A MORPHOLOGICAL AND GENETIC INVESTIGATION OF THE HIGHEST-LATITUDE ENDEMIC PASSERINE: MCKAY'S BUNTING

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ABSTRACT

I used two different approaches to investigate different aspects of the highest latitude endemic passerine, McKay's Bunting (*Plectrophenax hyperboreus*). I tested whether or not the juvenal plumage of McKay's Bunting is different from its closest relative, Snow Bunting (*P. nivalis*). Using light reflectance spectrophotometry to quantify visual differences, I found that McKay's and Snow buntings have significantly different juvenal plumages. This analysis supports their separation into two distinct species. Second, I investigated the genetic consequences of refugial isolation and the model of speciation that the genetic data fit. This species pair provides an excellent opportunity to investigate the genetic effects of speciation at high latitudes in a region known to be significantly impacted by Pleistocene climatic oscillations. Using a mitochondrial marker and anonymous nuclear markers, I found evidence for recent divergence and a very small founding population size of McKay's. After the founder event, there is evidence of a population expansion and a subsequent reduction of the McKay's population, probably as a result of rising sea levels and asymmetric hybridization into Snow Buntings postglacially colonizing Beringia. This recent, high latitude speciation event fits a model of founder effect peripatric speciation driven by a small founding population size and genetic drift.

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GENERAL INTRODUCTION

This thesis investigates the highest-latitude endemic songbird, McKay's Bunting, in relation to its closest relative, Snow Bunting. My advisor, Dr. Kevin Winker, went to St. Matthew Island, the sole breeding range of McKay's Buntings, in the summer of 1997 and collected a series of McKay's Buntings, taking tissue samples and preparing the birds as museum study specimens. This series is easily the largest tissue and skin collection of this species in the world, certainly from their breeding grounds. With this comparative wealth in study materials, I seized the opportunity to study this little-known species in relation to Snow Buntings, which are found in suitable habitat on both the Alaskan and Russian mainlands and all surrounding islands in the Bering Sea region.

First, because the juvenal plumage, or first plumage after downy, had never been described as different, I assembled nearly all existing skins in this plumage for analysis. This plumage is also considered to be conservative, as it is worn for a brief time, and is not heavily influenced by sexual selection. Once I had assembled a large series of juvenal-plumaged Snow and McKay's buntings, I noticed clear "average" differences between the two species. Because plumage color differences are often considered subjective, I wanted to quantify these differences and statistically test the hypothesis that these two species have the same juvenal plumage. I used a light reflectance spectrophotometer to measure color and light/dark characters for each specimen on the back and throat. I took 15 total measures per skin, and then averaged these measures to get single values of red to green, blue to yellow, and light to dark, for both the throat and the back. I then ran statistical analyses and determined that they were indeed significantly

different, and I could reject the null hypothesis. I also ran a discriminant analysis to determine if these characters could be used to separate the two populations and found that they could be separated with 100% accuracy. This previously undescribed difference in juvenal plumage is a useful character for determining species limits, and this method should be used in future studies when comparing closely related species if adult basic and alternate plumages are similar.

The second part of my study investigates the genetics of the speciation history of McKay's Buntings in relation to Snow Buntings. I chose to sequence a mitochondrial gene in 40 McKay's Buntings and eight Snow Buntings, adding these data to an additional 32 sequences of Snow Buntings available on GenBank. The gene I chose, cytochrome *b* (cyt *b*), is a well understood marker and allowed me to make a number of inferences about the population history of both species in relation to each other. I also used an anonymous nuclear marker system, Amplified Fragment Length Polymorphisms (AFLPs), to infer the most likely number of populations given the sampling strategy and the data. I also tested for natural selection or genetic drift driving the divergence between the two species. I determined that these two species are genetically distinct and have diverged very recently, probably during the Last Glacial Maximum (LGM). I also found evidence using coalescent analyses and overall lower genetic diversity that points to very few individuals from the ancestral population becoming isolated in Beringia and founding the McKay's population. I found evidence as well that genetic drift likely drove the divergence between the two species, not strong selection. I found evidence that the McKay's population expanded after the founder event and subsequently reduced in size,

a change that appears to coincide with changes in postglacial Beringia that included hybridization with Snow Buntings and rising sea levels increasingly restricting the amount of available habitat in the vicinity of St. Matthew Island. This evidence provides insight into the processes of rapid high-latitude speciation, especially in light of Pleistocene oscillations in available habitat and refugial isolation.

This study using morphology and genetics on the highest-latitude endemic songbird provides the characterization of a previously undescribed plumage stage and also uncovers some of the genetic underpinnings of rapid speciation driven by vicariance at high-latitude.

Chapter 1: The utility of juvenal plumage in diagnosing species limits: an example using *Plectrophenax* buntings[1]

1.1 Abstract

Species limits in *Plectrophenax* buntings have been difficult to assess. McKay's Buntings (*Plectrophenax hyperboreus*) are very similar both morphologically and behaviorally to Snow Buntings (*P. nivalis*). However, their breeding ranges are allopatric, and there is evidence of limited g[1]ene flow. The juvenal plumage of McKay's Buntings has never been described as different from the Snow Bunting's juvenal plumage. Comparison of series of McKay's and Snow buntings in juvenal plumage showed clear differences between the two species. We used color spectrophotometry to quantify the differences between the two taxa in two areas that appeared to be consistently different, the throat and back. The relative magnitude of the difference between McKay's and Snow buntings was greater than homologous differences between two subspecies of Snow Buntings (*P. n. nivalis* and *P. n. townsendi*). Four of six light reflectance variables were significantly different between McKay's and Snow buntings, whereas none of the variables were significantly different between the two subspecies of Snow Buntings. Bonferroni corrected *t*-tests showed that potential sexual dimorphism did not significantly bias our results. Regression of the variables against year of collection showed that fading or foxing did not introduce significant bias in our analyses. Discriminant analysis accurately separated 100% of the specimens into their respective species. These differences are notable given the evolutionarily conservative nature of juvenal plumage.

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[1] Authors: J. M. Maley and K. Winker, accepted pending minor revision at The Auk.

Our results support continued recognition of McKay's Buntings as a species and reconfirm the utility of juvenal plumage to help determine species limits.

1.2 Introduction

In closely related forms that have distinct but subtly different adult characters, juvenal plumage differences can be a useful indicator of species limits. Phillips and Dickerman (1965) argued that in certain instances juvenal plumage is just as useful, if not more so, than adult plumages for diagnosing taxon limits and evolutionary affinities, especially in taxa that have extremely similar adult plumages. In one species of passerine, consistent juvenal plumage differences in the complete absence of adult differences were been used to separate forms into subspecies (Phillips and Dickerman 1965). The primary reason for this utility is the evolutionarily conservative nature of the juvenal plumage. There are several reasons that juvenal plumage is more evolutionarily conservative than adult plumages. First, it is generally worn for a brief period, but selection at this stage is likely to be strong to maintain cryptic plumage for avoiding predator detection. There is also probably a complete lack of sexual selection on this plumage, because birds molt out of juvenal plumage well before reaching sexual maturity. Finally, there are clear examples of this evolutionary conservatism in passerines such as among the Turdidae, in which spot-breasted juvenal plumage is essentially ubiquitous, even among species in which adults have no breast-spotting. The evolutionarily conservative nature of juvenal plumage makes it a potentially useful source of characters for determining species limits, especially in groups of birds that are closely related or have subtle adult plumage differences.

Phillips (1969) demonstrated that the juvenal plumages of *Catharus occidentalis* and *C. frantzii*, two sympatric species that are difficult to distinguish as adults, show distinct differences, enough to support the recognition of these two forms as distinct species. Shortt (1951) noted that juvenal plumages of the two North American *Anthus* species are much more distinct than adult plumages. Some families are characterized by evolutionarily conservative juvenal plumage, and within these families this plumage can have characters that are unique and independent of adult plumages (e.g., Turdidae, Traylor 1972; and Emberizidae, Graber 1955, Paynter 1964). This previous work has demonstrated the usefulness of juvenal plumage for determining species limits in closely related forms of passerines. However, this work analyzed juvenal plumages qualitatively, not quantitatively as we have done here.

Species limits in *Plectrophenax* buntings have long been contentious. The American Ornithologists' Union (AOU 1998) recognizes two species within the genus: McKay's Bunting (*P. hyperboreus*) and Snow Bunting (*P. nivalis*). Both species breed at high latitudes. The Snow Bunting is distributed holarctically, whereas McKay's Bunting breeds allopatrically from the Snow Bunting on two islands in the Bering Sea, St. Matthew and Hall islands. McKay's Buntings have been discussed as a strongly demarcated subspecies on the basis of plumage similarity and evidence of hybridization (Sealy 1969; see Paynter and Storer 1970). Other authors have referred to McKay's Buntings as a subspecies of Snow Buntings without explanation (Salomonsen 1931, Vaurie 1959). These treatments of species limits in *Plectrophenax* have never resulted in the submersion of *hyperboreus* into *nivalis* by the AOU (1957, 1983, 1998), but the

majority of these publications stressed the need for further research. Indeed, the adult plumage characteristics of the two taxa enabling diagnosis are subtle outside of a single striking difference between adult males: white back in *hyperboreus* and a black back in *nivalis*. Other than slight and variable differences in the extent of black on the wing, tail, and crown, females are only easily separable in basic plumage, when they are considerably lighter. Evidence of hybridization (Sealy 1969), coupled with recognized plumage differences that are no more pronounced than among many passerine subspecies, leaves open the question of species limits.

The original description of McKay's Bunting (Ridgway 1884) was based on two adult males and two adult females in basic plumage, which were collected on the wintering grounds of western Alaska. The juvenal plumage of McKay's Buntings has only been marginally discussed in the literature and usually on the basis of little or no information. The first mention came from Ridgway (1901, pg. 153). He noted, "*young* very similar to that of *P. n. townsendi*, and not with certainty distinguishable" (original italics). He made no mention of the specimens used for this statement, but in an earlier publication (Ridgway 1886) he noted the first juvenal-plumaged specimen collected on Hall Island in 1885 by C. H. Townsend and mentioned his imminent description of this specimen. But this was apparently never published; he made no other mention of juvenal-plumaged buntings until 1901. Lyon and Montgomerie (1995) purportedly described the juvenal plumage of *hyperboreus*, but they confused a source (cited as Anonymous 1980, but here cited as Arbib 1980) as reporting on juvenal-plumaged McKay's, but this source described *juvenile* plumage (which we recognize as first basic), not *juvenal* plumage, as

suggested (for definition see Eisenmann 1965). The specimens used for Arbib (1980) were collected in Nome and are deposited at the University of Alaska Museum (UAM); they are in first basic plumage.

Here we conduct a thorough analysis of the juvenal plumages of McKay's and Snow buntings. We contrast differences between currently recognized subspecies of Snow Buntings (described based on adult measurements) and the relative differences between McKay's and Snow buntings. We also test for other factors that could be influencing our analyses (e.g., year specimen was collected and sexual differences). We quantify observed visual differences between the juvenal plumages of these two forms using reflectance spectrophotometry and demonstrate that they can be quantitatively and confidently separated. We also demonstrate the utility of juvenal plumage as a character that can be used to define species limits within this group by applying classic concepts (Mayr 1969) on relative differentiation between forms.

1.3 Methods

Fifty juvenal-plumaged bunting specimens were assembled for our spectrophotometric analyses. All 20 McKay's Buntings were collected on St. Matthew Island, Alaska. The 30 Snow Buntings were from a variety of locations in Alaska and Canada (see Appendix 1.A). Individuals were selected for analysis only if they clearly retained the majority of their juvenal plumage (determined by visual comparison with birds in first basic plumage) and possessed unbroken juvenal plumage on the back and throat. Most specimens (48) used in the analysis were collected during July or August,

with just two collected in early September. Specimens ranged from nestlings with some downy feathers to fledged birds that had just begun their first prebasic molt.

Spectrophotometry has been used to quantify subtle plumage differences in a variety of taxa (e.g., Graves 1997, Winker 1997). We used the Colortron™ II Digital Color Ruler (Light Source Computer Images, Inc., San Rafael, California) to obtain reflectance measurements. This instrument has been demonstrated to quantify subtle plumage differences accurately (Hill 1998, 2000, McGraw and Hill 2001). Colors are measured precisely with a 3×3 mm measurement aperture, and the quantified values then compared objectively using the colorimeter function in the accompanying Colorshop™ software (Light Source Computer Images, Inc., San Rafael, California). The software can output a variety of variables describing color. We followed Graves (1997) and Winker (1997) in choosing the set that most accurately represents light/dark and color: CIE Lab (Light Source 1996). Three variables were obtained for each measure, L (dark-to-light), a (red-to-green) and b (blue-to-yellow). The L value represents light reflectance on a scale from 0 to 300 (0 = no light reflected, 300 = all light reflected), and the a and b values are chromaticity coordinates on axes with scales from -300 to 300 (Light Source 1996, Graves 1997).

Visual examination of specimens suggested that the back and throat plumage were the most consistently different between *hyperboreus* and *nivalis*. Light reflectance spectrophotometric analysis was conducted on these two areas. On the back, three areas (upper, middle, and lower) were chosen and reflectance values averaged to minimize the influence of streaking and to obtain a more robust value per specimen (Graves 1997, Hill

1998). Each specimen was placed against the aperture, measured, and then it was removed and placed back against the aperture for each successive measurement. Three sequential measurements were made for each area, giving a total of nine measures of the back per specimen. The throat was more uniform than the back, but to incorporate light streaking six sequential measurements were made, again removing and repositioning the specimen for each measurement. Specimens were chosen at random regardless of species. All measurements were made on the same day under uniform conditions.

We made 750 measurements total on the 50 specimens, resulting in a total of 2,250 values in the data set (3 values per measurement). Measurements were then averaged to give six values per specimen: back L, a, and b and throat L, a, and b. We then tested for normality within the samples. The values were classified into two separate groups representing each species. Levene's test for equality of variances was conducted first to determine the appropriate assumption of variance for t-tests (Brown and Forsythe 1974). Independent sample t-tests, assuming either equal or unequal variance depending on the results of Levene's test, were conducted for each pair of values using SPSS 13.0 (SPSS Inc., Chicago, Illinois) to test for differences between the two species. In all cases using multiple tests, we used Bonferroni correction to maintain an experimentwise $\alpha = 0.05$ (Johnson and Wichern 1988, Beal and Khamis 1991).

Snow Bunting specimens were then classified into two currently recognized subspecies, determined by the collection locality and the last AOU checklist to treat subspecies (American Ornithologists' Union 1957). Because our specimens were all in juvenal plumage and not fully grown, we used geographic range to classify the

subspecies, which were described based on plumage and morphometric characteristics of adults only. We examined specimens of *P. n. nivalis* ($N = 16$) and *P. n. townsendi* ($N = 14$). Levene's test and independent sample *t*-tests were again conducted to test for differences. To help visualize the relative difference between species and between subspecies, we plotted the three most significant variables between McKay's and Snow buntings and the mean value per form on a 3-D scatterplot for both the species-level and subspecies-level comparisons using SPSS 13.0 (SPSS Inc., Chicago, Illinois).

Because sexual dichromatism and/or foxing could bias our results, we tested for these effects. Specimens of some species are known to change color over time, whether through fading or foxing (for definitions see Gabrielson and Lincoln 1951). Levene's test and independent sample *t*-tests were used to test for sexual differences both within subspecies and species, and then we combined all of the specimens to test for overall sexual differences. We also regressed reflectance variables against the year the specimen was collected using linear regression.

We first tested for significant differences between the two species, then, to determine how well the reflectance characters separated these taxa, a discriminant analysis was conducted using SPSS 13.0 (SPSS Inc., Chicago, Illinois). Discriminant analysis is a useful statistical tool for determining the ability with which overlapping characters can be used to separate groups (e.g., Mayr and Ashlock 1991, Winker 1997, Figuerola et al. 1999). Analyses were conducted using both equal prior probabilities and probabilities calculated using group size.

1.4 Results

Our first null hypothesis was that there is no difference between the juvenal plumages of McKay's and Snow buntings. We determined that the data were normally distributed using the Shapiro-Wilk W test for normality (Shapiro and Wilk 1965) after multiple test correction (no $P < 0.012$). When compared visually the juvenal plumages of these two species appear to have clear "average" differences in the shades of throat and back plumage (Fig. 1.1), with McKay's being overall lighter. Light reflectance spectrophotometry revealed overlap in every measured character (Table 1.1). However, on average McKay's Buntings were significantly lighter than Snow Buntings on the back and the throat (Table 1.1, variable *L*). They were also a significantly different shade on one axis of color for the throat (Table 1.1, variable *a*), and on one axis of color on the back (Table 1.1, variable *b*). We thus reject the null hypothesis; the juvenal plumages of McKay's and Snow buntings are significantly different. A three-dimensional multivariate plot of three variables showed that the degree of difference between McKay's and Snow buntings was greater than that between the two subspecies of Snow Buntings (Fig. 1.2).

To compare the level of differentiation between McKay's and Snow buntings with the level of differentiation between the two subspecies of Snow Buntings sampled, we hypothesized that nominate *nivalis* was not significantly different from *P. n. townsendi.* There were no differences among the 6 variables examined, although for both the throat and the back, *a* was significantly different between the two subspecies before multiple test corrections (Table 1.2, variable *a*). This difference was not consistently

evident visually. Here, we cannot reject the null hypothesis that these two subspecies are not different.

We also tested for sexual dichromatism within McKay's Buntings on the back and throat. We also tested for sexual dichromatism within Snow Buntings, and then we combined and analyzed all specimens grouped by sex. There was no sexual dichromatism in any analysis (before or after Bonferroni-correction of α), in throat or back plumage (Table 1.3). Here, we cannot reject the null hypothesis that there are no sexually dichromatic differences in the measured aspects of the juvenal plumage of *Plectrophenax* buntings.

Regression of the reflectance variables against the year of specimen collection showed that only one of the six variables had a significant relationship with the year the specimen was collected. Variable *a* on the back was significantly positively correlated with specimen age after Bonferroni-correction of α ($F = 14.598$, $P = 0.0004$; all other values $F < 3.5000$, $P > 0.0670$). Upon visual examination of the specimens, it appears that slight foxing may account for this relationship. This character was not one showing a difference between McKay's and Snow buntings (Table 1.1).

Discriminant analysis was able to classify 100% of the specimens into the correct species group using all six variables. The analysis was conducted not only using prior probabilities computed from group size but also assuming all group sizes equal; both yielded 100% correct classification. Cross-validation (removing an individual and classifying it based on coefficients calculated from the rest of the individuals) yielded 92% correct classification. This supports the contention that McKay's and Snow buntings

are consistently different in juvenal plumage. If they were not different we would expect a considerable number to be misclassified.

1.5 Discussion

The juvenal plumages of McKay's and Snow buntings are significantly different, both visually and quantitatively using reflectance spectrophotometry. Visually, on the back, Snow Buntings appear dark gray, whereas the McKay's Buntings are light brown/gray. On the throat, this difference appears to be caused by a lack of dark pigment in most McKay's Buntings, whereas Snow Buntings generally have a dark bib that is gray with buff feather edges.

Two factors could potentially bias our results, sexual dichromatism and plumage changes over time (e.g., foxing). Several species that have sexually dichromatic adult plumages also exhibit sexual differences in juvenal plumage (Graber 1955). *Plectrophenax* buntings have sexually dichromatic adult plumages, which are obvious in the remiges and rectrices of birds in first basic plumage, but we determined that sex did not bias the analysis of the throat and back juvenal plumage. Slight foxing did not bias our results, because each species shared an equal proportion of older specimens; 20% of the specimens of each species were collected over 100 years ago. Further, both between the two species and in the subspecific comparison, this variable (a on the back) was not significant after multiple-test adjustment of α (Tables 1.1, 1.2). Thus, neither sexual differences nor foxing in the throat and back plumage contributed to the differences observed.

Analyses of subspecific differences within the Snow Bunting provided a comparison of the level of differentiation between subspecies and putative species in *Plectrophenax* buntings, and showed that one set of differences (McKay's vs. Snow) was strong and consistent, in contrast to homologous, nonsignificant (or at best very slight) differences between the Snow Bunting subspecies *nivalis* and *townsendi* (Fig. 1.2). Mayr (1969) discussed the clustering of forms and the relative positions of the means within those clusters as an accurate way to judge relationships between taxonomic groups. He described differences as being scalar from higher to lower taxonomic rank, and that the best way to judge species limits within a group is to compare the differences between taxonomic ranks. Based on his reasoning and our data showing pronounced and consistent differences in several characteristics of juvenal plumage (Fig. 1.2), we consider that McKay's Buntings and Snow Buntings are different species.

Juvenal plumage is largely overlooked in systematic studies of closely related species. Because juvenal plumage is worn for such a brief time, specimens in this plumage tend to be rather rare in collections. Our results suggest that it can be a useful tool for examining species limits, and an effort should be made to fill this collection gap. If juvenal plumage varies by population, we would expect recognized subspecies to show differences, but not if generally conserved. By analyzing this plumage between subspecies and putative species we can obtain a relative scale for defining species limits using the associated informative characters. Quantifying these differences using spectrophotometry allows for rigorous statistical analyses of differences and potential biases. Analysis of juvenal plumage in other groups should also prove useful in

determining species limits, especially when adult plumage characters are slightly different or equivocal between populations.

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Table 1.1. Statistical summary of reflectance values (*L*, *a*, and *b*) from two body regions on specimens of juvenal-plumaged McKay's and Snow buntings.

		McKay's Buntings (20)			Snow Buntings (30)				
Character		Mean	SD	(Min. – Max.)	Mean	SD	(Min. – Max.)	t^a	*P*
Throat	*L*	47.98	4.83	(38.85 – 55.80)	41.87	4.4	(34.48 – 51.61)	-4.63	**<0.0005**
	a	-1.46	0.63	(-2.81 – -0.48)	-0.97	0.6	(-2.29 – 0.39)	2.78	**0.0077**
	b	6.52	1.63	(2.92 – 8.62)	6.32	2.55	(3.02 – 11.09)	-0.30	0.7623
Back	*L*	38.38	3.27	(33.97 – 46.19)	33.38	1.9	(29.09 – 37.60)	-6.17^b	**<0.0005**
	a	-0.26	0.49	(-1.03 – 0.67)	0.04	0.43	(-0.59 – 0.92)	2.28	0.0269
	b	7.02	1.34	(5.04 – 9.77)	4.92	1.55	(2.34 – 8.27)	-4.94	**<0.0005**

[a] *t*-value and associated *P*-value from an independent-samples *t*-test. Bonferroni-corrected $\alpha = 0.0083$.

[b] Case where the two species' variances were significantly different based on Levene's test; the *t*-test was performed assuming unequal variance.

Table 1.2. Statistical summary of reflectance values (*L*, *a*, and *b*) from two body regions on specimens of juvenal-plumaged Snow Bunting subspecies.

		P. n. townsendi (14)			*P. n. nivalis* (16)				
Character		Mean	SD	(Min. – Max.)	Mean	SD	(Min. – Max.)	t^a	*P*
Throat	*L*	42.71	4.68	(35.57 – 51.61)	41.14	4.14	(34.48 – 48.66)	0.97	0.3395
	a	-1.20	0.49	(-2.29 – -0.6)	-0.77	0.63	(-2.06 – 0.39)	-2.06	0.0492
	b	5.80	2.57	(3.02 – 10.73)	6.78	2.53	(3.67 – 11.09)	-1.06	0.2983
Back	*L*	33.97	1.44	(30.83 – 35.85)	32.86	2.13	(29.09 – 37.60)	1.64	0.1128
	a	-0.14	0.38	(-0.59 – 0.57)	0.20	0.42	(-0.55 – 0.92)	-2.38	0.0243
	b	4.67	1.55	(2.34 – 7.30)	5.13	1.57	(2.36 – 8.27)	-0.79	0.4337

[a] *t*-value and associated *P*-value from an independent-samples *t*-test.

Bonferroni-corrected $\alpha = 0.0083$.

Table 1.3. *P*-values of independent sample *t*-tests testing for sexual dichromatism. Tests conducted on the plumage color of *Plectrophenax* buntings. Specimens that were unsexed were excluded from the analyses.

		Character					
		Throat			Back		
species	N	*L*	*a*	*b*	*L*	*a*	*b*
P. hyperboreus	27	0.3763	0.1660	0.8602	0.8635	0.1406	0.9209
P. nivalis	17	0.2154	0.5665	0.7316	0.7793	0.3663	0.8619
combined	44	0.3505	0.2845	0.7214	0.6096	0.1729	0.8257

Bonferroni-corrected $\alpha = 0.0083$.

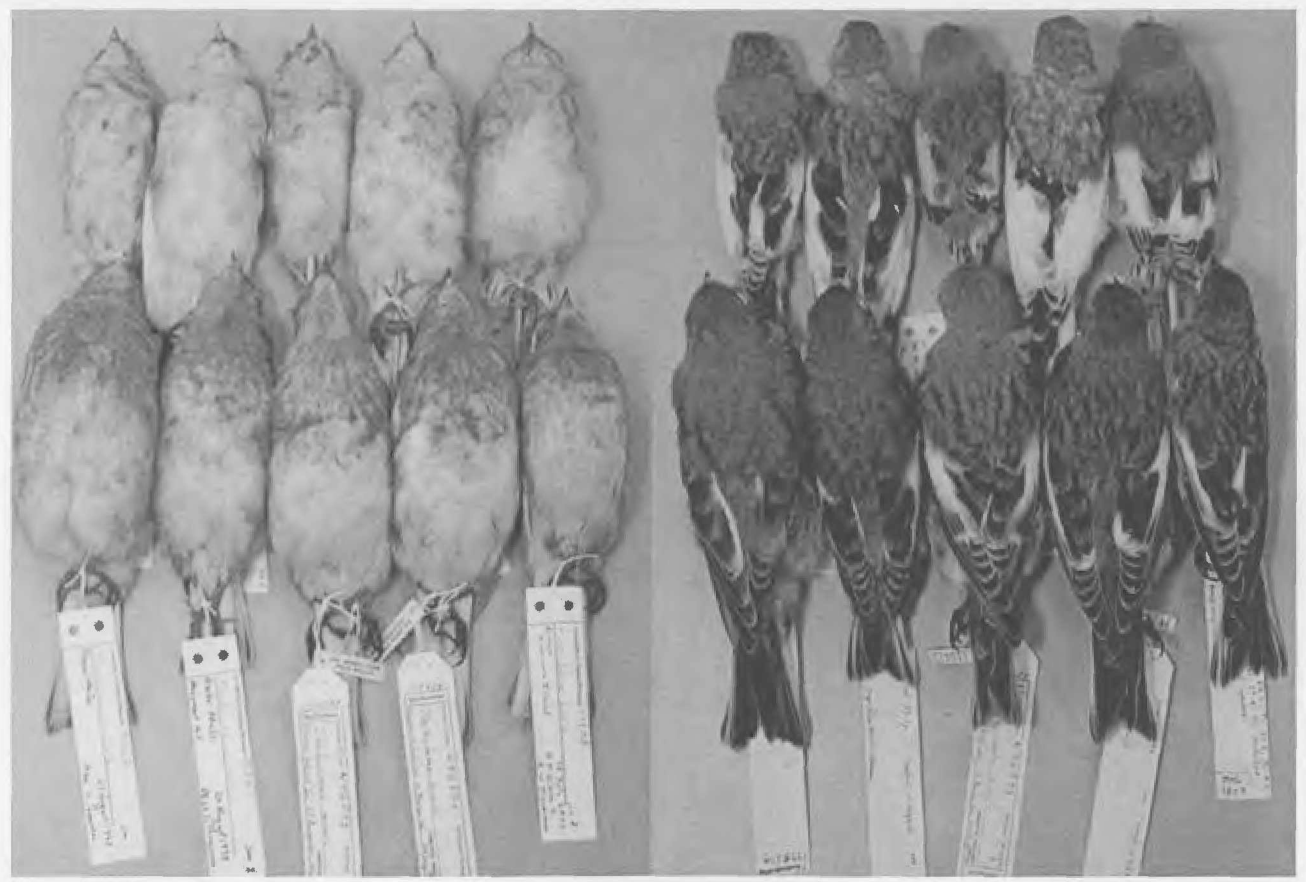

Figure 1.1 Image comparing McKay's and Snow buntings in juvenal plumage. The top five in each photo are McKay's Buntings, the bottom five are Snow Buntings. Note that McKay's are consistently lighter. The Snow Buntings are larger because they were collected on average several days older than the McKay's Buntings, but they are all in juvenal plumage.

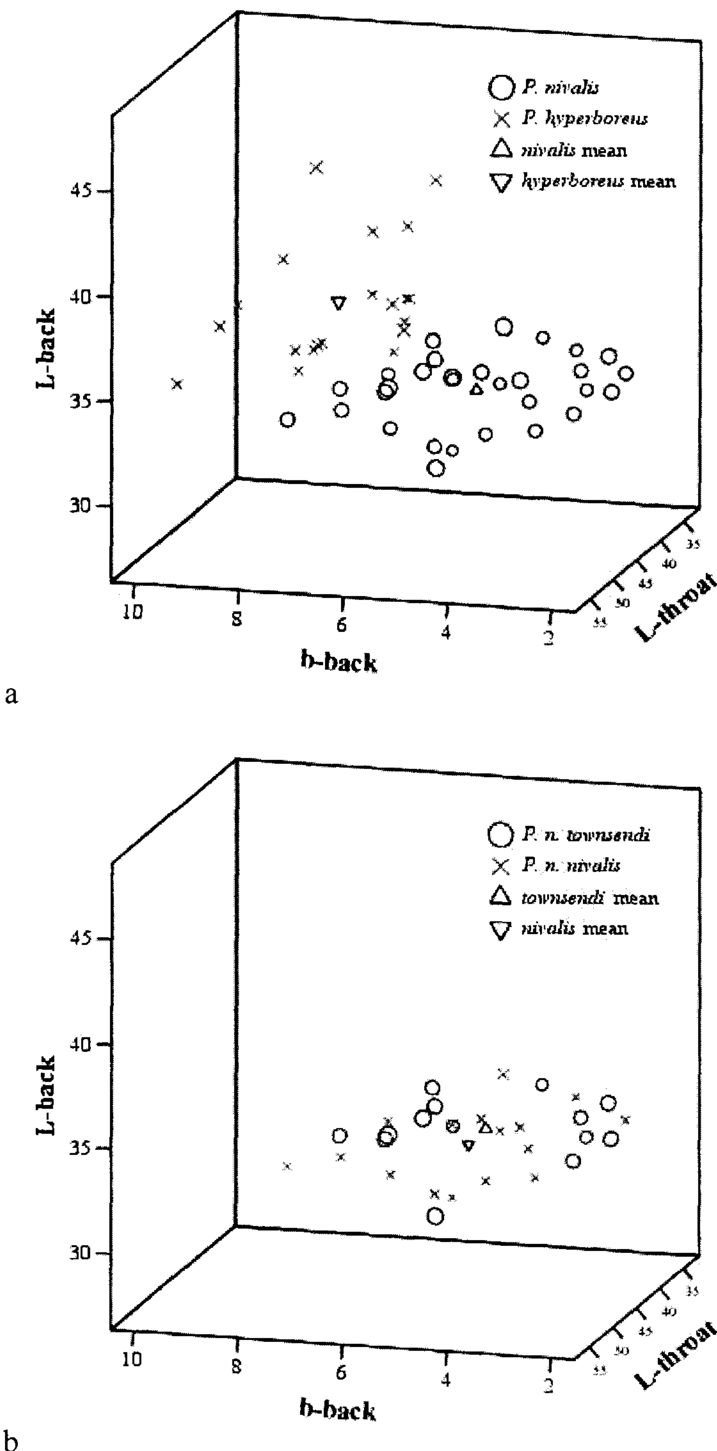

Figure 1.2 Two 3-D scatter plots showing the difference between clusters. Included are species (a) and subspecies (b) and relative positions of means within those clusters.

Appendix 1.A. Museum specimens used.

Species & locality	Specimen voucher number[a]
Plectrophenax nivalis	
Aleutian Islands	UAM 7275, 8425-8428, 8430, 13180.
Pribilof Islands	UAM 8478, 18516, 20644, 20645, 20723.
	USNM 496873, 496874.
Nunivak Island	UAM 11095, 11128, 11129.
Point Barrow	USNM 93114, 93117, 93118.
Canadian Arctic Archipelago	USNM 161877, 161879, 377129, 399703, 399704;
	401098, 401100, 423059, 423060, 572745.
Plectrophenax hyperboreus	
St. Matthew Island	UAM 4888, 11094-11096, 17490, 18042, 20646;
	20647, 20649-20651.
	USNM 164885, 164887, 595515, 595524-595526.
Hall Island	UAM 20648; USNM 164889, 164890.

[a] UAM = University of Alaska Museum; USNM = U. S. National Museum.

Chapter 2: Speciation at high latitudes: evidence for founder effect speciation and neutral divergence between snow and McKay's buntings[1]

2.1 Abstract

McKay's bunting, (*Calcarius hyperboreus*) is the highest latitude endemic songbird, and its global range is restricted to the region known as Beringia. Its closest relative, the snow bunting (*C. nivalis*) is Holarctic in its distribution and breeds in tundra habitat surrounding the island breeding range of McKay's bunting. This species pair provides an excellent opportunity to investigate the genetics of speciation at high latitudes in a region known to be significantly impacted by Pleistocene climatic oscillations. We studied 40 individuals of each species (80 individuals total) from Beringia using 1123 bp of mtDNA sequence (cyt *b*); we also analyzed a total of 1000 loci generated using amplified fragment length polymorphisms (AFLPs) for 57 of these individuals (32 McKay's and 25 snow buntings). There are six species in the genus, we also included two individuals of the other four members of the genus as an outgroup. Mitochondrial DNA data showed a high overlap of haplotypes, but population genetic differentiation estimates showed low but nonetheless significant differences between the two bunting species (cyt *b* $F_{ST} = 0.0784$; AFLP $F_{ST} = 0.0448$). AFLP data suggested that the most likely number of populations involved was two, and only one individual from each taxon was misassigned. Coalescent analysis of mtDNA data suggested that the two buntings diverged very recently (~13,000 to ~80,000 ybp), during the las[2]t glacial maximum. There appeared to be asymmetric gene flow, with substantially more gene

[2] Authors: J. M. Maley and K. Winker, currently being prepared for Evolution.

flow from *hyperboreus* into *nivalis*. Coalescent analysis also estimated that just one to six females founded the *hyperboreus* population. Effective population size estimates were roughly consistent with reported demographic data values, although they were rather high in *hyperboreus*. Effective population size estimates and a mismatch distribution support a founder event in *hyperboreus* followed by population expansion. The broad genome sweep yielded only five AFLP loci that appeared to diverge in a manner consistent with selection, a value lower than expected by chance, suggesting that this divergence was driven by genetic drift. Morphological divergence between the two species, apparently limited to plumage characters, appears to have occurred rapidly. After the founder event, there is evidence of a population expansion, followed by a reduction of the McKay's population, which coincides with rising sea levels as well as asymmetric hybridization with snow buntings following this species' postglacial colonization of Beringia. This recent, high-latitude speciation event best fits a model of founder effect peripatric speciation driven by a small founding population size and genetic drift.

2.2 Introduction

Differentiation in allopatry has long been considered the dominant process of speciation (Jordan 1905; Mayr 1942, 1963; Coyne and Orr 2004). This putative mode of speciation is especially relevant to diversification at high latitudes during the Quaternary through vicariance events caused by repeated glacial cycles, creating the mechanism for population isolation and divergence in glacial refugia (Hewitt 1996, 2000; Stewart and Lister 2001; Taberlet and Cheddadi 2002). The genetic effects of refugial isolation and population expansion and contraction have been explored in an array of taxa, using a

variety of molecular markers (e.g., Zink and Dittmann 1993; Cooper et al. 1995; Santucci et al. 1998; Runck and Cook 2005). With the advent of fine-scale genetic markers, complex modeling systems, and detailed climate history we can attempt to uncover the genetic signal of recent large-scale vicariance, postglacial population expansion, differentiation in the absence of morphological differences, infer population histories of affected biota, and put all of these into a geoclimatic context (Hewitt 2000).

The expansive lowlands of east of the Lena River in northeastern Russia and southwestern, central, and northern Alaska and the shallow continental shelf and islands of the Bering Sea are collectively known as Beringia (Hopkins 1967). This area was subjected to massive change during the Wisconsin glaciation (10,000 – 117,000 ybp) due to changes in sea level, and, while isolated from the rest of North America by ice sheets, remained ice free (Hamilton et al. 1986; Williams et al. 1998). In Beringia, different signals of population divergence, contraction, extinction, expansion, and origins of postglacial colonists have emerged among a broad spectrum of taxa hypothesized to have been isolated in the region (Tremblay and Schoen 1999; Flagstad and Røed 2003; Eddingsaas et al. 2004; Galbreath and Cook 2004; Shapiro et al. 2004a; Alsos et al. 2005; Loehr et al. 2005; Pruett and Winker 2005; Van Houdt et al. 2005). Despite these different signals, all of these studies share a common thread: the organisms that lived in Beringia during the last glacial maximum and the organisms that live there today were all significantly impacted by major climate change affecting the region.

The *Calcarius* buntings and longspurs represent a clade of comparatively high-latitude origin in the family Emberizidae (Klicka et al. 2003). They are currently

recognized as two genera by the American Ornithologists' Union (1998), but because the genus *Calcarius* is paraphyletic, with *Plectrophenax* nested within, we follow Klicka et al. (2003) in recognizing it as a single, monophyletic genus. The two buntings, *C. hyperboreus* and *C. nivalis*, are the least differentiated of the clade, both morphologically and genetically (Lyon and Montgomerie 1995; Klicka et al. 2003). The two have allopatric breeding distributions: *hyperboreus* breeds only on two small islands, St. Matthew and Hall islands in the Bering Sea, and *nivalis*, in the region, breeds on every other major island in the North Pacific and along the entire Russian and Alaskan Bering Sea coast (Fig. 2.1, Paynter and Storer 1970; Gibson and Kessel 1997; Winker et al. 2002). The *hyperboreus* population is an island population in two senses: it is a small population endemic to St. Matthew and Hall islands, and an island of morphologically different buntings allopatrically breeding entirely within the range of *nivalis*. The wintering range of *hyperboreus* is restricted to the western coast of Alaska; *hyperboreus* is entirely encapsulated within Beringia. In contrast *nivalis* typically migrates much farther south (Lyon and Montgomerie 1995).

As with any allopatric species, it is difficult to assess whether these two taxa are reproductively isolated, especially given limited evidence for secondary contact. There are reports of male *hyperboreus* occurring on islands peripheral to their breeding range and hybridizing with *nivalis* (Sealy 1967, 1969). Interestingly, *nivalis* has been found to be common on St. Matthew Island early in breeding season, with most individuals leaving before the fledging period, but only one pair of *nivalis* has ever been recorded on

the island during fledging (Winker et al. 2002). Lack of such data may reflect lack of study on this remote island complex.

This apparently recently diverged species pair represents an excellent system in which to examine the nature of high latitude speciation in an area known for its dynamic vicariant history. Our first goal was to determine whether these two species are genetically distinct. Our second goal was to estimate how recently they diverged, as this is critical to determining the model of speciation that best explains what occurred between this taxon pair. Finally, we wanted to investigate key aspects of the mechanisms of speciation at high latitude, e.g., evidence for a founder event followed by population expansion, and whether this particular example of divergence is accompanied by the genetic footprints of drift or selection.

2.3 Materials and methods

To achieve the goals set out in this study we used two types of molecular data, sequence from the mitochondrial gene cytochrome *b* (cyt *b*) and amplified fragment length polymorphisms (AFLPs; Vos et al. 1995). Klicka et al. (2003) sequenced cyt *b* and found low-level differentiation consistent with recent speciation (0.18% pairwise divergence); we decided to use the same marker with a larger sample size for population genetic analyses. The advantage of using cyt *b*, as opposed to the more variable control region (Peters et al. 2005), is that this gene is a well-understood marker in birds, especially passerines, giving us confidence in the mutation rate used to estimate population parameters (Lovette 2004). AFLPs are gaining prominence in studies of wild populations, especially when divergence between populations is assumed or known to be

recent (Wang et al. 2003), but they have not been used extensively within a population genetic context in birds (e.g. Busch et al. 2000; Bensch and Åkesson 2005). This marker system provides the advantage of an extensive sweep of the genome, uncovering substantial DNA polymorphism at a large number of loci (Vos et al. 1995). Development of AFLP markers is not difficult, and with the advent of fluorescently labeled primers and automated sequencers, a large number of loci can be analyzed and scored easily (Wang et al. 2003). The increased precision of automated sequencers, combined with a variety of analytical software, allows greater ease in handling of large quantities of data (although we found that we could not trust automated scoring software). There are obvious problems with the AFLP technique, e.g., the forced assumption of dominance (Vos et al. 1995), but this problem manifests itself in the limited power of analytical methods in which heterozygotes cannot be reported. This problem can be overcome somewhat, because a large number of polymorphic loci can be generated without any extra development costs. Other recently developed molecular techniques, such as microsatellites and single nucleotide polymorphisms (SNPs; Sunnucks 2000; Brumfield et al. 2003), overcome the dominance problem; however, development of a large number of loci using these techniques in non-model organisms is both costly and time prohibitive. We decided that it would be most valuable to combine mtDNA sequence data and AFLPs to answer the questions posed in this study.

2.3.1 Mitochondrial sequence data and sampling

We sequenced 1123 bases of the mtDNA gene cytochrome *b* (cyt *b*) of 40 *hyperboreus*, eight *nivalis*, and two individuals of each of the following species (which

are the buntings' closest relatives, Klicka et al. 2003): *Calcarius lapponicus*, *C. ornatus*, *C. pictus*, and *C. mccownii*. An additional 33 sequences of *nivalis* were obtained from GenBank. The *C. nivalis* sequences were obtained and selected from a variety of locations surrounding the range of McKay's buntings (Fig. 2.1). The majority of *hyperboreus* samples were collected from their breeding range, but four were from phenotypically-identified wintering individuals obtained near the Bering Sea coast (Bethel, Fig. 2.1). All sampled individuals are preserved as vouchered museum specimens at the University of Alaska Museum (UAM). Tissue extractions were performed using a Qiagen (Valencia, CA) DNeasy Tissue Kit following manufacturer's protocols. DNA amplifications followed standard PCR protocols, using the primers L14851 (Kornegay et al. 1993) and H16064 (Harshman 1996). Cycle-sequencing reactions were run on an ABI 3100 (Applied Biosystems, Foster City, CA). We sequenced in both directions, obtaining a high degree of base overlap. Sequences were aligned using the sequences obtained from GenBank and checked by eye using Sequencher v 3.1 (Gene Codes, Ann Arbor, MI). All DNA sequences have been deposited on GenBank (see Appendix 2.A).

2.3.2 Amplified fragment length polymorphisms

We report our modified protocol of Vos et al. (1995) that we used to generate AFLP data. The same 88 birds sequenced for cyt *b* were initially chosen for AFLP analysis. DNA concentration was quantified on a spectrophotometer, and extractions were diluted to obtain 0.05μg DNA per 6 μL of water. Samples were restricted and ligated in the same step using 1 U *Mse*I and 5 U *Eco*RI restriction enzymes (ABI) and 1

µL each of *Mse*I and *Eco*RI adapter pairs (New England Biolabs, Ipswich, MA). We made the adapter pair master mix first, on ice, with the following volumes per sample: 1 µL 10X T4 Buffer (NEB), 0.5 µL 1M NaCl, 0.5 µL 1 mg/mL BSA (NEB), 1 µL *Mse*I adapter, and 1 µL *Eco*RI adapter, totaling 4 µL per sample. This was vortexed and added to each sample. Then we made the restriction enzyme master mix, also on ice, with the following volumes per sample: 0.2825 µL H_20, 0.1 µL 10X T4 Buffer, 0.05 µL 1M NaCl, 0.05 1 mg/mL BSA, 0.1 µL 10 U/µL *Eco*RI, 0.25 µL 20 U/µL *Eco*RI, and 0.1675 µL 400 U/µL T4 DNA Ligase (NEB), for a total volume of 1 µL per sample. This mix was vortexed and added to the samples totaling 11 µL, which was then vortexed, centrifuged, and incubated in an MJ Research® thermocycler (Bio-Rad Laboratories, Inc. Hercules, CA) at 37°C overnight with a heated lid. The sample was then diluted with 189 µL of 0.1X TE buffer.

For PCR reactions we mixed our own "core mix," consisting of 385 µL H_2O, 68 µL Promega (Madison, WI) 10X reaction buffer, 41 µL $MgCl^{2+}$, and 6.8 µL 40 mM dNTPs for a total volume of 500 µL per tube. For the preselective amplification, we mixed 2.0 µL of the diluted restriction/ligation reaction with 8.0 µL of master mix consisting of the following volumes per reaction: 0.5 µL of the preselective primer pair, 7.375 µL of the core mix, and 0.125 µL of Promega Taq Polymerase for a total volume of 10.0 µL. The reaction was then run on the following thermal cycle: 72°C for 1 min, then 10 cycles of 94°C for 20 sec, 56°C for 30 sec, and 72°C for 2 min, with a 30 min extension at 60°C. The reaction was then diluted with 190 µL of 0.1X TE buffer.

For the selective reaction, two selective primers were chosen from the Applied Biosystems (Foster City, CA) AFLP Plant Mapping Kit for large genomes. The primers were labeled with letters and numbers to simplify the protocol (Table 2.1). We used 1.5 μL of the diluted preselective product and mixed it with 8.5 μL of a master mix consisting of the following volumes per reaction: 0.5 μL of the *Mse*I primer, 0.5 μL of the *Eco*RI primer, 7.375 μL of the core mix, and 0.125 μL Promega Taq Polymerase for a total volume of 10 μL. The reaction was then run with the following protocol: 94°C for 2 min, then 10 cycles ramping the annealing temperature down 1 degree per cycle starting with 94°C for 20 sec, 66°C for 30 sec, and 72°C for 2 min. Following the 10th cycle, we ran 20 cycles of 94°C for 20 sec, 56°C for 30 sec, and 72°C for 2 min. The reaction was finished with a 30 min extension at 60°C. Because we multiplexed all reactions, we then combined 5-FAM and NED dye reactions to increase the selective product to 20 μL total volume.

Multiplexed samples were vortexed and centrifuged, then 2.0 μL of each sample was added to a 96-well plate on ice. We then made a master mix consisting of 0.5 μL Applied Biosystems ROX 500 size standard and 7.5 μL of deionized formamide per sample. These were then added to the samples, vortexed, centrifuged, covered, and denatured at 95°C for 2 min without a heated lid. Samples were then placed on ice and run immediately on an ABI 3100 capillary automated sequencer. To ensure that runs were sufficient in duration and analyzed appropriately on a 50 cm array, we modified the run module to: Oven Temp 60°C, Prerun Voltage 15kVolts, Prerun Time 180 sec, Inject

Voltage 3.0 kVolts, Inject Time 22 sec, Voltage # Steps 10, Voltage Step Interval 60 sec, Data Delay Time 1 sec, Run Voltage 15 kVolts, and Run Time 5500 sec.

2.3.3 Multiplexing and scoring of amplified fragment length polymorphisms

To determine whether multiplexing would have an effect on data scoring, we ran the same four samples for each primer pair; then we ran a combination of 5-FAM and NED, 5-FAM and JOE, JOE and NED, and, finally, all three combined. We determined that the NED dye interfered with the other two dyes and caused pull-up (sensor overload), which would substantially influence final scoring of the data. There was no detectable difference between individuals analyzed using only either 5-FAM or NED or a combination of both. Following this experiment, samples were multiplexed using only 5-FAM and NED dyes. The experiment also allowed for checking replicability, as independent, randomly chosen individuals were run several times and checked for consistency. No major inconsistencies were encountered.

All fragments within a predefined range were binned for all samples using ABI GeneMapper®, and all loci were scored by eye. If loci were ambiguous they were deleted from the entire data set; if two fragments were sized within a single base pair, both loci were deleted; all loci that aligned with size standard peaks were deleted to ensure no effects from pull-up. Scoring began at 75 base pairs and was cut off when peaks became too short to score with confidence (~400 bp). Only individuals with good-quality amplifications were used; if the quality was determined by comparison to be low, then the sample was either discarded entirely or rerun. We discarded a total of 23 samples, resulting in 57 individuals included in the final analysis, 32 *hyperboreus* and 25 *nivalis*.

Most of the 23 discarded samples were from tissues preserved on buffer in the field, which may have influenced total genomic DNA quality, because cyt *b* would sequence without trouble but AFLPs would not amplify properly despite multiple extractions. In many cases the differences were immediately obvious; the reason that the remainder of the samples did not amplify properly was unclear, but it was likely due to a DNA quality issue associated with specimen care in the field or lab.

2.3.4 Mitochondrial sequence data analysis

Because many of the individuals sequenced shared a common haplotype, data were best visualized using haplotype networks. Median joining networks were generated using the program NETWORK 4.1.1.2 (Bandelt et al. 1999). The pairwise fixation index F_{ST} was estimated using DnaSP 4.10 (Rozas et al. 2003) which was also used to construct a genetic distance matrix. We also used DnaSP (Rozas et al. 2003) to calculate haplotype diversity (H_d) and nucleotide diversity (π), and to conduct a χ^2 test of genetic differentiation based on haplotype frequencies following Nei (1987). We used a simple χ^2 test with Yates continuity correction as implemented in PopTools 2.6.9 (Hood 2005), an add-in for Microsoft Excel®, to test for a significant difference in nucleotide diversity between the two species. We conducted an analysis to test for significant pairwise differences between the bunting species using Arlequin 3.0.1 (Excoffier et al. 1992). We also used this program to calculate a pairwise mismatch distribution under a sudden expansion model for *hyperboreus* to test for goodness of fit using 10,000 parametric bootstrap replicates (Schneider and Excoffier 1999).

We ran a coalescent analysis on the bunting mitochondrial sequence data using a Markov Chain Monte Carlo (MCMC) approach under an Isolation with Migration (IM) model (Nielsen and Wakeley 2001; Hey and Nielsen 2004, Hey 2005, Won and Hey 2005). We ran several simulations to determine the most appropriate burnin of steps to discard, maximum values of the parameters, and the best number of chains given the data. We determined that only one chain was necessary to achieve stationarity and ran four independent runs using the initial parameter starting maxima of $\theta_{nivalis}$: 500, $\theta_{hyperboreus}$: 30, $\theta_{ancestral}$: 30, m_1: 20, m_2: 15, and t: 20, the lower bound of the s parameter set at 0.5, with a burnin period of 500,000 updates and a different random number seed for each run using the HKY model of evolution and an inheritance of 0.25. We let each run proceed for more than 100 million updates to achieve a minimum effective sample size of 75 for any given parameter estimate, with most being much higher. Because the results of the four runs were very similar, we only report the parameters estimated in the longest run of 114,119,006 updates following burnin. A series of demographic parameter estimates was then calculated using four different divergence rates: one, two, four, and six percent sequence divergence per million years (μ is calculated as the mutation rate per year for the entire gene in a single diverging lineage, thus a 1% divergence has a μ = 1123 base pairs X 0.005 mutations / 10^6 years = 5.62×10^{-6}). While the best estimate of the divergence rate of cyt *b* in passerines is ~1.6% per million years (Fleischer et al. 1998), we used four different rates to incorporate uncertainty in this estimate (Lovette 2004; Ho et al. 2005). These mutation rates were thus calculated as $\mu_1 = 5.62 \times 10^{-6}$, $\mu_2 = 1.12 \times 10^{-5}$, $\mu_3 = 2.25 \times 10^{-5}$, and $\mu_4 = 3.37 \times 10^{-5}$. Because of a lack of demographic data

for these species, we assumed a generation time of one year in our calculations of effective population size (N_e). Following calculations outlined in Hey (2005), we calculated effective population sizes of *nivalis*, *hyperboreus*, and the ancestral population (N_1, N_2, and N_a respectively), the number of individuals coming into a population from the other population per year (N_1m_1 and N_2m_2), the time since divergence (t), and the number of individuals from the ancestral population that founded each diverged population (sN_a and $(s\text{-}1)N_a$).

2.3.6 Amplified fragment length polymorphism analyses

We analyzed each fragment locus as a dominant allele with two states, presence or absence. All bands scored were considered independent, homologous loci. Data generated by ABI GeneMapper® and scored by eye were aligned and transformed into a binary state matrix for each individual with primer and band size information using a Microsoft Excel® macro, which then transformed the matrix into nexus format (Rinehart 2004). We then used the program AFLP-SURV 1.0 (Vekemans et al. 2002) to calculate the fixation index Φ_{ST} and to construct a pairwise genetic distance matrix. We used the program Arlequin 3.0.1 (Excoffier et al. 1992) to test for significant pairwise differences between the two species and to run an Analysis of Molecular Variance (AMOVA). We counted the number of loci that were fixed in our samples of one species but polymorphic in our samples of the other, as well as the number of loci that were present in our samples of one species but missing from our samples of the other. We ran Pearson's χ^2 tests using PopTools 2.6.9 (Hood 2005) to test for significant differences in the number of fixed loci, and another to test for significant differences in the number of loci present in one species

but absent in the other. We used TFPGA 1.3 (Miller 1998) to test for significant differences in overall marker frequencies between the two species, using an exact test for population genetic differentiation. This program uses a Markov Chain Monte Carlo simulation to provide an approximation of the exact probability of the differences observed in marker frequencies (Raymond and Rousset 1995). We ran 20 batches, 2000 permutations per batch, and 1000 dememorization steps to estimate the *P*-value (Miller 1998).

We analyzed the bunting AFLP data under a Bayesian framework using MCMC simulations to determine the most likely number of populations involved and assign individuals to populations using the program STRUCTURE 2.1 (Pritchard et al. 2000). Because AFLP data necessarily incorporate a large number of gene histories, it is important for the model to be independent of the mutational history of the loci used (Wang et al. 2003). This program does not assume a particular mutation process, so it is capable of analyzing AFLP data and assigning individuals or determining whether they are hybrids and the degree to which these hybrid genotypes are admixed. Because of the forced dominance assumption inherent in AFLP data, each locus must be treated as a haploid allele. This treatment is considered valid under the no-admixture model. After running several experimental simulations, we determined that the most appropriate burnin was 30,000 iterations, and we ran four independent simulations for 100,000 iterations using a no-admixture model with the number of K populations set from one to six, then calculated the likelihood of K given the data as $P(K|X)$. To assess the ability of the data to infer population structure using the model, we decided not to use prior

population origin information in the model, despite being able to use both phenotypic and geographic information to identify the individuals used in the study. This program uses a model-based clustering method to assign the individuals in a study to a population and determine if the genotype of each individual is admixed. We used the program Distruct (Rosenberg et al. 2002) to transform and apply information from the STRUCTURE output and convert it into a figure.

To determine whether divergence in the genes we analyzed (only) between these two taxa had occurred by genetic drift or selection, we tested the AFLP loci for evidence of selection. We searched for and estimated the number of loci under selection in the AFLP dataset by using a simulated dataset acting under drift alone using an infinite alleles model. To conduct this test, we used the program fdist 2 (Beaumont and Nichols 1996). This program uses an average divergence to simulate predictions under an infinite alleles model of the expected distribution of differentiation across loci (Wilding et al. 2001; Campbell and Bernatchez 2004). The program does not require a specific mutation rate to be included, but rather generates a roughly uniform distribution of heterozygosities. Using the method of Nei (1977), the differentiation is estimated and measured by F_{ST} for each locus and corrected using the method of Nei and Chesser (1983). The program also calculates expected heterozygosity (H_S) for each simulated locus. This simulated distribution is then used to calculate quantiles of the median and 99% confidence intervals of the distribution of loci for the populations diverging under drift alone. It can then be used to calculate F_{ST} and H_S for all loci in the dataset, which are plotted with the quantiles estimated under the model. Loci that fall outside of the

quantiles are considered either under selection or linked to loci under selection (Beaumont and Nichols 1996). The data were analyzed to get an estimate of the average F_{ST} across loci, and then the model was fit to this F_{ST} and used to calculate the quantiles. We ran the simulation under the same conditions 20 times: with two demes total, sampling two populations, using an expected F_{ST} for the infinite alleles model of 0.095, an average sample size per population of 29, and 20,000 loci generated. We calculated the quantiles for each simulation and averaged them; our data were then plotted with the quantiles to search for loci considered statistically to be under selection (Beaumont and Nichols 1996).

2.4 Results

2.4.1 Genetic differentiation and population structure

There were no fixed nucleotide differences in cyt *b* sequence between snow and McKay's buntings. The cyt *b* haplotype network of *nivalis* and *hyperboreus* showed that a substantial number of individuals we sampled of each species shared an identical common haplotype, with each species possessing discrete haplotypes (Fig. 2.2a). There was a total of 13 different haplotypes found in the *nivalis* sampled and a total of seven haplotypes found in *hyperboreus*. This difference was evident in the haplotype networks for these two species alone (Figs. 2.2b and 2.2c). The buntings are much more closely related than any other member of the genus, with a pairwise F_{ST} between them of 0.0784, as opposed to a range of 0.9565 – 0.9959 of pairwise divergence between any other members of the genus (Table 2.2). This level of divergence between the buntings was significant ($P < 0.01$).

The AFLP data (Appendix 2.B) showed largely the same signal as the cyt *b* data: *hyperboreus* and *nivalis* are the most closely related members of the genus (Table 2.2). Of the 1000 loci analyzed, 784 loci (78.4%) were polymorphic when all members of the genus were included. Between the two buntings, 580 loci (58%) were polymorphic and ranged from 52.1 – 80.8% polymorphic bands per primer pair, whereas within species variation occurred at 42.4 – 76.8% frequencies, depending on primer pair (Table 2.3). We calculated Φ_{ST} for all pairwise comparisons and found that it was much lower between the buntings (0.0176) than the rest of the genus (0.2095 – 0.4833, Table 2.2). We also calculated F_{ST} between the two bunting species (0.04475) and found that this value was significantly different from zero ($P < 0.01$). The result of the exact test for differences between the two species in marker frequencies in the AFLP data was significant ($\chi^2 = 1483.6$, df = 1164, $P < 0.01$).

STRUCTURE analysis of the AFLP data estimated that the most likely number of bunting populations involved in our samples was two (Table 2.4). Two individuals were misassigned, one from each population. Species were not substantially admixed, but there was more admixture from *hyperboreus* into *nivalis* than from *nivalis* into *hyperboreus* (Fig. 2.3). The *nivalis* that was misassigned (UAM 7774) was estimated to have 92.9% of its genome originating from the *hyperboreus* population. It was collected from Cape Pierce, the closest sampling locality of *nivalis* to St. Matthew Island in our study. The *hyperboreus* that was misassigned (UAM 8199) was estimated to have 72.6% of its genome originating from the *nivalis* population. It was collected during the breeding season from St. Matthew Island. We examined each of these specimens, but because they

are both adult females, positive phenotypic differentiation is difficult to assess. However, when we examined them in a series, they both fit phenotypically with their putative population of origin, rather than their apparent genotypic population of origin. Because they are adult females it would be extremely difficult to phenotypically distinguish hybrids; plumage differences between females of these two species are too subtle to confidently identify an intermediate phenotype.

2.4.2 Divergence time and gene flow

The coalescent analysis we used to estimate the time since divergence yielded consistent results, and the distribution of the posterior probabilities of the *t* parameter (time since divergence = $t \times \mu$) did not include zero for any run (Figs. 2.4a). We calculated the estimates of divergence time using four different mutation rates (μ), and all four estimates suggested divergence during the Last Glacial Maximum (LGM), ranging from ~13,000 to ~80,000 ybp (*t*, Table 2.5). Even under a very high sequence divergence rate (6%), the buntings appear to have diverged during the LGM (Fig. 2.1).

We also used IM to obtain an estimate of historic levels of gene flow by calculating the effective number of migrants from one population into another. The estimated effective number of migrants per year of *hyperboreus* into *nivalis* in the coalescent was calculated as $N_1m_1 = 229$ (Table 2.5). This estimate is about 8% of the total current population of *hyperboreus* based on demographic estimates (2800, Lyon and Montgomerie 1995). The estimated effective number of migrants per year of *nivalis* into *hyperboreus* in the coalescent was calculated as $N_2m_2 = 0.0055$ (Table 2.5). These numbers represent a calculated estimate and should not be taken as the “true” number of

individuals migrating from one population to another; however, the pattern of asymmetric gene flow is striking. This level of asymmetry is an estimate of gene flow traced back through time in the coalescent, not an estimate of current rates, which can explain why it is not evident in our analysis of population structure using AFLP data (Fig. 2.3).

2.4.3 Genetic evidence for a founder event and population expansion

Coalescent simulations to estimate the effective population sizes of both species and the ancestral population were highly congruent (Fig. 2.4). Because mtDNA is maternally inherited, all effective population size estimates are breeding females only. The effective population size estimates of *nivalis* were very high, ranging from ~ 1.5 X 10^6 to 8.9 X 10^6 (N_1, Table 2.5). The estimates of the effective population size of *hyperboreus* were much smaller, ranging from ~22,000 to 130,000 (N_2, Table 2.5). The effective size of the ancestral population (N_A) ranged from 2135 to 12,809, depending on μ (Table 2.5). We also calculated the proportion of the ancestral population that founded each population and found that very few individuals apparently founded *hyperboreus* ($(1-s)N_a$ = 1 to 6, Table 2.5), whereas the majority of the ancestral population established *nivalis* (sN_a = 2134 to 12,803, Table 2.5). Nonsignificant results for the Sum of Squared Deviation (SSD) of the test of goodness of fit for the mismatch distribution did not allow us to reject the sudden expansion model: P(Sim. SSD $\geq$ Obs. SSD) = 0.623 (Schneider and Excoffier 1999). These results suggest that *hyperboreus* was founded by very few individuals and subsequently expanded to an effective population size (~22,000 to ~131,000) exceeding the estimated census size of the modern population (2800, Lyon and Montgomerie 1995).

Concordant with evidence for a *hyperboreus* founder event, *hyperboreus* had a lower haplotype diversity than *nivalis* (*hyperboreus*: $H_d = 0.51$, *nivalis*: $H_d = 0.73$; $\chi^2 = 28.73$, df = 16, $P = 0.0259$). Nucleotide diversity was also lower in *hyperboreus* than in *nivalis* (*hyperboreus*: $\pi = 0.00076$, *nivalis*: $\pi = 0.00107$; $\chi^2 = 546.12$, df = 1, $P < 0.01$). There were 33 AFLP loci that were fixed in *hyperboreus* but polymorphic in *nivalis*, and *nivalis* had 42 fixed alleles that were polymorphic in *hyperboreus*, but these differences were nonsignificant ($\chi^2 = 1.97$, df = 2, $P = 0.37$). There were 80 AFLP loci that were found in *nivalis* but absent from *hyperboreus*, and 45 alleles that were found in *hyperboreus* but missing in *nivalis*, and these differences were significant ($\chi^2 = 11.80$, df = 2, $P = 0.0027$). These results suggest a loss in genetic diversity consistent with a founder event (Mayr 1942).

2.4.4 Selection versus drift

To test whether drift or selection have been operating in the divergence of the AFLP loci we analyzed between these two taxa, we simulated loci diverging under drift alone, calculated quantiles from 20 simulations of the distribution of differentiation per locus, and plotted the AFLP data with these modeled results (Fig. 5). Only polymorphic loci were plotted ($N = 580$), as monomorphic loci would not provide any information in this context. The data fit the simulation very well, with the exception of a lack of loci with high heterozygosities, which is the result of assuming dominance and not being able to report true heterozygotes with AFLP data. Five loci fell outside of the 99% confidence intervals (Figs. 2.5); this is fewer than expected by chance (for just polymorphic loci expected $N = 5.8$). Statistically, there is thus little evidence for strong selection among the

1000 loci examined. We conclude that during a likely initial period of geographic isolation, drift was the predominant force operating in the process of differentiation between these two taxa.

2.5 Discussion

2.5.1 Founder-flush-crash

Our data suggest that very few founding individuals became isolated in Beringia to establish the *hyperboreus* population. Founder events have not been considered contentious in species formation, but the genetic consequences of a founder event have been debated (see Coyne and Orr 2004 and references therein). Our evidence from both mtDNA and AFLP data show that a loss of genetic variation occurred and has persisted in *hyperboreus*, concordant with Mayr's (1954) and Carson's (1971) predictions of the genetic consequences of founder events.

Our data suggest that genetic drift, not selection, has been the primary factor in the divergence between these two species. Strong genetic drift due to a loss of genetic variation is expected following a founder event, enhancing the rapid fixation of neutral mutations (Carson and Templeton 1984). If there had been strong selection, e.g., for adaptation to a novel habitat, strong sexual selection, or divergent selection due to ecological factors, we would have likely uncovered this signal through analysis of the large number of loci included in our study (Wilding et al. 2001). The expected mode of speciation under a general model of peripatry involves either divergent selection or genetic drift, requiring a complete cessation of gene flow (Haldane 1930; Mayr 1954). Under divergent selection, we would not expect genetic drift to be the driving force

causing reproductive isolation (Coyne and Orr 2004). Postulating selection differences between the Beringia and the southern edge of the ice sheets seems unrealistic. There is no evidence to suggest that the tundra plains of Beringia were a novel habitat during the LGM, and vegetation was not considerably different than it is today (Ager 2003). Our results also indicated population expansion of *hyperboreus* following the founder event. Population expansion is an important component of the founder-flush-crash model (Carson and Templeton 1984) of speciation, because selection is considered to relax as the population expands without intraspecific competition. If the habitat into which the population was expanding was novel, we would expect a strong selective force driving divergence, but we have little evidence to support strong selection driving the divergence.

Our estimates of the effective population size of *hyperboreus*, which are much higher than recent demographic estimates of the population size (2800 birds total in June, Lyon and Montgomerie 1995), indicate a population reduction (Roman and Palumbi 2003). Genetic evidence of asymmetric gene flow from *hyperboreus* into *nivalis*, combined with a dramatic reduction in available habitat caused by rising sea levels in the Bering Sea, support this conclusion. Our results suggest that gene flow is primarily unidirectional from *hyperboreus* into *nivalis*. There has been some recent study of passerines focusing on hybridization and "swamping" of one species by another once secondary contact occurs (Gill 1998; Rohwer and Wood 1998; Rohwer et al. 2001; Gill 2004; Shapiro et al. 2004b). Rohwer et al. (2001) studied Townsend's warblers (*Dendroica townsendi*) hybridizing with hermit warblers (*D. occidentalis*) and found that in some areas where only *townsendi* phenotypes occurred, the majority of their mtDNA

haplotypes were of *occidentalis* origin, consistent with asymmetric hybridization. Because of incomplete lineage sorting or a high level of asymmetric gene flow, we cannot distinguish between *Calcarius nivalis* and *C. hyperboreus* mtDNA haplotypes, but our IM results are consistent with the scenario presented by Rohwer et al. (2001). We would expect a signal of asymmetric gene flow if *nivalis* has been hybridizing *hyperboreus* out of the majority of the latter's historic range. Such a high rate of asymmetric hybridization is consistent with a scenario that *hyperboreus* was once a more widespread Beringian species that became reduced due to being overrun by postglacial colonization of *nivalis* and a reduction in available habitat as a result of climate change.

2.5.2 Speciation at high latitudes

Calcarius hyperboreus is a recently diverged species, sister to *C. nivalis*. Our estimate of divergence time using a 2% sequence divergence rate (~ 40,000 ybp), combined with a low level of divergence found using both marker systems, support a very recent divergence, the most recent reported for any currently recognized species of North American bird (American Ornithologists' Union 1998, and supplements; Klicka et al. 1999; Johnson and Cicero 2004). Under a model of genetic drift alone, because drift is not considered a strong force in the evolution of divergent traits, we would expect reproductive isolation to take a much longer time to occur (Coyne and Orr 2004). If drift, not strong selection, has driven the divergence, then we have to explain the rapid morphological differentiation that is evident today. In this case we appear to have relatively rapid speciation without a strong signal of divergent selection. We can conclude that speciation between these two taxa is complete or nearing completion,

because there is evidence of secondary contact on the breeding grounds of *hyperboreus* during the breeding season (Winker et al. 2002), but the two taxa have maintained their morphological and genetic integrity.

Our study suggests that founder effect speciation can occur in a relatively short time frame due to climatic oscillations at high latitudes. McKay's buntings were likely founded by very few individuals that apparently differentiated from snow buntings largely through genetic drift. During this process morphological differentiation became fixed in McKay's buntings. In postglacial Beringia there has been gene flow between the two taxa, but very asymmetrically. The extant population of *hyperboreus* has retained its morphological and genetic integrity in the face of gene flow. McKay's buntings appear to be a good example of recent, rapid speciation, driven largely by genetic drift on a small founding population.

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Zink, R. M., and D. L. Dittmann. 1993. Gene flow, refugia, and evolution of geographic variation in the song sparrow (*Melospiza melodia*). Evolution 47:717-729.

Table 2.1. Coded AFLP selective primers used.

Primer	Dye	3 Letter extension	Code
*Eco*RI	FAM	ACT	A
	FAM	ACA	B
	NED	AAC	C
	NED	ACC	D
	NED	AGC	E
*Mse*I	-	CAA	1
	-	CAC	2
	-	CAG	3
	-	CAT	4
	-	CTA	5
	-	CTC	6
	-	CTG	7
	-	CTT	8

Table 2.2. Pairwise genetic distance matrix of all members of the genus *Calcarius*. Above the diagonal are F_{ST} values calculated from cyt *b* data, and below the diagonal are Φ_{ST} values calculated from AFLP data.

species	*hyperboreus*	*nivalis*	*mccownii*	*pictus*	*ornatus*	*lapponicus*
C. hyperboreus	——	0.0784	0.9800	0.9959	0.9803	0.9838
C. nivalis	0.0176	——	0.9775	0.9942	0.9785	0.9819
C. mccownii	0.3647	0.3516	——	0.9896	0.9733	0.9779
C. pictus	0.4833	0.4612	0.3528	——	0.9565	0.9903
C. ornatus	0.4218	0.3990	0.2948	0.2095	——	0.9746
C. lapponicus	0.4245	0.4093	0.3304	0.3758	0.3043	——

Table 2.3. AFLP loci amplification and scoring results for each primer pair and total. Total bands (T), the number of polymorphic bands (P), and the percent of bands that were polymorphic (% P).

Primer pair[1]	All *Calcarius* species			Both buntings			Within *hyperboreus*			Within *nivalis*		
	T	P	% P	T	P	% P	T	P	% P	T	P	% P
A1	167	125	74.9	157	99	63.1	146	85	58.2	148	84	56.8
A4	162	135	83.3	146	98	67.1	131	80	61.1	136	80	58.8
B3	130	102	78.5	120	79	65.8	114	67	58.8	119	71	59.7
B8	137	111	81.0	118	79	66.9	102	61	59.8	109	62	56.9
C2	81	58	71.6	73	38	52.1	66	28	42.4	66	28	42.4
D6	114	101	88.6	99	80	80.8	87	63	72.4	95	73	76.8
E5	79	62	78.5	73	38	52.1	69	31	44.9	71	32	45.1
E7	130	90	69.2	127	69	54.3	119	53	44.5	125	64	51.2
Total	1000	784	78.4	913	580	63.5	834	468	56.1	869	494	56.8

[1] See Table 2.1

Table 2.4. Estimate of the number of clusters or likely populations involved (*K*). We used STRUCTURE without using prior population information.

K	Ln Pr(*X*|*K*)	P(*K*|*X*)
1	-12151.5	~0
2	-12003.4	~1
3	-12130.2	~0
4	-12028.8	~0
5	-12390.0	~0
6	-12585.3	~0

Table 2.5. Demographic parameters calculated from parameters estimated using an Isolation with Migration model (IM, Hey 2005). Effective size estimates of snow (N_1), McKay's (N_2), and ancestral populations (N_a), the number of migrants from McKay's into the snow population (N_1m_1) and from snow into McKay's (N_2m_2), and the number of ancestors that founded snow (sN_a) and McKay's populations ($(1\text{-}s)N_a$) are in units of individuals. The estimates of time since divergence (t) are in years. Mutation rates are calculated from 1% (μ_1), 2% (μ_2), 4% (μ_3), and 6% (μ_4) sequence divergence per million years.

	Mutation Rate (μ)			
Parameter	μ_1	μ_2	μ_3	μ_4
N_1	8,924,083	4,462,041	2,231,021	1,487,347
N_2	130,658	65,329	32,665	21,776
N_a	12,809	6405	3202	2135
N_1m_1[1]	229	-	-	-
N_2m_2[1]	0.0055	-	-	-
t	80,142	40,071	20,035	13,357
sN_a	12,803	6402	3201	2134
$(1\text{-}s)N_a$	6	3	2	1

[1] - These parameters are estimated independently of mutation rate.

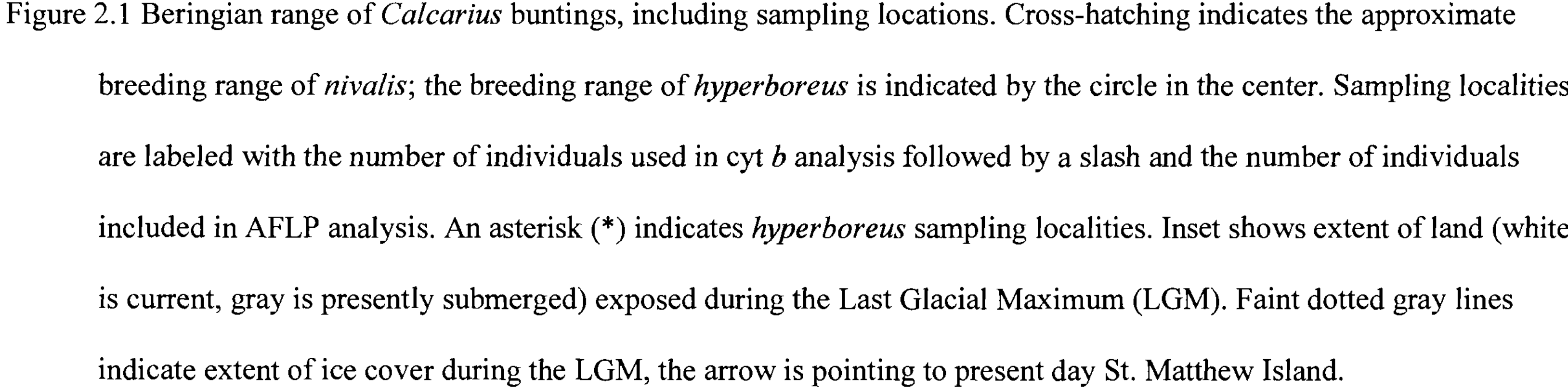
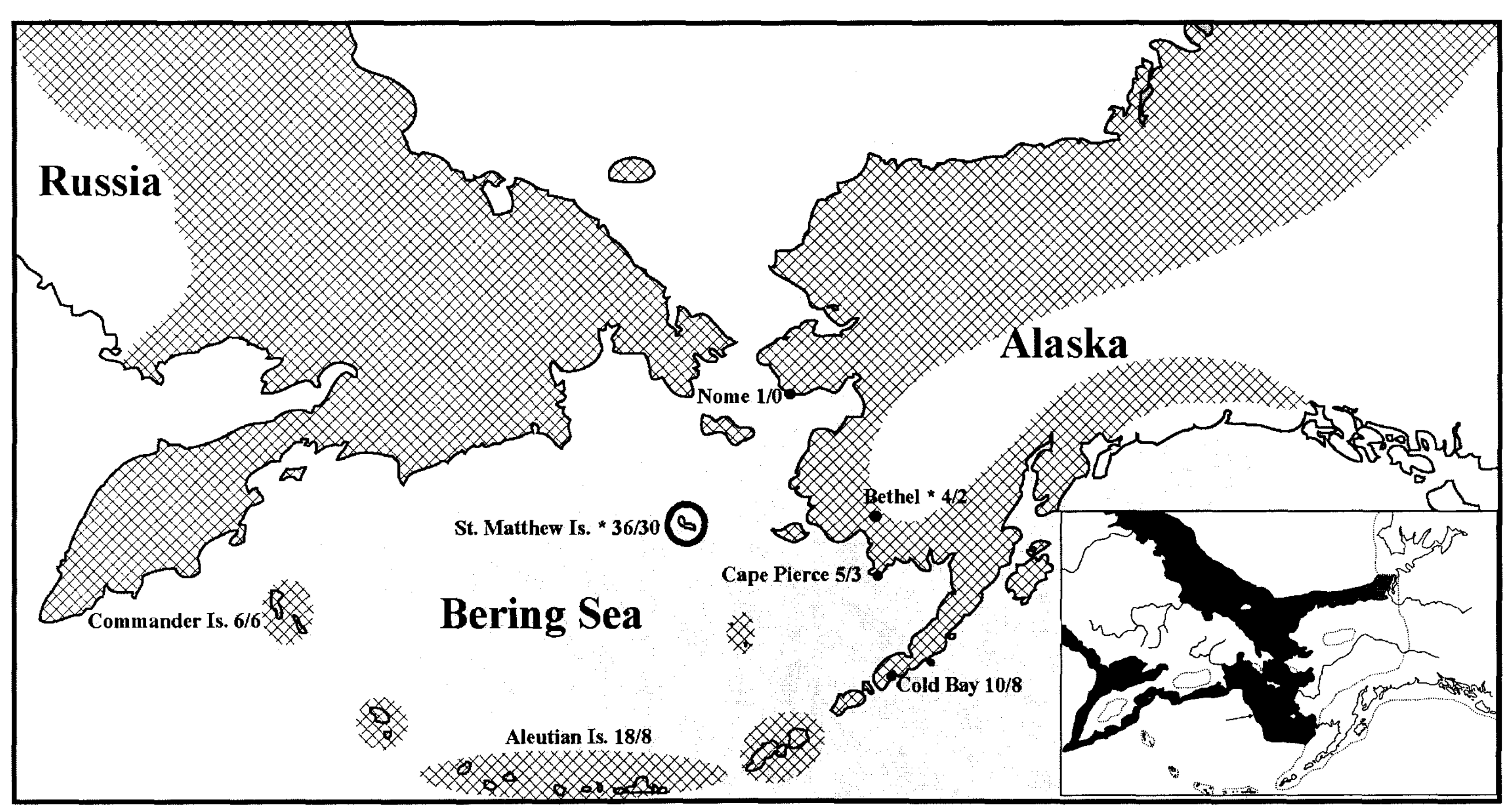


Figure 2.1 Beringian range of *Calcarius* buntings, including sampling locations. Cross-hatching indicates the approximate breeding range of *nivalis*; the breeding range of *hyperboreus* is indicated by the circle in the center. Sampling localities are labeled with the number of individuals used in cyt *b* analysis followed by a slash and the number of individuals included in AFLP analysis. An asterisk (*) indicates *hyperboreus* sampling localities. Inset shows extent of land (white is current, gray is presently submerged) exposed during the Last Glacial Maximum (LGM). Faint dotted gray lines indicate extent of ice cover during the LGM, the arrow is pointing to present day St. Matthew Island.


Russia
Alaska
Nome 1/0
Bethel * 4/2
St. Matthew Is. * 36/30
Cape Pierce 5/3
Commander Is. 6/6
Bering Sea
Cold Bay 10/8
Aleutian Is. 18/8

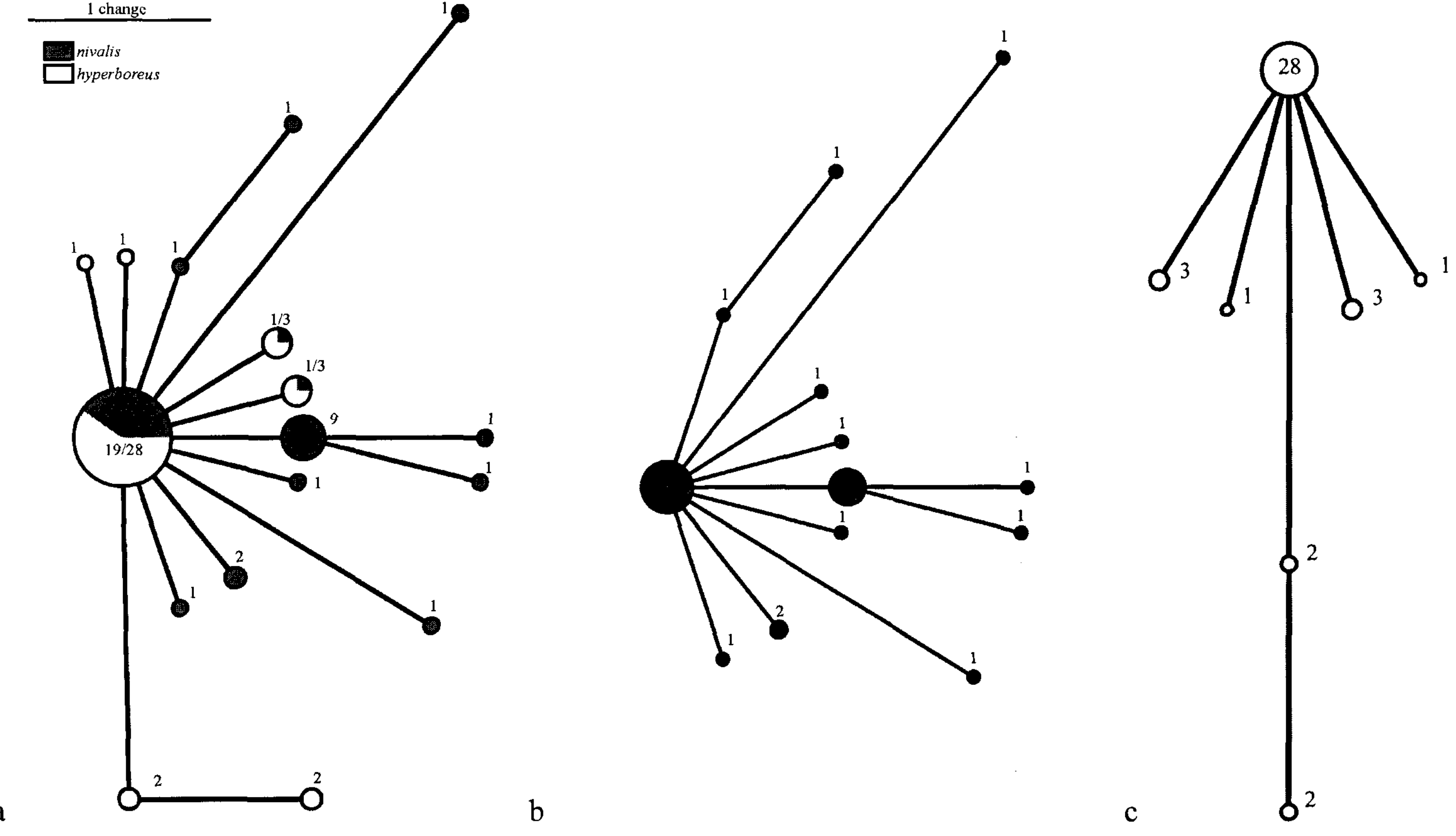


Figure 2.2. Three haplotype networks depicting the number and relation of cyt *b* haplotypes. The first network (a) displays both *hyperboreus* and *nivalis*, the second network (b) shows only *nivalis*, and the third network (c) shows only *hyperboreus*.

Figure 2.3. The genotypic makeup of the two populations inferred by STRUCTURE. All AFLP loci were included in the analysis. Each individual is represented by a single bar; the *nivalis* genotype is represented by gray, while *hyperboreus* is represented in white. The two misassigned individuals are the closest to the boundary between the two populations represented by a black line.

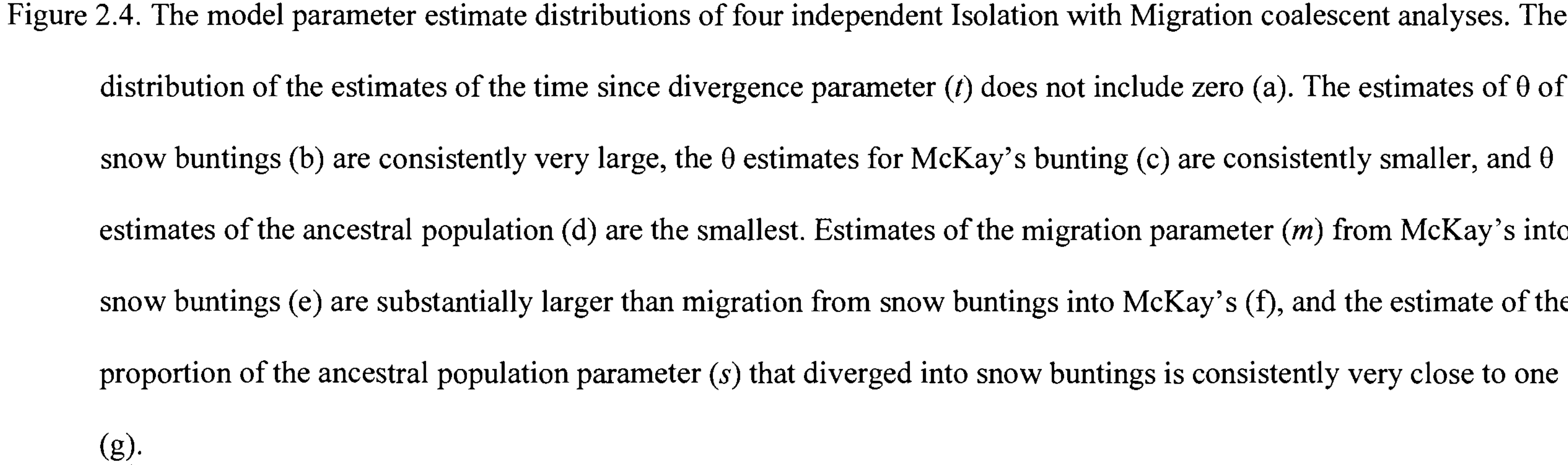


Figure 2.4. The model parameter estimate distributions of four independent Isolation with Migration coalescent analyses. The distribution of the estimates of the time since divergence parameter (*t*) does not include zero (a). The estimates of θ of snow buntings (b) are consistently very large, the θ estimates for McKay's bunting (c) are consistently smaller, and θ estimates of the ancestral population (d) are the smallest. Estimates of the migration parameter (*m*) from McKay's into snow buntings (e) are substantially larger than migration from snow buntings into McKay's (f), and the estimate of the proportion of the ancestral population parameter (*s*) that diverged into snow buntings is consistently very close to one (g).

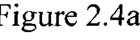


Figure 2.4a

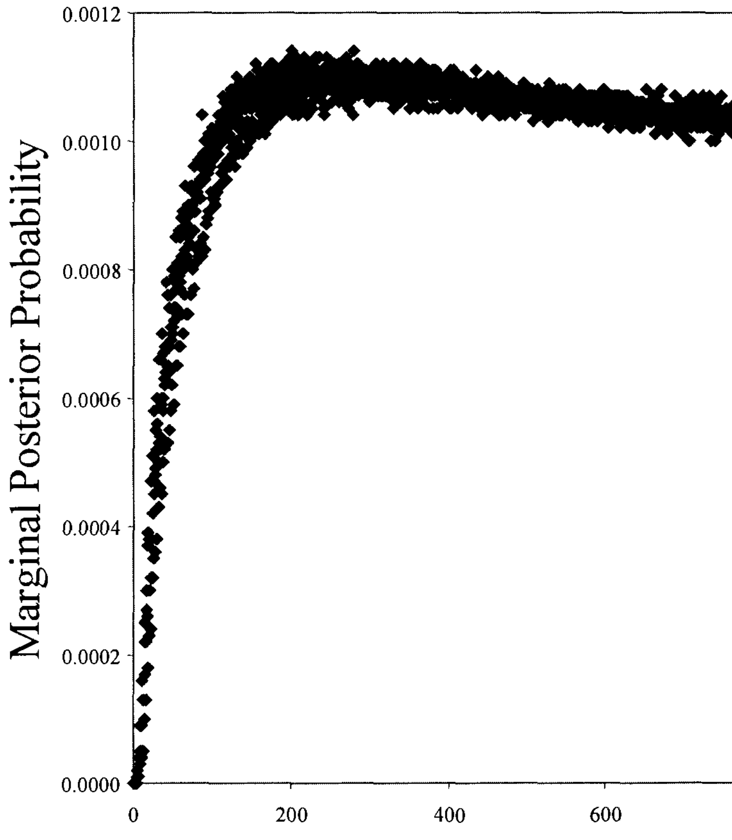
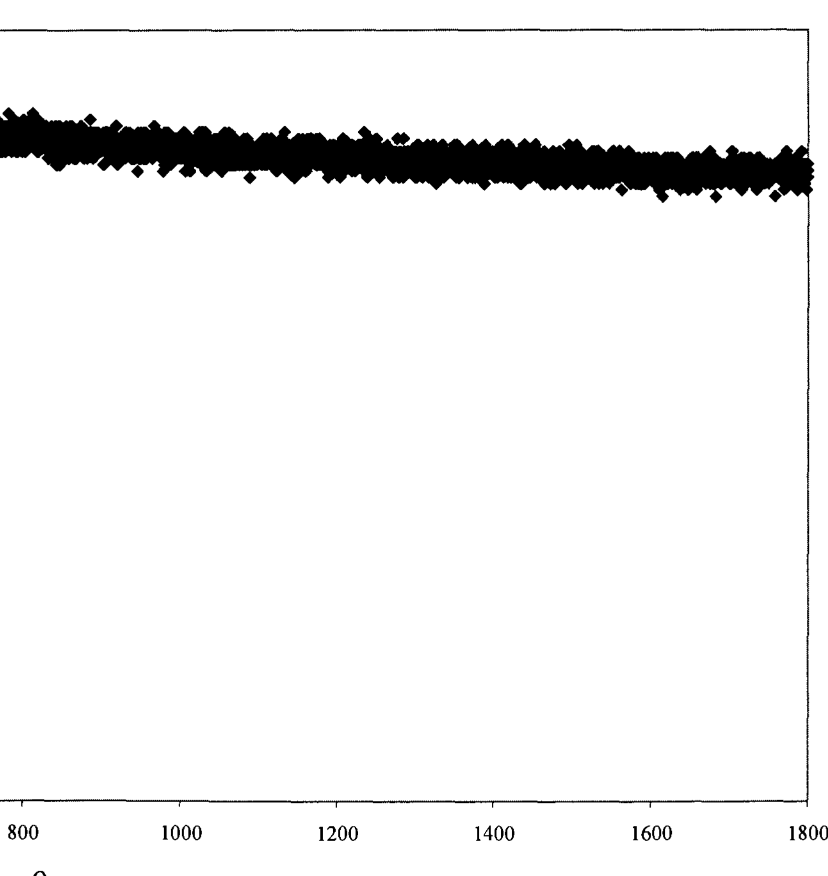


Figure 2.4b


800
1000
1200
1400
1600
1800
θnivalis

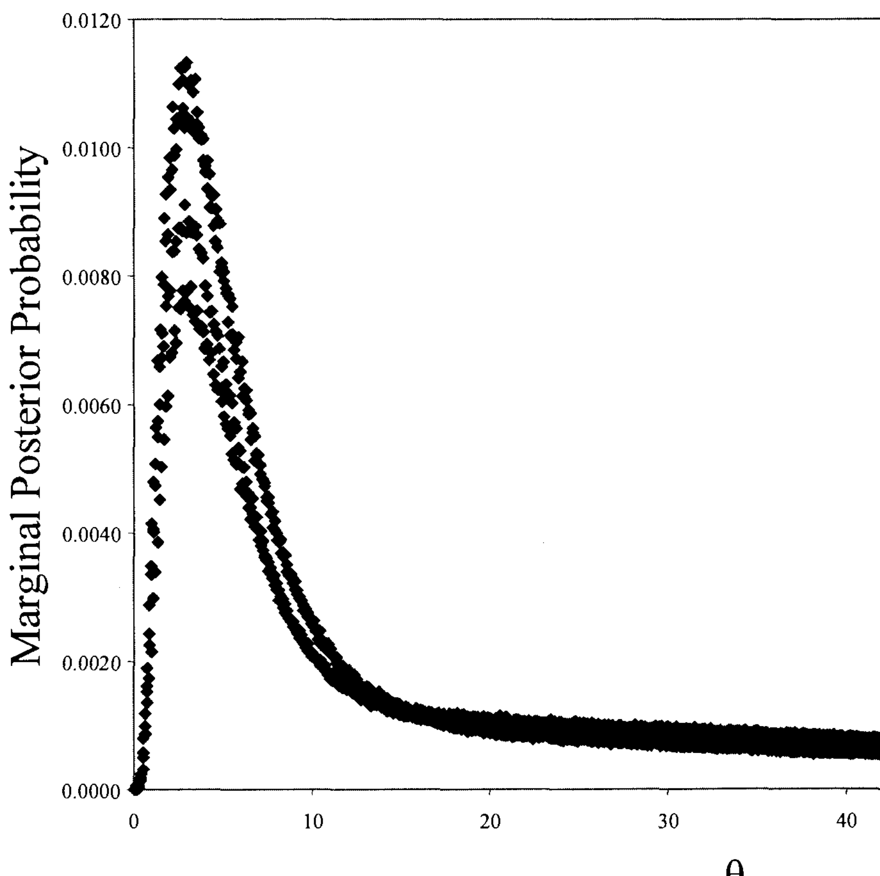
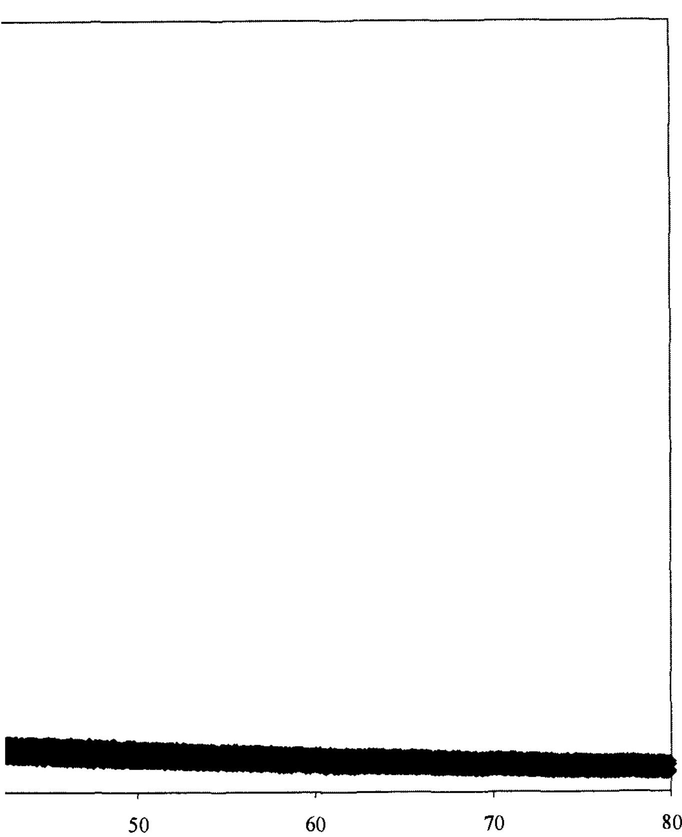


Figure 2.4c


50
60
70
80

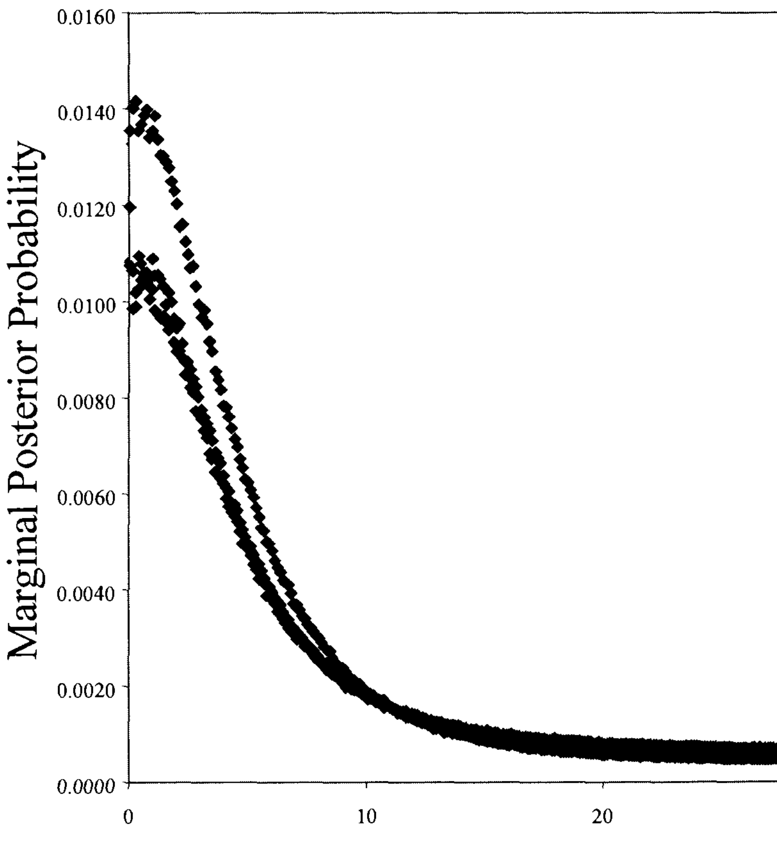
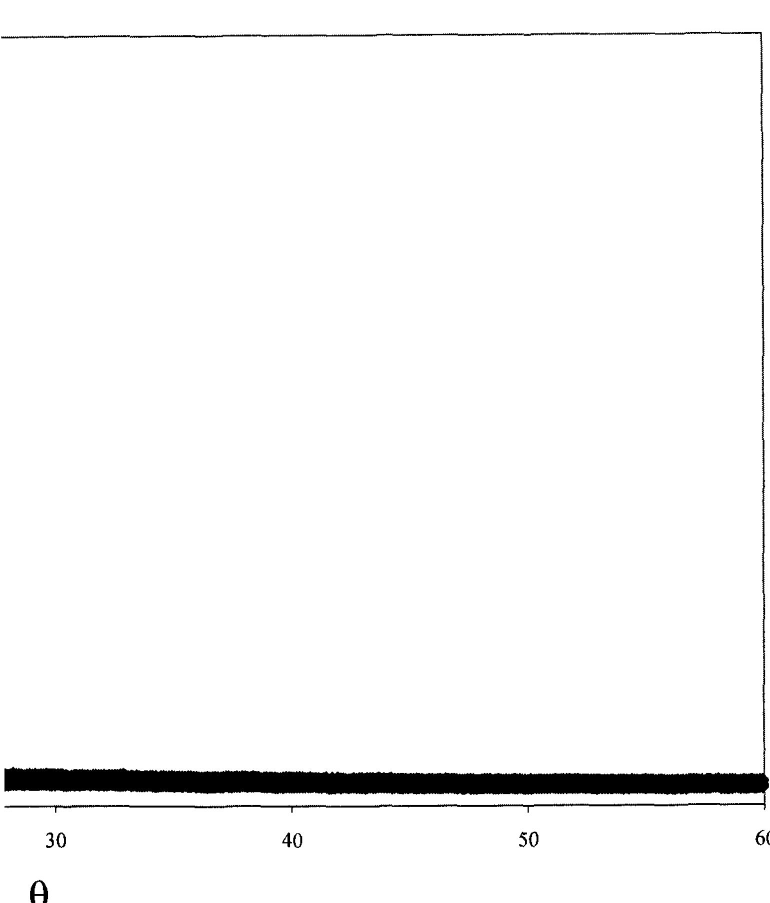


Figure 2.4d


30
40
50
60
$\theta_{ancestral}$

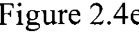


Figure 2.4e

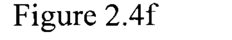


Figure 2.4f

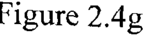


Figure 2.4g

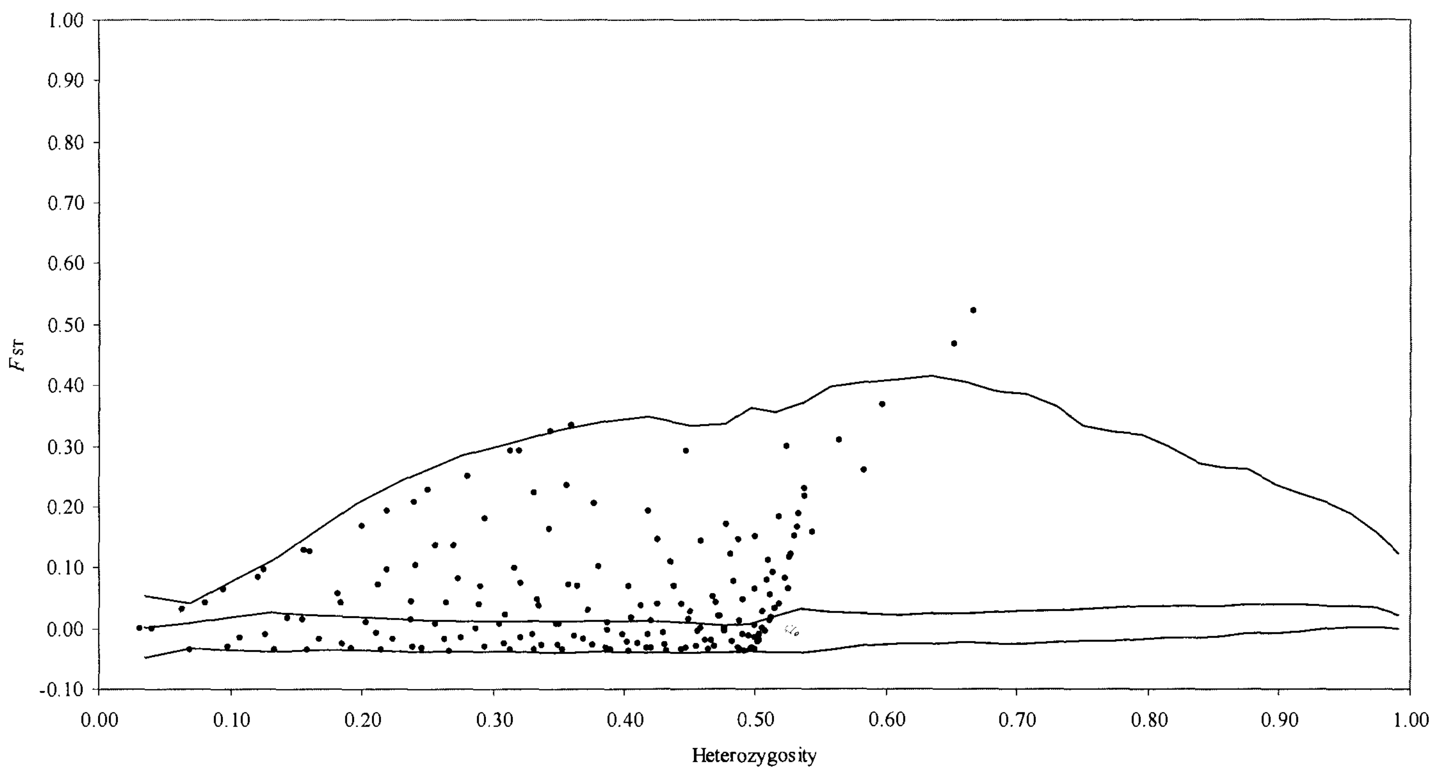


Figure 2.5. The distribution of the AFLP data plotted with quantiles. Quantiles were calculated using simulated data diverging under a model of drift alone using the program fdist 2. The top line is the upper 99% confidence interval, the middle line is the median, and the bottom line is the lower 99% confidence interval of the distribution of loci generated using the simulation.

Appendix 2.A.

Species	Location	Voucher numbers	GenBank numbers
C. hyperboreus	Bethel, AK	UAM[1] 8473, 11864, 13166, 13167	DQ489335-489337, 489364
	St. Matthew Is, AK	UAM 7403-7407, 7524, 7746, 8198-8205, 8210, 8211, 8479, 8480, 8537-8539, 10683, 17489, 17495-17499, 17502, 17547-17550, 17878, 17879	DQ489327-489334, 489338-489363 489365,489366
C. nivalis	Attu Is, AK	UAM 7260, 7275, 7655, 8430, 9307	AY156428, 156430-156432, DQ489326
	Shemya Is, AK	UAM 9863, 9873, 9900	AY156433-156435
	Adak Is, AK	UAM 9319, 9320, 9864, 10038, 10039, 10046, 14610, 14675, 14676, 14712	AY156436-156445
	Cold Bay, AK	UAM 8474, 8476, 10043-10045, 11841-11843, 11855, 11856	AY156446-156455
	Cape Pierce, AK	UAM 7335, 7774, 7775, 7806, 14147	AY156461-156465
	Nome, AK	UAM 8621	AY156460
	Commander Is, Russia	UAM 17398, 17400, 17404, 17406, 17407, 17412	DQ489320-489325
C. mccownii	USA	JK[2]94-073, JK94-074	DQ489373, 489374
C. pictus	Brooks Range, AK	UAM 19611, 19612	DQ489367, 489368
C. ornatus	USA	JK94-052, JK95-016	DQ489371, 489372
C. lapponicus	St. Matthew Is, AK	UAM 18453, 18860	DQ489369, 489370

[1] - University of Alaska Museum (UAM), [2] - John Klicka (JK) field number

Appendix 2.B. AFLP data. Numbers refer to UAM accession numbers except for loaned samples. Species and location can be found in Appendix 2.A.

7275	1010100111101111011110111001001011111101100000010000110000010110000001101010101010000010000101000100000011101110110010100111110010110000010000100011011000110001001001001000101110101101101
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	100011001011001
7335	01101101111011110010111111011000110111010000000000000110000010010000101000010100000001001000010110000000011110111111101101001111100001100000100001100100111011000000010011010000111101011101101
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	1010001011001111001010000000100110000000101001101000100000000000100010000000100101110111111110111111100100100100001100010001001000101000000110101111101111111011011011110101001100111001000 01
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	101010000011000
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	000001000010000
7404	0010100111101111000011111001100001011101100100000000110000010010000000100101000000000000100001001001000000011111110100110000011110000101000010000111010011100110000101001001000100011100101101
	0110001000000011001100010100100000011001000100010000011110111100001111111111011111101100000111010011100111001001000010000000010000000010010001100010100000111110011011110100101000010000 0101
	10110000110011110110000000001011100000000100001001000110000000000000100000000000011110111111110111111100100100100001000010000001000101000000110001111101111111011111010110011001100100000000 00
	010001000000000000000000010000100100000010011010000101111011111101110110010110101011111101111110010000001000101100000111111111110111111111110011111000110111100010110011011100001101000101 1010
	101011001010000
7405	10101001111011110001110110010000110111010000000010001100001100010100010010101010000100110010100001100000111111111101011100111110010100000010000100010111100100000101001001000001010100101101
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	000001000000000100100100100001001000001100110100001011110111111111011001011010101111100111100010001001110101100001111111111111101111111011011011010110110100011110111001100001011000101 0010
	100001000011000

Appendix 2.B continued.

7406

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10000000001 1000

7407

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7774

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100010101011000

Appendix 2.B continued.

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8201	*0011100101101101000111111101000011011101101000000000110000000101010001001010001000010010000101101100000011111111100011100011110010110000010000100011011100110000001001101010010010100101101* 0110001000000111011000000100100000001000000010011000001111011110010111111110110111100011000011110100111100111110010000100000011000000000110101001010110010111110011011101010111000000000101 1010001011001111011010000000101110000000100001001000100010000000000100000000111111001111111101111111011001001010011000100000000000101000000110001111011111110101110101101010011001100000000 0 0011010000100010001000011000000001010000000001010000101111011111111110010110110101011111101111110010000000111000010000111111111110111111011010111011000110100100010110001010100001001100101001 0 010000000011000
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Appendix 2.B continued.

8203
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8204
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8205
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8210
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8474
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Appendix 2.B continued.

8476	0010110111110111110011111111101101010110111010111000010001100000101000000101010101010000000010000100000100000111101110100110000111110010100000010000100011011100110001001001001000001010110101101011000100000001110100001010010000001000000010001000001101110110000111111010110111011011010011110110111011111010011000111000001101000011110101100000110010111111011011101110101010010100000110100000110001110110100000110011010000110100100000010000000000000100000000011011101111111111011111100100100100000100011000000000101000000110101111100101110011110101101010111001010010000000000010010100000000100000100001001000000000001010000101111111111111101101101101010101111110111110001001000101000110010011111111101011111111110100111110001100101000111100110110000110000000101001010000100000010000
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Appendix 2.B continued.

9319	1011100101101001001111111001100011011101100100001000111000010110000011001010101001010010001100011100000011101110110010100111110000100000000100100011111100100000000100100100011001010010110101100010000000111000100000100100000000000000001000100000111101110000011111101011001110101101001111010011110101011001000110111001100100001011011010000011101011011001101110110010100001000001011011000011001111101010000000100110000001101000000000110000000000000100011000011011110111111110011111001001001000011000110000001010101000000110101111101110110101110101101010111001100000010000000100000000000000001000100001001000000000110100011010111111110111011001011010101111110111100010010001101010000110111111111011111111111010011111110110010100110110011110100001100100101001010001000001000
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9864	0010110111101001001111111101100011011101100000000100011000001011000001010101010100001001000110011010000001110111010101010011111000010000000010010001001110011000000010010010001100101101010110100100010000000111011000010100100000001000010010001010001111011111000111111010110111001011000011110100111101101110011000100010000101000010110101100000011001011111001101110110010101001010001011010000001100111100101000000010011000010110100000100111000000000000001000000000101111011111111100111111010010010000100001100000000010100010011010111110111111101011001011010100110010100001000001101001000000000010000010000100100000000011010000101011011111111011011011011101111110111100010110000101100100101111111111111111111110111100111110001100101001101110110011000111100001010110100011001011000
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Appendix 2.B continued.

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Appendix 2.B continued.

11855	10101101111110110111111111001100011101111110000010000110010110010000100101010101000010010000101010100000011101110100010100111110000110000010100100011011101100010101001001000100110110101101 00100010000000110011000001001000000110000001000100000111111111000011111111011011110101100001111010011100111101001000000110001100100000111010110101011101010111001111110111010101001000000101 10100010111011110110100000000100110000000100101000000100010000000000010000000001111111011111111011111100100100100010101010100001000101001100110001111101110110100110101101110011001100001000 01000100000100000110000010000100100000000111010000101111011111111110111110110101011111101111000100000001000100100101111111111011110111111111101111101011010010011111001101010000111100010100 10 00000000011000
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13167	00101001111011110000111110011000110111011000000000001110000101000000101010100010010100100011010001000000011101110100010000111110010110000000000100011011100110000011001001001001011100101101 11100010000000110111000101010000000100010001000100000111011110000011111101111011101101100001111010011100011111001000010000101100100011011010110000011001011111001101110110010100000000000101 10100010110001110110100000000100110000000100001000000010000000000000010000000001101110011111111011111110110010010000000001000000100010100000011000111110110111011110010110101001100110000000 00 00110100000000011000110010000100100001100110000100101011011111011101101001011010101111100111110001000000011001011000011111111011011111111111101111110001101101000101100110101000010110001010 110 100000000111000
14147	01101011111011110011111111010000110111010011100010001110000100100100001010100010000000010000100000000000011110111010011110011011000110000000100001000110111001000000101001001000101010100101101 01100010000000111011000010100100010010000010100010100011110111100001101110101101111010110000011101101110011111000110001000000001001000010010010101000010000111110011011101100101000010000010 1 10100000110011100110101000000100110000000100001001000110000000010100100000001101111111001111111011111110010010010000010001100000101011100000011000111110110111010111010110111011100111001000 00 00000100001000000000000001000010010000000000110000010111101111111111011001011010101101100111100010011001000101100001111111111010111111111101001101111011010010001011001101010000100100010100 11 100010000010000

Appendix 2.B continued.

14676	10101001111011010010111111010000111111011001000110000100000100000000011010100010000000100001000100000000011101110110110100111110000110000010000110011111100110001001001001000111010100101101 011000100000001100100001010010000000101000001000100000111111101000011111111111001100101101000111010011110001111010100111010001100000001111010110100011000011111011101110111001010000101000101 1000000011101111111111000000010011010000010100100100001000000000000010000000101101111011111111001011110010010010000110001100000100010100010011000111110110111010111010110011001100111000000001 01000101000000000001000001000010010000000001110000001010110111111111101101101101010101111101111000100010010100011001101111111110101111101111100110111001101001000101100111111001011001001010110 100011000011000
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17400	00101101111011110001111110110001100110110010000110001000000101111000110010110001010010100000000001001000111011101000101001101100001110000101001000110111001000000101011001001110010101101101 0110101000000001001100001100100011010100000100010000011110111000001011111101100111011110000111101001101011111001011001000000010001100111001011010000100101111101110111011101010111100000001 1010011011111111101101000000010011000000011100010010011100010100000001001000000110111011111111110111111100100100100000100001000110000101100000110001110100101110101110101101010011101010000000000 001001001000000000000000000000100101000000011000001001011011111101110111010111101011111111111000000000001110101100001111111111001111111111101011111111101100111000101100010001001011011001010011 000000010111000
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Appendix 2.B continued.

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	01100010000000110111100101001000100000000000100010100011111111100101011110111100111011110110111101011110100111010111110011001101000011100010010110101101000110010111111011011101100101000010000 0101
	1010001011111111101101000010010111000000010000100100011001000000000001000000000110111101111111110011111110100100100001100010001001000101010000110001111101111111111010010110111001100101001001 00
	0001010000000000000010010010000100101000000111000000101111011111111101101101101111111111001111100100010010101011000011111111111111111111111101001101100011010010101011001101110000110100010100 10
	100010001011000
17549	00101001111011010010111111010000110111010010000001001100100101100000100011111010001000100010010011000000111111110101110011111100101100100101001000110111001000000001001001010101010101 01101101
	0110001000000011001000010101100010010000001010001010001111011111000011111101011011110101100011111010011100011111001000010000101100000000111010110001010001011111100110111011101010000101000101
	10100010110011111011010000000100110010000101000001000100000000000000010000000111111110011111111011111101100100100001100010000000101010100000011000111110011111001011011110111011100100000000000
	00000100000000010000010001000010010100010011101000010111101111110111011001011010111111110111110100000000010100101000011111111101101111111111110011011000110100100010110001011100001111000101101 0
	100011000011000
17550	*0010100111101101001011111001000011011101110000000000110000010000000001010101110000000100100001011001000001111011101000101001111100001000000101001100111111001100000010010010101010101110010 1101*
	0110001000000011001000010111100010010000001010001010001111111000001111111111101111111111000011110100111000011100010000100000000100100000111010010000011001011111001101110110010110001000001 01
	10110000110011110110100000000100110000001101001000000110000000000000100000000010011110111111111100111101100100100000100010000000101010100000011000111010111011010110011110111001100110001000 00
	0000010000001000000000000010000100100001000101000000100111011111111100100101101110111111011110001000100001010110000111111111111111111111111010011011000110100100010110011001100001010100101001 0
	100011001011100

Appendix 2.B continued.

17878	001011011111011111001111111110110001111110111000000010001000000000110000001101110001011100010001100100100000111101110101011100111110000110000010100110011011100110000011000001010100011100101101 01100010000000110110000000101100000001000100011001000011111011110010111111110110111101111100001111010011100011001011100110000000100100000111010110100000100101111101110111011001010100100000101 1010000001100110101101000000001011100000010000000000000100000000000000100000001011011100111111111011111110010010010000010001000000100010100000011000111010110010011111011110101001000101000000000 00110000001000000000000001000010010000000001001000010101101111111110110000110111011011001111000100000010101011000011111111111101111110111100110111001101101000111100110111000011010001010010 100111100011001
17879	0010100111101111000111111101100011011101010000001000110100010100000010101011111000110010001101111110000011110111010101110011011001011000001010110001101110011000000100110101010101010100101101 011000100001001101110001010010000001000100011001000011111011100000111111101100111010110000111101101111001110100110001000000001001000001010101100000110010111110110111011101011000100000101 1010000001100111101101000000001001100000001000010010001000100000000000100000001011011110111111111011111100100100100001100010000000010101000000110101111101111101010110010110111100110010100000100 00000110101000000000000001000000010100010011100000010001101111111110110000110101111011001111100100000011100001000011111111111111111111110100110110101101101000101100110101000010010001010010 100001000000101
18453	00000000111100010101100010111011100110000001111001001010010000000010000000100010010000101000000000000000011100100110001001100101000100001100111100000010010010100001100001001010011100001111 0011000010101000100011000100000101000000000100110011101111011011101011011000010001110011000000000110001110110101010010100000000001001000100000010000000100000111010000011111101101110101100011 00101000010111101000000000000000111101000000001001001010100001001000000001000010011100111000100100011101001010111001100000100000010010100000001010011011011010110000000010001011110010110000010 010000000010000010100010000001000000000000000100000101111110100111010110110000000111010000000010001100010000001010101111001100001111011000101011100110111001010000000010111001110000100010000 100100000010000
18860	0011100010110001010110011011100111010101100111001000000100100001000100000101000000100000001000000000001000111101001100010011111110011010000000010100100111110010110101101001001011011100001111 111110001100101000110100110011011000000000001000100101011111010111010111100010000111101100110110110011110110111001101100000000001000100100010000000101001000111110100011111101101110101101011 1000110011011111000101000100010010110001110001000000010100001001000010011000001110111100101010001110100101010101100100000100010100110000001001110101110010110000000110001011111010111000010 1000000000000111001100100000010000010000000010000010111110011011101011011010000011101000001011011101110000001011100111000100001111011000101011100110111001010100110000011001111100011010001 100101001010000
19611	0110100101011101010111000101110011101100011110000011001001000000000110010001100100000001001000000000010000111000100010010000110000110100101000100100010011110010100010000001011100011100100111 0100010001000000000000001010010000000000000010000010001011110110101011111011101010110001111111100101110011010111001000010000010001101000101100001000011000010110111101101010011111111101100000 111000010001110001000000000101001010001000000000100100100100010010010101000101001110110111111110001111000101001010001001100000000000101010000100100100101101110100110001000101101001111000000 000000010110000100110000100001000000010000000001000101111110010011010111111001111110011001000000000000110000100000010001110111011111101111011001111101000000101000100101011100000100000000100 10 100110000010000

Appendix 2.B continued.

19612	0110100101011101010101000101111011101000011110001001011001000000000100000111001100000000000000000001000001100010101001000111000011110010100010010001001101001010001000100100010011111111100111 01000100000010000000001001001000000000000000110010100101111001000101111101111011110100011111110010111001101011100100001000011000111000010100011000000001000111111111001010110111110101100000 10100001100111010100000001101001000001010000001000001001000100101001000000111111011011111110001111000101001000001001100000000001010100001001001011011011101001100010001011111010110000000 00000001001010110011000010010100010010000000000000101011110010011010101111001111110011000100000000011100010000000000000111110111101011110101000111010000001010000000010001000001100001110010 0001000000000000
JK94-052	00101000011110010111010001111100110010000111101000100011010000100001001000110010000000000000000000000001111001010100100011101000111001011001001000000110100011000110000010001000101001100010 11000000000000001000000101001000000000000000100000000111111110001111111111011010001000011111101101110011010110001000010000100001101000101000000000000100010011101110111110101111111001100000 10100011100101000000000100010100100000111000000000101100000011110100100000100011110111011111110110111000100001010001000000000000000010110000110010111110110101110011100100010111010100100000000 10000001011000000000100001000010000001000000000100010111111001111001001011010101111001000000001000010010000100100010111111111010111111111011101011111011010101000101001101100000110000011000 000100101000100
JK94-073	01101000011011010101010110011000111011000111101000001110100100111000001010100000100100000000010000000100011101100010011010110110001000000010010110000000100001000000000101100001000110010001 01100000000000000000001101001001000000100001000000000111101111001101010111111101010101011101010101101001101101010000001000000000000100010100101000000110000110101110111011101111111100100001 10100001100111110110001100001011101010000000000100000000001100010001100100000010111011111111100011110001111011000100110000100001011000010100001011101110010100100001010101111000000000000001 00000100010000001001101011000100001000000000001010001111011101100110000001011011100010110101101010010010011000001011011011001100111101110101011110010001001010000001101110010110100001010100 10000000010110000
JK94-074	01101000010011010101011010011001111010011111101010001101000101101001001100110000000101001100000000010000011011010110110101101100110001001001111000001011000100000001001011000011111100100011 01100000000000000100000101001001001010000000000101001111011111001111010111111101110001011100010101101101101101000100001000000000011111001100010100100110001001010111011101110111111101101100001 10100001100111010111100100001110001000000101000100100000000100010011000010000110111111111111110101101000111100100010001000000000000101000000100000101111110110100110001000101111001011000100 000100000000000000001000100101000001000001000101000111111111011001100010010111111011101101011000000000000101000100001011101111011111011111011101010101110010011101010011010111110100000100110100 01100000100010
JK95-016	00101100111001111101010001101100111010010011100001110100110001100011000000110010000001010000000000000001110011101001000111000001110010100110010000001101000010001000000000110001010010001 11000001000000011000000010001000000000000000100001000000011111100010111111101101000000000101101011110100110101100010000110000000010000000000000100000010000011111100001010101011111111100100000 11100001100101000000000100000100100000011000000000100100000011110100100000111011110111100111110010011000100001001001101110000000000101111000101101111101111110100000101100101111101011000100 01000001001000000001100001111010000010100000000000100101111110011010010010101101000111000000001000001000100000000000010011111110101111111110101000111000100101110000000011011010011000101100100 000100000000010

GENERAL CONCLUSION

My study has shown that the juvenal plumage of McKay's and Snow Buntings are different, and the combination of characters I analyzed can be used to accurately separate the juvenal plumages of the two species using a discriminant analysis. This difference is especially pronounced with respect to the two subspecies of Snow Buntings included in the study. The analysis of this conservative set of characters can be a useful tool in defining species limits.

The genetic characterization of these two species in the Beringian region provides insight into processes of high-latitude speciation. I have shown evidence that this species pair is genetically distinct and diverged very recently. I have also provided evidence that the divergence of McKay's Buntings fits a model of peripatric, founder effect speciation, with a very small founder population size, rapid morphological divergence likely driven by genetic drift. We also found evidence of likely population expansion followed by a reduction coinciding with asymmetric hybridization and climate change.