GENETIC DIFFERENTIATION OF THE KITTLITZ'S MURRELET *BRACHYRAMPHUS BREVIROSTRIS* IN THE ALEUTIAN ISLANDS AND GULF OF ALASKA

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Received 13 April 2010, accepted 12 May 2011

SUMMARY

BIRT, T.P., MACKINNON, D., PIATT, J.F. & FRIESEN, V.L. 2011. Genetic differentiation of the Kittlitz's Murrelet *Brachyramphus brevirostris* in the Aleutian Islands and Gulf of Alaska. *Marine Ornithology* 39: 45–51.

Information about the distribution of genetic variation within and among local populations of the Kittlitz's Murrelet *Brachyramphus brevirostris* is needed for effective conservation of this rare and declining species. We compared variation in a 429 base pair fragment of the mitochondrial control region and 11 microsatellite loci among 53 Kittlitz's Murrelets from three sites in the western Aleutian Islands (Attu Island) and Gulf of Alaska (Glacier Bay and Kachemak Bay). We found that birds in these two regions differ genetically in three assessments: (1) global and pairwise indices of genetic differentiation were significantly greater than zero, (2) mitochondrial haplotypes differed by a minimum of nine substitutions, and (3) molecular assignments indicated little gene flow between regions. The data suggest that birds in these regions have been genetically isolated for an extended period. We conclude that Kittlitz's Murrelets from Attu Island and from the Gulf of Alaska represent separate evolutionarily significant units, and should be treated as such for conservation. Genetic data for Kittlitz's Murrelets from the remainder of the breeding range are urgently needed.

Key words: Kittlitz's Murrelet, *Brachyramphus brevirostris*, conservation genetics, population genetics, evolutionarily significant units

INTRODUCTION

The Kittlitz's Murrelet *Brachyramphus brevirostris* is a small alcid with a fragmented breeding distribution in coastal Alaska and the northeastern Russia (Day *et al*. 1999). Owing to its rarity and secretive nesting habits, its basic biology is not well known. Reports of small and declining numbers led to consideration for listing under the US *Endangered Species Act* (US Fish and Wildlife Service 2010) and have stimulated research into factors influencing populations (e.g. Romano *et al*. 2007, Agness *et al*. 2008, Kaler *et al*. 2009). One important knowledge gap is how genetic variation is distributed within and among local breeding populations.

Population genetic information is critical for evaluating the conservation status of a species for several reasons (see reviews by Allendorf & Luikart 2006, Frankham 2010). At a basic level, it is useful for resolving taxonomic issues. For example, molecular genetic data can reveal cryptic species, i.e. species that are closely related and morphologically similar but reproductively isolated. The Long-billed Murrelet *B. perdix* (Friesen *et al*. 1992) and Monteiro's Storm-petrel *Oceanodroma monteiroi* (Bolton *et al*. 2008) are examples of cryptic species whose distinctness was confirmed using population genetic data. Knowledge of the extent to which local breeding populations differ genetically is also critical for protecting a species' genetic resources, including local adaptations and the potential for adaptation to future changes such as anthropogenic stressors (Allendorf & Luikart 2006). Finally, population genetic information can be used to infer patterns of gene flow among local breeding populations; populations that are not differentiated genetically likely have high gene flow, whereas populations with strong genetic differences likely have little gene flow (Allendorf & Luikart 2006, but see Whitlock & McCauley 1999). Because gene flow is a result of successful dispersal, it may indicate a species' ability to recover from local reductions or extirpations; species with high gene flow are expected to recover more rapidly than species with low gene flow because of greater potential for recruitment from outside areas. The importance of population genetic structure to conservation is recognized in many pieces of legislation, including the US *Endangered Species Act*, that provide special protection to distinct population segments of vertebrate species (Fay & Nammack 1996).

Due to differences in parameters such as mode of inheritance and rate of evolution, different types of genes provide information on different historical time scales and permit inferences about different aspects of a species' biology (Allendorf & Luikart 2006). Rapidly evolving nuclear loci, such as microsatellites, provide information about recent or contemporary demography, while more slowly evolving loci, such as nuclear introns and most protein-coding genes, are informative about more ancient demographic history. Because mitochondrial DNA (mtDNA) is inherited maternally (i.e. clonally) and does not appear to recombine, it is useful for construction of gene trees, which are informative about evolutionary relationships among populations. Some regions of the mitochondrial genome,

particularly the control region, evolve especially rapidly and are therefore highly variable within many species. Studies that incorporate data from both nuclear and mitochondrial genomes can be especially informative because they take advantage of strengths offered by both types of markers.

To aid understanding of the biology of the Kittlitz's Murrelet, we investigated population genetic variation across part of the species' range where it is most abundant (US Fish and Wildlife Service 2010). Specifically, we assayed variation in mtDNA and nuclear microsatellite loci to evaluate relationships among local populations of Kittlitz's Murrelet in the western Aleutian Islands and Gulf of Alaska. Our study was limited by small sample sizes, owing to the scarcity of genetic material collected on this species to date. Nonetheless, material was sufficient to provide preliminary estimates of genetic variation within the species and to assess whether there are major genetic discontinuities within the area sampled.

STUDY AREA AND METHODS

Between 1989 and 2002, 53 Kittlitz's Murrelets were sampled at three breeding locations roughly 3200 km apart, from Attu Island in the west to Glacier Bay in the east (Fig. 1, Table 1). Samples from Attu Island (n = 15) and Kachemak Bay (n = 18) consisted of freshly frozen liver and/or heart tissue from birds collected at sea for dietary analysis (Hobson *et al*. 1994). Samples from Glacier Bay (n = 20) consisted of whole blood dried onto filter paper from birds netted on the water at night from small boats as part of a radiotelemetry study (Romano *et al*. 2007). All samples were from adults in breeding plumage captured near known breeding areas during the breeding season (May–August; Day *et al*. 1999). We prepared DNA using either standard protease K/phenol-chloroform extraction (Sambrook *et al*. 1989) or DNeasy spin columns (Qiagen, Mississauga, Ontario) according to the manufacturer's instructions.

Mitochondrial control region

We amplified an approximately 700 base pair (bp) segment of mtDNA containing a small portion of the ND6 gene, the gene for tRNA for glutamic acid, and approximately 600 bp of the control region (domains I and II; Baker & Marshall 1997) using polymerase chain reaction (PCR) primers and protocols developed for the closely related Marbled Murrelet *B. marmoratus* (Friesen *et al*. 2005). We subjected PCR products to electrophoresis through 2% agarose gels, purified them using QIAquik Gel Extraction Kits (Qiagen, Montreal, Québec), and sequenced them using ThermoSequenase sequencing kits (GE Health Care, Montreal, Québec) according to the manufacturer's directions. Sequencing products were run through 6% polyacrylamide gels and visualized using autoradiography. For population-level analyses, we trimmed sequences to include only the control region (429 bp).

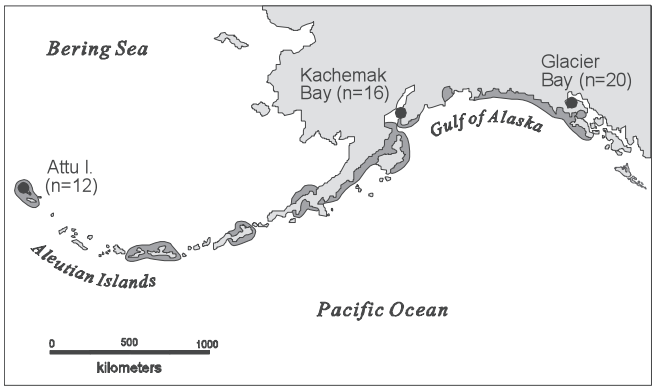

Fig. 1. Map showing approximate breeding range of Kittlitz's Murrelet in the Aleutian Islands and Gulf of Alaska (dark grey; from US Fish and Wildlife Service 2010) and sampling sites for genetic analyses (black circles), Alaska, 1990–2004. Sample sizes in parentheses.

Microsatellites

We assessed length variation in 11 microsatellite loci containing tetranucleotide repeats originally isolated from the Marbled Murrelet (Rew *et al*. 2006; Appendix 1, available online) according to protocols described by Ibarguchi *et al*. (2000). The annealing temperature for all microsatellites was 59 °C except for locus Bma555, which was annealed at 50 °C.

Tests of assumptions, and variabilities

We tested for deviations from neutrality in control region variation using the tests of Ewens-Watterson (Ewens 1972, Watterson 1978) and Chakraborty (Chakraborty 1990), and for deviations from Hardy-Weinberg and gametic equilibrium in microsatellite variation using Fisher's exact tests implemented in the software package Arlequin (v3.1; Excoffier *et al*. 2005) with 10 000 randomizations of the data. Arlequin also was used to estimate haplotypic (h) and nucleotide diversities (π) for control region variation, and expected heterozygosities (H_E) and inbreeding coefficients (F_{IS}) for microsatellite variation.

Population genetic structure and gene flow

We used analysis of molecular variance (AMOVA; Excoffier *et al*. 1992), also implemented in Arlequin, to index the geographic distribution of variation in control region sequences (Φ_{ST} and

TABLE 1
Sample and genetic data for Kittlitz's Murrelets from three sampling locations in Alaska

Data	Attu Island	Kachemak Bay	Glacier Bay
Sample size, n	15	18	20
Collection dates	2 August 1992 24 June 1999	26 July 1990 16–17 July 1996	6–14 May 2004
Haplotypic diversity, h (SD)	0.79 (0.09)	0.89 (0.05)	0.68 (0.09)
Nucleotide diversity π (SD), %	2.82 (2.21)	3.00 (2.4)	1.90 (1.74)
Expected heterozygosity, H_E (SD)	0.79 (0.06)	0.77 (0.17)	0.79 (0.15)
Wright's inbreeding coefficient,[a] FIS	0.02	-0.02	0.06[b]

[a] Averaged across 11 microsatellite loci.
[b] Significantly different from zero (P = 0.05).

pairwise sequence divergence [δ]) and microsatellite loci (F_{ST}) both globally and for population pairs. Because of the small number of populations, we did not make any corrections for Type I errors. For the control region data, we applied Kimura's two-parameter model of substitution (Kimura 1980) with a γ parameter of 0.45. In all of these analyses, we determined significance by randomization using 10 000 permutations of the data. To infer the extent of phylogeographic structure in control region haplotypes, we constructed a gene tree using the method of statistical parsimony implemented in TCS (v1.21; Clement *et al*. 2000) with α = 0.05. We also analyzed microsatellite variation using the program Structure (v2.3; Pritchard *et al*. 2000, Falush *et al*. 2003, Hubisz *et al*. 2009), which uses a Bayesian maximum likelihood approach to determine the number of genetic populations (*k*) represented in a sample set, i.e. the number of groups of samples needed to minimize deviations from Hardy-Weinberg and gametic equilibria. We ran the program with settings that are best able to detect weak population structure; specifically, the no admixture model with correlated allele frequencies using sampling site as prior information; use of alternative settings did not change the most likely value of *k*, although assignment probabilities for individual birds tended to be lower. Preliminary analyses indicated that a burn-in of 10 000 iterations and 100 000 replications after burn-in were sufficient for likelihood values to stabilize. We ran the program 20 times for $k = 1$ to 3 genetic populations and estimated the most likely value of *k* from the posterior probabilities, as described by Pritchard *et al*. (2010). Assuming that the maximum potential value of *k* was 3 (the number of sampling locations), the method of Evanno *et al*. (2005) could not be applied.

We used the program GeneClass (v2.0.h; Piry *et al*. 2004) to detect potential first generation migrants into each of the two genetic populations identified by Structure. We used Bayesian methods (Rannala & Mountain 1997) to estimate L_{home}, the likelihood that an individual's genotype originated within the genetic population where it was sampled. We determined the probability that an individual was a migrant by comparing L_{home} to a distribution for 10 000 multilocus genotypes created by Monte Carlo re-sampling of the original data (Paetkau *et al*. 2004). We used a rejection level (α) of 0.05 (Hall *et al*. 2009). Given that only part of the species' range was sampled, we did not consider assignment of immigrant individuals to specific source locations to be appropriate.

TABLE 2
Matrix of pairwise estimates of Φ_{ST} (below diagonal, upper value) and δ (below diagonal, lower value, as a %) from mitochondrial control region variation, and F_{ST} (above diagonal) from variation at 11 microsatellite loci for Kittlitz's Murrelets from three sampling locations in Alaska

	Attu Island	Kachemak Bay	Glacier Bay
Attu Island	—	0.06[b]	0.06[b]
Kachemak Bay	0.94[b] 4.71[b]	—	0.01[a]
Glacier Bay	0.96[b] 4.83[b]	0.00 0.00	—

[a] $P < 0.05$
[b] $P < 0.001$.

RESULTS

Tests of assumptions, and variabilities

Several lines of evidence indicate that the DNA sequences we obtained represent true mtDNA rather than nuclear homologs: (1) sequences were similar to those previously published for the Marbled Murrelet (Friesen *et al*. 2005); (2) the putative tRNA for glutamic acid could be folded into a typical clover-leaf structure (Desjardin & Morais 1990); (3) conserved sequence blocks were identical in sequence to those of the Marbled Murrelet (Friesen *et al*. 2005); (4) base composition of the light strand was biased against Gs; and (5) variable sites were concentrated in domain I of the control region (GenBank Accession Nos. JN257122–JN257134).

We detected 13 haplotypes among 47 birds (Appendix 2, available online; unambiguous sequence was not obtained for six birds). We found no evidence for deviations from neutrality (Ewens-Watterson and Chakraborty's tests, all $P > 0.36$). Haplotype diversity estimates ranged from 0.68 in Glacier Bay to 0.89 in Kachemak Bay; nucleotide diversity estimates ranged from 1.90 in Glacier Bay to 3.00 in Kachemak Bay (Table 1).

All microsatellite loci were variable, with between 6 and 16 alleles per locus. Allele sizes differed by 4 bp in a step-wise fashion, as expected for tetranucleotide repeats. Significant heterozygote deficiencies were found for only two loci in only one population (Glacier Bay; Appendix 1, available online), providing no evidence for null (non-amplifying) alleles or allelic drop-out (see also Rew *et al*. 2006). We found no consistent evidence for linkage among these loci: significant deviations from gametic equilibrium existed for only one pair of loci within the Attu Island population (BmaACCT555 and BmaTATC356), and for three different pairs of loci within the Glacier Bay population (BmaGGAT368 and BmaTGAA523; BmaTATC453 and BmaGATA553; and BmaGATA553 and

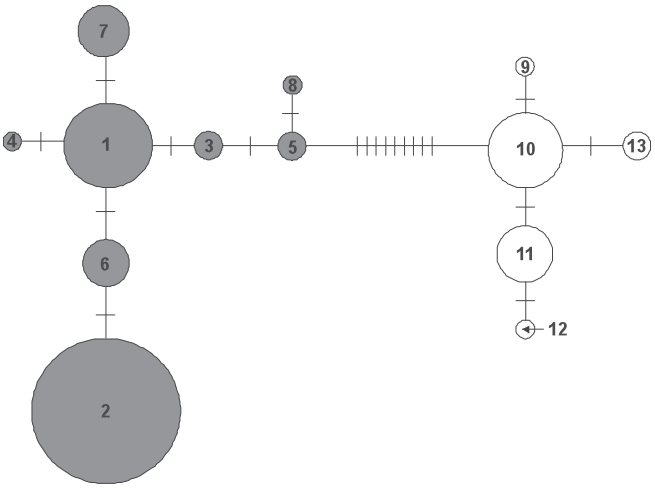

Fig. 2. Statistical parsimony tree showing substitutional relationships among control region haplotypes for Kittlitz's Murrelets, Alaska, 1990–2004. Haplotypes found in Attu Island samples are shown in light grey; haplotypes found in the Gulf of Alaska sites are shown in dark grey. Numbers in circles are haplotype numbers, and sizes of circles are proportional to haplotype frequencies. Cross-hatches represent substitutions.

BmaACCT555). Heterozygosities (H_E) averaged across the 11 loci did not differ among the three sampling sites (range from 0.77 to 0.79; Table 1). However, Wright's F_{IS} suggested the possibility of mild but statistically significant inbreeding within the Glacier Bay population (F_{IS} = 0.06; Table 1).

Population genetic structure and gene flow

Control region variation was strongly structured among sampling locations. The global estimate of Φ_{ST} was large and significantly different from zero (0.92, $P < 0.001$). Pairwise estimates of Φ_{ST} and mean sequence divergence (δ) indicated that population structure was due almost entirely to a strong genetic discontinuity between the Attu Island and the Gulf of Alaska populations (Glacier Bay and Kachemak Bay; Table 2). This discontinuity was also evident in the statistical parsimony tree: haplotypes from Attu Island samples differed from those from the Gulf of Alaska by a minimum of nine substitutions (Fig. 2). Furthermore, strong phylogeographic structuring was indicated by the complete sorting of haplotypes from these two populations on the haplotype tree.

We also found strong differentiation in microsatellite variation; the global F_{ST} estimate was highly significant (0.039, $P < 0.001$), although lower than the global estimate of Φ_{ST} (above). As with control region variation, population genetic structuring appeared to be due mainly to a genetic discontinuity between samples from Attu Island and the Gulf of Alaska (Table 2). Results from Structure indicated that the most probable number of genetic populations was two ($P > 0.999$). All 15 birds sampled from Attu Island were assigned to one genetic population with $P > 0.70$ (and all but 2 were assigned with $P > 0.99$), while all 38 birds sampled from Kachemak Bay and Glacier Bay were assigned to the other genetic population with $P > 0.92$ (Fig. 3). Subsequent analyses including only samples from the Gulf of Alaska did not detect any genetic substructure within this region (Pr[k=1] > 0.999). Results from the GeneClass analysis suggested that five individuals (two from Attu Island and three from the Gulf of Alaska) were unlikely ($P < 0.05$) to have originated within the area where they were sampled, and so represent potential immigrants.

DISCUSSION

Although the geographic sampling of Kittlitz's Murrelets in this study was incomplete, and sample sizes were small, our data clearly indicate that at least two strongly differentiated genetic populations exist: one on Attu Island and the other in the Gulf of Alaska. This division is supported by several types of analysis on both mitochondrial and nuclear data, including traditional indices of population genetic structure (Table 2), the structure of the mitochondrial gene tree (Fig. 2), and Bayesian inference of both population genetic structure (Fig. 3) and contemporary gene flow. Population differentiation in control region sequences in Kittlitz's Murrelet is among the highest of any seabird species studied to date (reviewed in Friesen *et al*. 2007).

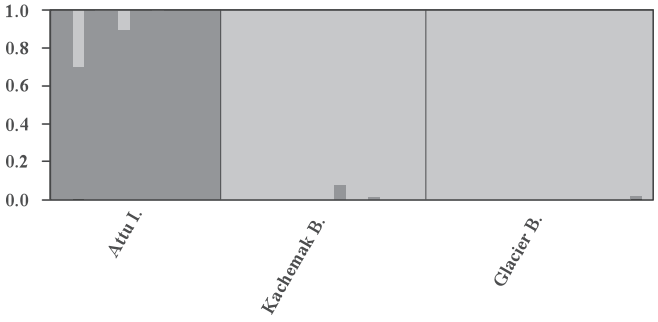

Fig. 3. Output from Structure showing probabilities of assignment of individual Kittlitz's Murrelets (vertical bars) to each of two genetic populations (shades of grey), Alaska, 1990–2004. Sampling locations are indicated on the horizontal axis and assignment probabilities on the vertical axis.

Because the Kittlitz's Murrelet is non-colonial and its nests are very difficult to locate (Day *et al*. 1999), we were limited to sampling birds on the water and therefore assume the sampled birds in our study were part of the local breeding population. We believe this was a reasonable assumption because birds were caught during the breeding season (May–August; Table 1) in the vicinity of alpine habitat suitable for nesting (Day *et al*. 1999). In addition, all sampled birds showed partial to full breeding plumage and evidence of brood patches (Romano *et al*. 2007). Although these characteristics are not always associated with breeding in Marbled Murrelet (MacFarlane Tranquilla *et al*. 2003), and the breeding propensity of Kittlitz's Murrelets is presumed to be low (Day & Nigro 2004), large movements within the breeding season are uncommon for *Brachyramphus* murrelets (Lougheed *et al*. 2002, Hebert & Golightly 2008, M. Kissling, unpublished data). Therefore, we infer that our assumption that sampled birds belonged to the local breeding population was reasonable. Regardless, inclusion of immature birds or pre-breeders or both in our study would have resulted in under-estimation of population genetic structure and over-estimation of gene flow.

While the mtDNA variation was strongly differentiated among sampling locations (global Φ_{ST} = 0.92), only moderate differentiation was evident in microsatellites (F_{ST} = 0.039). The level of differentiation was similar to that found in the Marbled Murrelet (maximum pairwise F_{ST} = 0.039; Hall *et al*. 2009) despite lower population structure in mtDNA in the Marbled Murrelet (maximum pairwise Φ_{ST} = 0.18; Friesen *et al*. 2005). Indices of population genetic structure based on microsatellites are generally lower than those based on mitochondrial genes because of the higher effective population size and typically higher variabilities of microsatellites (Hedrick 1999, Whitlock & McCauley 1999). However, moderate population differentiation in nuclear genes in the presence of very strong differentiation in mtDNA could indicate that gene flow is mediated by males in this species, although other factors (e.g., population bottlenecks) are possible. Male-mediated gene flow would be inconsistent with the observation that gene flow is female-mediated in most species of birds (Greenwood 1980, Clarke *et al*. 1997), including the Marbled Murrelet (Hall *et al*. 2009); this possibility warrants further investigation.

Genetic assignments (i.e. results from Structure and GeneClass) identified up to five birds with low probability of originating from the locations where they were sampled. Such "misassigned" birds may represent immigrants. Whether these birds successfully reproduced and thereby generated gene flow is uncertain, especially in light of apparent poor reproductive success of immigrant Great Frigatebirds *Fregata minor* in the Indo-Pacific (Dearborn *et al*. 2003) and Marbled Murrelets in central California (Hall *et al*. 2009, Peery *et al*. 2010).

While Kittlitz's and Marbled murrelets are closely related and have widely overlapping distributions, results of the present study suggest that the two species have different demographic histories. In addition to differences in the extent of population genetic structure (above), haplotype diversity estimates for Kittlitz's Murrelet (Table 1) were generally lower than those reported for Marbled Murrelet (up to 1.00; Friesen *et al*. 2005), and nucleotide diversity estimates were higher (Table 1, versus up to 1.04 in Marbled Murrelet, Friesen *et al*. 2005). The deep branch in the statistical parsimony tree for Kittlitz's Murrelet (Fig. 2) suggests that the species' distribution was fragmented historically (Templeton 1998), probably during the most recent glaciations, and the lower haplotypic diversities in this species suggest that fragmentation may have been coincident with small effective population sizes. In contrast, no similar deep branch is seen in the statistical parsimony tree for the Marbled Murrelet control region, and haplotypic diversities are higher (Friesen *et al*. 2005; V. Friesen, unpublished data). Thus, although peripheral populations of Marbled Murrelets (i.e., in central California and the western Aleutian Islands) are differentiated genetically from the central population, the Marbled Murrelet does not appear to have undergone historic population fragmentation or severe reductions in effective population size as has Kittlitz's Murrelet (but see Peery *et al*. 2010).

Several lines of evidence suggest that Kittlitz's Murrelets sampled in Glacier Bay may not be pairing randomly: (1) heterozygote deficiencies were detected for three loci (Appendix 1); (2) three pairs of loci showed significant deviations from gametic equilibrium; and (3) Wright's inbreeding coefficient was significantly greater than zero (Table 1). Although such deviations may result from technical artifacts such as null (nonamplifying) alleles, their occurrence in several loci within the Glacier Bay sample and absence in birds sampled from other locations argues against technical artifacts and suggests that population genetic structure may exist within Glacier Bay. This possibility should be investigated with larger numbers of samples and loci.

Conservation implications

In 1996, the US Department of the Interior (DOI) defined "distinct population segments" under the US *Endangered Species Act* as populations that are "markedly separated from other populations of the same taxon," and proposed that "Quantitative measures of genetic or morphological discontinuity may provide evidence of this separation" (Fay & Nammack 1996). DOI argued that a discrete population may merit special conservation status given "evidence that the discrete population segment differs markedly from other populations of the species in its genetic characteristics." Similarly, Moritz (1994) defined evolutionarily significant units for conservation as populations that are "reciprocally monophyletic for mtDNA alleles and also differ significantly for the frequency of alleles at nuclear loci." This definition has become widely applied to birds and other vertebrates (Holder *et al*. 2004). Although our results were derived from limited geographic coverage and relatively small sample sizes, high sequence divergence between the control region haplotypes of birds from Attu Island versus those from the Gulf of Alaska, combined with differentiation in nuclear DNA, suggests that Kittlitz's Murrelets in these locations have been isolated for a prolonged period and thus represent significant components of the species' evolutionary history. Furthermore, results from several analyses indicate that contemporary gene flow between these regions is restricted, and thus that murrelets in these regions are largely independent both genetically and demographically. We conclude that Kittlitz's Murrelets breeding at Attu Island versus those at the Gulf of Alaska constitute separate evolutionarily significant units and recommend that efforts to monitor and manage this species should reflect this finding. Further sampling is needed to determine the geographic location and abruptness of the transition between these genetic units.

Additional genetic populations may exist within Kittlitz's Murrelet because the breeding range extends farther north and west than the area we sampled, including portions of Alaska and the Russian Far East bordering the Bering and Chukchi seas (Day *et al*. 1999). Small numbers of birds have also been reported to breed around the Sea of Okhotsk (Artukhin *et al*. 2011) and in northwestern Alaska (Day *et al*. 2011). How murrelets in these locations relate genetically to those represented in this study is worthy of future study because small population sizes and large gaps in the breeding range enhance the likelihood of genetic divergence. However the species is difficult to sample owing to its dispersed, non-colonial nature, so obtaining a thorough representation of murrelets from across its entire range will be a significant challenge for future investigations.

ACKNOWLEDGEMENTS

We gratefully acknowledge the contributions of many staff members of US Geological Survey, US Fish and Wildlife Service, Alaska Maritime National Wildlife Refuge and crew of the *M/V Tiglax* for assistance in collecting samples. In particular, we thank K. Bell, G. Drew, A.B. Johnson, D.D. Gibson, R. Kaler, L. Kenny, M. Kissling, T. van Pelt, J. Pitoccheli, D.A. Rocque, M. Romano, M. St. Peters, J. Williams and K. Winker for help collecting or shipping specimens. M. Kissling, B. Day, K. Kuletz, E. Lance and G. van Vliet provided helpful discussions. We also thank M. Kissling, Z. Peery and one anonymous reviewer for providing useful comments on the manuscript, and M. Kissling for organizing the Kittlitz's Murrelet special symposium. Funding was provided by the US Geological Survey and the Natural Sciences and Engineering Research Council of Canada. Any use of trade, product or firm names in this publication is for descriptive purposes only and does not imply endorsement by the US government.

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