The Condor 98:681–690

EVIDENCE FROM CYTOCHROME *B* SEQUENCES AND ALLOZYMES FOR A 'NEW' SPECIES OF ALCID: THE LONG-BILLED MURRELET (*BRACHYRAMPHUS PERDIX*)[1]

V. L. FRIESEN[2]
Department of Ornithology, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario M5S 2C6, Canada

J. F. PIATT
National Biological Survey, Alaska Fish and Wildlife Research Center, United States Department of the Interior, 1011 East Tudor Road, Anchorage, AK 99503

A. J. BAKER
Department of Ornithology, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario M5S 2C6, Canada

Abstract. Marbled Murrelets (*Brachyramphus marmoratus*) are coastal seabirds that breed predominantly in old-growth forest throughout the North Pacific. Presently they are classified into two phenotypically distinct subspecies: one in North America (*B. m. marmoratus*) and one in Asia (*B. m. perdix*). The Asian form was classified as a separate species in 1811, but was lumped with *B. marmoratus* during the 20th century. Populations of both types are considered threatened or endangered and information about the extent of genetic differentiation among birds from different sites is required for their conservation. We compared variation in 1,045 base pairs of the mitochondrial cytochrome *b* gene and 39 allozyme loci among Marbled Murrelets and the closely related Kittlitz's Murrelets (*B. brevirostris*) from throughout the North Pacific. All analyses indicted that North American and Asian Marbled Murrelets are genetically distinct: cytochrome *b* sequences were highly divergent, fixed allele differences occurred at two allozyme loci, and estimated gene flow was essentially zero. Phylogenetic analyses of cytochrome *b* sequences and allozymes both provided strong support for a monophyletic relationship among North American Marbled Murrelets and Kittlitz's Murrelets, with Long-billed Murrelets forming the basal lineage. Long-billed and North American Marbled Murrelets clearly represent distinct species by any definition, and must be managed independently. Significant genetic differentiation also was found among both Marbled and Kittlitz's Murrelets from different sites within North America.

Key words: allozyme; Brachyramphus*; cytochrome b; heteroduplex; murrelet; population genetics; phylogenetics; SSCP.*

INTRODUCTION

Marbled Murrelets (*Brachyramphus marmoratus*) are coastal seabirds that breed predominantly in old-growth forest throughout the North Pacific (Ralph et al. 1995). Presently they are classified into two subspecies: the nominate form (*B. m. marmoratus*) has a partial white neck collar and rufous back, and breeds from California to the Aleutian Islands; a distinctly larger, long-billed form with a white eye-ring (the Long-billed, Partridge, or Asiatic Murrelet, *B. m. perdix*) breeds from Japan through the Sea of Okhotsk to the Kamchatka Peninsula (Konyukhov and Kitaysky 1995; Fig. 1). Long-billed Murrelets originally were classified as a species of guillemot (*Cepphus perdix;* Pallas 1831), but were reclassified as *B. perdix* by Ridgeway in 1919, and eventually were lumped with North American Marbled Murrelets by the American Ornithologists' Union (AOU 1983). Zink et al. (1995) and Friesen et al. (1996a) documented differences in mitochondrial DNA (mtDNA) between Long-billed and North American Marbled Murrelets, and suggested that the two forms may represent separate species.

Marbled Murrelets are declining in numbers throughout most of their range due to conflict with logging interests, vulnerability to oil pollution, and drowning in gill nets (Sealy and Carter 1984, Carter and Kuletz 1995, Carter et al. 1995). Murrelets have almost disappeared from several parts of their range, and are under pressure from loss of habitat in other areas. The spe-

[1] Received 30 April 1996. Accepted 17 July 1996.
[2] Present address and corresponding author: Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada, e-mail: friesenv@biology.queensu.ca

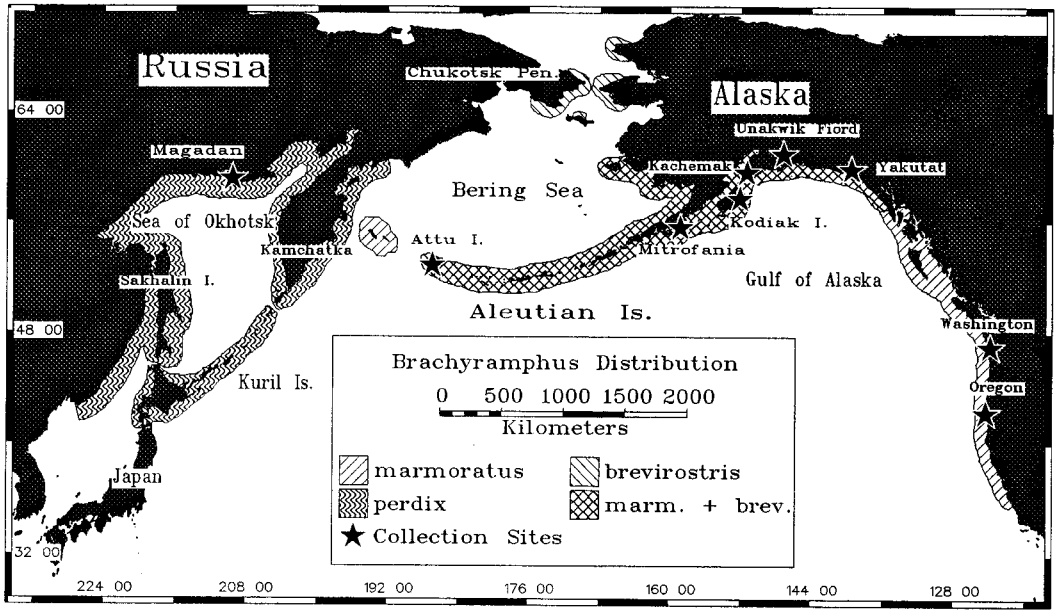

FIGURE 1. Breeding distributions and sampling sites for murrelets.

cies is listed officially as Threatened in Canada and Endangered in California, Oregon and Washington; the size of the Asian population is unknown, but it also is under increasing threat from logging. Design of a successful conservation plan for murrelets requires knowledge of the extent of genetic differentiation among animals from different sites: if subpopulations differ genetically, then loss of a population will reduce the species' genetic resources, which may increase its vulnerability to extinction (Allendorf and Leary 1986, Gilpin and Soulé 1986). Furthermore, if animals from different sites are genetically isolated, they may not naturally recolonize areas from which they are extirpated. Genetic information also can lead to the identification of cryptic species—animals that are similar in appearance but that represent distinct, reproductively isolated species. Pittochelli et al. (1995) compared variation in morphology and mtDNA among Marbled Murrelets and the closely related Kittlitz's Murrelet (*B. brevirostris*) from several sites in Alaska, but no large-scale surveys have been conducted. In the present paper, we compared variation in the mitochondrial cytochrome *b* gene and allozymes among brachyramphine murrelets from throughout the North Pacific. Our objectives were to measure the level of genetic divergence between Asian and North American Marbled Murrelets, and to obtain a preliminary indication of the extent of differentiation among Marbled Murrelets from different areas within North America.

METHODS

SAMPLING

Tissue samples were obtained from murrelets from throughout their range (Fig. 1; Table 1). Most samples comprised freshly frozen heart, liver and striated muscle from birds shot at sea for dietary analyses, but some samples were derived from dead or dying murrelets collected by the Marbled Murrelet Recovery Team of the Pacific Seabird Group. Samples from California consisted of stomachs from murrelets collected in 1982 for dietary analysis and preserved in ethanol; these samples could not be used for protein electrophoresis. Tissue from four Long-billed Murrelets from Magadan, Russia, were donated by the Burke Museum of Natural History, Seattle. Tissues were stored between −70°C and −80°C until use.

CYTOCHROME *B*

Variation in cytochrome *b* was assayed both by direct nucleotide sequencing and by analyses of single-stranded conformation polymorphisms

TABLE 1. Collection sites, and frequencies of cytochrome *b* genotypes for murrelets.

Site	Genotype																	
	Bm1	Bm2	Bm3	Bm4	Bm5	Bm6	Bm7	Bm8	Bm9	Bm 10	Bm 11	Bm 12	Bm 13	Bp1	Bb1	Bb2	Bb3	Bb4	Total
Marbled Murrelets																			
Magadan, Russia	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	4
Attu I., AK	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Mitrofania B., AK	8	0	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	14
Kodiak I., AK	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2
Kachemak B., AK	3	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	5
Unakwik F., AK	6	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	9
Yakutat, AK	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Humboldt In., WA	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Southwest OR	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Kittlitz's Murrelet																			
Attu I., AK	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	3
Kachemak B., AK	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	4

(SSCPs) and heteroduplexes (HDs; Lessa and Applebaum 1993). Combination of these techniques provides a relatively rapid and inexpensive method of screening for variation. Analysis of SSCPs is based on the fact that single-stranded DNA forms a characteristic secondary structure that depends on the sequence of nucleotides, and that the rate at which a segment of DNA migrates through a non-denaturing gel in response to an electrical current is determined in part by its secondary structure. Segments of DNA that differ by even a single base have slightly different secondary structures and migrate at different rates. In HD analysis, DNA from a sample that serves as a standard is mixed with an equal amount of DNA from an unknown sample. The DNA is denatured, then cooled slowly to allow complementary strands to reanneal. The result is a mixture of double-stranded products consisting of two types of homoduplexes (with complementary strands from the same samples), and two types of heteroduplexes (with complementary strands from different samples). If nucleotide sequences of the two samples are the same, then homo- and heteroduplexes will have identical eletrophoretic profiles, but if samples differ by even a single mutation, electrophoretic profiles of the four products will differ.

In the present study, DNA was extracted from tissue samples, and a 1,045 base pair (bp) fragment of cytochrome *b* was amplified using the primers L14841 (Kocher et al. 1989) and H16065 (located in the $tRNA^{thr}$; Birt and Baker, unpubl. data) using standard protocols (Birt and Baker, unpubl. data). Amplification products were subjected to electrophoresis in 2% agarose gels containing 1 μg/mL ethidium bromide, then excised from the gel. Gel slices were diluted with 100 μL H_2O and melted at 90°C, and 1 μL was used as template for a second amplification that included 1 μCi ^{35}S-α-dATP. Because the efficiency of detection of either SSCPs or HDs declines as the length of the amplification product increases, the fragment analyzed in the present study was cleaved into two segments, one ~500 bp and one ~550 bp, using the restriction endonuclease *Mbo I*. For analysis of SSCPs, 1 μL of radiolabeled DNA was combined with 9 μL of a loading buffer containing 95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol. Samples were heated to 95°C for 5 min, then plunged into ice-water for 2 min. 5 μL of the product was subjected to electrophoresis in a 20 cm × 45 cm, 0.5 × HydroLink® MDE (J. T. Baker, Phillipsburg, NJ) polyacrylamide gel at 6 W for 48 hr at ambient temperatures. Results were visualized by autoradiography for ~18 hr. For HD analysis, 1 μL of radiolabeled DNA was combined with 1 μL of a standard and 2 μL water, heated to 95 °C for 3 min, then cooled to 25°C over 30 min. 1 μL of tracking dye (50% sucrose, 5 × TBE buffer, 0.05% bromophenol blue and 0.05% xylene cyanol) was added and samples were run in a 1 × MDE gel at 600 V for 24 hours. Results were visualized by autoradiography for ~18 hr.

Complete nucleotide sequences were determined for individuals with variant genotypes using standard protocols (Birt and Baker, unpubl. data). Briefly, DNA remaining from the first set

of amplifications (above) was purified using GeneClean® II kits (Bio-101; Vista, CA) according to the manufacturer's directions, and double-stranded sequencing was conducted with Sequenase® kits (U.S. Biochemicals; Cleveland, OH) using one of the amplification primers or four internal primers as described in Birt and Baker (unpubl. data). Ten birds that appeared from analysis of SSCPs and HDs to possess the most common genotype for North American Marbled Murrelets (type Bm1) also were sequenced to estimate the proportion of variation that was undetected from SSCPs and HDs. Sequences were aligned by eye and were confirmed by sequencing complementary strands (approximately 40% of base pairs).

The extent of differentiation of cytochrome *b* sequences among sampling sites was assessed using three indices. The among-site component of variation (G_{ST}) was calculated using the program AMOVA and was tested for significance by randomization (Excoffier et al. 1992). Because G_{ST} tends to decline as the number of genotypes increases, we also calculated the mutational divergence among sites (γ), which is the probability that any two individuals chosen at random from different sites will have different genotypes (Lynch and Baker 1994, Friesen et al. 1996b). γ was tested for significance using a computerized randomization program (A. Lynch, University of Toronto, unpubl. data). G_{ST} and γ do not incorporate information on sequence divergence among genotypes, so may under-represent the extent of genetic differentiation among sites; we therefore used an analysis of molecular variance (AMOVA) to calculate ϕ_{ST}, or the extent to which sequence variation is partitioned among sites (Excoffier et al. 1992). ϕ_{ST} was tested for significance using a randomization procedure (Excoffier et al. 1992). Gene flow (N_em, females per generation) was calculated from G_{ST} as described by Birky et al. (1983). Although other methods for calculating gene flow from sequence data have been developed recently (e.g., Slatkin and Maddison 1989, Neigel and Avise 1993), the present data were not amenable to these approaches due to the lack of clear phylogenetic relationships among many genotypes (see Results).

Maximum parsimony analysis of cytochrome *b* sequences was conducted on PAUP (version 3.1.1; Swofford and Begle 1993) using the branch-and-bound search algorithm with mid-point rooting and no specified outgroup. Starting trees were generated using the 'closest' addition option, and 'tree bisection-reconstruction' was used for branch-swapping. Support for phylogenetic relationships was evaluated by bootstrapping. A neighbor-joining tree (Saitou and Nei 1987) also was constructed using Kimura's (1980) two-parameter correction for multiple hits (MEGA version 1.0; Kumar et al. 1993). Support for phylogenetic relationships was assessed using both standard error tests (Rzhetsky and Nei 1992, 1993) and bootstrap values (Felsenstein 1985).

ALLOZYMES

A preliminary screening of electrophoretic variation in allozymes in 10 murrelets (five Marbled, one Long-billed and four Kittlitz's Murrelets) was conducted for 39 presumptive loci using standard starch gel protocols (Baker et al. 1985, Friesen et al. 1996a; Table 2); 36 additional samples were assayed for variation at those loci found to be variable in the preliminary survey. Allele frequencies were tested for deviation from Hardy-Weinberg equilibrium using a Chi-square analysis with pooling of classes and Levene's correction for small sample sizes (Biosys-1; Swofford and Selander 1981). Wright's F_{ST} was calculated as a weighted mean (Kirby 1975) using Biosys-1, and was tested for significance by jackknifing across loci (Weir 1990). Gene flow (N_em; individuals per generation) was estimated as $((1/F_{ST}) - 1)/4$ (Wright 1965). Phylogenetic relationships among sites were investigated using a maximum likelihood analysis of allele frequencies (Felsenstein 1981) on PHYLIP (version 3.41; Felsenstein 1989).

RESULTS

CYTOCHROME *B*

Analyses of SSCPs and HDs clearly distinguished cytochrome *b* genotypes of Long-billed, North American Marbled, and Kittlitz's Murrelets. Nucleotide sequencing revealed that the most common genotypes of Long-billed and North American Marbled Murrelets differed by 100 substitutions in 1045 bp (including 11 transversions and nine amino acid replacements), and those of Long-billed and Kittlitz's Murrelets differed by 84 substitutions (including 9 transversions and nine amino acid replacements), whereas those of North American Marbled and Kittlitz's Murrelets differed only by 59 substitutions (including four transversions and one amino acid

TABLE 2. Loci, sample sizes (*n*), and allele frequencies at eight polymorphic loci for populations of Marbled and Kittlitz's Murrelets.

	Marbled Murrelet						Kittlitz's Murrelet	
Locus[1]/Allele	Attu	Kodiak	Kachemak	Unakwik	Oregon	Magadan	Attu	Kachemak
Ada (*n*)	5	12	5	9	4	4	3	4
A	1.00	1.00	1.00	0.89	1.00	0.88	1.00	1.00
B	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00
C	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00
D	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00
Est-1 (*n*)	5	12	5	9	3	4	1	3
A	1.00	1.00	0.90	0.94	1.00	1.00	1.00	0.83
B	0.00	0.00	0.10	0.06	0.00	0.00	0.00	0.17
Got-1 (*n*)	5	12	5	9	3	4	3	4
A	0.90	0.42	0.30	0.50	0.50	1.00	0.00	0.62
B	0.10	0.42	0.50	0.28	0.33	0.00	0.00	0.00
C	0.00	0.16	0.20	0.22	0.17	0.00	1.00	0.38
α-Gpd (*n*)	5	12	5	9	2	4	3	4
A	0.50	0.71	0.50	0.56	0.25	0.12	0.00	0.00
B	0.50	0.29	0.50	0.44	0.75	0.75	0.67	0.12
C	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00
D	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.88
Mdh-2 (*n*)	5	12	5	9	4	4	3	4
A	0.60	0.92	0.90	0.94	1.00	1.00	1.00	1.00
B	0.40	0.04	0.10	0.06	0.00	0.00	0.00	0.00
C	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00
Me (*n*)	3	12	5	9	2	4	3	4
A	0.33	0.33	0.50	0.50	0.25	0.00	0.17	1.00
B	0.67	0.67	0.50	0.50	0.75	0.00	0.83	0.00
C	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
6-Pgd (*n*)	5	12	5	9	1	4	3	4
A	0.80	0.92	0.80	0.89	1.00	0.00	1.00	1.00
B	0.20	0.08	0.20	0.11	0.00	0.00	0.00	0.00
C	0.00	0.00	0.00	0.00	0.00	0.88	0.00	0.00
D	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00
Sod (*n*)	5	12	5	9	4	4	3	4
A	1.00	0.96	1.00	1.00	1.00	0.12	0.00	0.00
B	0.00	0.04	0.00	0.00	0.00	0.88	0.00	0.00
C	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00

[1] Loci surveyed: *Acon* (enzyme commission number 4.2.1.3), *Ada* (3.5.4.4), *Adh* (1.1.1.1), *Ak-1* (2.7.4.3), *Ak-2* (2.7.4.3), *Ca-1* (4.2.1.1), *Ca-2* (4.2.1.1), *Ck-1* (2.7.3.2), *Ck-2* (2.7.3.2), *Eap-1* (3.1.3.2), *Eap-2* (3.1.3.2), *Est-1* (3.1.1.1), *Est-4* (3.1.1.1), *Gda* (3.5.4.3), *Glud* (1.4.1.3), *Got-1* (2.6.1.1), *Got-2* (2.6.1.1), *GP-1, GP-2, GP-3, α-Gpd* (1.1.1.8), *Idh-1* (1.1.1.42), *Idh-2* (1.1.1.42), *Ldh-1* (1.1.1.27), *Ldh-2* (1.1.1.27), *Mdh-1* (1.1.1.37), *Mdh-2* (1.1.1.37), *Me* (1.1.1.40), *Mpi* (5.3.1.8), *Pep-A* (3.4.11), *Pep-B* (3.4.11), *Pep-D* (3.4.11), *6-Pgd* (1.1.1.44), *Pgi* (5.3.1.9), *Pgm-1* (2.7.5.1), *Pgm-2* (2.7.5.1), *Sdh* (1.1.1.14), *Sod-1* (1.15.1.1), and *Sod-2* (1.15.1.1).

replacement; sequences have been deposited in GenBank, accession numbers U63044-61).

No variation was found among cytochrome *b* sequences of the four Long-billed Murrelets. Thirteen genotypes, differing by one to four substitutions, were found among North American Marbled Murrelets. One substitution resulted in replacement of an alanine by a threonine in type Bm2; all other substitutions were silent. Most genotypes occurred in a single individual each (Table 1). Two lineages that differed by a minimum of 11 substitutions were found among Kittlitz's Murrelets: one lineage comprised two genotypes that differed by two replacement substitutions and that were confined to the sample from Attu Island; the other included two genotypes that differed by one silent and one replacement transition and that occurred only in the sample from Kachemak Bay (Table 1). Direct sequence analysis revealed one new genotype among ten murrelets that appeared from SSCPs and HDs to possess type Bm1; this new genotype

TABLE 3. Estimates of among-site components of genetic variance (G_{ST}), among-site components of sequence variation (ϕ_{ST}), mutational divergence (γ), and gene flow ($N_f m$, females per generation) for populations of murrelets.

Species	Comparison	G_{ST}	ϕ_{ST}	γ	$N_f m$
Marbled Murrelet	All populations	0.24***	0.97***	0.36***	1.6
	Magadan/North America	0.59***	0.99***	1.00***	0.3
	North America populations	0.01	0.02	0.00	50
Kittlitz's Murrelet	Attu I./Kachemak Bay	0.43***	0.91***	1.00***	0.7

*** $P < 0.001$.

(type Bm11) differed from the most common type by a single silent transition.

All three indices of genetic structure indicated strong differentiation among Marbled Murrelets from different sites (Table 3). Most of this variation was due to the Long-billed Murrelets; no significant differentiation was found among Marbled Murrelets from different sites within North America. Kittlitz's Murrelets from the two sites were clearly differentiated despite small sample sizes.

Both maximum parsimony analysis and neighbor-joining grouped sequences of North American Marbled and Kittlitz's Murrelets together to the exclusion of the Long-billed Murrelet (Fig. 2). This relationship received strong support both from bootstrap analyses and from standard error tests. Genotypes of Kittlitz's Murrelets from Attu Island and Kachemak Bay also were clearly distinct, but relationships among genotypes of North American Marbled Murrelets could not be resolved. No evidence of phylogeographic structure was found among Marbled Murrelets from different sites within North America.

ALLOZYMES

Electrophoretic variation was found at eight presumptive protein loci in a preliminary analysis of five North American Marbled Murrelets ($\bar{x} \pm$

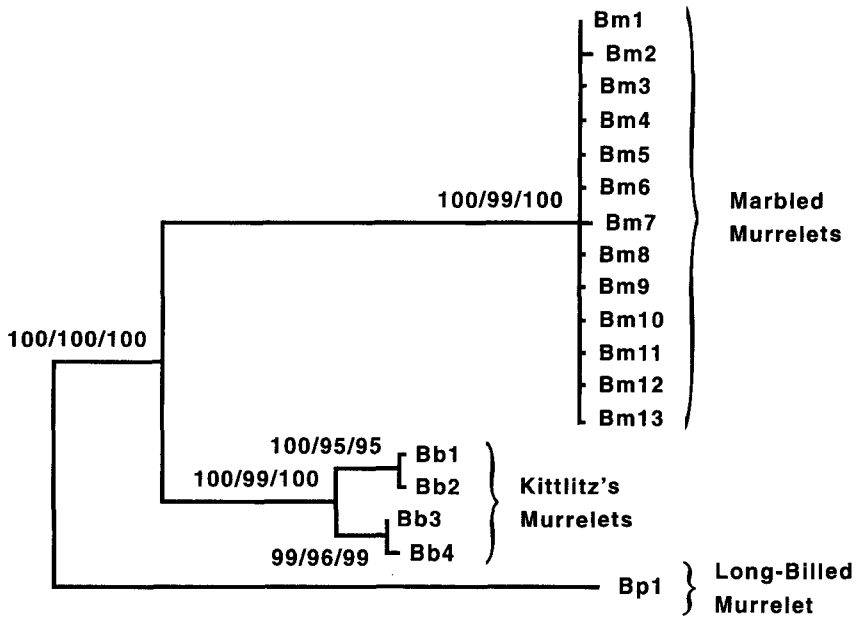

FIGURE 2. Consensus of nine shortest trees obtained by parsimony analysis with mid-point rooting of cytochrome *b* sequences of murrelets (length = 144 steps; consistency index excluding uninformative sites = 0.09). Branch lengths are proportional to numbers of substitutions. Numbers at nodes are indices of support from bootstrap analysis (100 replications) of the maximum parsimony trees, and standard error tests and bootstrap analysis (100 replications) of the neighbor-joining tree, respectively.

SE heterozygosity $\bar{H} = 2.6 \pm 1.1\%$; Table 3), one Long-billed Murrelet ($\bar{H} = 1.6 \pm 6.6\%$) and four Kittlitz's Murrelets ($\bar{H} = 2.6 \pm 1.3\%$; Friesen et al. 1996a). The remaining murrelets were screened for variation at these eight loci, as well as at *Mdh-2* and *Sod,* which appear during staining for *Me* and *Sdh,* respectively. Because the larger survey does not represent a random selection of loci, it could not be used to estimate heterozygosity.

Chi-square analysis of allele frequencies revealed that almost all loci were in Hardy-Weinberg equilibrium within each sampling site for Marbled Murrelets (Table 3). However, analysis of the total sample of Marbled Murrelets, including both North American and Asian forms, indicated significant deficiencies of heterozygotes for *Got-1* ($\chi^2_1 = 8.8$, $P < 0.01$) and *6-Pgd* ($\chi^2_1 = 5.1$, $P < 0.05$), suggesting population subdivision. Chi-square analysis revealed that the distributions of alleles for *Me* and *6-Pgd* were significantly non-random: no alleles were shared between the samples from Magadan and those from North America. Furthermore, jackknife analysis suggested that murrelets from different sites are genetically differentiated ($\bar{F}_{ST} = 0.27$, SE = 0.036, Student's $t = 7.39$, $P < 0.001$). Most of this variation was due to the Long-billed Murrelets: $\bar{F}_{ST}$ between Magadan and North America was 0.39. Assuming that populations have attained equilibrium between mutation, migration and genetic drift, this value of $\bar{F}_{ST}$ suggests essentially no genetic exchange ($N_e m = 0.6$ individuals per generation) between the two forms.

Chi-square tests did not reveal any differences in allele frequencies among Marbled Murrelets from different sites within North America; however, a significant deficiency of heterozygotes was found for the total North American sample for *Got-1* ($\chi^2_1 = 6.2$, $P < 0.01$), suggesting population subdivision. $\bar{F}_{ST}$ for murrelets within North America was low, but jackknife analysis indicated that it was significantly greater than zero and was not due to any one site ($\bar{F}_{ST} = 0.09$, SE = 0.009, Student's $t = 9.55$, $P < 0.001$). Assuming that the populations are in genetic equilibrium, this value of $\bar{F}_{ST}$ suggests genetic exchange of ~2.6 individuals per generation.

Although the number of Kittlitz's Murrelets that were sampled was extremely low, no loci appeared to be out of Hardy-Weinberg equilibrium within either of the two sampling sites. However, a significant deficiency of heterozygotes was found for *Me* for the total sample ($\chi^2_1 = 4.27$, $P < 0.05$). Chi-square tests revealed significant differences in allele frequencies for both *α-Gpd* ($\chi^2_1 = 7.2$, $P < 0.05$) and *Me* ($\chi^2_1 = 10.4$, $P < 0.01$), and $\bar{F}_{ST}$ was high (0.40), suggesting genetic exchange of only ~0.40 individuals per generation between these sites.

Maximum likelihood analysis of allele frequencies provided strong support for a monophyletic relationship among Kittlitz's Murrelets and North American Marbled Murrelets, with Long-billed Murrelets forming the basal lineage (Fig. 3). Marbled Murrelets from Alaska formed a monophyletic group distinct from the sample from Oregon; other relationships were not strongly supported.

DISCUSSION

Analyses both of cytochrome *b* sequences and of allozymes suggest that Long-billed Murrelets are genetically distinct from other brachyramphine murrelets. Surveys of both genetic systems (Tables 2 and 3) indicated that Long-billed and North American Marbled Murrelets are strongly differentiated, and that gene flow is less than one individual per generation. This level of exchange is insufficient to counter the effects of drift (Wright 1965), and indicates that birds from the two sites probably are genetically isolated. Furthermore, phylogenetic analyses both of cytochrome *b* sequences and of allozymes provided strong evidence for a monophyletic relationship among North American Marbled and Kittlitz's Murrelets to the exclusion of Long-billed Murrelets (Figs. 2 and 3): this grouping received 100% support from bootstrap and standard error tests on cytochrome *b* sequences, and > 95% support from maximum likelihood analysis of allozyme variation. *Brachyramphus marmoratus* clearly is a paraphyletic taxon: either Kittlitz's Murrelet represents a subspecies of the Marbled Murrelet, or the Long-billed Murrelet represents a full species.

The taxonomic status of Kittlitz's Murrelet is unequivocal by any definition of a species (Zink and McKirtrick 1995). They are distinctly smaller than Marbled Murrelets (mass ≅ 240 g for North American Marbled Murrelets and ~ 225 g for Kittlitz's Murrelets; Sealy et al. 1982), have shorter bills (culmen length ≅ 16 mm for North American Marbled Murrelets and ~ 11 mm for Kittlitz's Murrelets; Sealy et al. 1982, Pittochelli

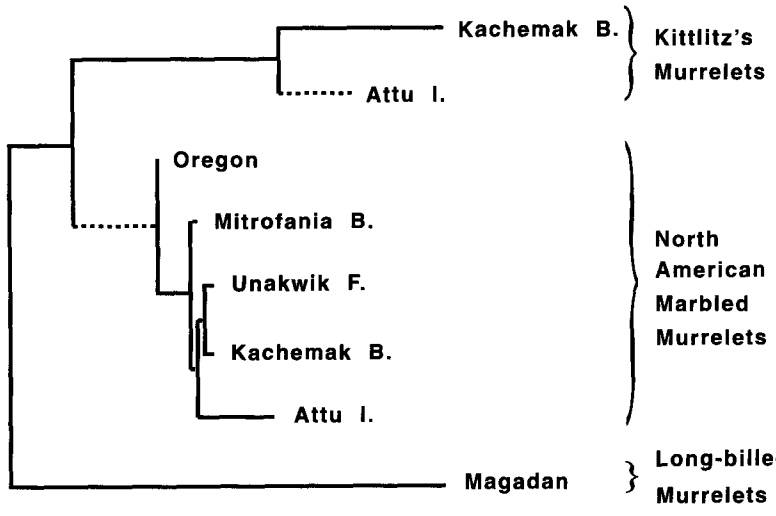

FIGURE 3. Maximum likelihood analysis of allozyme allele frequencies for populations of murrelets. Dotted lines indicate branches with lengths that do not differ significantly from 0.

et al. 1995), nest in the open on high alpine slopes (Day et al. 1983), and generally feed near glaciers. Despite extensive overlap in the breeding ranges of Kittlitz's and Marbled Murrelets (Fig. 1), hybrids have not been reported, probably because of ecological segregation of nesting habitats (Ralph et al. 1995). The presence of fixed allele differences between Kittlitz's and Marbled Murrelets further supports the contention that they are reproductively isolated and represent biological species.

Long-billed and North American Marbled Murrelets also are morphologically distinct (mass $\cong$ 300 g for Long-billed Murrelets; culmen length $\cong$ 20 mm; Sealy et al. 1982; see Introduction for plumage differences). Although the two populations are allopatric and their ability to interbreed has not been tested, the presence of fixed allele differences at two loci, as well as significant differences in allele frequencies at three other loci, suggests that they have been reproductively isolated for an extended period. Furthermore, percent sequence divergence between cytochrome *b* sequences of Long-billed and other murrelets (9.6%) exceeds that between cytochrome *b* sequences of most other congeneric alcids (Friesen et al. 1996a), and the number of transversions between these two lineages (1.1/100 bp) is greater than for any other congeneric alcids (Friesen et al. 1996a). Zink et al. (1995) also found high levels of sequence divergence in a comparison of restriction site variation in mtDNA among Marbled Murrelets from the eastern and western Pacific. Assuming that transversions within cytochrome *b* accumulate at a rate of 0.2/100 bp/10^6 yr (Irwin et al. 1991; Friesen et al., unpubl. data), the number of transversions among cytochrome *b* sequences of the brachyramphine murrelets suggests that North American Marbled and Kittlitz's Murrelets diverged about 1.6 million years ago (see also Pitocchelli et al. 1995), and that Long-billed Murrelets diverged from other brachyramphine murrelets approximately 5–6 million years ago. Although considerable debate has surrounded the definition of a species (e.g., Zink and McKitrick 1995), the Long-billed Murrelet clearly merits full species status according to phylogenetic, evolutionary or biological criteria. Thus, the two populations must be managed independently.

Despite small sample sizes, genetic differentiation also was evident among both Kittlitz's Murrelets and Marbled Murrelets from different sites within North America (Tables 2, 3; Figs. 2, 3). Kittlitz's Murrelets from Attu Island and Kachemak Bay exhibited differences in cytochrome *b* sequences and allozymes, indicating a subdivided population structure in this species. Marbled Murrelets from different sites within North America also displayed significant variation in allozymes. Although cytochrome *b* sequences did not exhibit any evidence of differentiation among sites, substitutional relationships among genotypes suggest a 'starburst' phylogeny (Fig. 2), in-

dicative of recent expansion of a bottlenecked population. These sites all occur in areas that were glaciated during the Pleistocene, so are probably less than 10,000 years old. Thus, insufficient time may have passed for the evolution of population-specific markers in cytochrome *b*. Nuclear DNA is less vulnerable to population bottlenecks than is mtDNA, and so will retain more variation from the ancestral population. Sufficient variation would have been present for the evolution of population markers through founder effects and genetic drift. Results for murrelets contrast markedly with those for the Atlantic population of Thick-billed Murres (*Uria lomvia,* a related alcid), which exhibited no differentiation among colonies in either cytochrome *b* sequences (G_{ST} = 0.002, Friesen et al. 1996c) or allozymes ($\bar{F}_{ST}$ = 0.02; Friesen, unpubl. data), despite evidence from band returns of strong natal philopatry (e.g., Noble et al. 1991). A thorough assessment of the extent of population genetic differentiation in both North American Marbled and Kittlitz's Murrelets is clearly merited, especially since both species are in decline. Such a study will require analysis of more rapidly evolving loci, such as the mitochondrial control region, nuclear introns, or microsatellite loci, from a larger number of birds.

ACKNOWLEDGMENTS

This project would not have been possible without donations of tissue samples by S. Rohwer and C. Wood (the Burke Museum of Natural History, WA), C. J. Ralph and S. Miller (U.S. Forest Service, Arcata, CA) and K. Nelson (Oregon State University, Corvallis, OR). J. Pitocchelli, T. van Pelt and crew of USFW research vessel "Tiglax" helped with tissue collections. M. Peck, O. Haddrath, H. Jones, S. Doran, and T. Birt helped with laboratory work and data analysis. T. Birt, M. Peck, and G. van Vliet provided helpful discussions. We thank Kathy Kultez and Nancy Naslund (USFWS, Migratory Bird Management) for support of work in Prince William Sound. Financial and/or logistical support was provided by the U.S. National Biological Service and the USFWS Alaska Maritime National Wildlife Refuge, NSERC (operating grants held by AJB and VLF and a post-doctoral fellowship held by VLF) and the Endangered Species Recovery Fund of the World Wildlife Fund.

LITERATURE CITED

ALLENDORF, F. W., AND R. F. LEARY. 1986. Heterozygosity and fitness in natural populations of animals, p. 57–76. *In* M. R. Soulé [ed.], Conservation biology, the science of scarcity and diversity. Sinauer, Sunderland, MA.

AMERICAN ORNITHOLOGISTS' UNION. 1983. Check-list of North American birds. 6th ed. American Ornithologists' Union, Washington, DC.

BAKER, A. J., A. M. LYNCH, AND C. E. EDWARDS. 1985. Biochemical genetic studies of shorebirds: methods and applications. Wader Study Group Bull. 44:34–39.

BIRKY, C. W., JR., T. MARUYAMA, AND P. FUERST. 1983. An approach to population and evolutionary theory for genes in mitochondria and chloroplasts, and some results. Genetics 103:513–527.

CARTER, H. R., AND K. J. KULETZ. 1995. Mortality of Marbled Murrelets due to oil pollution in North America, p. 261–270. *In* C. J. Ralph, G. Hunt, M. Raphael, and J. F. Piatt [eds.], Ecology and conservation of the Marbled Murrelet. Gen. Tech. Rep. PSW-GTR-152. Albany, CA.

CARTER, H. R., M. L. C. MCALLISTER, AND M. E. ISLEIB. 1995. Mortality of Marbled Murrelets in gill nets in North America, p. 271–284. *In* C. J. Ralph, G. Hunt, M. Raphael, and J. F. Piatt [eds.], Ecology and conservation of the Marbled Murrelet. Gen. Tech. Rep. PSW-GTR-152. Albany, CA.

DAY, R. H., K. L. OAKLEY, AND D. R. BARNARD. 1983. Nest sites and eggs of Kittlitz's and Marbled Murrelets. Condor 85:265–273.

EXCOFFIER, L., P. E. SMOUSE, AND J. M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

FELSENSTEIN, J. 1981. Evolutionary trees from gene frequencies and quantitative characters: finding maximum likelihood estimates. Evolution 35: 1229–1242.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

FELSENSTEIN, J. 1989. PHYLIP—Phylogeny inference package, Ver. 3.2. Cladistics 5:164–166.

FRIESEN, V. L., A. J. BAKER, AND J. F. PIATT. 1996a. Phylogenetic relationships within the Alcidae (Charadriiformes: Aves) inferred from total molecular evidence. Mol. Biol. Evol. 13: 359–367.

FRIESEN, V. L., W. A. MONTEVECCHI, R. T. BARRETT, AND W. S. DAVIDSON. 1996b. Molecular evidence for kin groups in the absence of large-scale genetic differentiation in a migratory bird. Evolution 50:924–930.

FRIESEN, V. L., W. A. MONTEVECCHI, A. J. BAKER, R. T. BARRETT, AND W. S. DAVIDSON. 1996c. Population differentiation and evolution in the Common Guillemot (*Uria aalge*). Mol. Ecol.: in press.

GILPIN, M. E. AND M. E. SOULÉ. 1986. Minimum viable populations: processes of species extinction, p. 19–34. *In* M. R. Soulé [ed.], Conservation biology, the science of scarcity and diversity. Sinauer, Sunderland, MA.

IRWIN, D. M., T. D. KOCHER, AND A. C. WILSON. 1991. Evolution of the cytochrome *b* gene of mammals. J. Mol. Evol. 32:128–144.

KIMURA, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111–120.

KIRBY, G. C. 1975. Heterozygote frequencies in small subpopulations. Theor. Pop. Biol. 8:31–48.

KOCHER, T. D., W. K. THOMAS, A. MEYER, S. V. EDWARDS, S. PÄÄBO, F. X. VILLABLANCA, AND A. C. WILSON. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. 86:6196–6200.

KONYUKHOV, N. B., AND A. S. KITAYSKY. 1995. The Asian race of the Marbled Murrelet, p. 23–32. *In* C. J. Ralph, C. J., G. Hunt, M. Raphael, and J. F. Piatt [eds.], Ecology and conservation of the Marbled Murrelet. Gen. Tech. Rep. PSW-GTR-152. Albany, CA.

KUMAR, S., K. TAMURA, AND M. Nei. 1993. MEGA: Molecular evolutionary genetics analysis, Ver. 1.0. Pennsylvania State. Univ., Univ. Park, PA.

LESSA, E. P., AND G. APPLEBAUM. 1993. Screening techniques for detecting allelic variation in DNA sequences. Mol. Ecol. 2:121–129.

LYNCH, A., AND A. J. BAKER. 1994. A population memetics approach to cultural evolution in chaffinch song: differentiation among populations. Evolution 48:351–359.

NEIGEL, J. E., AND J. C. AVISE. 1993. Application of a random walk model to geographic distributions of animal mitochondrial DNA variation. Genetics 135:1209–1220.

NOBLE D. G., A. J. GASTON, AND R. D. ELLIOT. 1991. Preliminary estimates of survivorship and recruitment for Thick-billed Murres at Coats Island, p. 45–51. *In* A. J. Gaston and R. D. Elliot [eds.], Studies of high latitude seabirds, Vol. 2. Can. Wildl. Serv. Occas. Pap. No. 69, Ottawa.

PALLAS, P. 1831. Zoographia Rosso-Asiatica, Vol. 2. Academiae Scientiarum Impress, Petropoli.

PITOCCHELLI, J., J. PIATT, AND M. CRONIN. 1995. Morphological and genetic divergence among Alaskan populations of *Brachyramphus* murrelets. Wilson Bull. 107:235–250.

RALPH, C. J., G. HUNT, M. RAPHAEL, AND J. F. PIATT [EDS.]. 1995. Ecology and conservation of the Marbled Murrelet. General Technical Report PSW-GTR-152. Albany, CA

RIDGEWAY, R. 1919. The birds of Middle and North America, part 8. U.S. Natl. Mus. Bull. No. 50.

RZHETSKY, A., AND M. Nei. 1992. A simple method for estimating and testing minimum-evolution trees. Mol. Biol. Evol. 9:945–967.

RZHETSKY, A., AND M. Nei. 1993. Theoretical foundation of the minimum-evolution method of phylogenetic inference. Mol. Biol. Evol. 10:1073–1095.

SAITOU, N., AND M. NEI. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

SEALY, S. G., AND H. R. CARTER. 1984. At-sea distribution and nesting habitat of the Marbled Murrelet in British Columbia: problems in the conservation of a solitary nesting seabird, p. 737–756. *In* J. P. Croxall, P. G. H. Evans, and R. W. Schreiber [eds.], Status and conservation of the world's seabirds. Int. Comm. Bird Protect. Tech. Publ. No. 2.

SEALY, S. G., H. R. CARTER, W. D. SHUFORD, AND D. ALISON. 1982. Occurrences of the Asiatic Marbled Murrelet [*Brachyramphus marmoratus perdix* (Pallas)] in North America. Auk 99:778–781.

SLATKIN, M., AND W. P. MADDISON. 1989. A cladistic measure of gene flow inferred from the phylogenies of alleles. Genetics 123:603–613.

SWOFFORD, D. L., AND D. P. BEGLE. 1993. PAUP: Phylogenetic analysis using parsimony, version 3.1. Illinois Nat. Hist. Surv., Champaign, IL

SWOFFORD, D. L., AND R. B. SELANDER. 1981. BIOSYS-1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J. Hered. 72:281–283.

WEIR, B. S. 1990. Intraspecific differentiation, p. 371–410. *In* D. M. Hillis and C. Moritz [eds.], Molecular systematics. Sinauer, Sunderland, MA.

WRIGHT, S. 1965. The interpretation of population structure by *F*-statistics with special regard to systems of mating. Evolution 9:395–420.

ZINK, R. M., AND M. C. MCKITRICK. 1995. The debate over species concepts and its implications for ornithology. Auk 112:701–719.

ZINK, R. M., S. ROHWER, A. V. ANDREEV, AND D. L. DITTMANN. 1995. Trans-Beringia comparisons of mitochondrial DNA differentiation in birds. Condor 97:639–649.