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MECHANISMS OF POPULATION DIFFERENTIATION IN MARBLED MURRELETS: HISTORICAL VERSUS CONTEMPORARY PROCESSES

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Abstract.—Mechanisms of population differentiation in highly vagile species such as seabirds are poorly understood. Previous studies of marbled murrelets (*Brachyramphus marmoratus*; Charadriiformes: Alcidae) found significant population genetic structure, but could not determine whether this structure is due to historical vicariance (e.g., due to Pleistocene glaciers), isolation by distance, drift or selection in peripheral populations, or nesting habitat selection. To discriminate among these possibilities, we analyzed sequence variation in nine nuclear introns from 120 marbled murrelets sampled from British Columbia to the western Aleutian Islands. Mismatch distributions indicated that murrelets underwent at least one population expansion during the Pleistocene and probably are not in genetic equilibrium. Maximum-likelihood analysis of allele frequencies suggested that murrelets from ''mainland'' sites (from the Alaskan Peninsula east) are genetically different from those in the Aleutians and that these two lineages diverged prior to the last glaciation. Analyses of molecular variance, as well as estimates of gene flow derived using coalescent theory, indicate that population genetic structure is best explained by peripheral isolation of murrelets in the Aleutian Islands, rather than by selection associated with different nesting habitats. No isolation-by-distance effects could be detected. Our results are consistent with a rapid expansion of murrelets from a single refugium during the early–mid Pleistocene, subsequent isolation and divergence in two or more refugia during the final Pleistocene glacial advance, and secondary contact following retreat of the ice sheets. Population genetic structure now appears to be maintained by distance effects combined with small populations and a highly fragmented habitat in the Aleutian Islands.

Key words.—*Brachyramphus marmoratus,* coalescent theory, intron, marbled murrelet, population differentiation.

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Mechanisms of population differentiation in broadly distributed, highly vagile species such as seabirds are poorly understood. Allopatric models of divergence require extrinsic barriers to dispersal usually associated with large-scale vicariant events (Mayr 1963). However, for the majority of seabirds, vicariant events or extrinsic barriers to dispersal either are nonexistent or are limited to a small number of geological events. Whether the level of philopatry in these species is sufficient for population differentiation is generally unknown, but a number of highly philopatric species show little or no population genetic structure (e.g., Austin et al. 1994; Friesen et al. 1996a). Peripatric, parapatric, and sympatric models of divergence are theoretical alternatives that do not require prolonged geographic isolation (Mayr 1963; Maynard Smith 1966; Bush 1975; Endler 1977; Barton and Charlesworth 1984; Carson and Templeton 1984). Peripatric models involve the differentiation of small peripheral populations; in this case, the spatial arrangement of populations and effective population sizes are thought to be the primary factors promoting divergence, with or without natural selection (Barton and Charlesworth 1984; Carson and Templeton 1984). In contrast, parapatric models invoke a combination of restricted gene flow and/or disruptive selection as the primary mechanism of divergence; in these models, differentiation occurs without geographic isolation if the intensity of selection is large relative to gene flow (Endler 1977). Despite the theoretical plausibility of these alternatives, demonstrated examples of nonallopatric divergence are rare (e.g., Wood and Foote 1996), and the potential for nonallopatric processes to promote and maintain genetic divergence is controversial (Otte and Endler 1989; Duffy 1996; Wood and Foote 1996).

Current patterns of intraspecific genetic variation can be used to evaluate the applicability of alternate models of divergence to specific taxa. Species of seabirds that lack extrinsic barriers to dispersal are particularly suited to this type of analysis. Both the spatial arrangement of breeding sites and the resources used in specific locations vary widely for many seabird species. Intraspecific variation in these factors provides the opportunity for diversification through multiple evolutionary processes and allows divergence due to different processes to be partitioned and examined independently. We investigated the mechanisms promoting divergence in one such species, the marbled murrelet (*Brachyramphus marmoratus*; Charadriiformes: Alcidae).

Marbled murrelets are distributed widely along the North American coast from central California to the outer Aleutian Islands (Piatt and Ford 1993; Ralph et al. 1995). Much of this area was glaciated during the late Pleistocene (Thorson and Hamilton 1986; Hamilton 1994; Hughes and Hughes 1994). Throughout most of their range, murrelets nest predominantly in large, old-growth conifers (Carter and Morrison 1992; Ralph et al. 1995), although about 3% of birds nest on the ground in the Aleutian Islands and Alaskan Peninsula (Mendenhall 1992; Piatt and Ford 1993). Murrelets nest both in trees and on the ground along the Alaskan Peninsula (Piatt and Ford 1993; Pitocchelli et al. 1995; Fig. 1). Murrelet nesting habitat becomes increasingly fragmented and numbers of birds decline dramatically in the Aleutian Archipelago (Piatt and Ford 1993). Because marbled murrelets are highly cryptic, little is known about their basic

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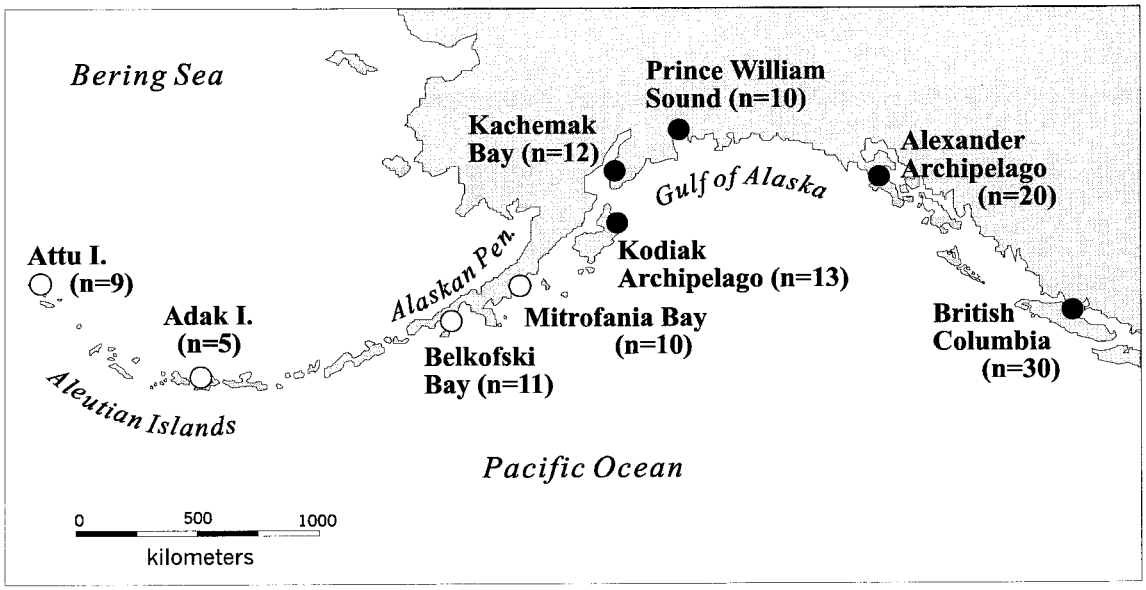

FIG. 1. Marbled murrelet sampling sites. Sample sizes at each location are given in parentheses. Filled circles, tree-nesting sites; open circles, ground-nesting sites.

breeding biology or other life-history parameters, although major advances have been made in recent years (e.g. Cooke 1999). The ecologically similar long-billed murrelets (*B. perdix*) are genetically highly divergent from marbled murrelets, and probably became reproductively isolated 5–6 million years ago (Zink et al. 1995; Friesen et al. 1996b, 1997).

Previous studies using nuclear markers showed low but statistically significant population structuring in marbled murrelets (Friesen et al. 1996b, 1997); these studies could not discern the details of the structuring because of small sample sizes. Slight but significant morphometric differences also were found between tree- and ground-nesting murrelets (Pitocchelli et al. 1995). In contrast, no significant population structure was found in two studies of variation in mitochondrial DNA (Pitocchelli et al. 1995; Friesen et al. 1996b). Conflicting results from nuclear and mitochondrial DNA have yet to be reconciled, although Friesen et al. (1996b) proposed that the lack of spatial variation in cytochrome *b* sequences may be due to a population bottleneck during the Pleistocene glaciations. If murrelets did undergo a bottleneck, the value of mitochondrial markers for inferring historical relationships among populations may be limited.

The glacial history of northwestern North America, spatial variation in murrelet numbers, and distribution of nesting habitat suggest four possible hypotheses for the existence of population genetic structuring in murrelets: (1) secondary contact following historical vicariance; (2) isolation by distance associated with natal philopatry; (3) genetic divergence of small, peripherally isolated populations; and (4) parapatric divergence in situ associated with nesting habitat selection. These possibilities are neither exhaustive nor mutually exclusive. Our purpose was to evaluate which, if any, of these hypotheses explain population differentiation in murrelets. This required separating the effects of divergence associated with historical isolation from isolation by distance, relative population size and position, and habitat heterogeneity.

Separating historical from contemporary evolutionary processes requires analysis of sequence variation across multiple loci. Until recently the high resolution nuclear markers and interpretation methods needed for this type of analysis have been unavailable. Combining the targeted amplification of nuclear introns (Palumbi and Baker 1994) with analyses of single- stranded conformational polymorphism (SSCPs; Hayashi 1991; Lessa and Applebaum 1992) offers a potential solution to this problem. We have developed primers and protocols for rapid analysis of sequence variation in a range of nuclear introns (Friesen et al. 1997, 1999). Furthermore, recent developments in coalescent theory are providing more sensitive methods for inferring demographic histories from DNA sequences. In this study we analyzed sequence variation in nuclear introns, and used both traditional approaches and methods based on coalescent theory to derive a detailed temporal and spatial picture of the evolutionary processes responsible for intraspecific divergence in marbled murrelets.

MATERIALS AND METHODS

Sampling

Tissue samples were obtained from 120 marbled murrelets from nine sites from British Columbia to the western Aleutian Islands (Fig. 1). Samples were collected at sea near breeding sites during the breeding season, and most comprised freshly frozen tissue taken from birds shot for dietary analysis between 1989 and 1997. Samples from southern British Columbia consisted of whole blood that was collected from birds netted at sea and stored in ethanol (Vanderkist et al. 1999). Sampling included two peripheral sites where murrelets nest on the ground in the Aleutian Islands (Attu and Adak Islands), two mainland sites (including offshore islands) where murrelets nest on the ground (Mitrofania and Belkofski Bays), and five mainland sites where murrelets nest predominantly in trees (remaining sites; Fig. 1). Numbers of murrelets in

Mitrofania and Kachemak Bays are low. Samples were not available from sites south of British Columbia.

Molecular Analyses

Total cellular DNA was extracted from tissue and blood samples by protease digestion and phenol/chloroform extraction (Friesen et al. 1997). Samples were screened for sequence variation at nine nuclear introns using a combination of targeted amplification and analyses of SSCPs using polymerase chain reaction (PCR) primers and protocols described in Friesen et al. (1997, 1999). Nucleotide sequences of alleles were determined using Thermo-Sequenase cycle sequencing kits (Amersham, Baié d'Urfe, Quebec, PQ), which use ^{33}P-labeled dideoxy termination mixes. These kits allowed variable sites in a heterozygous sample to be visualized as multiple autoradiograph bands at a single nucleotide position. Sequences of common alleles were determined directly from homozygous samples. To obtain sequences for alleles that were seen only as heterozygotes, samples that were heterozygous for the rare allele and a common allele were sequenced; the sequence of the rare allele then was determined by subtracting the sequence of the common allele from the composite sequence. To determine if analysis of SSCPs identified all nucleotide variants, samples that were collected from different locations but that appeared from SSCP autoradiograms to possess the same allele also were sequenced.

Variation in three microsatellite loci also was examined; however, none of these loci appeared to follow a stepwise mutation model (unpubl. data), so all were excluded from further analyses.

Tests of Assumptions

All methods of estimating population genetic structure and/or gene flow from molecular data make several simplifying assumptions. Most importantly, most assume that loci are unlinked, recombination does not occur within loci (i.e., that each allele has a single evolutionary history), variation is neutral to selection, and population sizes are stable (i.e. that populations have not undergone recent bottlenecks or expansions). Popgen (vers. 1.31; Yeh et al. 1997) was used to test for linkage disequilibrium among loci, and Recombine (Kuhner et al. 1998) was used to estimate recombination rates for individual loci. Although variation can never be proven to be selectively neutral (because neutrality represents a null hypothesis), a number of statistics can be used together to infer whether patterns of variation differ from expectations of the neutral theory. Specifically, selection, nonrandom mating, population subdivision, small population size, mutation, and null alleles all can result in deviations of genotype frequencies from Hardy-Weinberg frequencies; selection, mutation rate heterogeneity, and population expansions can cause significant departures of Tajima's *D* from zero (Tajima 1989a,b; Aris-Brosou and Excoffier 1996); and population bottlenecks and/or expansions, selective sweeps, and mutation rate heterogeneity can cause ''waves'' in the frequency distributions of substitutional differences among pairs of individuals (mismatch distributions; Slatkin and Hudson 1991; Rogers and Harpending 1992; Rogers et al. 1996). Genotype frequencies for each locus were tested for deviations from Hardy-Weinberg equilibrium both for individual sampling sites and for the total sample (Guo and Thompson 1992). Tajima's *D* statistic was estimated for the combined data after randomizing the order in which alleles were recorded for each individual to avoid establishing pseudo-linkages across loci; because mutation rates vary from less than 0.25% per million years to more than 0.65% per million years (unpubl. data) and because selection may differ among loci, loci also were tested independently. (Note that nonsignificant values of Tajima's *D* may result from a combination of population expansion and mutation rate heterogeneity, and so do not necessarily support neutrality.) Mismatch distributions were derived for all loci combined as well as for individual loci with sufficient levels of variability. All tests were done using Arlequin (Schneider et al. 1997), with sequential Bonferroni adjustments to control for Type I errors associated with multiple comparisons involving single sampling sites (Rice 1989).

Population Genetic Structure

Phylogenetic analyses

As a first approximation of population genetic structure, the evolutionary relationships among sampling sites were investigated using the CONTML algorithm of PHYLIP (Felsenstein 1989). This program applies a maximum-likelihood procedure to allele frequencies to reconstruct the relationships among populations and to test whether branch lengths are significantly greater than zero (Felsenstein 1981). Time since divergence of populations was estimated as $t = \delta 10^6/r$, where $\delta = \pi_{xy} - 0.5\ (\pi_x + \pi_y)$, π_{xy} is the mean pairwise nucleotide divergence between populations x and y, π_x and π_y are the mean pairwise divergence within populations x and y, and r is the nucleotide divergence rate for the sequence being analyzed (Wilson et al. 1985). Divergence rates were approximated from percent sequence divergence between the most common alleles for marbled and Kittlitz's murrelets (*Brachyramphus brevirostris*; unpubl. data), assuming a divergence date of 2.2 million years ago (Pitocchelli et al. 1995; Friesen et al. 1996b).

Analysis of molecular variance

Population genetic structure in murrelets also was examined using an analog of Wright's F_{ST}, ϕ_{ST}, estimated by analysis of molecular variance (AMOVA; Excoffier et al. 1992; Michalakis and Excoffier 1996) using Arlequin (Schneider et al. 1997). Because AMOVA incorporates information on sequence divergence among haplotypes for a single locus only, effective use of the sequence information from all loci required inputting the data for all loci for each individual as two ''haplotypes'' and randomizing the order in which alleles were recorded for each individual to avoid establishing pseudo-linkages across loci. Initially, all samples were classified as a single group so that variation among sampling sites could be estimated; ϕ_{ST} also was estimated for pairs of sites. The significance of estimates of ϕ_{ST} was determined via a Markov chain analysis as described by Raymond and Rousset (1995) using 5000 permutations (Schnei-

der et al. 1997); *P*-values did not change significantly beyond this number of permutations. Sequential Bonferroni adjustments were applied (see above).

The components of genetic variation associated with nesting habitat and peripheral sites were determined by altering the a priori group definitions in AMOVA and examining changes in the among-group variance components (Stanley et al. 1996). Sampling locations were grouped in three ways: (1) predominantly ground- (Attu Island to Mitrofania Bay) versus predominantly tree- (British Columbia to Kodiak Islands) nesting sites; (2) Aleutian Island (Attu and Adak Islands) versus ''mainland'' (all other) sites; and (3) Attu versus Adak versus ''mainland'' (all other) sites.

Analyses based on coalescent theory

Use of Wright's F_{ST} and its analogues for estimating population genetic structure and gene flow involves several assumptions, including equal population sizes and symmetrical gene flow (Beerli 1998). Because some of these assumptions probably do not hold for murrelets (see Results), gene flow also was estimated using a maximum-likelihood approach based on coalescent theory (Beerli and Felsentstein 1999). Specifically, magnitudes and directions of gene flow were estimated from intron sequences using Migrate (Beerli 1997). For all analyses, the default settings of Migrate were used except that the numbers of trees sampled for the short and long chains were increased to 100,000 and 1,000,000 respectively to avoid local maxima on the likelihood surface. Unfortunately, the time required to estimate gene flow using this program becomes prohibitive as the number of populations and/or loci increases. Therefore, only the five most variable introns were used, and sampling sites were grouped in three ways for separate analyses: (1) Alaskan Peninsula (Belkofski and Mitrofania Bays) versus Gulf of Alaska (Kachemak Bay, Kodiak Archipelago, Prince William Sound) versus Alexander Archipelago versus British Columbia; (2) mainland sites (all of the above) versus Adak versus Attu; and (3) tree-nesters (British Columbia to Kachemak Bay) versus ground-nesters (Mitrofania Bay to Attu Island). To test whether effective population size is reduced in peripheral sites, Migrate also was used to estimate Υ ($4N_e\mu$; Kuhner et al. 1995), which is an index of genetic variability directly related to the genetically effective population size.

Isolation by distance

Isolation by distance was examined using log-log regressions of *M* versus geographic distance (Slatkin 1993), where *M* is an index of gene flow equivalent to N_em, and is given by the equation $M = 0.25[(1/\phi_{ST})-1]$. Linearized ϕ_{ST} values (Slatkin 1995) from pairwise comparisons of sampling sites (above) were used, and intersite geographic distances were calculated using Distance Finder (http://www.indo.com/distance [1997]). All analyses were repeated for both shortest geographic distance between sites, and distance along the shoreline. Pairwise geographic distances ranged from 722 km to 3974 km, and shoreline distances ranged from 722 km to 4269 km. ϕ_{ST} values of zero indicate panmixia between sampling locations ($M = \sim$ infinite), and were not included in the regression analyses. The significance of regression coefficients from each analysis was tested by nonparametric resampling (Resampling Stats®) as per Congdon et al. (1997). Correlations between estimates of gene flow derived by Migrate and geographic distance also were tested. Finally, Migrate provides a maximum- likelihood method to test whether patterns of migration fit the expectations of a specified model; we used this option to test whether gene flow in murrelets fits the expectations of a stepping-stone model.

RESULTS

Patterns of Variation

All nine introns were variable, with the number of alleles per locus ranging from four to 14 (Fig. 2). Most differences between alleles involved transitional substitutions, although several differences involve transversions and some involved indels (insertions or deletions). Four multibase insertion/deletions (indels) were found in three loci. At least three of these indels appeared to originate from replication errors that repeated or removed a single block of sequence (unpubl. data); therefore, each indel was treated as a single mutational event for subsequent analyses. For most loci, substitutional relationships among alleles described a star, with most alleles differing from the most common allele by a single substitution; however, alleles for the enolase intron formed two distinct groups that differed by a minimum of three substitutions. Mean sequence divergence between these two groups (δ) suggests that they diverged approximately two million years ago, at about the time that marbled and Kittlitz's murrelets diverged (Pitocchelli et al. 1995; Friesen et al. 1996b). No evidence for phylogeographic structuring was found because most alleles occurred at most sampling sites (see Appendix).

Tests of Assumptions

No significant cases of linkage disequilibrium were found within sampling sites (all $P > 0.05$ after Bonferroni corrections), and recombination rates were at least an order of magnitude lower than mutation rates for all nine introns (range $= 0.07$–$0.28\ \mu$). A heterozygote deficiency occurred in the aldolase intron in the sample from British Columbia ($P < 0.0001$); otherwise, genotype frequencies did not differ from Hardy-Weinberg expectations for any intron, either for individual sites or for the total sample (although the power of the tests was low due to small sample sizes and large numbers of alleles). Tajima's *D* for the total sample was slightly negative but not significant ($D = -0.012$, $P > 0.10$). Estimates of *D* for individual introns were slightly negative but nonsignificant for most loci, but were slightly positive for the tropomyosin intron and significantly positive for both the enolase ($D = 1.958$, $P = 0.034$) and P40 introns ($D = 3.8$, $P = 0.002$). The mismatch distribution combining all sampling sites and all nine introns produced a distinctly unimodal distribution (Fig. 3a). Although this curve was significantly different from a Poisson distribution ($P < 0.001$), its shape contrasted markedly with distributions generated in populations at mutation-drift equilibrium (Slatkin and Hudson 1991; Aris-Brosou and Excoffier 1996). Of the seven introns with enough variation to be analyzed independently, those

allele/	*Ornithine Decarboxylase*								
/ site	37	39	83	153	325	413	508-527	535	587
1	G	A	A	A	T	T	I	C	T
2	G	A	A	A	T	T	I	C	C
3	G	A	G	A	T	T	I	C	T
4	G	A	A	G	T	T	I	T	T
5	G	A	A	A	T	T	-	C	T
6	A	G	G	A	T	G	I	C	T
7	A	G	G	A	T	T	I	C	T
8	G	A	A	A	A	T	I	C	T

allele/	*Ribosomal Protien 40*									
/ site	60	88	154	176	177	195	209	234	252-260	307
1	C	G	T	C	G	G	C	C	-	T
2	C	A	C	T	G	G	C	-	I	C
3	C	G	C	C	G	A	T	-	I	C
4	T	G	C	C	G	G	T	-	I	T
5	C	G	C	T	G	G	C	-	I	C
6	T	G	C	C	G	G	T	C	-	T
7	C	G	T	C	A	G	C	C	-	T
8	C	G	C	C	G	G	T	-	I	C

allele/	*Tropomyosin*						
/ site	93	386	527	819	891	951	983
1	T	C	C	C	C	A	T
2	C	C	C	T	C	A	T
3	C	C	C	C	C	G	C
4	C	C	T	C	C	A	T
5	C	C	C	C	C	A	T
6	C	T	C	C	C	A	T
7	C	C	C	C	T	A	T

allele/	*MPP*		
/ site	122	293	294
3	G	C	G
4	G	C	A
5	G	T	G
6	A	C	G

allele/	*Lamin*					
/ site	85	119	131	134	183	301
2	T	C	C	A	A	C
3	T	C	C	A	G	C
4	T	C	T	A	G	C
5	C	T	C	A	G	C
6	T	C	C	G	G	G

allele/	*Enolase*					
/ site	12	85	126	170	242	301
1	C	T	A	G	T	C
2	C	T	A	A	T	C
3	T	T	G	G	C	C
5	T	C	G	G	C	C
6	T	T	G	G	C	G

allele/	*Aldolase*			
/ site	42	76	318	325
2	A	C	C	G
3	G	C	T	C
4	A	C	C	C
6	A	A	C	C

allele/	*Lactate dehydrogenase*						
/ site	95	125	175	178	212	340	381
1	A	A	A	G	G	C	G
2	C	A	G	A	G	C	G
3	C	A	A	G	G	C	G
4	C	A	A	G	G	T	G
5	C	A	A	G	A	C	A
7	C	T	A	G	G	T	G

allele/	*Glyceraldehyde-3-phosphate dehydrogenase*														
/ site	12	95	100	123	124	163	216	219	220	232	247-250	275-287	277	278	279
2	C	G	C	T	A	G	A	A	T	A	-	I	T	G	C
3	C	G	C	T	A	G	A	A	T	G	I	I	T	G	C
4	C	A	C	T	A	G	A	A	T	G	I	I	T	A	C
5	C	A	C	T	A	G	A	A	T	G	I	I	T	G	C
6	C	A	C	C	G	G	A	A	T	G	I	I	T	G	C
7	T	G	C	T	A	G	A	A	T	G	I	I	T	G	T
8	C	G	C	T	A	G	A	A	T	A	I	I	T	G	C
9	C	G	C	T	A	G	C	A	T	G	I	I	T	G	C
10	C	A	C	T	A	G	A	A	C	G	I	I	T	G	C
11	C	A	G	T	A	G	A	A	T	G	I	I	T	G	C
12	C	A	C	T	A	G	A	A	T	G	I	I	C	G	C
13	C	G	C	T	A	G	A	G	T	G	I	I	T	G	C
14	C	A	C	T	A	G	A	A	T	G	I	-	T	G	C
15	C	G	C	T	A	C	A	A	T	G	I	I	T	G	C

FIG. 2. Sequence variation in nine nuclear introns in marbled murrelets. Site designations are nucleotide positions relative to the end of the forward primer. ''Hyphen'' and ''vertical band'' indicate deletions and multibase insertions, respectively.

for ornithine decarboxylase and *Gpdh* also showed unimodal distributions (Fig. 3b,c), whereas those for aldolase and lactate dehydrogenase had bimodal distributions differing only slightly from the expected Poisson form (Fig. 3d,e). The remaining three loci (the enolase, P40, and tropomyosin introns) had distinctly bimodal distributions (Fig. 3f–h).

Population Genetic Structure

Phylogenetic analyses

Maximum-likelihood analyses of intron allele frequencies grouped mainland sites together to the exclusion of Aleutian Island samples; this branch received strong support (Fig. 4). Other relationships received poor support, and changed when different loci were excluded from the analyses. Given a mean ($\pm$ SE) pairwise sequence divergence between the samples from Attu and British Columbia (the two most distant sites) of 0.21% ($\pm$ 0.003), mean sequence variation within the Attu samples of 0.21% ($\pm$ 0.006), and mean sequence variation within the British Columbian samples of 0.19% ($\pm$ 0.003), then δ between these two groups is approximately 1.3×10^{-4}. Assuming a mean mutation rate of 0.45% per million years, these two lineages diverged $\sim$ 0.03 million years ago (95% CI = 0 to 0.06 million years ago).

Analysis of molecular variance

AMOVA on intron variation with all sampling sites treated as one group indicated significant differentiation among sites (Table 1). ϕ_{ST} estimates for most pairwise comparisons involving Aleutian Island versus mainland sites were statistically significant (Table 2). Murrelets from Attu were genetically distinct from those at all mainland sites east of Mitrofania Bay, and birds from Adak differed significantly from those at mainland sites east of Belkofski Bay. The two Aleutian Island sites did not differ significantly from each other. Given the potential sampling error associated with the small sample for Adak, pairwise ϕ_{ST} comparisons involving this site must be interpreted cautiously. However, combining the Aleutian samples and repeating this analysis produced equivalent results ($\phi_{ST} = 0.031$, $P < 0.001$). Pairwise ϕ_{ST} values for this analysis also indicated that the Aleutian samples were

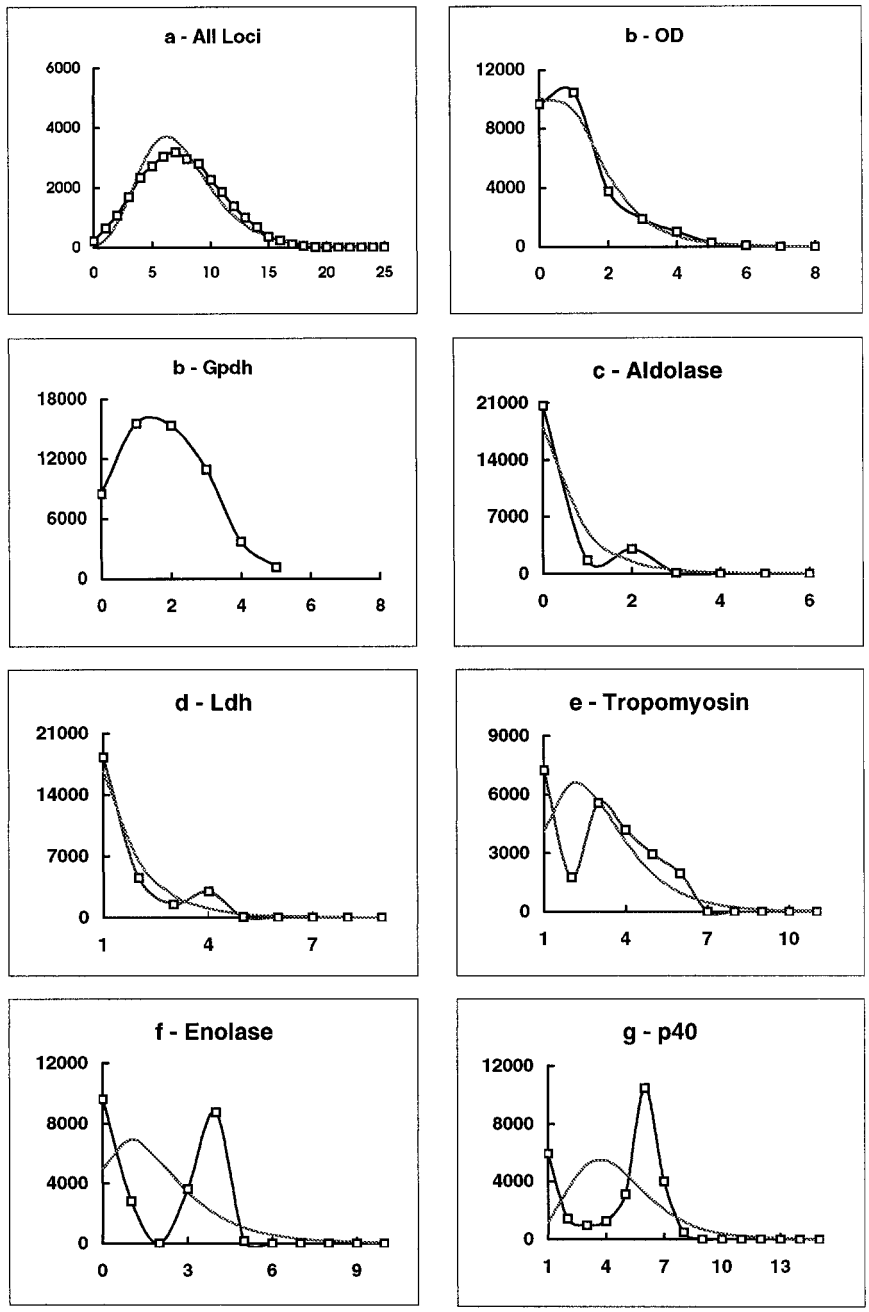

FIG. 3. Mismatch distributions for all introns combined and for individual introns. The darker curves represent the observed distributions and the and lighter represent distributions expected for an expanding population.

genetically distinct from all mainland sites east of Belkofski Bay. Following Bonferroni corrections, no significant ϕ_{ST} values were observed among any two mainland sites in either of these analyses.

All three AMOVAs using different a priori group definitions showed statistically significant among-group variation (Table 1). However, AMOVA based on different nesting types had significant among-site variation within groups. Combining mainland sites but separating Aleutian sites maximized the among-group variation. Variation among sites within groups was not significant in this analysis, suggesting that variation among Aleutian sites was responsible for the significance of this value in AMOVAs based on nesting habitat. This possibility is further supported by the lack of significant population structure in analyses of mainland sites only (not shown).

Analyses based on coalescent theory

Analyses of intron sequences using Migrate indicated that mean gene flow among mainland sites ranged from 0.19 to 4.31 immigrants per generation (Fig. 5a). Gene flow was generally symmetrical, but significant asymmetries in gene

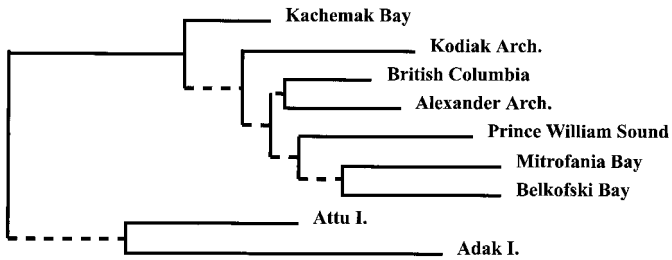

FIG. 4. Maximum-likelihood tree derived from allele frequencies at nine introns for marbled murrelets. Dashed lines indicate that the likelihood support for a branch is less than 95%.

flow occurred between some sites: Specifically, the 0.01 confidence limit for gene flow from the Alexander Peninsula into the Alaskan Peninsula was greater than the 0.99 confidence limit for gene flow from the Alaskan Peninsula into the Alexander Peninsula; the 0.01 confidence limit for gene flow from British Columbia into the Gulf of Alaska was greater than the 0.99 confidence limit for gene flow back; and the 0.01 confidence limit for gene flow from British Columbia into the Alexander Peninsula was greater than the 0.99 confidence limit for gene flow back. Υ was an order of magnitude lower for the sample from the Alexander Peninsula than for the other sites.

Theoretically, estimates of gene flow derived using coalescent theory will always exceed zero, since conspecific populations must have exchanged at least one migrant (or more specifically, one founder) at some time in their history; however, Wright (1931) argued that, in the absence of selection and regardless of the model of dispersal (island vs. stepping stone), one *immigrant* every two generations (i.e., 0.5 immigrants per generation) is sufficient to counteract genetic drift and homogenize genotype frequencies between two populations. Although this rate does not accomodate selection and does not provide an absolute value at which gene flow will or will not prevent divergence of populations through genetic drift, we used it as a general guideline to infer whether gene flow among sites is biologically meaningful. The 0.01 confidence limit on estimates of gene flow among most mainland sites exceeded 0.5 immigrants per generation, suggesting that gene flow is probably sufficient to counteract divergence of these populations through drift; given the logistical constraints on the number of populations that could be analyzed by Migrate, mainland samples therefore were pooled for further analyses.

When samples from Attu, Adak, and mainland sites were treated separately, results from Migrate indicated that gene flow is lower than among mainland sites, but is probably still sufficient to counteract genetic drift between all sites except Attu and Adak. Significant asymmetries in gene flow also exist: specifically, the 0.01 confidence limits for estimates of gene flow from either Attu or Adak into the mainland exceed the 0.99 confidence limits for gene flow back (Fig. 5b). Υ for the sample from Adak was an order of magnitude lower than that for Attu, and two orders of magnitude lower than that for the mainland sites. Although coalescent analyses are thought to be less sensitive to small samples sizes than are other approaches, only four birds were sampled from Adak, therefore these results should be interpreted with caution.

Results from Migrate indicated that gene flow among murrelets from different nesting habitats is relatively high, and almost certainly enough to counteract drift (Fig. 5c). Comparisons of confidence limits indicated that gene flow from tree-nesting sites into ground-nesting sites (which include some mainland sites) is almost certainly greater than gene flow back. Estimates of Υ for the two nesting habitats are similar.

Isolation by distance

Significant isolation by distance effects were observed from a log-log regression of gene flow (derived from ϕ_{ST} estimates) versus shortest geographic distance for all sites ($F_{1,22} = 11.4$, $R^2 = 0.34$, $P < 0.001$, Fig. 6). This relationship was heavily influenced by a few widely spaced data points for intersite distance less than 750 km (log distance < 2.7). To check the overall validity of this relationship, regression analyses were undertaken on various sub-components of the data. The relationship was not found to be significant when the two Aleutian Island populations were removed ($F_{1,7} = 0.16$, $R^2 = 0.02$, $P = 0.71$), but remained significant for a subsample of sites from Kachemak Bay to Attu ($F_{1,7} = 45.63$, $R^2 = 0.85$, $P = 0.02$). (Analyses could not be performed on

TABLE 1. AMOVA results for different subdivisions of marbled murrelet populations for nine introns. *P*-values are based on 4970 permutations. AG, AP/WG, and WP are the among-groups, among-sites/within-groups, and within-sites components of variation, respectively.

Groupings	Divisions	Variance component	% Total variance	*F*-Statistics	*P*
Global	none	AP	2.10	$F_{ST} = 0.021$	0.003
	all populations	WP	97.9		
Habitat divisions	ground-nesting	AG	2.83	$F_{CT} = 0.017$	0.007
	tree-nesting	AP/WG	1.62	$F_{SC} = 0.045$	0.007
		WP	95.55	$F_{ST} = 0.028$	< 0.001
Geographic divisions	Aleutian Islands	AG	8.94	$F_{CT} = 0.089$	0.027
	mainland	AP/WG	−0.19	$F_{SC} = -0.002$	0.580
		WP	91.25	$F_{ST} = 0.088$	0.005
Geographic divisions	Attu	AG	9.45	$F_{CT} = 0.094$	< 0.001
	Adak	AP/WG	−0.39	$F_{SC} = -0.004$	0.714
	mainland	WP	90.94	$F_{ST} = 0.091$	0.004

TABLE 2. Pairwise ϕ_{ST} (upper number) and *P*-values (lower number) below the diagonal, and intersite distances for shortest (upper number) and shoreline (lower number) distance above the diagonal. Asterisks indicate values that are significant at $P < 0.05$ following sequential Bonferroni corrections.

	Population								
	Attu Island	Adak Island	Belkofski Bay	Mitrofania Bay	Kodiak Arch.	Kachemak Bay	Prince William Sound	Alexander Arch.	British Columbia
Attu Island		720	1640	1840	2230	2320	2490	3190	3970
		720	1750	1960	2450	2590	2800	3530	4270
Adak Island	0.032		1020	1240	1690	1800	2000	2640	3330
	0.194		1020	1240	1720	1870	2080	2810	3550
Belkofski Bay	0.129	0.0520		220	690	820	1030	1620	2330
	0.002*	0.077		220	700	850	1050	1790	2520
Mitrofania Bay	0.087	0.055	0.000		490	620	830	1400	2140
	0.011*	0.080	0.646		490	630	840	1570	2310
Kodiak Archipelago	0.154	0.127	0.007	0.000		150	350	960	1800
	0.000*	0.001*	0.278	0.550		150	350	1090	1820
Kachemak Bay	0.134	0.110	0.000	0.000	0.000		210	870	1780
	0.000*	0.014*	0.396	0.572	0.993		210	940	1680
Prince William Sound	0.119	0.090	0.010	0.000	0.000	0.003		740	1700
	0.003*	0.022*	0.267	0.871	0.592	0.342		740	1470
Alexander Archipelago	0.126	0.807	0.001	0.003	0.000	0.000	0.007		740
	0.001*	0.015*	0.376	0.355	0.930	0.971	0.285		740
British Columbia	0.094	0.076	0.000	0.000	0.003	0.000	0.000	0.002	
	0.000*	0.013*	0.463	0.849	0.314	0.543	0.423	0.343	

Aleutian sites only due to the small number of sites.) Similar results were obtained for shoreline distance. Thus, potential distance effects were observed only at large geographic distances (> 750 km), and for mainland versus Aleutian Island sites. Importantly, Aleutian Island-to-mainland comparisons produced consistently lower values of *M* than mainland-to-mainland comparisons over similar geographic distances (Fig. 6). This suggests that greater gene flow occurs among mainland sites than between mainland and Aleutian Island sites over equivalent distances. Tests involving estimates of gene flow derived from Migrate also failed to detect isolation by distance among mainland sites (for correlations between

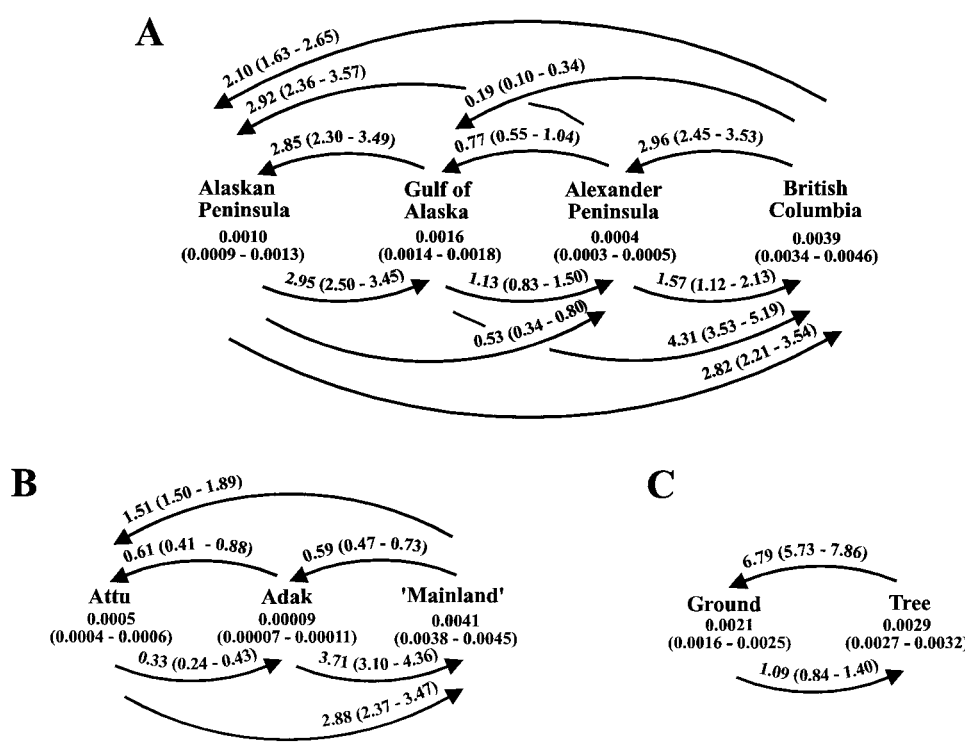

FIG. 5. Estimates of gene flow (above arrows, with 0.01 and 0.99 confidence limits in parentheses) among samples of marbled murrelets, and estimates of Y (below site names, with 0.01 and 0.99 confidence limits in parentheses) for individual sites. (A) Mainland sites only. (B) Attu versus Adak versus combined mainland sites. (C) Combined ground- versus tree-nesting sites.

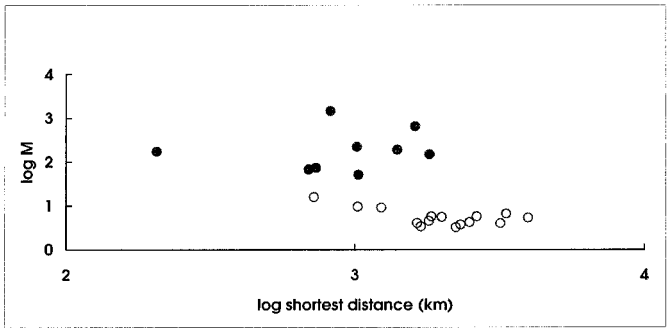

FIG. 6. Scatter plot of log (M) versus log (shortest geographic distance) for murrelets. Closed circles represent pairwise comparisons involving mainland populations only; open circles represent pairwise comparisons between Aleutian Island and mainland sites.

gene flow and geographic distance, all $P > 0.05$; for the maximum-likelihood test on Migrate, $\chi^2_{12} = 10{,}000$, $P < 0.0001$).

DISCUSSION

Results from phylogenetic analyses, AMOVA and Migrate all indicated that significant genetic differences exist among marbled murrelets from different sites, and that gene flow among some sites is restricted. Whereas estimation of population genetic structure and gene flow using Wright's F_{ST} and its analogues assumes that population sizes are equal and that migration between populations is symmetrical, results from Migrate indicate that genetically effective population sizes (as indexed by θ) vary by two orders of magnitude among sites, and that significant asymmetries in gene flow exist (Fig. 5). Furthermore, ϕ_{ST} is more sensitive to small samples sizes (such as for the Aleutian sites) than are methods based on coalescent theory. Thus, estimates of population genetic structure and gene flow derived from AMOVA are probably less accurate than those obtained from Migrate in the present study. All of these analyses involve a number of other assumptions (above), most of which appear not to be violated in the present study: No significant levels of either recombination or linkage were found, and results of most tests for selection were nonsignificant. Two loci (the enolase and P40 introns) had significantly positive values for Tajima's D, which suggests that they are affected by selection and/or mutation rate heterogeneity; thus, mismatch distributions for these loci may not provide reliable indicators of population genetic processes. However, exclusion of these loci from analyses of population genetic structure and gene flow did not alter the conclusions (results not shown).

In contrast, mismatch distributions for four loci suggest that murrelets underwent one or more rapid population expansions following either a population bottleneck or a selective sweep. (Population bottlenecks and selective sweeps produce equivalent reductions in effective population size and genetic variation, and so are difficult to distinguish.) A population expansion also is supported by the starlike substitutional relationships among alleles for most introns (Slatkin and Hudson 1991; unpubl. data). Estimates of τ derived from the mismatch distributions for different loci (Rogers 1995) suggest that this expansion occurred between 80,000 and 800,000 years ago. Although this estimate has a large range, it suggests that the expansion predated the last glaciation. Sequence variation in cytochrome *b* also indicates that murrelets underwent a population bottleneck and expansion, but dates the expansion to ~ 16,000 years ago (unpubl. data). This estimate correlates with the late Pleistocene glaciations (Thorson and Hamilton 1986; Hamilton 1994; Hughes and Hughes 1994), and may be associated with colonization of suitable breeding habitat either following the final Pleistocene glacial retreat ~ 15,000 years ago, or during the interstade that followed the glacial maximum (~ 60,000–25,000 years ago). Regardless of the number and timing of population expansions, mismatch distributions suggest that murrelet populations may not be in equilibrium between mutation, migration, and genetic drift. Estimates of population genetic structure and gene flow derived from molecular markers therefore will not provide accurate estimates of contemporary gene flow or effective population sizes, but instead will reflect a combination of historical and contemporary conditions. Nonetheless, results of the present study enable evaluation of the four hypotheses presented in the introduction.

Mechanisms of Population Differentiation in Murrelets

Historical vicariance

According to the classical allopatric model, population differentiation and speciation require prolonged isolation of populations by extrinsic barriers to gene flow (Mayr 1963). Previous studies of other species of alcids implicated vicariance associated with Pleistocene glaciations in both speciation (Friesen et al. 1996b) and the origin of population genetic structure (Birt-Friesen 1992; Friesen et al. 1996a,b; Kidd and Friesen 1997). Thus, population genetic structure in murrelets could result from isolation of birds in two or more glacial refugia, followed by population expansion and secondary contact after retreat of the glaciers. For species such as murrelets that are not in equilibrium between mutation, migration and drift, historical vicariance may be difficult to detect if contemporary gene flow is high, because contemporary gene flow would homogenize genotype frequencies. However, the signature of historical vicariance should remain in the form of deep branches in gene trees. With the exception of the enolase intron (in which two groups of alleles appear to have diverged ~ 2 mya), substitutional relationships among intron alleles described 'stars' and did not provide any evidence of historical subdivision; however, substitution rates of these loci are probably too low to detect vicariance during the late Pleistocene. Analyses of mitochondrial control regions (in progress) should be more informative. In contrast, maximum likelihood analysis of allele frequencies suggested that murrelets from mainland sites are genetically different from those from the Aleutian Islands, and estimates of δ based on sequence variation suggest that these two populations diverged ~ 33,000 years ago; although the error associated with this estimate is large, the date is consistent with divergence of murrelets in two or more glacial refugia during the late Pleistocene.

Results from Migrate indicate that most gene flow from Attu and Adak to the 'mainland' exceeds gene flow back (Fig. 5b); because populations of murrelets may not be in

genetic equilibrium, these estimates probably retain a component of historical gene flow and suggest that murrelets may have had a refugial population in the Bering Sea area. Geological evidence for an Aleutian refugium is scarce. The westward limit of the ice sheet that covered the Alaskan Peninsula and inner Aleutian Islands during the final Pleistocene glacial episode is unknown (Hamilton 1994), so the availability of nesting habitat is unknown. Similarly, little is known about the availability of food resources. Current information suggests that biological activity has been continuous in the southwest Bering Sea for the last 50,000 years (Morel and Robinson 1986). However, Sancetta and Robinson (1983) argued that during the last Pleistocene glacial advance spring temperatures were cooler, the productive ice-free season was short, and perennial sea ice was present around the southeast margins of the Bering basin. Alternatively, coastal areas of the ice-free area in Beringia could have provided suitable breeding habitat for ground-nesting murrelets.

Isolation by distance

According to parapatric models of divergence, a continuously distributed population may become genetically structured through restricted gene flow (e.g., due to poor dispersal abilities or philopatry) and/or strong selection (Endler 1977). Such populations should show evidence of isolation by distance. Accordingly, gene flow estimated from ϕ_{ST} in murrelets was inversely correlated with distance between sampling sites (Fig. 6). However, this relationship was weak and was not statistically significant when samples from the Aleutian Islands were removed, even though the mainland sites spanned a distance greater than that from Attu to the Alaskan Peninsula. Isolation by distance also was not evident in the results from Migrate. Thus, natal philopatry and/or strong selection alone do not appear to explain population genetic structure in murrelets.

Peripheral isolation

According to peripatric models of divergence, genetic drift and selection may be accentuated at the periphery of a species' range due to small population size, restricted gene flow, and adaptation to suboptimal conditions (e.g., Barton and Charlesworth 1984; Carson and Templeton 1984). Thus, differences in the spatial arrangement of breeding sites and numbers of murrelets at Attu and Adak versus the mainland may influence levels of differentiation. Populations of murrelets nesting in the Aleutian Islands are small compared with mainland Alaska and British Columbia, and so may be more subject to divergence through genetic drift. Furthermore, suitable breeding sites within the Aleutian Islands are scattered over large distances, so mainland emigrants entering the area may be unlikely to find suitable breeding habitat. Thus, genetic drift could be accentuated in the Aleutian populations. This possibility is supported by the small values of Y for Attu and Adak relative to most mainland sites (Fig. 5). Also, results from both AMOVA (Table 1) and Migrate (Fig. 5) suggest that gene flow between mainland and Aleutian sites is relatively low, and that population genetic structure is best described when samples from Attu, Adak, and mainland sites are treated separately. Thus, murrelets from Attu and Adak may have diverged through genetic drift alone.

Nesting habitat selection

Gene flow between ground- and tree-nesting murrelets could be restricted either by birds choosing to nest in habitat similar to the one in which they were raised or by mortality of birds attempting to nest on their nonnatal substrate. However, results from both AMOVA and Migrate suggest that gene flow between ground- and tree-nesting murrelets is probably sufficient to counteract differentiation through genetic drift (Table 1; Fig. 5c). Although weak or even moderate selection on characters associated with nesting habit would not be detected using neutral molecular markers, strong selection would reduce the movement of even these markers, and is contraindicated by results of the present study.

Conclusions

The above explanations are not mutually exclusive, and our results are most consistent with the following scenario: (1) population expansion between 800,000 and 80,000 years ago; (2) isolation and genetic divergence of marbled murrelets in two refugia during the late Pleistocene; (3) population expansion and secondary contact following retreat of the glaciers; and (4) maintenance of population genetic structure through distance effects combined with small populations and a highly fragmented habitat in the Aleutian Islands. Population genetic structure in marbled murrelets does not appear to relate to nesting habit; however, other explanations are possible. For example, if murrelets from Aleutian and mainland breeding areas have different wintering areas, then philopatric associations between breeding and wintering areas could restrict gene flow between regions; this possibility cannot be addressed at present due to a lack of information on murrelet site fidelity or winter ranges. More definitive conclusions may be obtained by analysis of peripheral tree-nesting locations in Washington, Oregon, and California; inclusion of these sites will allow fragmentation effects (in this case, due to human activities) to be examined across sites where nesting habit does not vary. This work is continuing.

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Appendix

Intron allele frequencies in populations of marbled murrelets. Refer to Figure 1 for sampling locations.

Location	*Glyceraldehyde-3-phosphate dehydrogenase (Gapdh, EC No. 1.2.1.12)*														
/ Allele	2	3	4	5	6	8	9	7	10	11	12	13	14	15	N
Attu Island	0.00	0.44	0.00	0.17	0.22	0.11	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	18
Adak Island	0.00	0.00	0.00	0.50	0.20	0.10	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10
Belkofski Bay	0.05	0.36	0.09	0.36	0.00	0.05	0.00	0.05	0.05	0.00	0.00	0.00	0.00	0.00	22
Mitrofania Bay	0.00	0.45	0.00	0.35	0.05	0.05	0.00	0.05	0.05	0.00	0.00	0.00	0.00	0.00	20
Kodiak Archipelago	0.00	0.38	0.12	0.35	0.08	0.04	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	26
Kachemak Bay	0.04	0.33	0.04	0.33	0.04	0.04	0.04	0.04	0.04	0.04	0.00	0.00	0.00	0.00	24
Prince William Sound	0.00	0.40	0.20	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20
Alexander Archipelago	0.05	0.34	0.13	0.39	0.05	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	38
British Columbia	0.08	0.50	0.02	0.22	0.05	0.05	0.00	0.02	0.00	0.00	0.02	0.02	0.02	0.02	60

Location	*Lamin*						*Lactate dehydrogenase (Ldh)*						
/ Allele	2	3	4	5	6	N	1	2	3	4	5	7	To
Attu Island	0.00	0.63	0.00	0.00	0.38	16	0.06	0.00	0.06	0.89	0.00	0.00	18
Adak Island	0.00	0.40	0.00	0.00	0.60	10	0.00	0.00	0.13	0.88	0.00	0.00	8
Belkofski Bay	0.00	0.74	0.14	0.00	0.14	22	0.00	0.00	0.00	0.91	0.09	0.00	22
Mitrofania Bay	0.00	0.72	0.11	0.06	0.11	18	0.05	0.00	0.10	0.70	0.10	0.05	20
Kodiak Archipelago	0.00	1.00	0.00	0.00	0.00	24	0.00	0.04	0.15	0.81	0.00	0.00	26
Kachemak Bay	0.00	0.83	0.08	0.00	0.08	24	0.04	0.00	0.25	0.71	0.00	0.00	24
Prince William Sound	0.05	0.70	0.10	0.10	0.05	20	0.00	0.00	0.17	0.83	0.00	0.00	18
Alexander Archipelago	0.00	0.78	0.03	0.08	0.13	40	0.08	0.10	0.00	0.80	0.03	0.00	40
British Columbia	0.04	0.75	0.04	0.05	0.13	56	0.00	0.07	0.09	0.83	0.02	0.00	58

Location	*Myelin Proteolipid Protein*					*Ornithine Decarboxylase*								
/ Allele	3	4	5	6	N	1	2	3	4	5	6	7	8	N
Attu Island	0.56	0.00	0.44	0.00	16	0.72	0.00	0.22	0.00	0.00	0.06	0.00	0.00	18
Adak Island	0.50	0.10	0.40	0.00	10	0.50	0.00	0.10	0.00	0.30	0.10	0.00	0.00	10
Belkofski Bay	0.69	0.04	0.27	0.00	20	0.48	0.19	0.17	0.04	0.05	0.04	0.04	0.00	22
Mitrofania Bay	0.78	0.06	0.11	0.06	18	0.45	0.10	0.15	0.10	0.05	0.00	0.00	0.10	20
Kodiak Archipelago	0.79	0.21	0.00	0.00	24	0.65	0.00	0.19	0.04	0.08	0.00	0.00	0.04	26
Kachemak Bay	0.92	0.00	0.08	0.00	24	0.67	0.04	0.21	0.04	0.04	0.00	0.00	0.00	24
Prince William Sound	0.70	0.15	0.15	0.00	20	0.40	0.15	0.15	0.20	0.00	0.10	0.00	0.00	20
Alexander Archipelago	0.75	0.10	0.10	0.05	40	0.58	0.03	0.25	0.05	0.03	0.05	0.00	0.03	40
British Columbia	0.78	0.13	0.07	0.02	54	0.52	0.07	0.22	0.04	0.09	0.00	0.06	0.00	54

Location	*Ribosomal Protien 40*									*Aldolase (EC No. 4.1.2.13)*				
/ Allele	1	2	3	4	5	6	7	8	N	3	4	2	6	N
Attu Island	0.00	0.44	0.06	0.50	0.00	0.00	0.00	0.00	18	0.00	1.00	0.00	0.00	16
Adak Island	0.20	0.50	0.00	0.30	0.00	0.00	0.00	0.00	10	0.00	1.00	0.00	0.00	10
Belkofski Bay	0.35	0.45	0.05	0.00	0.05	0.05	0.05	0.10	20	0.04	0.91	0.05	0.00	22
Mitrofania Bay	0.25	0.45	0.30	0.00	0.00	0.00	0.00	0.00	20	0.05	0.90	0.05	0.00	20
Kodiak Archipelago	0.19	0.23	0.27	0.04	0.04	0.00	0.15	0.08	26	0.09	0.91	0.00	0.00	22
Kachemak Bay	0.33	0.17	0.17	0.08	0.08	0.00	0.04	0.13	24	0.00	1.00	0.00	0.00	24
Prince William Sound	0.15	0.35	0.30	0.00	0.05	0.00	0.05	0.10	20	0.10	0.90	0.00	0.00	20
Alexander Archipelago	0.35	0.25	0.23	0.05	0.00	0.00	0.05	0.08	40	0.03	0.86	0.00	0.11	36
British Columbia	0.25	0.32	0.20	0.07	0.02	0.00	0.04	0.11	56	0.14	0.82	0.00	0.04	56

Location	*Enolase (EC No. 4.2.1.11)*						*Tropomyosin*							
/ Allele	1	2	3	5	6	N	1	2	3	4	5	6	7	N
Attu Island	0.13	0.63	0.25	0.00	0.00	16	0.00	0.06	0.28	0.17	0.11	0.39	0.00	18
Adak Island	0.50	0.50	0.00	0.00	0.00	8	0.00	0.00	0.50	0.10	0.30	0.10	0.00	10
Belkofski Bay	0.04	0.59	0.34	0.00	0.04	16	0.00	0.09	0.42	0.25	0.09	0.05	0.10	22
Mitrofania Bay	0.19	0.31	0.44	0.06	0.00	16	0.06	0.06	0.50	0.13	0.13	0.06	0.06	16
Kodiak Archipelago	0.31	0.31	0.38	0.00	0.00	26	0.00	0.17	0.33	0.13	0.25	0.08	0.04	24
Kachemak Bay	0.08	0.29	0.63	0.00	0.00	24	0.00	0.25	0.40	0.10	0.30	0.05	0.00	20
Prince William Sound	0.20	0.15	0.65	0.00	0.00	20	0.00	0.06	0.33	0.22	0.22	0.11	0.06	18
Alexander Archipelago	0.08	0.33	0.60	0.00	0.00	40	0.00	0.10	0.63	0.08	0.18	0.03	0.00	40
British Columbia	0.10	0.33	0.57	0.00	0.00	58	0.00	0.09	0.57	0.09	0.12	0.07	0.03	58