AFLP loci that were fixed in *fulva* but polymorphic in *dominica*, and there were 19 alleles fixed in *dominica* but polymorphic in *fulva*; these differences were not significantly different from one another ($\chi^2$ = 0.381, df = 2, $P$ = 0.899). There were nine AFLP loci present in *fulva* that were absent in *dominica*, and 11 loci present in *dominica* that were absent in *fulva*, and these differences were also not significant ($\chi^2$ = 0.200, df = 2, $P$ = 0.726). AFLP banding patterns appear in Appendix 2.B).

Five loci fell outside the simulated data set's 99% confidence interval (Fig. 1.8). The five loci had unusually high $F_{ST}$. This result includes more loci than would be expected by chance (expected N = 2.4; 1%), but this could be a result of drift operating to cause an increasing number of loci to go to fixation over an extended period following speciation or as a result of the uncertainty with which $F_{ST}$ is calculated in a

heterogeneously evolving genome ('n = 1 constraint'; Nosil *et al.* 2009, Buerkle *et al.* 2011).

**1.5 Discussion**

Genetically, American and Pacific golden plovers are very distinct in both mtDNA and genomic AFLP markers (Figs. 1.4, 1.6). Their mtDNA divergence of 4.7% was unexpectedly high given their morphological similarity. Nuclear alleles also clearly separate the two species, with every individual having a greater than 90% assignment to its putative population of origin (most were much higher; Table 1.2, Fig. 1.6). Despite our empirical evidence of hybridization, gene flow rates between these plovers are effectively zero (Fig. 1.7c), a rate suggesting that isolating mechanisms are very strong despite sympatry and parapatry. The coalescent analyses estimated a divergence date of 1.8 Mya. This divergence date was deeper than expected and suggests that this cryptic species pair has existed through many of the glacial cycles of the Pleistocene. We found that 2% of AFLP loci may have been under selection, a value higher than expected by chance (1% at $\alpha = 0.01$) but lower than that seen in other studies (Nosil *et al.* 2009). However, the linear distribution of loci once a heterozygosity of 0.5 is reached (Fig. 1.8) is consistent with a process of genetic drift acting over an extended period of time, and we do not infer evidence of selection at these loci from these results.

While it is clear that these two species diverged in the Pleistocene, it is not possible to pinpoint a specific glacial-interglacial cycle with which this speciation event was correlated. Paleotemperature records suggest that there were more than 40 glacial-interglacial cycles in the Quaternary (Ruddiman *et al.* 1986). However, the complex refugial history of Beringia (Hopkins 1967, Kaufman & Manley 2004), coupled with uncertainties in mutation rates (Lovette 2004, Weir & Schluter 2008), make precise correlation of their divergence with a particular glacial cycle unlikely. Further, our data suggest they probably speciated during a period when the cycles were occurring at a higher frequency (~every 40,000 yrs) than the later Pleistocene (~every 100,000 yr; Ruddiman *et al.* 1986), and the CI on the estimate was broad (Table 1.3). The precise historic breeding range(s) of the ancestor of this *Pluvialis* pair is of course unknown.

However, we can with confidence assume that this ancestor was a long-distance migrant breeding on high-latitude tundra and wintering at tropical and/or south-temperate latitudes.

Despite our initial hypothesis that these birds may have undergone a form of parapatric speciation, the unexpectedly deep divergence places the event too far back in time for an assessment of this. We found a pattern different from several other Beringian taxa. For example, *Anas crecca* (Green-winged Teal), a migratory species whose geographic distribution is similar to the plovers, with Old and New world forms meeting in Beringia, is experiencing ongoing parapatric speciation, with distinct Eurasian (*A. c. crecca*) and North American (*A. c. carolinensis*) forms (Peters *et al.* 2012). However, these teal fall short of achieving full speciation (due to ongoing gene flow at substantial levels), even though they appear to have been diverging for a longer period of time (~2.6 Mya) than *dominica* and *fulva*. In another case, a highly mobile group of Arctic-breeding gull species (*Larus* spp.) show limited genetic structuring, with a high number of shared alleles (Sonsthagen *et al.* 2012). This complex of migratory gulls apparently lacks the isolating mechanisms needed to prevent lineages from reticulating during interglacial periods of sympatry. The plovers successfully diverged long enough ago, and with such apparently effective isolating mechanisms, that shared alleles are rare and gene flow is very low. Our data cannot effectively address whether this divergence occurred in allopatry or in parapatry because the event occurred too long ago. As noted earlier, accomplishing long periods of strict allopatry would require some fundamental differences in ancestors in traits that occur now in the two lineages, e.g., long-distance migration, Beringia breeding range, etc.

The reasons for *dominica* and *fulva* having effectively ceased interbreeding while *A. crecca* and Arctic *Larus* species have not are likely due to differences in the selection pressures experienced by these diverging lineages. These are very different groups of birds. For example, most ducks, which have female-biased philopatry, form pair bonds on the wintering grounds (Carboneras 1992). Displaced males are therefore more likely to pair-bond with a member of a different population and follow their mate back to a different breeding ground, resulting in introgression. Plovers, however, form pair bonds

on their breeding grounds, diminishing chances for non-assortative mating when compared with ducks. The high rates of interspecific hybridization in ducks (Johnsgard 1960, Grant & Grant 1992, Tubaro & Lijtmaer 2002, Gonzalez *et al.* 2009) also suggest that isolating mechanisms may be weaker in general in this group. Similarly, in *Larus* species interspecific hybrids are common (Good *et al.* 2000, Crochet *et al.* 2003, Malling Olsen & Larsson 2004), suggesting that here as well isolating mechanisms are weak. Gulls also tend to be colonial nesters (Malling Olsen & Larsson 2004), which may also increase chances for non-assortative mating. Although breeding systems show differences in all three groups, all have some level of courtship display that affects pair bonding. The plovers exhibit differences in mating calls and displays (Connors *et al.* 1993, Miller 1996) between the forms that are genetically determined and tend to be evolutionarily conserved (Miller 1996), suggesting that these characters have contributed to speciation, a common occurrence generally in birds (Price 2008). But such mechanisms are also present to some degree in *Anas* and *Larus* species (e.g., McKinney *et al.* 1990, Tinbergen 1960).

There are several plausible ecological sources for divergent selection between these *Pluvialis* species. The most obvious difference between the two forms is migratory direction: the Pacific Ocean divides their wintering grounds (Fig. 1.1). It has been suggested that "requirements of juvenile migration might exert severe selection pressures against hybrid[s]" (Connors 1983:618). However, because golden plovers can modify their genetically determined pattern of migration by learning (Sauer 1963) and they often migrate in flocks (Johnson and Connors 2010), it is conceivable that migratory orientation is not an impervious isolating barrier causing strong selection against hybrid individuals. Our single hybrid individual is additional evidence of this. Although we failed to find evidence for substantial gene flow between these two species currently, when traits subject to divergent selection (like migration) contribute to non-random mating, speciation with gene flow is more likely to occur (Servedio & Kopp 2012).

Our discovery of a hybrid individual shows that it does occur, albeit rarely, despite previous unsubstantiated reports (Gray 1958, McCarthy 2006). Given the degree of haplotype divergence between the two species, we view the mismatch in mtDNA and

AFLP data in this individual as a case of hybridization and not incomplete lineage sorting (Peters *et al.* 2007). AFLPs have been used before to assess hybrids (Vallender *et al.* 2007, Rush *et al.* 2009, Irwin *et al.* 2009), and given that this individual showed a nearly identical banding pattern to its presumed male parent it is unlikely that it was an F1 hybrid. A first-generation hybrid would be expected to show an AFLP profile intermediate between the two populations and would show a more even distribution of nuclear alleles between the two genomic groups in the STRUCTURE analysis. Although we did not anticipate finding a hybrid, detection of one is not altogether surprising, given that reproductive isolation is often incomplete after speciation in birds (Price 2008), although it is less common in shorebirds (McCarthy 2006).

If migratory orientation alone failed to provide a definitive isolating barrier, flyway-specific ecological attributes of a particular migratory pattern might do so instead. Prevailing winds, weather, the distances involved, and potential stopover sites are all factors likely to be unique to a particular migratory route. These differences would in turn lead to differences in phenology. For example, *dominica* arrive in western Alaska in mid-May, a time when winter prevails on the west side of the Bering Strait (Kessel 1989). Even if hybrids were able to survive a migratory cycle, a strong tendency to migrate in one direction or the other would facilitate differentiation in phenology related to migratory route. Similarly, differences in migration destinations could lead to differing ecological selection pressures on wintering ground adaptations (Connors 1983, Byrkjedal & Thompson 1998). It is likely that these ecological factors (migratory patterns and the subsequent differences in phenology, wintering ground adaptations, and navigational requirements), combined with sexual selection on the breeding grounds, provide important isolating mechanisms in these plovers (Connors 1983, Connors *et al.* 1993). This might explain why there is one species of golden plover in each major flyway (*Pluvialis apricaria* occupies the Palearctic–African flyway; Cramp 1983). These selective pressures stemming from different migratory patterns would have similar effects under allopatric or parapatric speciation scenarios, reinforcing differences acquired largely in allopatry or mitigating the effects of low levels of gene flow.

## 1.6 Literature Cited

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Table 1.1: Amplified fragment length polymorphism amplification and scoring results for each primer pair and total.

| primer pair extensions and dye | | | both species | | | within *P. fulva* | | | within *P. dominica* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *EcoR1* | *MseI* | dye | T | P | %P | T | P | %P | T | P | %P |
| -ACT | -CAA | FAM | 32 | 19 | 59.4 | 31 | 16 | 51.6 | 31 | 13 | 41.9 |
| -ACT | -CAC | FAM | 38 | 20 | 55.6 | 30 | 13 | 43.3 | 34 | 15 | 44.1 |
| -ACT | -CAG | FAM | 45 | 15 | 33.3 | 44 | 11 | 25.0 | 44 | 14 | 31.8 |
| -ACT | -CAT | FAM | 34 | 23 | 67.7 | 33 | 14 | 42.4 | 33 | 18 | 56.3 |
| -ACA | -CAG | FAM | 23 | 13 | 56.5 | 23 | 10 | 43.5 | 20 | 8 | 40.0 |
| -ACA | -CAT | FAM | 40 | 24 | 63.2 | 36 | 16 | 44.4 | 38 | 17 | 44.7 |
| -ACA | -CTA | FAM | 30 | 23 | 76.7 | 27 | 14 | 51.9 | 27 | 16 | 59.3 |
| Totals | | | 242 | 137 | 58.9 | 224 | 94 | 43.2 | 227 | 101 | 45.4 |

Total peaks (*T*), the number of polymorphic peaks (*P*), and the percentage of peaks that were polymorphic (*%P*).

Table 1.2: STRUCTURE estimates of the number of clusters (*K*) in sampled plovers. We used SRUCTURE without using prior population information.

| K | Ln Pr(*X*\|*K*) | P(*K*\|*X*) |
|---|---|---|
| 1 | -3801.2 | ~0 |
| 2 | -2323.8 | ~1 |
| 3 | -2555.3 | ~0 |
| 4 | -2454.8 | ~0 |
| 5 | -2361.9 | ~0 |

Table 1.3: Demographic parameter estimates calculated from isolation with migration coalescent analysis (Hey 2005) with 95% highest posterior densities.

| | Substitution rate ($\mu$) | | | |
|---|---|---|---|---|
| | $\mu_{0.24\%}$ | $\mu_{0.5\%}$ | $\mu_{1.3\%}$ | $\mu_{2.16\%}$ |
| Parameter | | | | |
| Long term effective population size in thousands of individuals. | 91 (43 - 233) | 43 (20 – 111) | 16 (7.9 – 43) | 10 (4.7 – 24) |
| Migration rate* | 0.0023 (0.001 – 0.12) | - | - | - |
| Time since divergence in millions of years | 9.6 (7.2 – 19.6) | 4.6 (2.5 – 9.4) | 1.8 (1.3 – 3.6) | 1.0 (0.8 – 2.2) |

Estimates of effective population size and number of migrants are in units of individuals. Estimates of time since divergence are in years.

*This parameter is independent of mutation rate

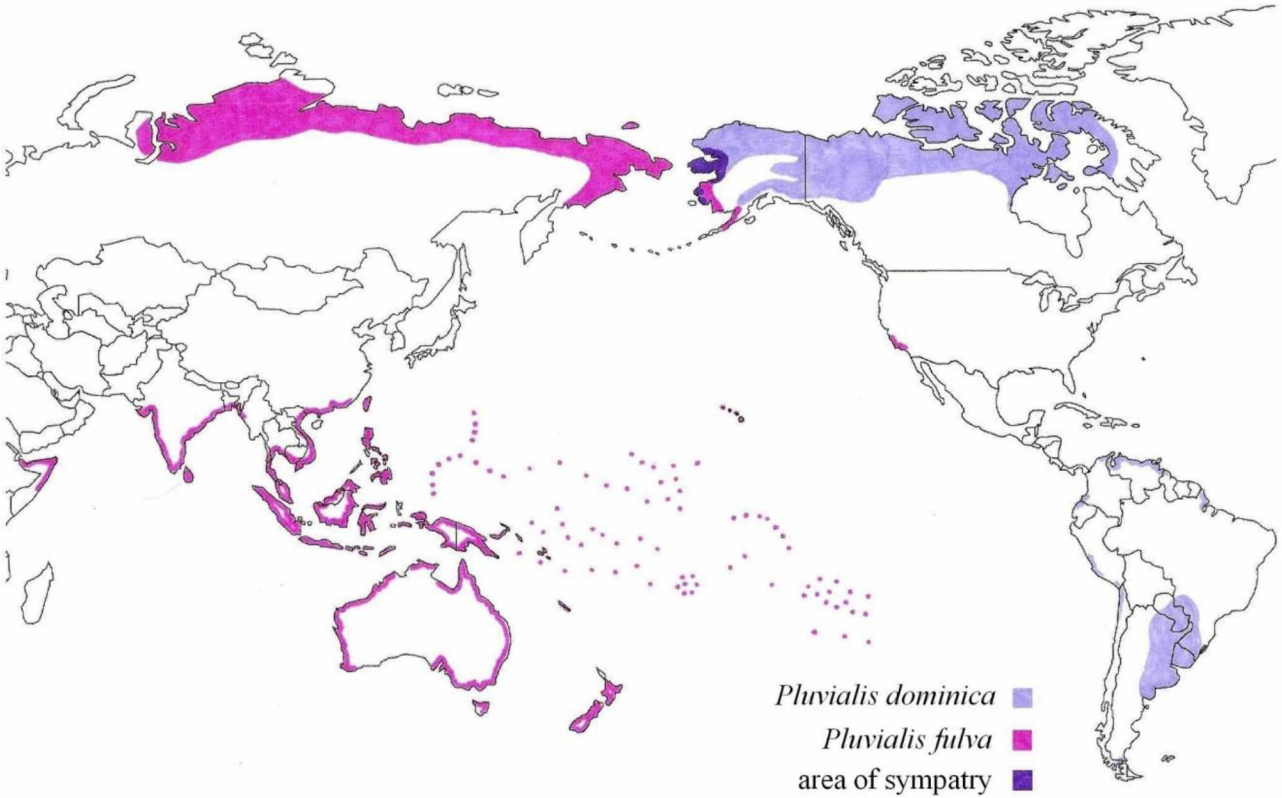


Figure 1.1: World range of *Pluvialis dominica* and *P. fulva,* showing arctic breeding grounds and temperate and tropical wintering grounds.

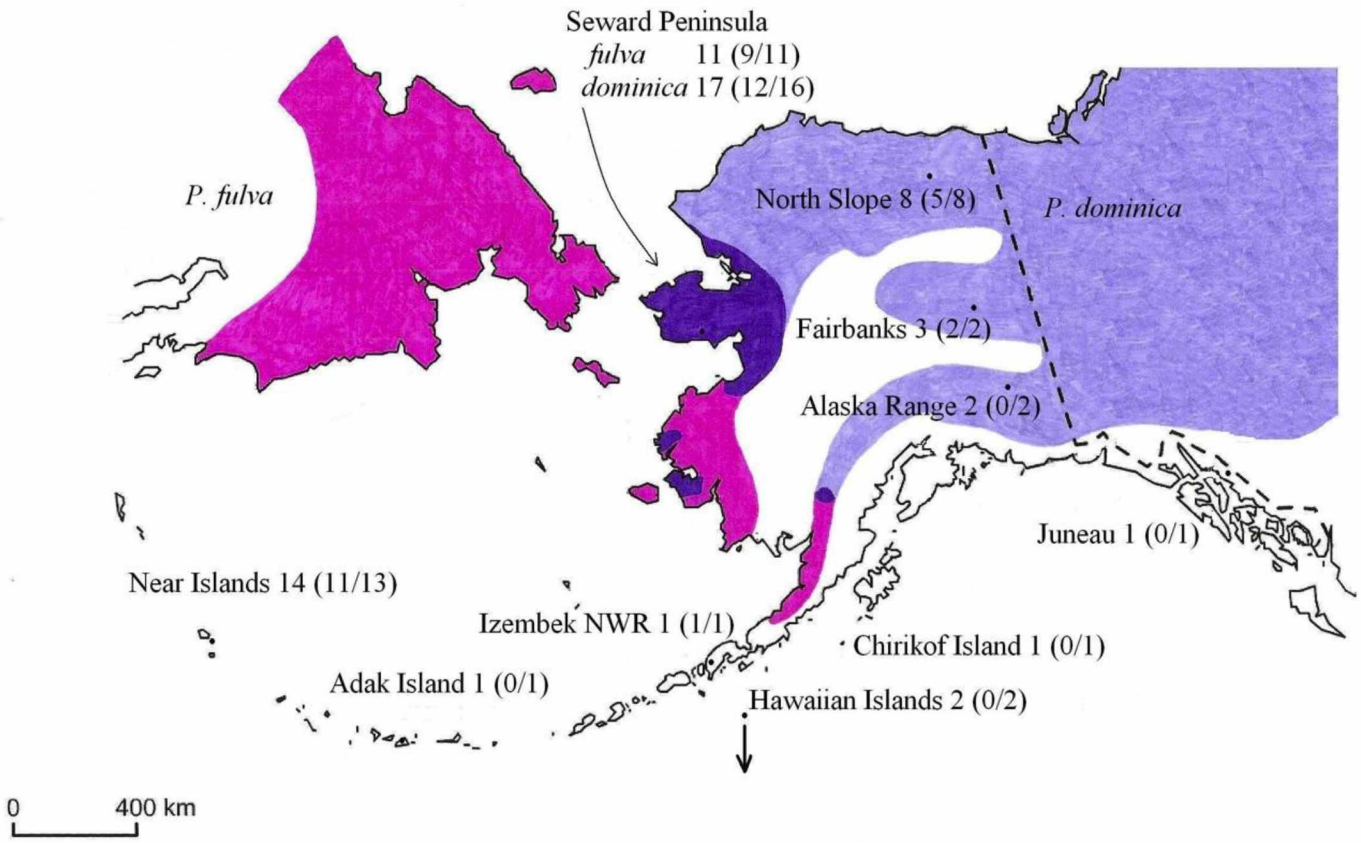


Figure 1.2: Beringian range of *Pluvialis dominica* and *P. fulva*, showing areas of sympatry (purple) and sampling sizes and locations, with samples sizes for NADH dehydrogenase subunit two and amplified fragment length polymorphisms, respectively, given parenthetically.

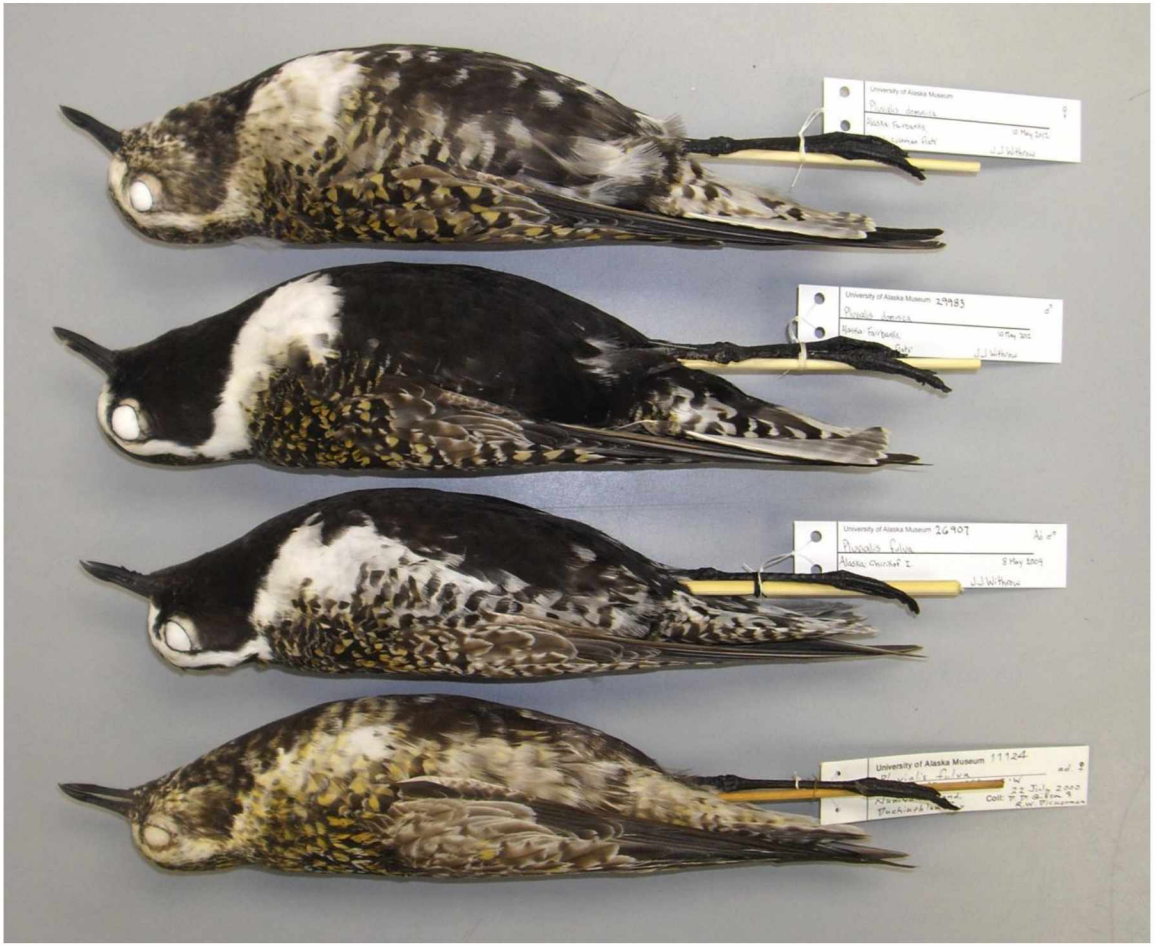


Figure 1.3: Typical breeding-plumage specimens of *Pluvialis dominica* (top two) and *P. fulva* (bottom two); males in center, females at top and bottom. Photo by J. J. Withrow

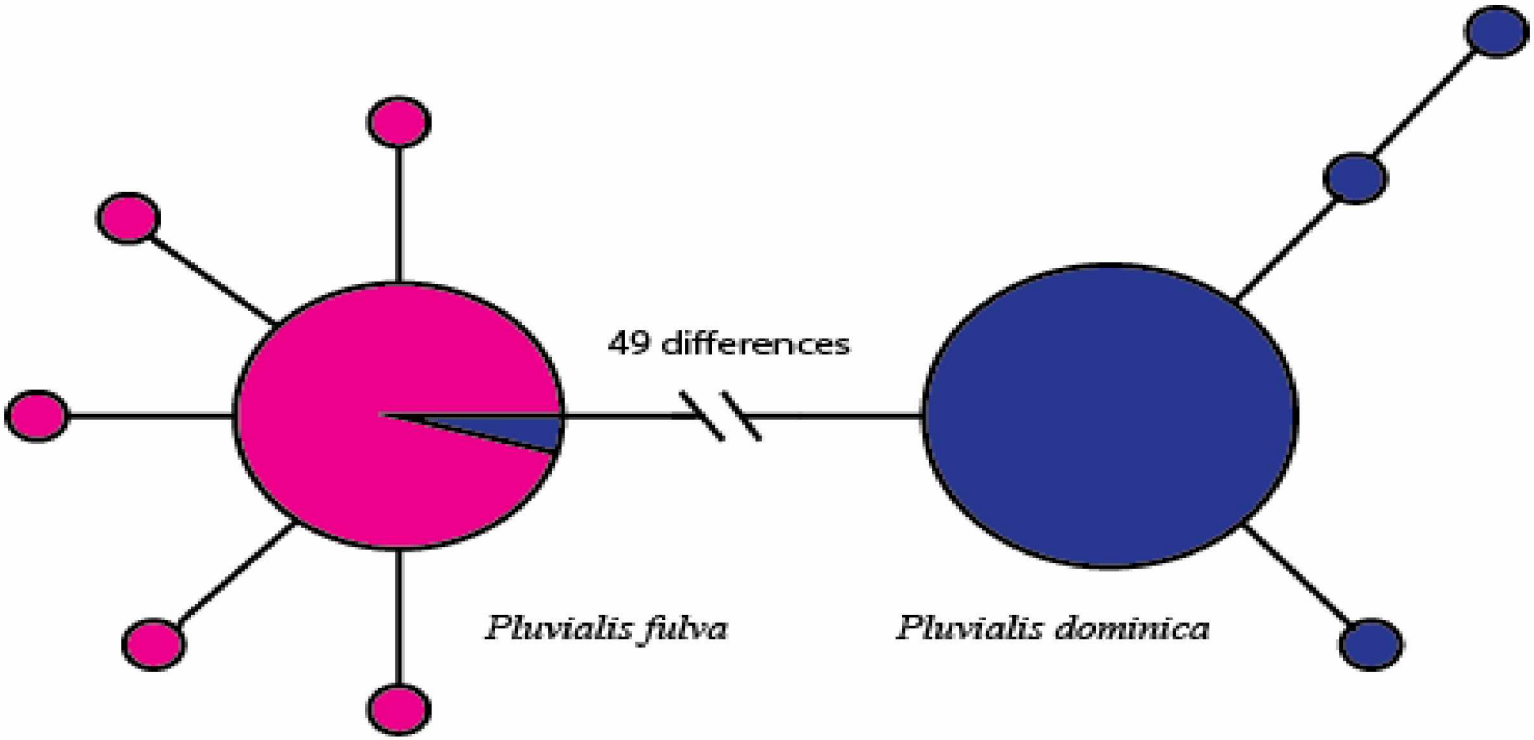


Figure 1.4: Haplotype network for 1041 bp of NADH dehydrogenase sub unit 2, depicting the number and relationships among haplotypes of both species. Circles are scaled in size to the number of each haplotype occurring in our specimens. The single hybrid individual is indicated by a wedge within the most common *P. fulva* haplotype.

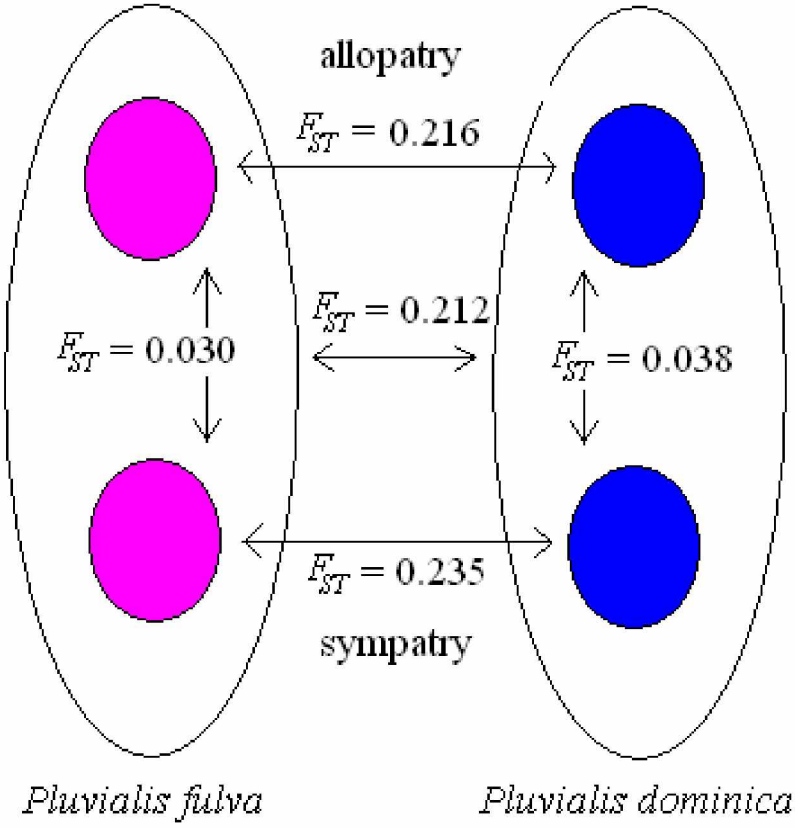


Figure 1.5: Amplified fragment length polymorphisms $F_{ST}$ values between and within populations of *P. dominica* and *P. fulva*. Colored circles represent samples of Seward Peninsula and non-Seward Peninsula birds to roughly assess sympatric and allopatric breeding populations.

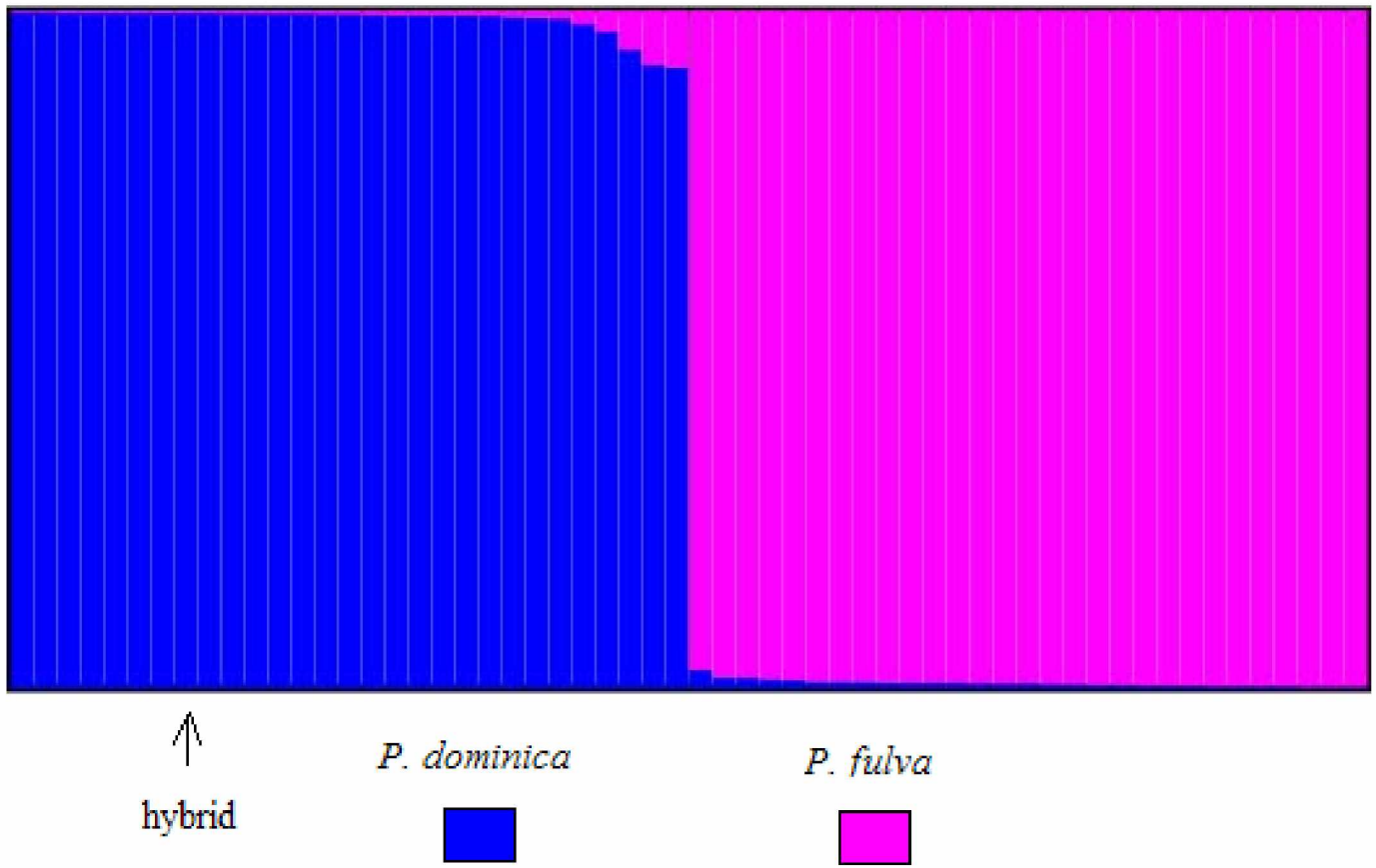


Figure 1.6: The genotypic makeup of 29 *P. dominica* and 29 *P. fulva.* Inferred using amplified fragment length polymorphisms and STRUCTURE (Pritchard *et al.* 2000). Each bar represents a phenotypically identified individual. The hybrid is indicated by an arrow.

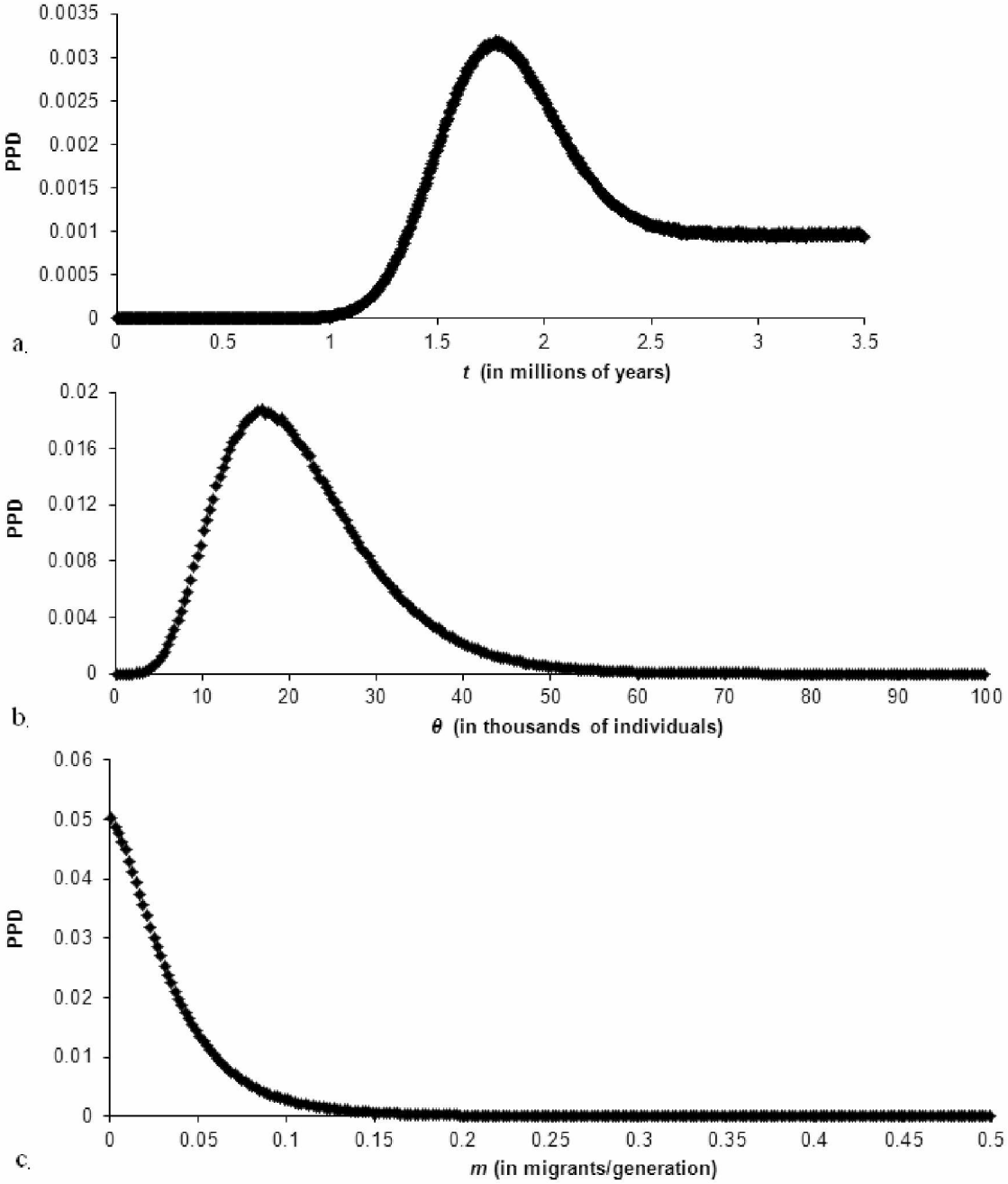


Figure 1.7: Model parameter estimate distributions of one run of isolation with migration coalescent analyses. Divergence time ($t$) is in years (a), effective populations size ($\theta$) in individuals (b), and migration rate ($m$) in migrants per generation (c).

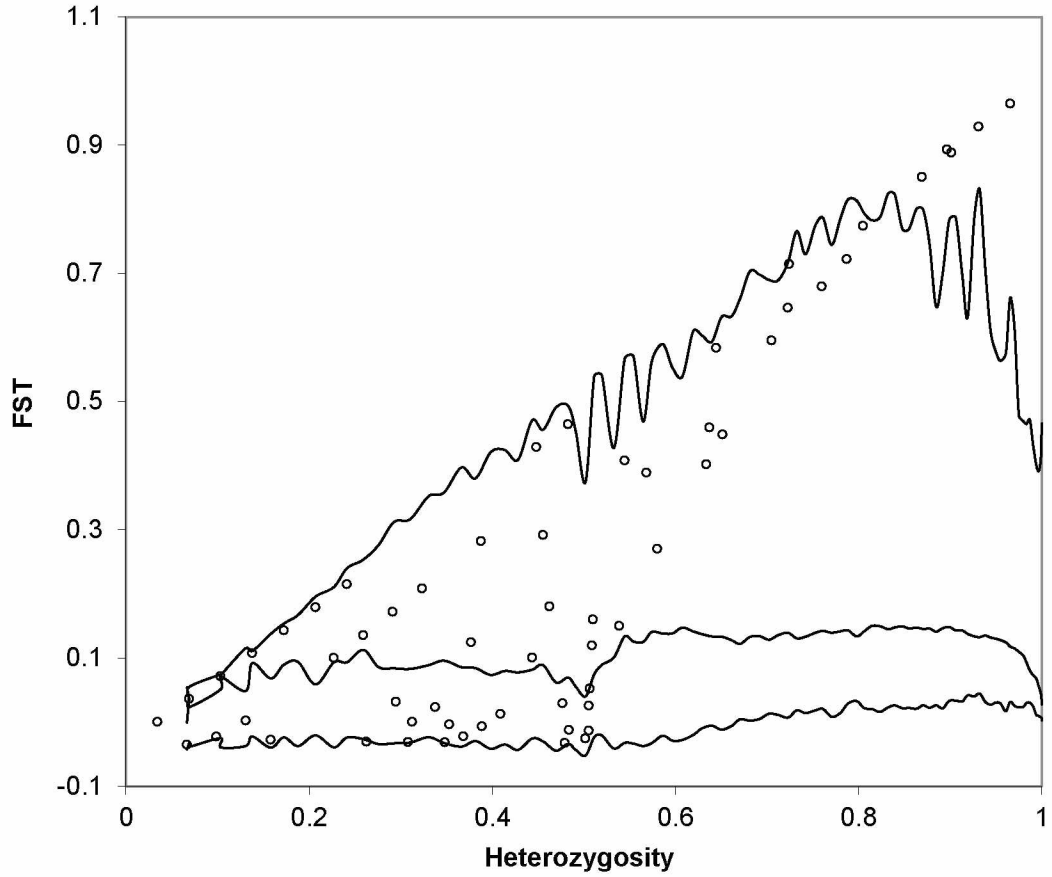


Figure 1.8: Distribution of amplified fragment length polymorphism data (circles) and quantiles. Upper (top line) and lower (bottom line) 99% and median (middle line) quantiles were calculated using simulated data diverging via drift alone using the program dfdist2 (Beaumont and Nichols 1996).

Appendix 1.A: Species, locations, University of Alaska Museum (UAM) specimen voucher numbers, and GenBank accession numbers for NADH dehydrogenase sub unit 2 and amplified fragment length polymorphism data. Alaska is abbreviated with AK, Hawaii with HI.

| Species | Location | Voucher numbers (UAM) | GenBank numbers by species |
|---|---|---|---|
| *P. dominica* | North Slope, AK | 13341, 13536, 13537, 13883, 13884, 18930* 18931*, 19412* | KC628677 – KC628696 |
| | Seward Pen., AK | 8550, 8783, 8784, 8938, 8939, 8941, 8995, 9510, 9511, 13181, 13576, 8551*, 8940*, 11758*, 12573*, 12572§ | |
| | Nulato Hills, AK | 24584 | |
| | Alaska Range, AK | 26934*, 26935* | |
| | Fairbanks, AK | 19498, 19497*, 14590§ | |
| | Juneau, AK | 17751* | |
| *P. fulva* | Aleutian Is., AK | 11110, 12442, 13370, 13545, 14175, 14671, 15177, 19275, 20111, 21830, 22577, 10492*, 11579*, 26906*, 15066§ | KC628697 – KC628718 |
| | Seward Pen., AK | 8555, 8785, 9509, 9512, 11307, 11308, 11392, 11756, 11757, 8798*, 9513* | |
| | Alaska Pen., AK | 20178 | |
| | Chirikof I., AK | 26907* | |
| | Hawaiian Is., HI | 8786*, 14602* | |

* denotes individuals for which AFLP but not ND2 data were generated.

§ denotes individuals for which only ND2 data were generated.

Appendix 1.B: Amplified fragment length polymorphisms scoring data for *Pluvialis*. Numbers refer to UAM accession numbers. Species and locations can be found in Appendix 1.A.

| | |
|---|---|
| | 1101011101100111101111111011111111111100111111110111011010111000011001111111111 |
| 9510 | 1011111111111111101110111011111111011111011111111011000101110111111011111111101111 |
| | 0011110101111111011101011111111111001111110101111011111111110110110011101110111111 |
| | 10111 |
| | 1111011101100111101111110011111111111111111111111011111101011100011100111111111111 |
| 11758 | 1101110111111111101110111011111111011111111111111101100101110111111111011111110111 |
| | 0110110001111110111110111111111111001111110101011011101111011101100010011101110 |
| | 00111 |
| | 1111011101100111101111100011111111111100111111110111111010111000011001111111111 |
| 12573 | 1111110111111111100111011101111111111111110111111111011000101110111111101111111110111 |
| | 0111110101111111011101111111111111001101110101111011110111111011011001110111010 |
| | 10111 |
| | 1101011101110111101111100111111111111100111111110111111010111000111001111111111 |
| 18930 | 1111111111111111101110111011111111011111111111111101101101110111111101111111110111 |
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| | 00111 |
| | 1111011101110111101111100111111111111101111111101111110101110000110011111111111 |
| 18931 | 1111110111111111111111011101111111111111110111111110111111011001111110111111110111 |
| | 0111110111111111111101011101111111001111110101111011101111101101100111011101010 |
| | 10111 |
| | 1101011101100111101111110111111111111110111111110111011010111000111101111111111 |
| 19412 | 1011111111111111101111011101111111101111101111111101100101110111111101111111110111 |
| | 0110110101111111011101011101111111001111110101111011010111111011011101110111011111 |
| | 10111 |
| | 1001011101100111001111100011111111111100111111110111011010111000011001111111111 |
| 19497 | 1011111111111111101111011101101111101111011101111011000101110111111101011111110111 |
| | 0110110101111111011101011100111111001111110101111001010111110010110011101110111010 |
| | 00111 |
| | 1001011101000111101111100111111111111001111111101111110101110000110011111111111 |
| 19498 | 1011111111111111101110111011111111011111011101111011000101110111011101011111110111 |
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| | 10111 |
| | 1101011101000111001111100011111111111100111111110110111010111000011001111111111 |
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| | 0111110001110110111010111001111100111111101011110010101111110110010001101110111010 |
| | 10111 |
| | 1001011101100111001111101011111111111100111111110111111010111000111001111111111 |
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| | 00111 |
| | 1001011101000111001111100011111101111100111111110111111010111001010001111111111 |
| 13884 | 1111111111111111101111011101111111111011101011101111111100101110111101000111110111 |
| | 0110110001111110111010110001111110010111101010110001101111101111001110111011010 |
| | 10111 |

Appendix 1.B continued.

| | |
|---|---|
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Appendix 1.B continued.

| | |
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| | 10111 |
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Appendix 1.B continued.

| | |
|---|---|
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| | 10111 |
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| | 01111011011101101111101110011111101101111011101100101010111111110111111111100101 |
| | 01011 |
| | 11111111011001111011111010111110101111001111111101100100101110011111011111111111 |
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| | 01111111111101101110101110111111100101111011101101101010111010011011110100010111 |
| | 10111 |
| | 11111111011001111011111110111111111111001111111101100100101110011111001111111111 |
| 21830 | 10111101111011111011001110111111111111111011101011011111111101111111111011110111 |
| | 01111111111101101110101111111111001101110011101100101010111110011011110100010100 |
| | 00111 |
| | 11111111011001111011111001010111111111100111111110110010010111001011101111111111 |
| 20111 | 10111111111011111011101110111111111111111011111111101101111110111111111111111110111 |
| | 01101111011101111110101111111111100101111011101101101010111110100110111101000111 |
| | 00111 |
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| | 11111111011101111011111101010111111111011011111111011101001011100111001111111111 |
| 15177 | 10111111111111111011101110111101111111111011111111101101111010111111101111111110111 |
| | 11111111111111101110101110111111101111111011101100111010110010011011110100011100 |
| | 00111 |
| | 11111111011101111011111100101111111110110111111111011001001011100111001111111111 |
| 19275 | 10111111111111111111101110111111111111111011101111011011101110111111101111110111 |
| | 01111111111101101110101110111111101111111011111100101010111011011001110100010100 |
| | 00111 |

Appendix 1.B continued.

| | |
|---|---|
| | 11111111011101111011111000111110111111101111111101110100101110011110011111111111 |
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| | 01111111111101101110101110111111101111111011111100101010111010011011111100010100 |
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| | 11111111011001110011111000111111111111001111111101100100101110010100011111111111 |
| 9513 | 10111111111011111011001110111111111111101011101111011011110100111111111111110111 |
| | 01111111011101101110001010111111000010111101010110011111011101001100111110001010 |
| | 00101 |
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| | 00101 |
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| 11392 | 10111111111011111011001110111111111111101111111101101111010111111111111011111111 |
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| | 00101 |
| | 11111111111001111011111101011111111100011111111011101001011100111000111110111 |
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| | 10101 |
| | 11111111011001111011111000111111111101101111111101110100101110111111001111111111 |
| 8798 | 10111111111111111011101110111111111101111101111111101101111010111111111101111110111 |
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| | 10111 |
| | 11111111011111111011111001111111111111001111111101110100101110011100011111111111 |
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| | 01111111111101111110101110111111100101111011101100101111111111101111111011001010 |
| | 10111 |
| | 11111111011101111011111111111111111111001111111101110100101110011100011111111111 |
| 26906 | 10111101111111111011101110111111111111101011101111011001110101111110001011111111 |
| | 01111111111101101110101110111111100101111011101101101011111111101111111010001010 |
| | 11111 |

## Chapter 2: Heteropatric differentiation in the Haida Gwaii owl, *Aegolius acadicus brooksi*.[2]

### 2.1 Abstract

The Northern Saw-whet Owl (*Aegolius acadicus*) occurs across much of North America but exhibits morphological and genetic differentiation in only one population: the one that is resident on the Queen Charlotte Islands (Haida Gwaii), British Columbia, Canada. We studied the genetic differentiation of this population (*A. a. brooksi*) from mainland populations (*A. a. acadicus*) using 1047 and 971 bp, respectively, of the mitochondrial genes NADH dehydrogenase subunit 2 (ND2) and cytochrome *b* (cyt *b*) and 405 amplified fragment length polymorphisms (AFLPs). Samples were drawn from 20 or more individuals for each marker and population. Both mitochondrial DNA and nuclear genomic markers showed significant differentiation between subspecies. MtDNA gene flow was estimated to be very low (< 1 individual per generation) between the subspecies, which likely diverged during the Wisconsin glacial maximum 16,000 years ago. We suggest that heteropatric differentiation, driven largely by a loss of migratory activity in *brooksi*, likely drove this divergence.

### 2.2 Introduction

Allopatric speciation has long been thought to be the predominant mode of speciation (Mayr 1963, Coyne & Orr 2004, Price 2008). Recently, some speciation research has focused on attributes other than geography as being important in the process of differentiation (Gavrilets 2003, Butlin *et al.* 2008, Li *et al.* 2010). Factors such as behavior, ecology, and the environment are increasingly seen as important drivers of speciation (Schluter 2001, McKinnon *et al.* 2004, Verzijden *et al.* 2012). These are indeed important attributes of the speciation process that can all operate within each category of geographic speciation (allopatric, parapatric, and sympatric; Gavrilets 2003). There is, however, room for refinement of the geographic context within which

[2] Jack J. Withrow, Spencer Sealy, and Kevin Winker, Heteropatric differentiation in the Haida Gwaii owl, *Aegolius acadicus brooksi*, in preparation for Molecular Ecology.

speciation takes place, and some evolutionary pressures may play a more common role in one context over another. One such refinement of geographic context is heteropatric speciation (Winker 2010), a model in which diverging migratory organisms occur in allopatry and sympatry cyclically through an annual cycle.

Many migratory birds have heteropatric distributions. Taxa exhibiting this pattern do not exhibit multigenerational spatial isolation, a key component of the allopatric speciation model. Nonetheless, many migratory lineages with heteropatric distributions have undergone differentiation and speciation, therefore speciation in these lineages may best be interpreted in a framework in which strict allopatry was not present (Winker 2010). This refinement of the geographic context of speciation better accommodates differentiation occurring in migratory taxa and requires a more important role for the behavioral, ecological, and environmental aspects of speciation that can be viewed, on average, as less important in an allopatric speciation framework.

Northern Saw-whet Owls (*Aegolius acadicus*) on Haida Gwaii (the Queen Charlotte Islands), British Columbia, Canada, exhibit a pattern of heteropatry with their more numerous mainland counterpart. Throughout most of its range, *A. acadicus* shows no significant variation in size or coloration (Cannings 1993). However, the population breeding on Haida Gwaii has been identified as a unique subspecies, *A. a. brooksi* (Fleming 1916; Fig. 2.1), although nominate birds are known to occur there during migration (Sealy 1998). This subspecies is darker in coloration, exhibits unique feeding habits, and is non-migratory (Hobson & Sealy 1991, Sealy 1998, 1999). It also shows genetic differentiation in mtDNA from mainland *acadicus* (Topp & Winker 2008).

The causes of this genetic and morphologic differentiation seem likely to be related to glacial cycles during the Pleistocene, as seen in many bird and other taxa (e.g., Avise & Walker 1998, Johnson & Cicero 2004, Weir & Schluter 2004, Shafer *et al.* 2010). The Haida Gwaii area in particular is thought to have constituted a refugium during the last glacial maximum (Warner *et al.* 1982, Heusser 1989, Pielou 1991). Endemic species and subspecies are described from Haida Gwaii for many types of organisms, including plants (Ogilvie 1989), insects (Kavanaugh 1989, Clarke *et al.* 2001), fish (Moodie & Reimchen 1976, O'Reilly *et al.* 1993), and mammals (Fleming &

Cook 2002). Birds in particular have been shown to exhibit genetic differentiation consistent with a refugial history there (e.g., Burg *et al.* 2005, Pruett & Winker 2005, Burg *et al.* 2006, Topp & Winker 2008), although some differentiation may have occurred after glaciers receded (e.g., Bull *et al.* 2010).

The mechanisms responsible for these divergences have often been left unstated or assumed to be a product of isolation (Avise & Walker 1998). Currently, Hecate Strait separates Haida Gwaii by ~70 km from the mainland. Historic isolation distances are unknown but the migratory capacity of birds and the glacial history of northwestern North America suggests isolation distances were not significantly different than today. Despite close geographic proximity, these two taxa are distinct and probably relatively young given that most taxa described from Haida Gwaii are subspecifically distinct, entirely so in birds. Thus, assumptions of historic allopatry, although possible, may not be the most parsimonious model of differentiation. Here the heteropatric model may apply, and it makes two key predictions: significant genetic differentiation will be present and gene flow will be low (Winker 2010). We ask a series of questions designed to determine the evolutionary history of *brooksi* relative to *acadicus*. First, they are morphologically distinct, but what is the level of genetic differentiation? Second, how might their divergence date correspond with the past climatic history of the area? Third, is there evidence of gene flow and at what level? Lastly, we synthesize the available evidence and propose hypotheses for how differentiation occurred between these taxa and the factors that drove it.

## 2.3 Materials and Methods:

### 2.3.1 Mitochondrial sequence data and sampling

We amplified 1047 bp of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene for 19 individual *acadicus* and 24 *brooksi*; one additional *acadicus* sequence was taken from GenBank (Appendix 2.A). We amplified 971 bp of the cytochrome *b* (cyt *b*) gene for 1 individual *acadicus* and 14 *brooksi* and supplemented these with 20 *acadicus* and 10 *brooksi* from Topp & Winker (2008; Appendix 2.A). Most *acadicus* individuals were from Alaska, but we also included individuals from New Mexico,

Oregon, and Washington in an attempt to capture broader geographic variation (Fig. 2.2, Appendix 2.A). ND2 is a well-known marker in birds and has been shown to be particularly informative and approximately neutrally evolving (Zink *et al.* 2005); similarly, cyt *b* is a well-studied gene with a fairly constant rate of evolution (Moore & DeFilippis 1997, Avise 2000), allowing population parameters to be estimated with reasonable confidence (Lovette 2004).

DNA was extracted from frozen tissues using a Qiagen (Valencia, CA) DNeasy Tissue Kit following the manufacturer's protocol. Initial amplifications were performed using cyt *b* primers L0-25 and H1117 (Topp & Winker 2008) and ND2 primers H6313 and L5219 (Sorenson *et al.* 1999). PCR amplification was conducted using 2.5 μL of each primer at 10 μM, 3 μL of a 10 μM solution of dNTPs , 0.2 μL (1 unit) of *Taq* DNA polymerase, 6μL of 25 mM $MgCl_2$, 5 μL of 5X *Taq* Buffer (Promega Corp., Madison, WI), 2.5 μL of 1 mg/mL BSA, and 23.3 μL water for a total reaction volume of 50 μL. PCR thermal regime started with 2 min. at 94° C followed by 29 cycles of 48° C for two min., 72° C for 2 min., and with a final elongation step at 72° C for 5 min.

PCR cleanup and sequencing were done at the High-Throughput Genomics Unit (University of Washington, Seattle, WA) using an ExoSAP cleaning process, cycle-sequenced using BigDye (Applied Biosystems Inc. [ABI], Foster City, CA) and sequenced on an ABI 3730KL high-throughput capillary sequencer (ABI). Cycle - sequencing amplifications were done using the initial sequencing primers and internal primers for some individuals. These internal primers were as follows: cyt *b* internal forward primer (5'-TTCTCAGCCGTACCATACATTGGC-3'), cyt *b* internal reverse primer (5'-ATCACAGCTGGATGGGATTCCT-3'), ND2 internal forward primer (5'-TCTTGCCTCCTCCTAACAACAGCA-3'), and ND2 internal reverse primer (5'-TGTTGATAGGATGGCCATGGAGGT-3'). Sequences were aligned and edited using Sequencher 4.7 (Gene Codes, Ann Arbor, MI).

### 2.3.2 Amplified fragment length polymorphisms data and sampling

Amplified fragment length polymorphisms (AFLPs) were generated using a protocol modified from Vos *et al.* (1995). These were generated for 24 *brooksi* and 22

*acadicus* from throughout the species' range (Fig. 2.2, Appendix 2.A). Initial sample DNA concentration was quantified on a spectrophotometer, and all samples had concentrations of 30-70 ng/μL. The restriction and ligation of adapter pairs was performed in one step using 0.28 μL water, 1.1 μL 10X T4 Buffer (New England BioLabs, Inc. [NEB], Ipswich, MA), 0.55 μL 1M NaCl, and 0.55 μL 1 mg/mL BSA (NEB), 0.1 μL MseI, and 0.25 μl EcoRI (ABI) per 6 μL of template DNA. The reaction mixture was vortexed and placed in a thermal cycler at 37° C for approximately 12 hrs. This reaction mixture was subsequently diluted with 94.5 μL of 0.1M TE buffer and frozen until preselective amplification.

Preselective amplification was done using the following: 5.7 μL water, 1.0 μL 5X buffered GoTaq, 0.6 μL 25mM MgCl, 0.1 μL 10 μM dNTPs, and 0.125 μL (0.625 units) *Taq*, which was mixed on ice and added to 2.0 μL of diluted template. The thermal cycler regime consisted of 1 min. at 72° C followed by 19 cycles of 94° C for 20 sec, 56° C for 30 sec, and 72° C for 2 min followed by 30 min at 60° C. After preselective amplification, reactions were diluted with 80 μL of 0.1X TE buffer and frozen until selective amplification.

Selective amplifications were done using the same PCR mix as in the preselective amplification, but dye-labeled MseI and EcoRI primers were used to selectively amplify a subset of DNA. All reagents were mixed on ice as quickly as possible. Thermal-cycler regime consisted of 2 min at 94° C followed by 11 cycles of 94° C for 20 sec, 66° C for 30 sec, and 72° C for 2 min. During each cycle the annealing temperature was dropped by one degree, and at 56° C 19 additional cycles were run at that annealing temperature followed by 30 min at 60° C. Samples were selectively amplified and 1.0 μL of each sample was loaded into a 96-well plate the day before running. This plate was vacuum centrifuged to remove moisture and then frozen overnight. The next morning 8.5 μL deionized formamide and 0.5μL GeneScan 500 LIZ size standard (ABI) were added to each sample. The samples were then heated to 95° C for 2 min immediately before running on an ABI 3100 automated sequencer (ABI).

We used 9 primer pair combinations for selective amplification (Table 1). GeneMapper ver. 3.7 (ABI) was used to score the chromatograms. Only unambiguous

loci with clean, well-defined peaks were scored. We used a minimum peak width of 1.5 bp and a minimum peak height of 75 as a starting point, but then examined each peak individually to maximize the useful phylogenetic signal (Holland *et al.* 2008). We discarded two samples that did not amplify larger fragments, likely due to tissue degradation related to the salvaged nature of these specimens. Data were transformed into a binary state matrix using a Microsoft Excel® macro, which also transformed the matrix into nexus format (Rinehart 2004). All bands were considered independent, homologous loci.

### 2.3.3 Genetic differentiation and population structure

A median joining network illustrating haplotype frequencies was generated using NETWORK 4.6.0.0 (Bandelt *et al.* 1999). We used Arlequin (Excoffier *et al.* 1992) to calculate pairwise $\Phi_{ST}$ values between populations for cyt *b* and ND2 sequences separately and for a combined mtDNA data set. Genotypes were permuted 1000x to obtain *P*-values determining whether $\Phi_{ST}$ values were significant. We calculated $F_{ST}$ (and *P*-values) following Lynch and Milligan (1994) for our AFLP data using AFLP-SURV 1.0 (Vekemans *et al.* 2002), assuming Hardy-Weinberg equilibrium using the Bayesian method with non-uniform priors, 10,000 random permutations, and 1,000 bootstraps for genetic distances.

We also analyzed AFLP data in STRUCTURE 2.2 (Pritchard *et al.* 2000, Falush *et al.* 2007) to determine the most likely number of populations and to identify any admixed individuals. STRUCTURE uses MCMC simulations in a Bayesian framework to assign individuals to populations and determine the most likely number of populations (Pritchard *et al.* 2000). The program's model-based clustering method effectively deals with the genotypic ambiguity present in dominant markers such as AFLPs (Falush *et al.* 2007). Preliminary runs indicated that a burnin of 20,000 iterations was sufficient. We then ran three independent simulations, using the admixture model, for 100,000 iterations for values of (*K*) from one to five. The likelihood of *K* given the data was calculated as $P(K|X)$. To avoid biasing the inference of population structure, we did not use population origin information, although most individuals were phenotypically identifiable. We used

the program Distruct (Rosenberg *et al.* 2002) to transform and apply information from one STRUCTURE run into a postscript file which was viewed and manipulated in Adobe Illustrator. Although Pritchard *et al.* (2000) demonstrated STRUCTURE's ability to correctly identify $K$ in data sets such as this, we also evaluated the log-likelihood values for $\Delta K$ (Evanno *et al.* 2005) using STRUCTURE HARVESTER v6.93 (Earl & vonHoldt 2012).

To independently assess STRUCTURE results we also performed an admixture analysis in BAPS 5.3 (Corander & Marttinen 2006, Corander *et al.* 2008). Like STRUCTURE, BAPS assesses population structure and individual admixture using Bayesian clustering algorithms.

### 2.3.4 Divergence time, effective population size, and gene flow

The program IM (Nielson & Wakeley 2001) was used on the concatenated mtDNA data to estimate seven parameters, most scaled to the neutral mutation rate, μ: $t$ ($T/\mu$, where $T$ is time since divergence in years before present), $\theta_2$ ($4N_e\mu$ for *A. a. acadicus*), $\theta_1$ ($4N_e\mu$ for *A. a. brooksi*), $\theta_a$ ($4N_e\mu$ for the ancestral population at time of divergence), $m_1$ ($2M/\theta_1$ where M is the effective number of migrants moving into Haida Gwaii per year), $m_2$ ($2M/\theta_2$, migration rate from Haida Gwaii to the rest of North America), and $s$ (a splitting parameter, the proportion of nominate *acadicus* that founded the *brooksi* population; 1-$s$ is the proportion of *brooksi* contributing to *acadicus*; Hey 2005). We ran three independent runs using the initial parameter starting maxima of $\theta$ = 100, $m$ = 3, and $t$ = 3, with a burnin of 500,000 steps and a different random number seed for each run. We calculated generation time ($G$) using the equation $G = \alpha + (s/1 + s)$, where α is the age of maturity and $s$ is the expected adult survival rate (Saether *et al.* 2005). Most *A. acadicus* probably begin breeding when one year old (α = 1; Cannings 1993), and we assumed a survivorship percentage of 0.66% based on Cannings (1993) and Marks & Doremus (2000), giving an estimated generation time of 3 years. The HKY model of molecular evolution (Hasegawa *et al.* 1985) was used in all runs; it takes into account multiple nucleotide substitutions at the same position, nucleotide frequency differences, and any transition/transversion bias. We let each run proceed for more than

10 million updates to achieve a minimum effective sample size of 100 for any given parameter estimate; most were several orders of magnitude higher than this. Results from the three runs were similar, and we report here only the parameters estimated in the longest run of 30,002,974 updates after burnin.

The parameters estimated by IM are dependent on the mutation rate, which is an uncertain quantity (Lovette 2004, Ho *et al.* 2005). A mutation rate of 2 % divergence per My (0.00002018 per year per 2018 bp) was used (following Weir & Schluter 2008).We also set a range of mutation rates at 1 - 4% divergence per My (0.00001 – 0.00004 per year per 2018 bp) to incorporate uncertainties in the mutation rate (Weir & Schluter 2008, Pacheco *et al.* 2011).

### 2.3.5 Genetic diversity and selection

We used DNAsp (Rozas *et al.* 2003) on mtDNA data to calculate haplotype diversity ($H_d$; Nei & Chesser 1983) and nucleotide diversity ($\pi$). We conducted $\chi^2$ tests of genetic differentiation between populations based on haplotype frequencies and nucleotide diversity indices. The $\chi^2$ tests with Yates continuity corrections were conducted in PopTools 2.6.9 (Hood 2005), an add-in for Microsoft Excel®.

To determine whether the genes sampled through AFLPs diverged via genetic drift or selection we compared $F_{ST}$ and heterozygosity estimates for each locus in our dataset with a simulated dataset acting under drift alone using an infinite-alleles model. To produce these simulated data we used the program dfdist2 (Beaumont & Nichols 1996, Beaumont 2000). This program uses an average divergence of $F_{ST}$ and expected heterozygosity ($H_S$) to simulate the expected distribution of differentiation across loci (Campbell & Bernatchez 2004, Bonin *et al.* 2006). It uses an $F_{ST}$ calculated by the method of Nei (1977) as modified by Nei & Chesser (1983) and generates a uniform distribution of heterozygosties in place of a specified mutation rate. This distribution is then used to calculate quantiles of the median and upper and lower 99% confidence intervals of the distribution of loci for the population diverging under drift alone. It also calculates $F_{ST}$ and $H_S$ for all polymorphic loci in the dataset, which are then plotted against the confidence intervals. Loci falling outside this distribution in excess of

expected false positives are assumed to be under selection or closely linked to loci under selection (Beaumont & Nichols 1996, Nosil *et al.* 2009). The data were analyzed to get an estimate of the average $F_{ST}$ across all loci. The model was then fit to this $F_{ST}$ for simulation. We ran the simulation for 20,000 realizations, with two demes total, sampling the two populations of *A. acadicus* with an expected $F_{ST} = 0.11$ and an average sample size per population of 22 individuals.

## 2.4 Results

### 2.4.1 Genetic differentiation and population structure

Haplotype networks of both ND2 and cyt *b* showed some structuring between populations. Interestingly, with only three haplotypes cyt *b* still exhibited a fixed difference between the populations, with two haplotypes in the larger *acadicus* population (Fig. 2.3). ND2 was less structured, and had 8 haplotypes, 3 in *brooksi* and 6 in *acadicus* (Fig. 2.3).

Population structuring in mtDNA was apparent in both markers separately and together, with $\Phi_{ST}$ for cyt $b = 0.96$ ($P < 0.001$), $\Phi_{ST}$ for ND2 = 0.67 ($P < 0.001$), and a combined $\Phi_{ST} = 0.67$ ($P < 0.001$). These are consistent with and reinforce the significant structure seen visually in the haplotype networks (Fig. 2.3). AFLP data also showed significant structure between the populations, with $F_{ST} = 0.074$ ($P < 0.0001$).

STRUCTURE analysis estimated that the most likely number of populations involved was two (ln Pr($K|X$) = -1438.4; P($X|K$) = ~1; Table 2.1). Most individuals (35/45, 78%) were estimated to have greater than 90% of their genomic alleles originating from their putative (phenotypic) population of origin (Fig. 2.4). Three Haida Gwaii birds (of 24, 12.5%) had more than half of their nuclear alleles matching those of *acadicus*, and two mainland birds (of 22, 9.0%) had more than half of their nuclear alleles matching *brooksi*. Six (25%) Haida Gwaii birds had >10% of nuclear alleles matching *acadicus*, while four (18%) mainland birds had similar amounts of nuclear alleles matching *brooksi*.

BAPS analysis also estimated the most likely number of populations to be two (ln Pr($K|X$) = -1631.1; Table 2.1). It also identified the same individuals as being

genomically admixed in the same proportions as STRUCTURE (Table 2.2; individual data not shown).

### 2.4.2 Divergence time, effective population size, and gene flow

Coalescent analysis in IM estimated the high point (95% confidence interval) of the probability distribution for the following model parameters (Fig. 2.5): effective female population size of *brooksi* ($\theta_1$) = 0.35 (0.65 – 96.15), effective female population size of *acadicus* ($\theta_2$) = 60.75 (8.45 – 97.75 ), effective ancestral female population size ($\theta_a$) = 0.05 (0.05 – 38.85), migrants from *acadicus* into *brooksi* ($m_1$) = 0.0015 (0.017 – 2.52), migrants from *brooksi* into *acadicus* ($m_2$) = 0.0045 (0.017 – 2.63), divergence time ($t$) = 0.33 (0.14 – 3.29) and the proportion of the ancestral population that gave rise to *brooksi* ($s$) = 0.0005 (0.0025 – 0.97). We converted these values into demographic units using a divergence rate of 2% per My (Table 2.2). The posterior distribution of $t$ did not include zero and estimated a divergence date of 8,114 - 32,457 years ago. The effective population size of *acadicus* ($\theta_2$; 250,867 females) did not converge well (Fig. 2.5c), likely because of its extremely large size relative to *brooksi* and inadequate signal in the dataset for an accurate estimate. The effective population size of *brooksi* ($\theta_1$) was estimated at 1,445 females. Estimates of mtDNA migration rates were very small, effectively zero ($m_1$ = 0.0026, $m_2$ = 0.14; Fig. 2.5e, f). The proportion of the ancestral population founding *brooksi* was estimated to be very small ($s$ = 0.05%; Fig. 2.5g).

### 2.4.3 Genetic diversity and selection

Nucleotide diversity was lower in *brooksi* than *acadicus* ($\pi$ = 0.00008 and $\pi$ = 0.00039; $\chi^2$ = 2342.8, df = 1, $P < 0.001$), but haplotype diversity was not significantly different between the two taxa (*brooksi* $H_d$ = 0.163, *acadicus* $H_d$ = 0.538; $\chi^2$ = 2.1, df = 9, $P$ = 0.144).

A total of 405 AFLP loci were produced by the nine primer pairs. Of these, 102 (25.2%) were polymorphic (Table 2.3, Appendix 2.B) when both subspecies were included. There were 19 AFLP loci that were fixed in *brooksi* but polymorphic in *acadicus*, and there were 23 alleles fixed in *acadicus* but polymorphic in *brooksi*; these

differences were not significant ($\chi^2 = 0.0477$, df = 2, $P = 0.977$). There were six AFLP loci present in *brooksi* that were absent in *acadicus*, and seven loci present in *acadicus* that were absent in *brooksi*, and these differences were also not significant ($\chi^2 = 0.844$, df = 2, $P = 0.981$).

One of 405 loci (0.25%) fell outside the simulated data set's 99% quantile (Fig. 2.6). This result includes fewer loci than would be expected by chance (expected $N = 4$; 1%), suggesting that these two populations are probably not experiencing strong divergent selection at the loci examined.

**2.5 Discussion**

These owl subspecies exhibit two of the key attributes predicted by the heteropatric speciation model. Their mtDNA differentiation of $\Phi_{ST} = 0.67$ ($P < 0.001$; Fig. 2.3) and genomic differentiation of $F_{ST} = 0.074$ ($P < 0.0001$; Fig. 2.4) suggest a significant period of assortative mating despite their occurrence together on Haida Gwaii today. This reinforces the validity of the phenotypically well-defined *brooksi* subspecies (Fig. 2.1). Our analyses of mtDNA data suggest that there is effectively no gene flow between populations (Table 2.2, Fig. 2.5e, f). In contrast, Bayesian clustering algorithms run on AFLP data suggested nuclear allele sharing between populations in some individuals (Fig. 2.4). We are not able to determine whether these alleles are a result of incomplete lineage sorting or gene flow. Most birds, including owls, have female-biased dispersal (Konig *et al*. 2009), and *A. acadicus* in particular appears to fit this pattern (Beckett & Proudfoot. 2012). It may also be nomadic to some extent (Marks & Doremus 2000, Bowman *et al.* 2010). We would therefore expect mtDNA to be a leading indicator of gene flow in this species. Thus, the very low estimates of gene flow seen in maternally inherited mtDNA (effectively zero), coupled with the shallow level of divergence observed, suggests that the shared nuclear alleles are due to incomplete lineage sorting rather than gene flow. This is a pattern similar to that seen between Snow and McKay's buntings, another case of island differentiation, in which mtDNA suggested that no gene flow was occurring, but in which AFLP markers showed a sharing of some nuclear alleles (Maley &Winker 2010).

IM simulations estimated a divergence date of ~16,000 years before present, about the time of the oldest known evidence for a terrestrial environment on Haida Gwaii (Warner *et al.* 1982) and at or just after the maximum extent of late Wisconsin glacial extent in the area (Clague & James 2002, Clark *et al.* 2009). However, uncertainties about substitution rate estimates make precise estimates of divergence dates from a single molecular marker difficult (Ho *et al.* 2005, Price 2008, Tinn & Oakley 2008). Thus, whether these owls were isolated on Haida Gwaii as sea levels rose after the Pleistocene or were isolated in a coastal refugium during an older, colder period is uncertain. Regardless, our estimate of their divergence peaked sharply at a time corresponding reasonably well with the last glacial maximum of the Pleistocene, making *brooksi* a recently derived taxon.

There is also evidence of a small effective population size and a possible founder effect in *brooksi*. Population size estimates for *brooksi* (1,445 females) and *acadicus* (250,867 females) correspond well with the current census size estimates of 1,900 individual *brooksi* (COSEWIC 2006) and 100,000 – 300,000 individual *acadicus* (Cannings 1993).

We did not find evidence of selection in our sample of nuclear loci. Our sampling of the genome is very small, however, so this does not mean that selection is absent. In fact, given differences in plumage, migration, and diet, the existence of some between-lineage selection can be inferred.

Glaciation, tectonic rebound, and fluctuating sea levels make geologic inferences of historical refugia complex, but much of the continental shelf of south coastal Alaska and coastal British Columbia north and south of Haida Gwaii remained ice free during the last glacial maximum (Hetherington *et al.* 2003, Kaufman & Manley 2004, Carrara *et al.* 2007). This history also suggests that at least during the last glacial maximum Haida Gwaii was connected to the mainland (Hetherington *et al.* 2003). Thus, a string of refugia along northwestern North America (Shafer *et al.* 2010) likely existed, with Haida Gwaii being only one of several ice-free areas. Therefore, we don't suggest that *brooksi* necessarily arose on Haida Gwaii, but rather that it arose on an unglaciated area in the region and likely shifted its range as sea levels rose and suitable habitats shifted. A

migratory *Aegolius* ancestor could have used this coastal region just as nominate *acadicus* does today. *Aegolius*' ability to colonize areas much more remote than a Haida Gwaii refugium is highlighted by *Aegolius gradyi* from Bermuda, a close relative of *A. acadicus*, which likely arose by peripatric speciation after an ancestral *A. acadicus* reached that island (Olson 2012).

It seems unlikely that a species with the high dispersal capability of *acadicus* would have been absent from a Haida Gwaii refugium long enough for multigenerational allopatry to occur. Current geographic and demographic patterns fit a model of heteropatric divergence well. Alternatively, *brooksi* might have been completely isolated in a Haida Gwaii refugium and had subsequent contact with nominate *acadicus* after differentiation in allopatry, in which case heteropatric differentiation mechanisms could still have acted to maintain differences acquired largely in allopatry. Our data cannot distinguish between these models. Although we consider it likely that the Haida Gwaii refugium was accessible and accessed by ancestral continental *acadicus* while a sedentary population was established and began diverging, it is possible that continental ice sheets assisted in lowering the number of continental immigrants while strong divergent selection occurred, creating sufficient isolating mechanisms that when the degree of heteropatry increased to what we observe today (there is, after all, a large continental population in the region that could have been there during the last glacial maximum (LGM)), lineage integrity was maintained. Further study of nuclear gene flow is warranted.

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Table 2.1: STRUCTURE and BAPS estimates of the number of clusters or likely populations involved ($K$) in *Aegolius acadicus*. We used SRUCTURE and BAPS without using prior population information. $\Delta K$ (Evanno et al. 2005) values calculated in STRUCTURE HARVESTER (Earl & vonHoldt 2012) are also shown.

| STRUCTURE | | | | BAPS |
|---|---|---|---|---|
| K | mean Ln Pr($X$\|$K$) | P($K$\|$X$) | $\Delta K$ | Ln Pr($X$\|$K$). |
| 1 | -1534.7 | ~0 | - | - |
| 2 | -1439.0 | ~1 | 110.2 | -1631.1 |
| 3 | -1463.9 | ~0 | 7.5 | -1662.4 |
| 4 | -1610.8 | ~0 | 0.9 | -1694.6 |
| 5 | -1823.0 | ~0 | - | -1739.2 |

Table 2.2: Demographic parameter estimates from one 30 million step isolation with migration run.

| Demographic description | model parameter | value in demographic units at 2% divergence | 95% lowest and highest densities |
|---|---|---|---|
| *A. a. brooksi* effective population size | $\theta_1$ | 1,445 | (34,894 – 403,658) |
| *A. a. acadicus* effective population size | $\theta_2$ | 250,867 | (2,684 – 397,051) |
| Ancestral effective population size | $\theta_a$ | 206 | (206 – 160,431) |
| Migrants from *acadicus* into *brooksi** | $m_1$ | 0.0026 | (0.0165 – 2.51) |
| Migrants from *brooksi* into *acadicus** | $m_2$ | 0.136 | (0.0165 – 2.63) |
| Time since divergence | $t$ | 16,228 | (6,813 – 163,156) |
| Number of founders of *acadicus* | $1 - s$ | 205.9 | -- |
| Number of founders of *brooksi* | $s$ | 0.1 | (0.0025 – 0.97) |

All estimates of effective population size and number of migrants are in units of individuals.

Estimates of time since divergence are in years.

*These parameters are independent of mutation rate.

Table 2.3: Amplified fragment length polymorphism amplification and scoring results for each primer pair and totals.

| primer pair extensions and dye | | | both subspecies | | | within *acadicus* | | | within *brooksi* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *EcoR1* | *MseI* | dye | T | P | %P | T | P | %P | T | P | %P |
| -ACT | -CAG | FAM | 34 | 13 | 38.2 | 34 | 8 | 23.5 | 34 | 10 | 29.4 |
| -ACT | -CAT | FAM | 22 | 4 | 18.2 | 22 | 2 | 9.1 | 22 | 1 | 4.5 |
| -ACT | -CTA | FAM | 55 | 11 | 20.0 | 55 | 9 | 16.4 | 53 | 8 | 15.1 |
| -ACA | -CAA | FAM | 62 | 18 | 29.0 | 60 | 15 | 25.0 | 59 | 11 | 18.6 |
| -ACA | -CAC | FAM | 51 | 16 | 31.4 | 50 | 10 | 20.0 | 51 | 13 | 25.3 |
| -ACA | -CAG | FAM | 67 | 11 | 16.4 | 63 | 6 | 9.5 | 66 | 8 | 12.1 |
| -ACA | -CAT | FAM | 27 | 5 | 18.5 | 27 | 2 | 7.4 | 26 | 4 | 15.4 |
| -ACA | -CTA | FAM | 51 | 18 | 35.3 | 51 | 14 | 27.5 | 51 | 12 | 23.5 |
| -AAG | -CAT | JOE | 36 | 6 | 16.7 | 36 | 5 | 13.9 | 36 | 5 | 13.9 |
| Totals | | | 403 | 102 | 25.2 | 398 | 71 | 17.8 | 398 | 72 | 18.1 |

Total peaks (*T*), the number of polymorphic peaks (*P*), and the percentage of peaks that were polymorphic (*%P*).

Figure 2.1: Dorsal and ventral views of *Aegolius acadicus brooksi* (left) and *A. a. acadicus* (right). Photos by J. J. Withrow

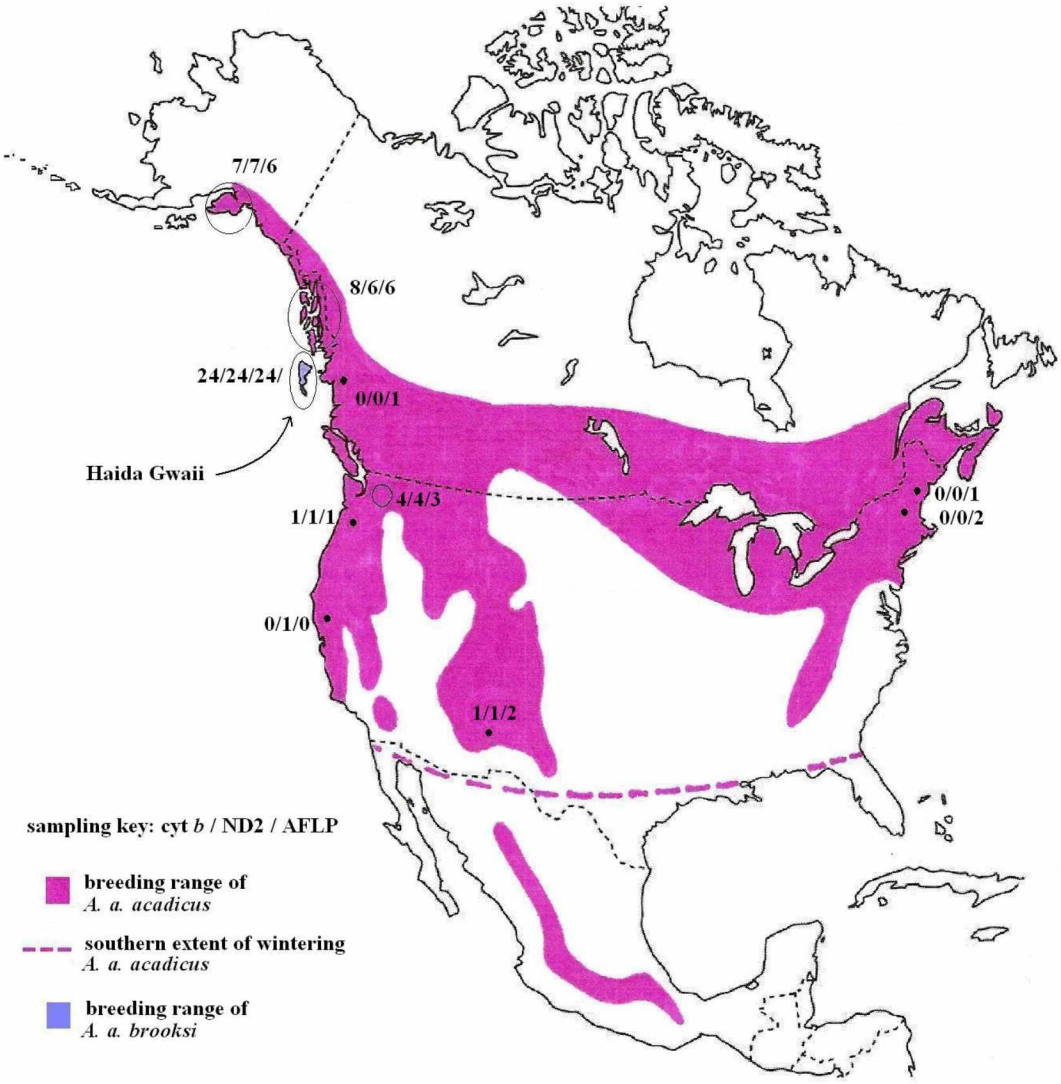


Figure 2.2: Breeding range and sampling locations of *Aegolius acadicus acadicus* and *A. a. brooksi*. Numbers indicate sample sizes for the three markers: cytochrome *b* (cyt *b*), NADH dehydrogenase subunit 2 (ND2), and amplified fragment length polymorphisms (AFLPs), respectively. Dashed line marks the southern extent of wintering *A. a. acadicus*.

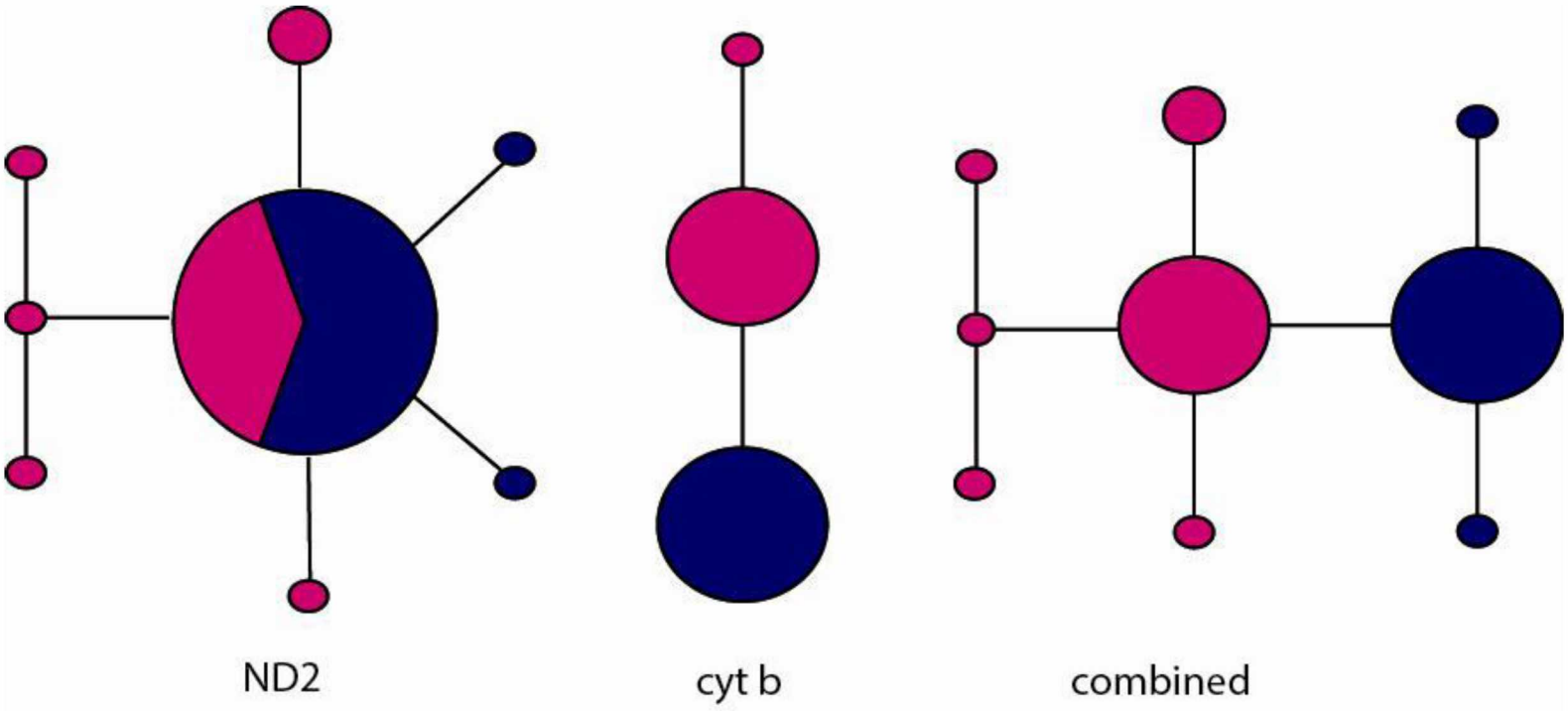


Figure 2.3: Haplotype networks showing ND2 sequences, cyt *b* sequences, and concatenated sequences from 24 *brooksi* (blue) and 21 *acadicus* (pink). Circle sizes are proportional to the number of individuals with that haplotype.

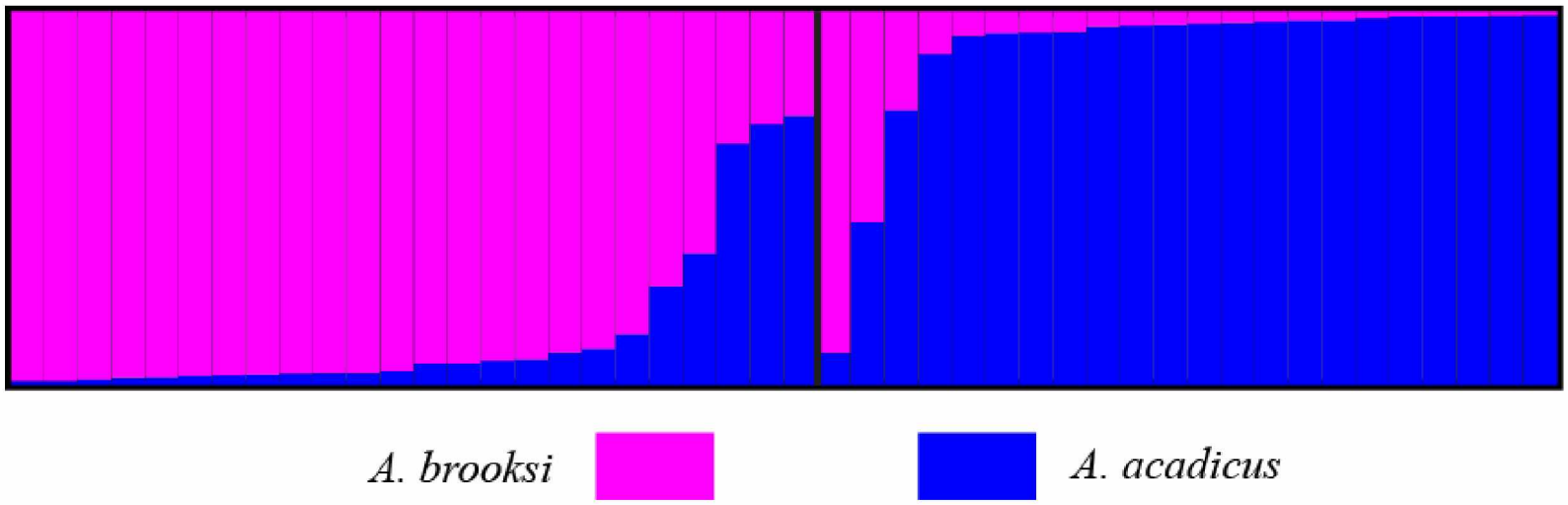


Figure 2.4: Genotypic make up of 22 *A. a. acadicus* and 24 *A. a. brooksi* inferred by STRUCTURE (Pritchard *et al.* 2000). Each bar represents a phenotypically identified individual.

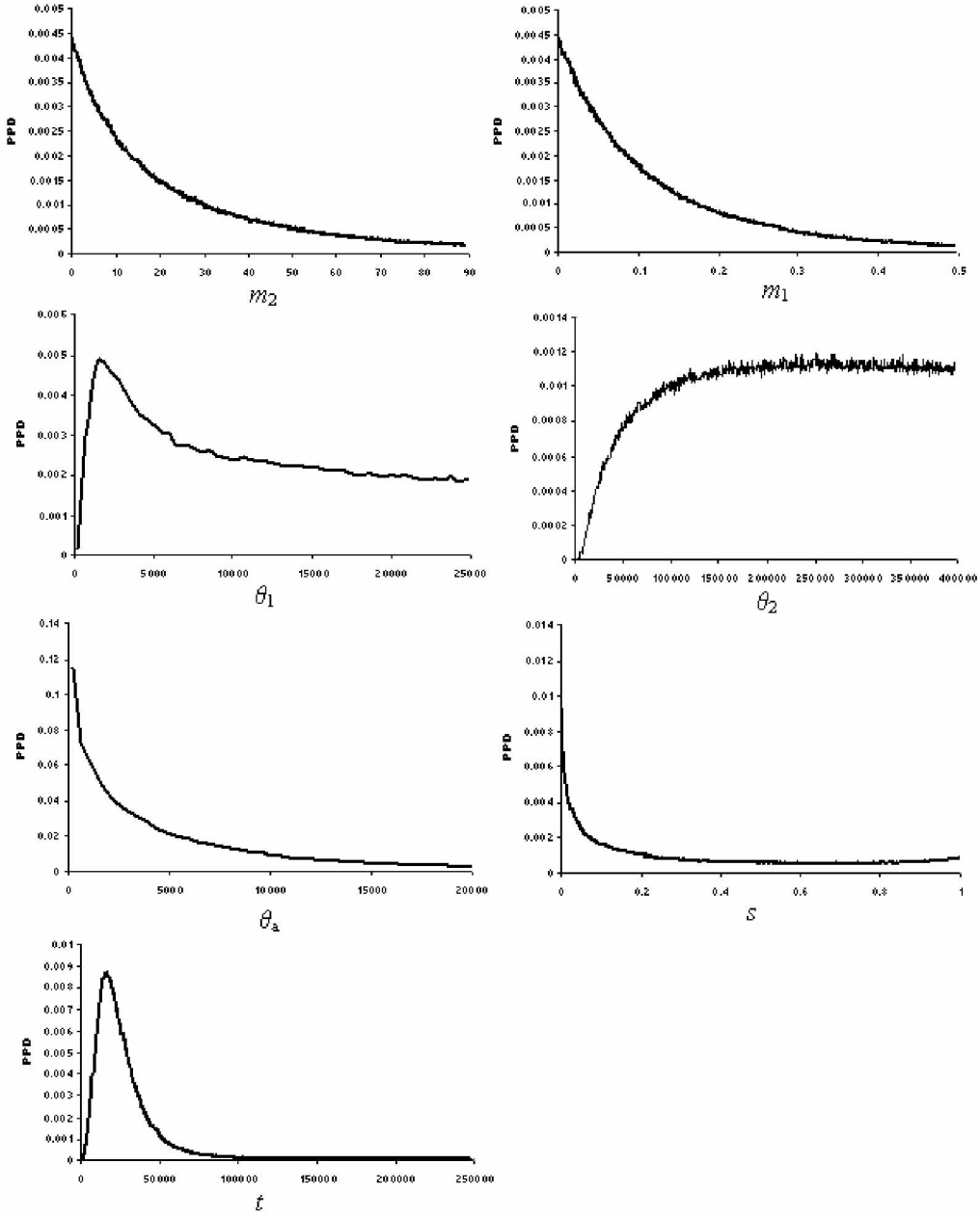


Figure 2.5: Parameter estimate distributions of a 30 million step isolation with migration coalescent analysis. A generation time of 3 years and a divergence rate of 2% per MY were used. Divergence ($t$) is in years, effective population sizes ($\theta_a$, $\theta_1$, and $\theta_2$) are in individuals, migration rates ($m_1$ and $m_2$) are individuals per generation, and the splitting parameter ($s$) is the proportion of $\theta_a$ contributing to $\theta_1$.

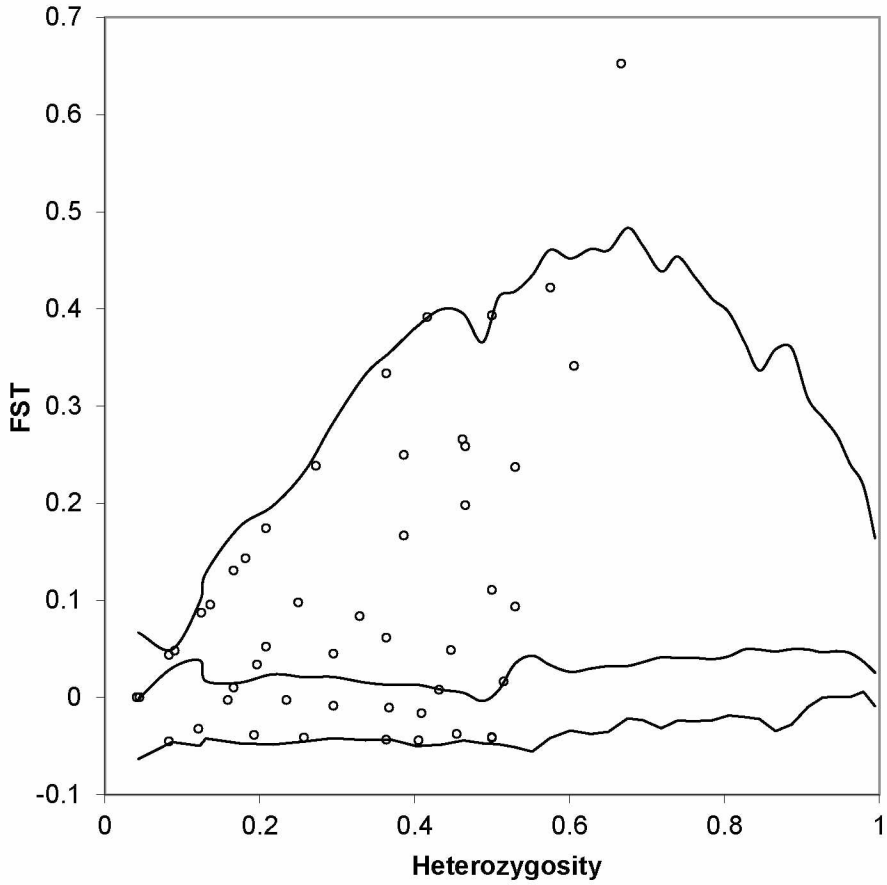


Figure 2.6: Distribution of amplified fragment length polymorphism data (circles) and quantiles. Upper (top line) and lower (bottom line) 99% and median (middle line) quantiles were calculated using simulated data diverging via drift alone using the program dfdist2 (Beaumont & Nichols 1996).

Appendix 2.A: Subspecies, voucher numbers, and GenBank accession numbers for cyt *b*, ND2, and AFLP data. All voucher numbers are University of Alaska Museum (UAM) unless otherwise noted.

| Voucher numbers | cyt *b* | ND2 |
|---|---|---|
| *A. a. acadicus* | | |
| 5488[§], 5851[§], 6500, 6501, 6901, 6904, 8990, 8989, 9180, 9181*, 9277[‡], 13949, 13995, 13996, 14940, 15184[‡], 17882, 17883, 17953[‡], 17954[‡], 17955[‡], 17957, UWBM68205, UWBM79081, UWBM67190, UWBM67021* MVZ181707[†] | EU075383-EU075387 EU075398-EU075412 EU601051, KC620183 | KC620150-KC620168 |
| *A. a. brooksi* | | |
| 10153, 10154, 19042, 19472, 19473[‡], 19474, 19479, 19481-19484*, 19485, 26388-26390, 27886-2789, 27891-27896 | EU075388-EU075397 KC620169-KC620182 | KC620126-KC620149 |

[§] denotes individuals for which only cyt *b* data were used.

* denotes individuals for which no AFLP data were generated.

[‡] denotes individuals for which only AFLP data were generated.

[†] denotes individual for which only ND2 data were used.

Appendix 2.B: Amplified fragment length polymorphism scoring data for *Aegolius*. Species, locations, and accession number acronyms can be found in Appendix 2.A.

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UAM 15184

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UAM 17953

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UAM 17955

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Appendix 2.B continued.

UAM 13995

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UAM 17954

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UAM 6500

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UAM 6501

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UAM 6901

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UWBM 67190

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Appendix 2.B continued.

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UWBM 79081

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UAM 27887

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UAM 27889

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UAM 27888

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UAM 27896

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Appendix 2.B continued.

UAM 27894

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UAM 27893

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UAM27895

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UAM 27886

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UAM 27891

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# GENERAL CONCLUSION

My study has shown that *P. dominica* and *P. fulva*, despite their recent elevation to species status and extreme phenotypic similarity, are a good example of high-latitude cryptic speciation, having diverged quite long ago. Despite this relatively deep divergence and strong evidence from mtDNA that gene flow has been very low historically, they appear to hybridize at a low frequency. The level of divergence and low gene flow (almost producing reciprocal monophyly in ND2) provide little evidence of speciation with gene flow, although their current natural history and range made this a speciation event likely to involve such a process.

Genetic characterization of the endemic Queen Charlotte Island saw-whet owl (*A. a. brooksi*) showcases a recent case of high-latitude island endemism. I have provided evidence that this subspecies pair is distinctive in both mtDNA and in the nuclear genome and is probably experiencing little to no gene flow with mainland populations despite cyclic sympatry of the two forms. The likely cause of their divergence is heteropatric differentiation, driven largely by a loss of migratory activity in *brooksi* and perhaps other divergent selection (e.g., in timing of breeding) that limits gene flow.