Molecular Phylogenetics and Evolution
Vol. 15, No. 2, May, pp. 319–326, 2000
doi:10.1006/mpev.1999.0730, available online at http://www.idealibrary.com on IDEAL®

Phylogeography of Mitochondrial DNA Variation in Brown Bears and Polar Bears

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Received June 4, 1999; revised September 9, 1999

We analyzed 286 nucleotides of the middle portion of the mitochondrial cytochrome *b* gene of 61 brown bears from three locations in Alaska and 55 polar bears from Arctic Canada and Arctic Siberia to test our earlier observations of paraphyly between polar bears and brown bears as well as to test the extreme uniqueness of mitochondrial DNA types of brown bears on Admiralty, Baranof, and Chichagof (ABC) islands of southeastern Alaska. We also investigated the phylogeography of brown bears of Alaska's Kenai Peninsula in relation to other Alaskan brown bears because the former are being threatened by increased human development. We predicted that: (1) mtDNA paraphyly between brown bears and polar bears would be upheld, (2) the mtDNA uniqueness of brown bears of the ABC islands would be upheld, and (3) brown bears of the Kenai Peninsula would belong to either clade II or clade III of brown bears of our earlier studies of mtDNA. All of our predictions were upheld through the analysis of these additional samples.

Key Words: **mitochondrial DNA; cytochrome *b*; bears; phylogeography; paraphyly**

INTRODUCTION

Although the brown bear (*Ursus arctos*) is believed to be a species that is both phylogenetically recent (Kurtén, 1968; Kurtén and Anderson, 1980; Mazza and Rustioni, 1994) and highly vagile (USFWS, 1987), it exhibits extensive morphological variation across its range of distribution (Merriam, 1918; Ognev, 1931; Kurtén, 1973; Hall, 1984). Phylogenetic recency and high vagility are characters usually associated with species displaying little or no phylogeographic substructure (Avise, 1986). Yet, we have described extensive phylogeographic substructure in the patterns of mitochondrial DNA (mtDNA) of brown bears in Alaska (Talbot and Shields, 1996a) and have described numerous clades of mtDNA across a broader range of distribution of the brown bear (Waits *et al.*, 2000).

Phylogeographic descriptions are accurate, however, only if species are sampled extensively across their range of distribution. If major geographic areas are unsampled or if sample sizes are small, phylogeographic descriptions and predictions about past phylogenetic relationships can be erroneous. For example, Byun *et al.* (1997) reported data on mtDNA of black bears (*Ursus americanus*) and claimed the existance of a glacial refugium of long duration for them during the Pleistocene on or near the Haida Gwaii Archipelago (Queen Charlotte Islands) of British Columbia. Foster (1965), Calder and Taylor (1968), Cowan (1989), Schofield (1989), Josenhans *et al.* (1995), and Heaton *et al.* (1996) have all proposed glacial refugia in southeast Alaska. However, with more extensive geographic sampling of populations of black bears to the east of Haida Gwaii, Wooding and Ward (1997), Stone and Cook (2000), and Demboski *et al.* (1999) have all described molecular data which were interpreted to support a possible postglacial expansion of southern populations of black bears into Haidi Gwaii rather than being taken as evidence for a refugium there.

We have previously described two major clades of brown bears in Alaska based on analysis of DNA sequences of three complete (mt) genes (Talbot and Shields, 1996a). Brown bears currently residing on Admiralty, Baranof, and Chichagof (ABC) islands of southeastern Alaska constitute the most genetically unique mitochondrial clade (clade I) of brown bears in the New World. They differ from the other major clade in Alaska (clade II/III, which includes brown bears of

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1055-7903/00 $35.00

the immediate coastal mainland to the east of the ABC islands) by a minimum of 23 fixed substitutions in the cytochrome *b* gene alone. Accordingly, we estimate that these two clades may have separated from a common ancestor about 300,000 years ago (Talbot and Shields, 1996a).

Surprisingly, we have never observed a brown bear of the clade I type anywhere other than on the ABC islands, even when we analyzed the DNA of 166 brown bears from 10 geographic locations in Alaska (Talbot and Shields, 1996a). This suggests that female brown bears on the ABC islands disperse very little, if at all, and that the isolation of their mtDNA from that of other brown bears has been long-term. We sampled only 24 brown bears of the ABC islands (clade I) and 2 brown bears (clade II) of the immediate coastal mainland of southeastern Alaska in our earlier study (Talbot and Shields, 1996a). Brown bears of the coastal mainland of southeastern Alaska have been difficult to sample because of: (1) their occurrence in low densities compared with populations on the nearby ABC islands, (2) low hunting pressure on them, and (3) the pausity of biological studies that have been conducted in that region. In association with long-term ecological research on brown bears of the ABC islands and an emphasis on obtaining tissue samples of mainland brown bears, we have now obtained much larger sample sizes for bears of both the coastal mainland of southeast Alaska and the ABC islands. We report on those data here and hope to more substantially test our earlier observation that mtDNAs of the ABC type were restricted to bears of the ABC islands and that clade II- and clade III-type mtDNAs were found elsewhere in Alaska but not on the ABC islands.

We have also compared the DNA sequences of Alaskan brown bears to those of brown bears from throughout the remainder of the distributional range (Montana, northwestern Canada, and Eurasia) of the species (Waits *et al.*, 2000). These comparisons of homologous gene segments across the range of the species have allowed us to describe the major phylogeographic associations within brown bears. Consequently, we are relatively certain about the uniqueness and geographic restriction of clade I mtDNA types of brown bears on the ABC islands.

In our earlier analyses we described a sister taxon association between brown bears of the ABC islands and the eight polar bears (*Ursus maritimus*) that we studied from Alaska (Shields and Kocher, 1991; Talbot and Shields, 1996a). Waits *et al.* (2000) included four different polar bears in their comparative study, two from Europe, one from Asia, and one from North America, and described the same sister taxon association between mtDNAs of ABC brown bears and polar bears. Because the polar bear has a circumarctic distribution, the phylogeographic sampling in all three of the above-mentioned studies was incomplete. Thus, our observation of a sister taxon association between brown bears of the ABC islands and all polar bears studied may not be supported when polar bears from additional regions of their distribution are studied. Finally, brown bears of the Kenai Peninsula of Alaska were not included in our earlier study (Talbot and Shields, 1996a). This population is important because it has experienced increased interactions with humans due to increased human habitation and tourism on the peninsula. Increasing numbers of brown bears are being destroyed because of these encounters (*c. c.* Schwartz, pers. comm.). If it becomes necessary to augment the brown bear population on the Kenai Peninsula with bears from other regions, the phylogenetic relationships among potential source populations must be known. We have obtained tissues of brown bears from the Kenai Peninsula and are now able to place those bears in a phylogeographic context with regard to all other brown bears. Thus, our analyses are also of considerable importance to the conservation of these large mammals.

MATERIALS AND METHODS

We compared 286 nucleotides of DNA from the middle portion (15243 to 15529; Irwin *et al.*, 1991) of the mitochondrial cytochrome *b* gene of 61 brown bears and 55 polar bears. We chose this region of DNA because: (1) we possessed a reference data set for the cytochrome *b* gene of 166 brown bears of Alaska (Talbot and Shields, 1996a; Waits *et al.*, 2000); (2) the DNA primers for this region demonstrated fidelity (Talbot and Shields, 1996a); (3) 15 nucleotide substitutions were identified in this region in our earlier studies, 11 of which were phylogenetically informative for brown bears and polar bears of Alaska (Talbot and Shields, 1996a); and (4) a similar association between brown bears and polar bears was observed when comparisons of DNA sequences of the control regions were analyzed (unpublished). This lessened our concern for a type 2 error if only cyt b sequences had been compared. All of the sequences analyzed herein are new to the literature. The sample of brown bears consisted of 30 bears from the ABC islands of southeast Alaska (Fig. 1), 11 bears from the coastal mainland of southeast Alaska (Fig. 1), and 20 bears from the Kenai Peninsula (Fig. 2). The sample of polar bears consisted of 40 bears from Arctic Canada (Figs. 3 and 4) and 15 bears from Arctic Siberia (Figs. 5 and 6).

We extracted DNA from tissue using proteinase K/salt based on the procedures of Medrano *et al.* (1990) as well as extractions using Chelex. DNA was purified using sodium chloride and ethanol precipitations based on the methods described by Talbot and Shields (1996a).

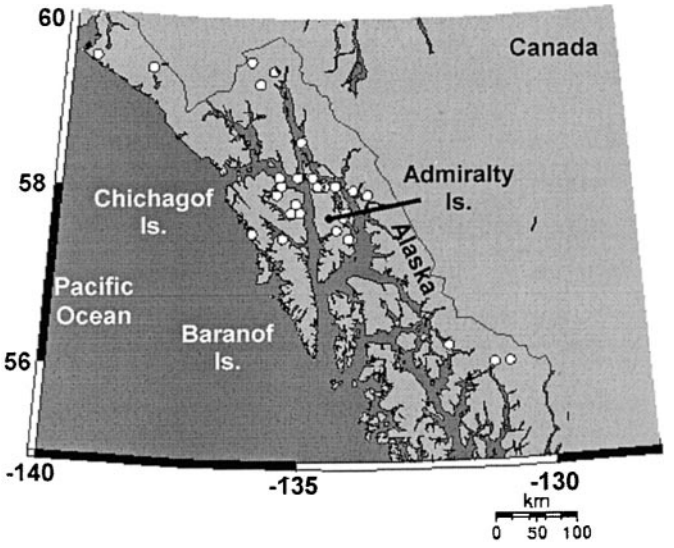

FIG. 1. Locations (open circles) on Admiralty, Baranof, and Chichagof islands and on the coastal mainland of southeast Alaska where tissues of brown bears were obtained.

The middle section of the cytochrome *b* gene was amplified using the primer set (L15168) 5′-TGAGGACAAATATCGTTCTG-3′ and (H15590) 5′-CTCCTAGTTTATTAGGGATGGATCG-3′, as described by Talbot and Shields (1996a). Amplifications took place in either 25- or 50-µl total reaction volumes with 35 cycles of the polymerase chain reaction using a cloned version of *Thermus aquaticus* DNA polymerase. The DNA was amplified by a Perkin–Elmer–Cetus thermal cycler, with denaturation at 94°C for 60 s, annealing at 45°C for 60 s, and extension at 72°C for 90 s. Amplification was verified by electrophoresis of 5.0 µl of the reaction product on a 1.5% agarose gel containing ethidium bromide in Tris–borate–ethylenediaminetetraacetate (TBE). Product bands were visualized under ultraviolet light. The size of the initial sequence amplified was about 350 bp. All samples were electrophoresed against

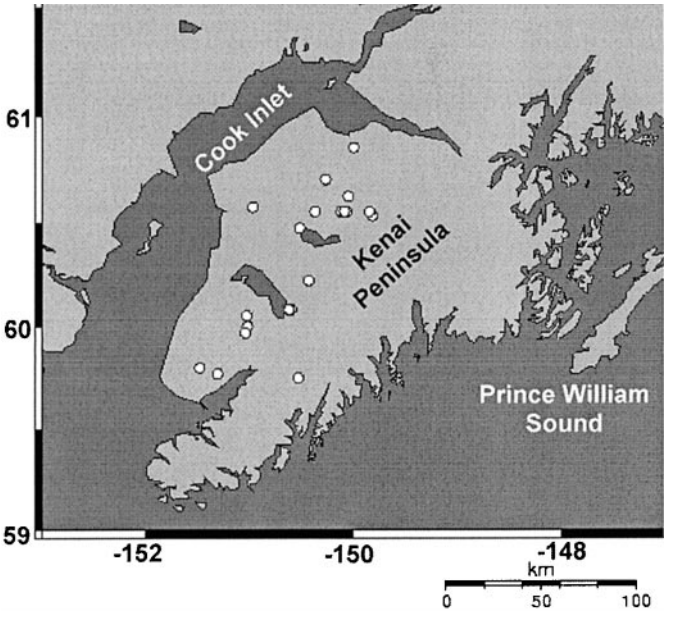

FIG. 2. Locations (open circles) on Alaska's Kenai Peninsula where tissues of brown bears were obtained.

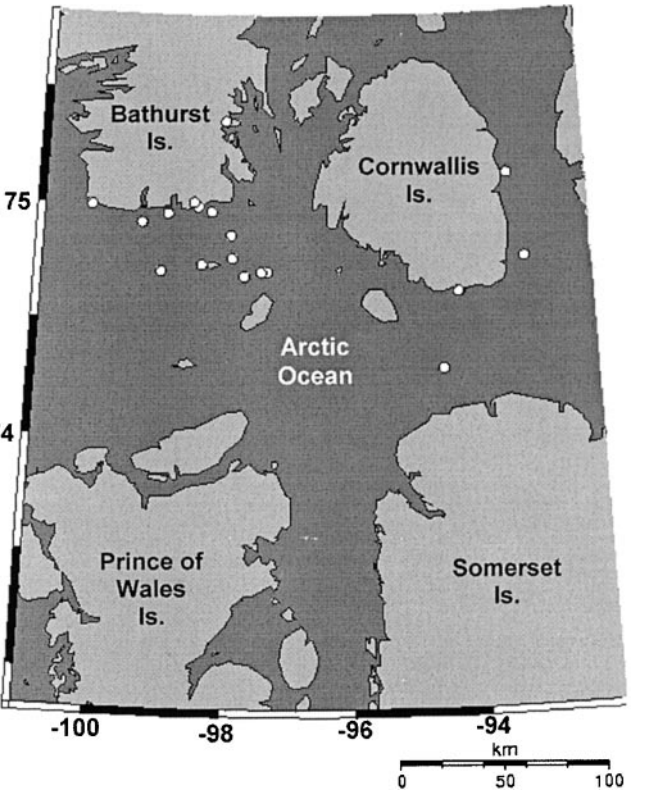

FIG. 3. Locations (open circles) in central Arctic Canada where tissues of polar bears were obtained.

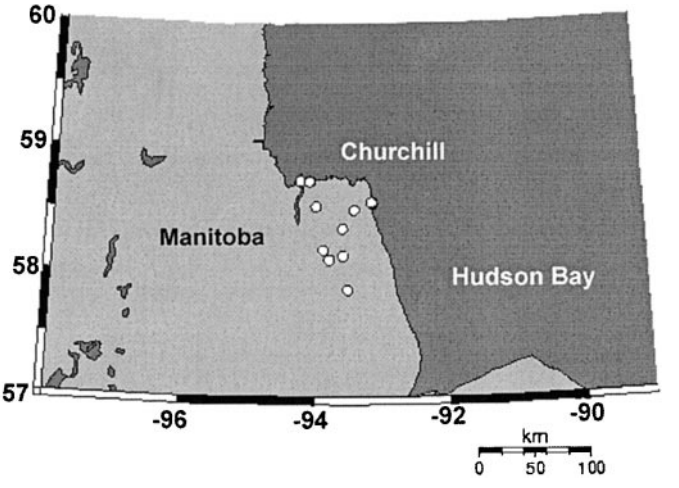

FIG. 4. Locations (open circles) in central Manitoba where tissues of polar bears were obtained.

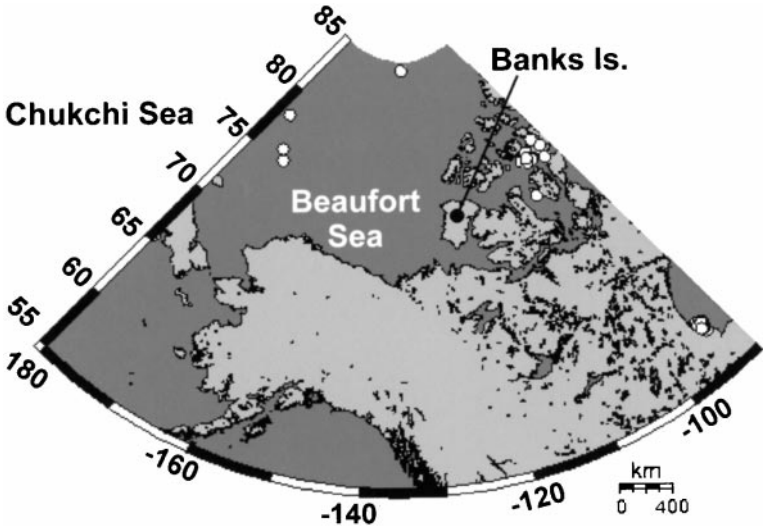

FIG. 5. Locations (open circles) in western Arctic Canada where tissues of polar bears were obtained.

a negative control to avoid contamination. The amplification product was sequenced using an Applied Biosystems 373-A automated DNA sequencer, which used the dideoxynucleotide method of sequence determination (Sanger *et al.*, 1977). DNAs were sequenced in both directions, aligned using Sequence Navigator, and compared to the 174 sequences of our earlier study (Talbot and Shields, 1996a) as well as to those sequences deposited in GenBank. For phylogenetic analysis, we used PAUP (Swafford, 1993). Branch-and-bound and exhaustive search options were used. Consensus trees based on majority rule and 500 bootstrap replicates were produced by the bootstrap option based on the methods of Eck and Dayhoff (1966) and Kluge and Farris (1969). DNA of the black bear was used as an outgroup. Analyses (not shown) using neighbor-joining (Saitou and Nei, 1987) gave essentially the same results as those using parsimony. All sequences were deposited in GenBank (Accession Nos. AF257168–AF257174).

RESULTS

Brown bears of the ABC islands. Sequences of DNA from 15243 to 15529 of the cytochrome *b* gene of the 17 brown bears from Admiralty Island, 12 brown bears from Chichagof Island, and 1 brown bear from Baranof Island analyzed here were identical (Fig. 7). This result places these 30 brown bears in clade I of our earlier study (Talbot and Shields, 1996a). Since the sequence comparisons herein are based only on a portion of the cytochrome *b* gene, unlike our earlier descriptions of the entire gene (Talbot and Shields, 1996a), we cannot assign any of these bears to a specific mtDNA lineage. However, they belong either to lineage GB01 or lineage

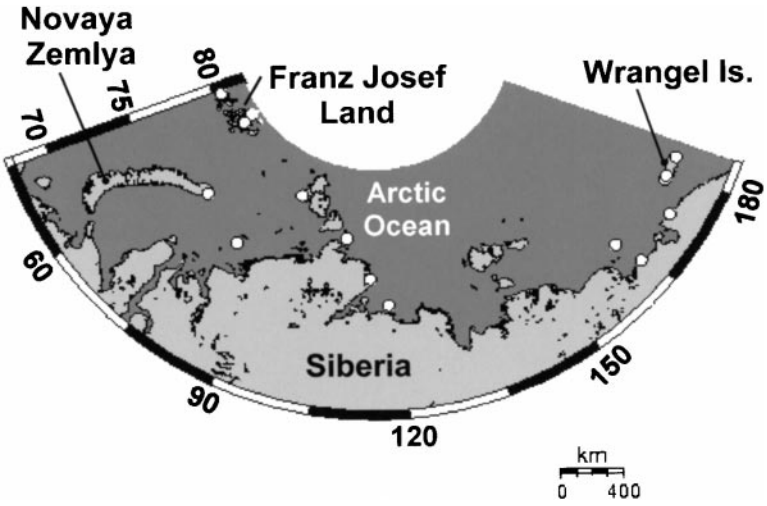

FIG. 6. Locations (open circles) in Arctic Siberia where tissues of polar bears were obtained.

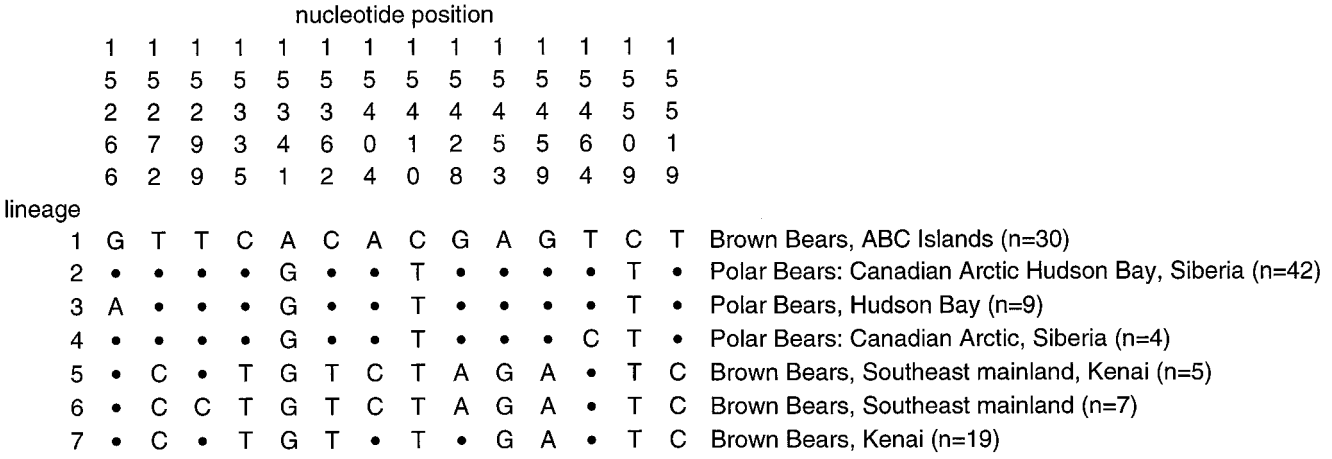

FIG. 7. Condensed dot matrix of variable nucleotide positions among 286 nucleotides (15243 to 15529) of the middle portion of the cytochrome *b* gene of bears of this study.

GB05 (see Fig. 3, Talbot and Shields, 1996a). Analyses of these new sequences together with our earlier observations indicate that all 56 brown bears of the ABC islands studied to this point possess the clade I type of mtDNA. The 30 brown bears of the ABC islands analyzed here are fixed at three nucleotide positions (15341, 15410, and 15509; Fig. 7) when compared to all other bears of this study.

Brown bears of the coastal mainland of southeast Alaska. DNA sequences of the middle portion of the cytochrome *b* gene from the 11 brown bears of the coastal mainland of southeast Alaska were not identical (Fig. 7). Seven of the bears possessed a C nucleotide at position 15299 and the other 4 bears possessed a T nucleotide at that position. With the exception of these variants, these 11 brown bears were identical in sequence to lineage GB09, a clade II lineage, of the coastal mainland of southeast Alaska (Talbot and Shields, 1996a). Compared to the brown bears of the ABC islands, brown bears of the coastal mainland of southeast Alaska are fixed for 11 nucleotide substitutions in the middle portion of the cytochrome *b* gene (Fig. 7). Extreme genetic uniqueness continues to be exhibited in brown bears of the mainland and those of the ABC islands with these new data. The ABC islands are isolated from the mainland by Icy Strait and Stephens Passage with varying distances of from 5 to 15 km.

Brown bears of the Kenai Peninsula of Alaska. Nineteen of the 20 brown bears of the Kenai Peninsula analyzed here had DNA sequences (Fig. 7) identical to lineage GB11 of clade III of Talbot and Shields (1996a). The remaining brown bear of the Kenai Peninsula had both an A–C transversion at position 15404 and a G–A transition at position 15428 (Fig. 7).

Polar bears of the Canadian Arctic. Nine of the 13 polar bears of the Hudson Bay region of Canada have an A nucleotide at position 15266 (Fig. 7); the other 4 bears from Hudson Bay along with the remaining 27 bears from the Canadian Arctic and 11 polar bears from Russia have a G at that position. Two of the 23 polar bears from Canada have a C at position 15462; the remaining 38 bears from Canada have a T at that position.

Polar bears of the Siberian Arctic. Two of the 15 Siberian polar bears have a C at position 15464 (Fig. 7); the remaining 13 Siberian polar bears have a T at that site. Unlike the populations of brown bears on the ABC islands and those on the coastal mainland of Alaska, there are no fixed substitutions among the 40 polar bears of the Canadian Arctic and the 15 polar bears from the Siberian Arctic analyzed in this study. Thus, for this study, only three lineages of polar bears were discovered across a broad range of the distribution of the species.

Polar bears vs brown bears. All 55 polar bears and the 54 brown bears of the ABC islands analyzed by us (24 in our earlier studies and 30 analyzed here) differ by only three fixed nucleotide transitions in the middle portion of the cytochrome *b* gene: G ↔ A at 15341, T ↔ C at 15410, and T ↔ C at 15509 (Fig. 7). A parsimony tree depicting these relationships is shown in Fig. 8. The same phylogeographic pattern resulted when 455 brown bears and 4 polar bears were compared (Waits *et al.*, 2000) and when 166 brown bears and 8 polar bears were compared (Talbot and Shields, 1996a).

DISCUSSION

With the addition of DNA sequences of the 41 brown bears from southeast Alaska, we have now analyzed the cytochrome *b* genes of 67 brown bears from this region. All 54 of the brown bears of the ABC islands belong to our clade I as expected, whereas all 13 brown bears of

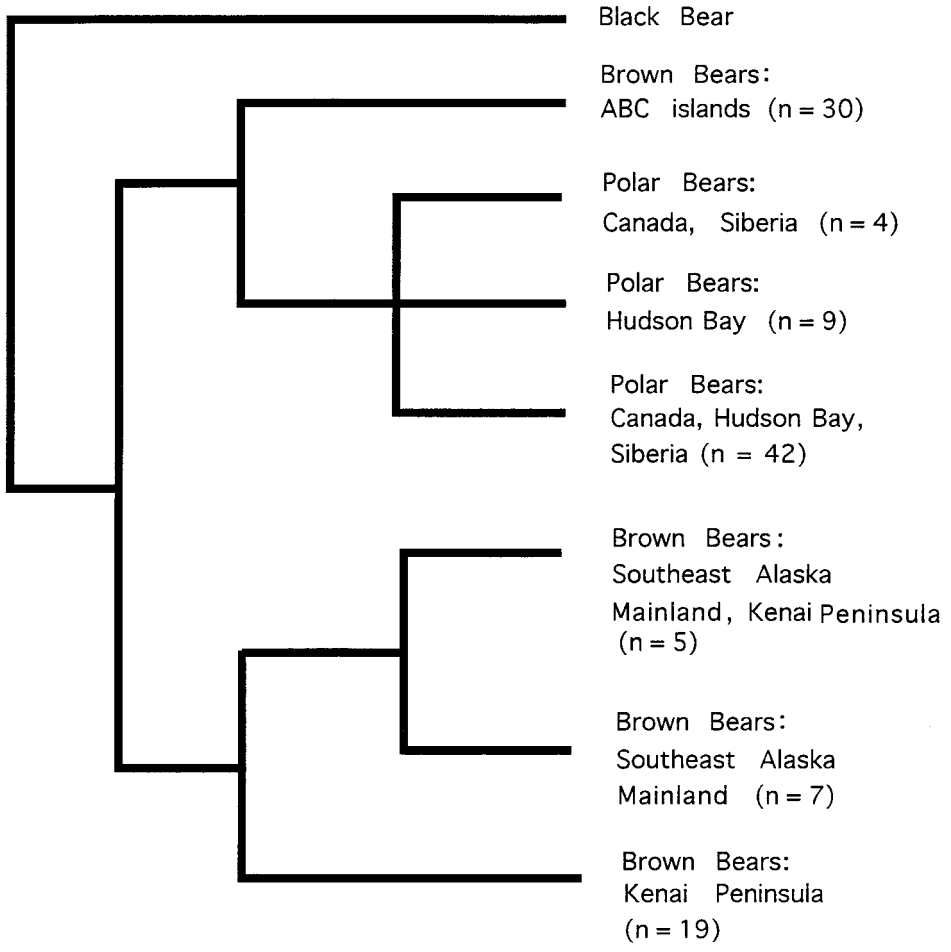

FIG. 8. Unweighted maximum-parsimony tree for the 286 nucleotides of the seven mtDNA lineages of bears of this study.

the immediate coastal mainland belong to clade II; we observed no exceptions to this separation. Moreover, we are unaware of such vast intraspecific divergence among the mtDNAs in any other species of mammal. This observation implies that there is no gene flow of mtDNA between bears of the mainland and those of the ABC islands. Thus, our first hypothesis, that the mtDNAs of brown bears of the ABC islands would remain unique when additional samples of the region were analyzed, has been sustained. The extreme uniqueness of the mtDNAs of brown bears of the ABC islands in 227 bears analyzed by us (Talbot and Shields, 1996a and this study), as well as their phylogeographic positioning among 455 brown bears across their range of distribution (Waits *et al.*, 2000), implies their early separation from an ancestral stock (estimated to have occurred 300,000 years ago) and a possible early entry into the New World from Asia across the Bering Strait. The recent discovery by Allan Cooper (pers. comm.) of ABC-like mtDNA in a fossil bone of a brown bear recovered in the Yukon Territory, Canada provides a geographic link between brown bears currently residing on the ABC islands and their ancient relatives in Eurasia. This also indicates a previous wider distribution of the ABC lineage in North America and subsequent extirpation over most of its range. Moreover, analysis of mtDNA of brown bears of Kluane National Park in Canada by Waits *et al.* (1998) indicates that those bears belong to our clade II, as do brown bears of the coastal region of southeast Alaska. We suggest that this clade entered the New World from Asia after the retreat of the Wisconsin glacial masses (Talbot and Shields, 1996a).

The analysis in this study of an additional 40 polar bears from the Canadian Arctic and 15 polar bears from the Siberian Arctic further supports the paraphyletic association between mtDNA of brown bears and polar bears. Indeed, while brown bears of the mainland of southeast Alaska differ in their mtDNAs from brown bears of the ABC islands by 11 fixed nucleotide substitutions in the middle portion of the cytochrome *b* gene, polar bears from Arctic Canada, Arctic Siberia, and Alaska differ from ABC brown bears at only 3 nucleotide positions in this portion of the gene. Thus, our second hypothesis, that mtDNA paraphyly between polar bears and brown bears of the ABC islands would

be sustained with the analysis of additional polar bears, also has been upheld.

Our observations of mtDNA paraphyly for brown bears with respect to polar bears (Shields and Kocher, 1991; Talbot and Shields, 1996a,b and this study) are also supported when other genes of the mitochondrial DNA molecule are analyzed. Waits *et al.* (1999) described a sister taxon association between the two species when DNA sequences of six segments (16S, control region, cyt b, NADH-4, NADH-5, and CO-II) of mtDNA were compared for all species of ursids. Brown bears of the ABC islands of southeastern Alaska may be descendants of ancient ursids that diverged from other lineages of brown bears and subsequently founded the polar bear lineage. It is possible that this paraphyletic association is based on shared common ancestry rather than introgressive hybridization (gene flow of mtDNA of female brown bears into polar bears) because Kurtén (1964, 1968) cites fossil evidence that polar bears may have arisen from a type of brown bear somewhere in coastal northeastern Asia.

The emergence of the brown bear may have been one million years ago (Waits *et al.*, 2000). The separation of the polar bear from a brown bear stock may have been as recent as 200,000 years ago (Talbot and Shields, 1996a), which implies that, as a species, the polar bear has been in existence about 1/5 the time of brown bears. This prediction of recency as a species for the polar bear is supported by other data. Though our sample size for polar bears is an order of magnitude less than that of Waits *et al.* (1999), we described only three separate lineages among 45 polar bears of Siberia and Canada. Waits *et al.* (1999) described 56 lineages of brown bears. Moreover, genetic distance (*D*s) among microsatellite loci (Paetkau *et al.*, 1997, 1998) of Alaskan brown bears (0.57) is about twice that of distances (0.33) among even the worldwide population of polar bears (Paetkau *et al.*, 1999). This could imply recency of species origin for polar bears.

Finally, analysis of the middle portion of the cytochrome *b* gene in 20 brown bears of the Kenai Peninsula suggests that these bears are recent derivatives of brown bears of clade II/III (Talbot and Shields, 1996a). Thus, our third hypothesis has been sustained. This observation suggests that if augmentation of the declining population of brown bears on the Kenai Peninsula is necessary, source populations on the immediate coastal mainland of southcentral Alaska should be used.

Although analysis of mtDNA can lead to accurate descriptions of phylogeographic patterns, it can provide an inaccurate measure of contemporary population structure. For example, we have compared the DNA of 17 microsatellite loci of the nuclei of eight populations of brown bears in Alaska and Canada, including bears of the ABC islands and those of the southeast coastal mainland of Alaska (Paetkau *et al.*, 1998). This analysis suggests nuclear gene flow (probably mediated by males) between brown bears of the coastal mainland of southeast Alaska and those of the ABC islands. Analysis of these two genetic systems, mtDNA on the one hand and nuclear microsatellites on the other, in the same populations emphasizes the special utility of studying historical patterns of phylogeography and current patterns of differential dispersal between the sexes.

ACKNOWLEDGMENTS

We thank Thomas LeCroy for technical assistance in sequencing the DNAs on the 373-A automated DNA sequencer. This research was funded by a grant from the Arctic Natural Sciences section of the Division of Polar Programs of the National Science Foundation (OPP 9707626FY97) to G. F. Shields. We thank Drs. Stanislav E. Belikov, Nature Conservation, Moscow, Russia and Mikhail S. Stishov, Wrangel Island Nature Reserve, Russia for their help in collecting tissues. Collection of tissue samples in Alaska was in association with long-term research and management programs supported by the Alaska Department of Fish and Game and various projects in Federal Aid to Restoration of Wildlife.

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