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Evolution

Hypervariable-control-region sequences reveal global population structuring in a long-distance migrant shorebird, the Dunlin (*Calidris alpina*)

(PCR/direct sequencing/genetic–morphometric correlation)

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ABSTRACT **Hypervariable segments of the control region of mtDNA as well as part of the cytochrome *b* gene of Dunlins were amplified with PCR and sequenced directly. The 910 base pairs (bp) obtained for each of 73 individuals complete another of the few sequencing studies that examine the global range of a vertebrate species. A total of 35 types of mtDNA were detected, 33 of which were defined by the hypervariable-control-region segments. Thirty of the latter were specific to populations of different geographic origin in the circumpolar breeding range of the species. The remaining three types indicate dispersal between populations in southern Norway and Siberia, but female-mediated flow of mtDNA apparently is too low to overcome the effects of high mutation rates of the control-region sequences, as well as population subdivision associated with historical range disjunctions. A genealogical tree relating the types grouped them into five populations: Alaska, West Coast of North America, Gulf of Mexico, western Europe, and the Taymyr Peninsula. The Dunlin is thus highly structured geographically, with measures of mutational divergence approaching 1.0 for fixation of alternative types in different populations. High diversity of types within populations as well as moderate long-term effective population sizes argue against severe population bottlenecks in promoting this differentiation. Instead, population fragmentation in Pleistocene refuges is the most plausible mechanism of mtDNA differentiation but at a much earlier time scale than suggested previously with morphometric data.**

The amplification and direct sequencing of highly polymorphic regions of mtDNA provide a potentially rich source of variation at the nucleotide level for determining the molecular population structure within species and the phylogeny of intraspecific lineages. mtDNA is the molecule of choice for such studies because it is nonrecombining and maternally inherited (but see ref. 1) and has a high average rate of evolution. Analyses of sequence variation of the human mtDNA genome, for example, have shown that the noncoding control region harbors the most variability (2–5) and that this variation is located principally in two hypervariable segments (6, 7). In this paper we report the nucleotide sequences¶ of two hypervariable segments of the control region of 73 individual Dunlins and contrast these results with those from a segment of the more slowly evolving cytochrome *b* gene. We demonstrate that these mtDNA segments can not only elucidate the population genetic structure of this long-distance migrant shorebird over much of its circumpolar breeding range in the arctic tundras of the Northern Hemisphere but also can distinguish subpopulations within composite flocks of birds at more southerly wintering sites or during migration. Additionally, gene flow between populations of breeding birds can be readily detected and quantified.

The Dunlin is among the most polytypic species of highly vagile shorebirds, with up to nine subspecies recognized on the basis of variation in plumage and external measurements (8). Previous attempts in defining subpopulations or subspecies have been frustrated by overlapping statistical distributions of these variates as well as pronounced sexual dimorphism in size, as in many other species of migrant shorebirds (9). Recoveries of banded birds have been instructive in revealing stopover sites along flyways, but detection of population subdivision remains elusive because of the low number of recoveries. This problem can now be circumvented with diagnostic nucleotide substitutions in the mtDNA segments we have amplified and sequenced. Our results are of considerable use in the conservation biology of migrant shorebirds because many populations are today threatened by pollution and human encroachment on vital staging areas for migration and in wintering habitats in major estuaries around the world.

MATERIALS AND METHODS

Populations Sampled. Blood or solid tissue samples were obtained from Dunlins caught on their arctic breeding grounds or at staging or wintering sites. Of the 33 North American birds, eight were sampled near Cordova in southern Alaska after their arrival on spring migration, nine were collected at West Coast wintering sites in Washington ($n = 4$) and California ($n = 5$), and 16 in Texas ($n = 13$) and Florida ($n = 3$). The Texas and Florida birds are part of a population breeding around Hudson Bay in Canada (10). Three breeding birds were sampled in Iceland, blood from 17 more was obtained in the Hardangervidda region of southern Norway, and also from 14 birds captured at their breeding sites in the Taymyr Peninsula in northern Russia. Six wintering birds were also sampled in western Europe, four in the German Waddensea, and two in the Dutch Waddensea.

Amplification and Sequencing of mtDNA. Template mtDNAs used in subsequent amplifications were either purified from liver with two CsCl gradient ultracentrifugations using conditions as described in ref. 11 or were isolated as total DNA extracted from whole blood with standard procedures (12). For both the control region and the cytochrome *b* gene, 0.5 ng of purified mtDNA or 0.5 μg of total DNA was subjected to 30 cycles of amplification in a thermal cycler (Perkin–Elmer/Cetus) to produce double-stranded mtDNA. Single-stranded

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L06721–L06755).

templates suitable for sequencing were then generated by using asymmetric amplification (13) of the light strand in a 60-μl reaction volume with 1 unit of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus). The two segments of the control region (I and II) and the cytochrome *b* segment were amplified separately for 40 and 35 cycles, respectively, using the following temperature profile: 93°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min. Based on the cloned control-region sequence of a Turnstone (*Arenaria interpres*) (unpublished data) and its homology to the published chicken (*Gallus domesticus*) sequence (14), we designed two sets of primers to amplify the hypervariable segments: control region I, L98 (5′-GCATGTAATTTGGGCATTTTTTG-3′) and H401 (5′-GTGAGGAGTCCGACTAATAAAT-3′), control region II, L438 (5′-TCACGTGAAATCAGCAACCC-3′) and H772 (5′-AAACACTTGAAACCGTCTCAT-3′). L and H refer to the light and heavy strands, respectively, and the numbers refer to the base at the 3′ end of the primer in the chicken mtDNA sequence (14). These primers amplified respective internal segments of 302 and 313 base pairs (bp) in the control region. To amplify a 307-bp internal fragment of the cytochrome *b* gene we used the primers in ref. 15. All amplified products were purified by selective isopropanol precipitation (16). Sequencing of single-stranded DNA was done by using a Sequenase kit (United States Biochemical) and 2′-deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate. The sequencing reaction products were separated electrophoretically in 8% acrylamide/7 M urea gels for 2 and 4 hr at 60 W in a BRL sequencing apparatus. After fixing, gels were dried and exposed to film for 48 hr.

Phylogenetic Analysis and Population Structure. Based on the 42 phylogenetically informative sites, genealogical relationships among mtDNA types were estimated with maximum-parsimony algorithms in the computer package PAUP 3.0 (17), in which the number of inferred substitutions is minimized. A consensus sequence for the homologous segments of three Turnstones (*A. interpres*) was used as an outgroup to root the tree because it is only 12.7% divergent from the Dunlin sequences. To guard against suboptimal solutions dictated by the input order of the types in a heuristic search (18, 19), we used the random addition sequence with tree-bisection reconnection branch swapping. Fifteen replicates were run, but all resulted in the same set of 20 trees of 96 steps. A strict consensus tree was computed to synthesize information in the 20 equally parsimonious trees. Based on the phylogeographic groupings thus identified, we assessed population structure in Dunlins with G_{ST}, the ratio of the among-region genetic diversity to the genetic diversity in the total sample (20). We also used a measure of mutational divergence (γ) that is appropriate for sequences with high mutation rates (21).

RESULTS

Variation in Control Region and Cytochrome *b*. As in mammals (6, 22, 23), the control region of the Dunlin is highly variable, and much of this variation is concentrated in two hypervariable segments located on either side of a central conserved sequence block. Thirty of the 42 variable sites in the two hypervariable segments are located in control region I, near the beginning of the control region. Relative to the reference sequence (ALA1), there are a total of 33 substitutions in this region, of which 27 are transitions and 6 are transversions. Control region II also harbors considerable site polymorphism, with 12 variable sites among the 73 birds we sequenced (Fig. 1). Eight of 10 substitutions are transitions, two are transversions, and there are also two deletions/insertions. Between control region I and the hypervariable part of control region II there is a highly conserved sequence block of 250 bases. Thirty-three types of mtDNA were identified in the total sample with the sequence variants in the two hypervariable segments of the control region.

The much more slowly evolving segment of the cytochrome *b* gene detected variation at only eight sites. Ten types of mtDNA were identified in the total sample of birds with this gene segment. Eight of these types were associated with the control-region types, and when the total 910 bp of sequence for the control region and cytochrome *b* were considered jointly, 35 mtDNA types were distinguished in the 73 Dunlins. All substitutions in the cytochrome *b* segment were transitions.

Phylogeographic Analysis. Because some of the birds analyzed in this study were captured on migration or at wintering sites where flocks are potentially of different genetic stocks or subspecies, it was necessary to construct a genealogical tree relating the 35 types of mtDNA. A strict consensus tree of the 20 maximum parsimony trees from PAUP is shown in Fig. 2. Five major phylogeographic clusters of mtDNA types are evident. Cluster I groups together the four types found in all eight Alaskan birds (presumably breeding birds) and six nonbreeding birds from Washington (n = 2) and California (n = 4), cluster II is composed of three types from nonbreeders in Washington (n = 2) and California (n = 1), cluster III includes all types from the breeding adults in the Taymyr Peninsula in Russia (n = 13) as well as two breeding birds with types TAY1 and TAY2 from the Hardangervidda tundra of southern Norway (see Fig. 1), cluster IV groups all the remaining types from birds that either breed, stage, or winter in western Europe together with one breeding bird from the Taymyr Peninsula (NOR4), and cluster V is composed of types from nonbreeding birds wintering in Texas and Florida. The latter are characterized by the most substitutions relative to the reference sequence (see Fig. 1) and thus clearly contain the most divergent mtDNA types of all the populations we examined. Except for southern Norway, where two Taymyr types noted above occur along with other western European types, and the Taymyr Peninsula, where one Norwegian type occurs, the mtDNAs have high geographic specificity indicative of strong phylogeographic subdivision. Each of these three examples involves a single bird with an "immigrant" mtDNA type. One bird from the German Waddensea (TAY3) caught on spring migration had a very high body weight (90 g) and large body parts (wing, 127 mm; bill, 37.7 mm) characteristic of Taymyr birds. It also had a Taymyr type of mtDNA and was thus allocated to the Russian population.

Population Structure. Within-population diversity of mtDNA types, which can range from 0 when all birds have the same type to 1 when all are different, is much greater for the hypervariable-control-region segments than for cytochrome *b*, as expected (Table 1). The lowest diversity is found in the birds with Alaskan types of mtDNA, and they also have the lowest average sequence divergence among individuals. The long-term effective population sizes (N_e) of the Dunlin populations identified above can be estimated approximately from the average sequence divergence in the fast-evolving part I of the control region of individuals in each population (with the exception of the West Coast of United States population, for which the sample size is too small). N_e can be estimated with the equation given in ref. 24, as follows:

$$N_e = 10^6 t_x / s \cdot g,$$

where δ_x is the mean pairwise distance (corrected for multiple hits) among individuals, and $t_x = 0.5\delta_x$. Applying the rate of evolution (s) for the hypervariable-control-region I sequences for Canada geese of 20.8% per million yr (25) to the Dunlin sequences, and an average generation time (g) of 5 yr, N_e for the Dunlin populations is moderate (Table 1). Although these estimates are considerably less than current census population sizes, they do indicate that the populations have

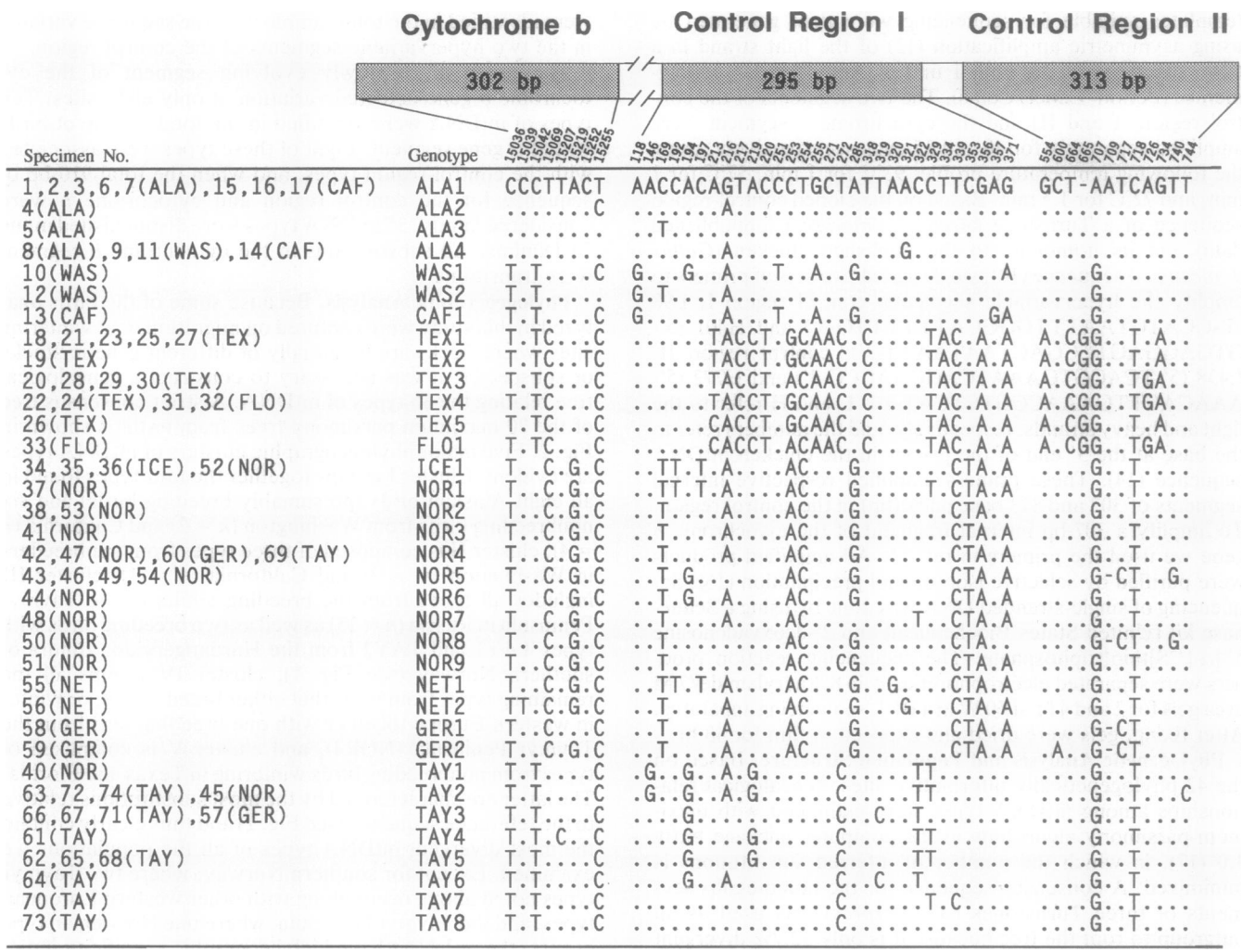

FIG. 1. Variable sites in cytochrome *b* and control-region segments of Dunlin mtDNA. Numbers above sites refer to standard numbers in chicken mtDNA (14). Identity with reference sequence (ALA1) is indicated with dots, and deletions are indicated with dashes. Types of mtDNA are designated by abbreviations based on their geographic origins as follows: ALA, Alaska; WAS, Washington; CAF, California; TEX, Texas; FLO, Florida; ICE, Iceland; NOR, Norway; NET, Netherlands; GER, Germany; and TAY, Taymyr Peninsula, Russia. Individuals listed in the same rows have the same sequences, and their sampling locations are indicated in parentheses.

not been subject to the extreme reductions in size in the Pleistocene that affected many other species of shorebirds (26, 27).

The degree of population subdivision in the Dunlin estimated with G_{ST} is greatest for the least-variable mtDNA segments (cytochrome *b* and control region II) and is much smaller for the hypervariable control region I and all segments together (Table 2). This result is counterintuitive because the control-region I segments are fixed for different types in all but the Taymyr and western Europe populations. Such artifacts commonly arise when rates of mutation are high (28, 29) and result in high within-population diversity, as in the control region of mtDNA. This problem can be circumvented with a measure of mutational divergence (γ) (21), which is based on the ratio of the probability of identity of sequence variants among populations relative to that within populations. Under an island model of population structure, γ approximates the ratio between mutation and migration rates. As mutation rates increase, γ will increase because most sequence variants will occur in single populations (private alleles) or be confined to a subset of them unless migration rates are exceptionally high. In the Dunlin, γ increases from the more conserved cytochrome *b* segment through the more variable control-region segments and is greatest for all segments combined (Table 2). These very high values reflect the near fixation of different sequence variants in each population and, more importantly, provide evidence of limited gene flow among population mtDNA gene pools.

Correlation of mtDNA and Morphometric Variation. Based on the means of the lengths of the wing, tail, tarsus, bill, and white vane as well as tarsus width in ref. 8, we estimated the amount of morphological divergence (average taxonomic distance) among the populations identified in the genealogical tree in Fig. 2. For the Alaska population (I) we used morphometric data for locality 14 (southern Alaska) in ref. 8, for the North American West Coast population (II) we used data for locality 15 (northern Alaska), for the Taymyr population (III), we used data for localities 11 and 12 (western Siberia), for western Europe (IV), we used data for localities 2–5 and 8–9 (Iceland, Great Britain, and southern Scandinavia), and for the population of birds wintering on the Gulf coast of the United States (V) we used data for localities 16 and 17 (Hudson Bay). Mantel's test (30) gave a matrix correlation of $r = -0.365$ ($P = 0.101$) between the morphological distance matrix and the among-population genetic distance δ_A, corrected for within-population variation (24). The negative correlation is induced primarily by the large mtDNA divergence of the Gulf coast population from other North American populations they closely resemble morphometrically. There is no tendency for morphological and mtDNA divergence to be correlated among the populations in our study.

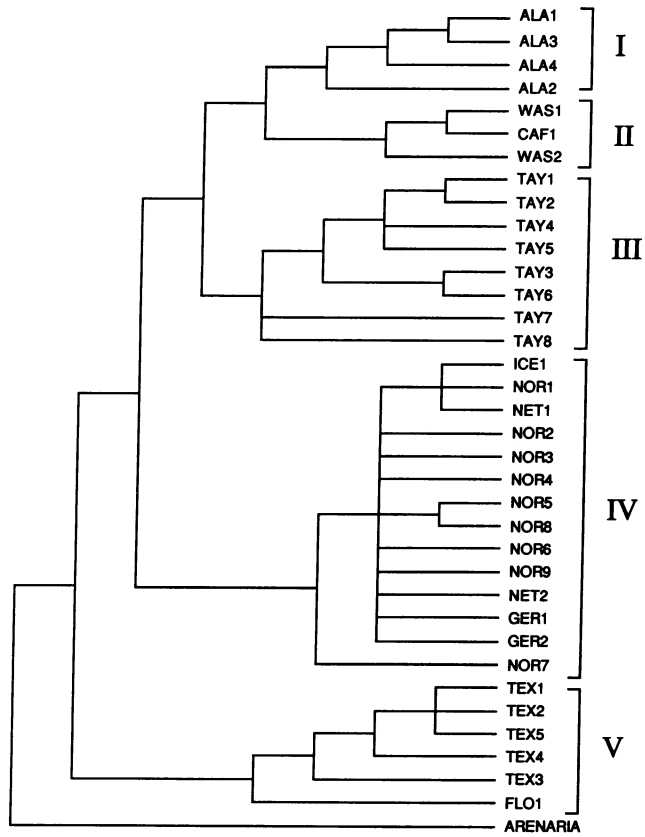

FIG. 2. Maximum-parsimony genealogical tree of the mtDNA types of 73 Dunlins rooted with homologous sequences from the Turnstone (*A. interpres*). This is the strict consensus tree of 20 trees, all of 96 steps in length. Types are identified with abbreviations as in Fig. 1. Roman numerals refer to clusters of types that can be allocated to different populations.

DISCUSSION

Geographic Specificity of mtDNA Types. The principal finding of this study is the very high geographic specificity of mtDNA types among populations of Dunlins sampled over widely dispersed portions of their circumpolar breeding range and in winter flocks at much more southerly sites in North America and western Europe. The two hypervariable segments of the control region contain nucleotide substitutions that are collectively diagnostic of broad phylogeographic groups or populations. Even the much less variable cytochrome *b* segment we sequenced is almost diagnostic for these populations; however, two of the three birds sampled at West Coast winter sites in Washington and California have an identical cytochrome *b* sequence to 14 birds sampled at breeding sites in southern Norway and the Taymyr Peninsula in Siberia. Hence, it is advisable to assay hypervariable-control-region sequences, even in species with pronounced population structuring, as less variable sequences may lack the resolution required to detect genetic subdivision. The unequivocal delineation of populations with their mtDNA types contrasts sharply with the confusing overlap in more traditionally employed morphometric and plumage characters that are the bane of intraspecific taxonomy, attesting to the efficacy of the amplification and sequencing of hypervariable segments of mtDNA in intraspecific phylogeographic studies.

Antiquity of Population Structure. Based on the mtDNA clock of 20.8% sequence divergence per million yr for part I of the Canada goose control region (25), it is possible to construct an approximate time frame for the splitting of the different populations identified in Fig. 1 on which corrected sequence divergence estimates for part I of Dunlins are superimposed. The deepest branch in the tree separates the North American birds that winter on the Gulf coast from all other birds. The corrected sequence divergence of 9.1% translates to a time of splitting of ≈440,000 yr ago. The most recent split is between the two clades in western North America, composed of southern Alaskan breeding birds, on the one hand, and birds wintering in California and Washington, on the other. The corrected sequence divergence of 1.9% suggests they diverged ≈90,000 yr ago. The divergence of the western European population from the Alaska and Taymyr populations dates to ≈350,000 yr, based on an average corrected sequence divergence of 7.3%. Even allowing for the imprecision of these estimates arising from potentially large stochastic errors, it seems certain from the magnitude of sequence divergence that contemporary population structure in the Dunlin coincides with the disruptive effects of the glaciations through the later half of the Pleistocene.

Phylogeography of Dunlins. Similar population structuring of mtDNA in Canada geese breeding in arctic latitudes in North America has been attributed to geographic fragmentation in arctic glacial refugia coupled with high breeding-site philopatry in females (11, 31, 32). This conjunction of demography and historical isolation of populations likely applies to Dunlins as well. Dunlins show high fidelity to their natal breeding sites (33), and major disjunctions in their circumpolar breeding range were formed by Pleistocene ice sheets. Based on an analysis of geographic variation in six morphometric characters of 17 samples over the world-wide breeding range of the Dunlin, Greenwood (8) hypothesized that the six geographic populations he distinguished originated in different refugia that existed in the last interglacial ≈25,000 yr ago. Although this is a plausible biogeographic scenario, it clearly is too recent to fit with the sequence-divergence data. Furthermore, it does not account for the deep branch in the genealogical tree, indicating the more ancient divergence of the population wintering in the Gulf coast of the U.S. that now breeds in central Canada. Instead, these sequence data suggest strongly that Alaskan and West

Table 1. Within-population variation in the mtDNAs of Dunlins and long-term effective population sizes (N_e)

Population	Origin	n	Types, no.	Diversity				Sequence difference,*%				N_e
				All	CR1	CR2	Cyt *b*	All	CR1	CR2	Cyt *b*	
I	Alaska	14	4	0.63	0.63	0.00	0.14	0.13	0.37	0.00	0.05	1800
II	West Coast N. America	3	3	1.00	1.00	0.00	0.67	0.66	1.83	0.00	0.22	—
III	Taymyr	14†	7	0.87	0.84	0.36	0.14	0.09	0.96	0.12	0.05	4600
IV	W. Europe	23‡	14	0.94	0.89	0.72	0.17	0.19	0.95	0.29	0.03	4570
V	Gulf coast N. America	16	6	0.82	0.58	0.52	0.12	0.19	0.37	0.17	0.04	1800

Diversity is given by $h = (1 - \Sigma x_i^2)n/(n - 1)$, where x_i is the frequency of a type and n is the sample size. CR, control region; N., North; Cyt, cytochrome.

*Percentage sequence difference is the average pairwise divergence among individuals.

†Specimen 69 is excluded because it is an immigrant from Norway.

‡Specimens 40 and 45 are excluded here because they are immigrants from Taymyr.

Table 2. Population subdivision for different segments of mtDNA of Dunlins

mtDNA segment	Types, no.	G_{ST}	γ^*
All segments	35	0.111	0.989
Control region I	28	0.165	0.985
Control region II	9	0.663	0.924
Cytochrome *b*	10	0.628	0.892

*$\gamma = (1 - I_B)/I_W$, where I_B is the probability of identity of sequences between populations, and I_W is the probability of identity within populations.

Coast wintering birds are derived from the isolation of populations in the vast Beringian refugium but at much earlier time scale than envisioned by Greenwood (8).

The only other study that approaches ours in the geographic scope of mtDNA variation is that of Ball *et al.* (34), who surveyed restriction fragment length polymorphisms in 127 red-winged blackbirds (*Agelaius phoeniceus*) sampled across North America. They attributed the wide geographic distribution of mtDNA types and lack of population subdivision to homogenizing gene flow in a highly vagile species. Furthermore, they hypothesized that the magnitude of phylogeographic structure in mtDNA within species of vertebrates was negatively correlated with their respective dispersal capabilities. A caveat suggested by our work on Dunlins is that relatively recently evolved population subdivision in species with high dispersal power can only be detected with rapidly evolving DNA sequences, and thus caution should be exercised in generalizing about the effects of dispersal power on population structure. Strong philopatry to breeding sites and high mutation rates can accumulate genetic variants within populations and prevent or retard their spread to other populations. For neutral genes the amount of realized gene flow rather than dispersal capability *per se* is the critical parameter in determining population structure. The only gene flow we detected in Dunlins was between neighboring populations centered in Norway and central Siberia, and this may reflect recent contact of these expanding populations rather than a breakdown of local philopatry via long-distance dispersal. Additionally, two of the three migrants were males, and thus the actual level of gene flow in maternally transmitted mtDNA is much lower than that predicted from the number of migrants exchanged between populations. We conclude that current levels of gene flow in the Dunlin are insufficient to overcome the effects of high mutation rates and historical subdivision.

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