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NORTH AMERICAN BLACK BEAR MTDNA PHYLOGEOGRAPHY: IMPLICATIONS FOR MORPHOLOGY AND THE HAIDA GWAII GLACIAL REFUGIUM CONTROVERSY

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Abstract.—The controversial role of Haida Gwaii (Queen Charlotte Islands) as a biological refugium on the northwestern coast of North America has been widely discussed for more than fifty years. The presence of morphologically divergent subspecies on Haida Gwaii is one of the major lines of evidence suggesting this archipelago's role as a refugium during the Wisconsin. However, since morphological distinction can be derived postglacially as well as in extended isolation, such evidence is ambiguous. To examine this question, we did a phylogenetic analysis of cytochrome *b* sequences (719 bp) of black bear (*Ursus americanus*), one of the distinctive endemics of Haida Gwaii, and compared these with conspecifics from across North America, focusing primarily on the northwestern coast. We found that the Haida Gwaii bear are indistinguishable from coastal bear of British Columbia and Vancouver Island, but are highly distinct from continental bear. Coastal and continental bears differ by 24 synapomorphies and an average sequence divergence of 3.6%. The coastal mitochondrial lineage occurs in each of the three recognized coastal subspecies suggesting that the morphological characteristics differentiating these taxa may be postglacially derived. The data are consistent with recent suggestions that a glacial refugium existed on the now submerged continental shelf connecting Haida Gwaii, Vancouver Island, and the coastal fringe of mainland British Columbia. This refugium would have been an additional source for postglacial recolonization of northwestern North America.

Key words.—Biogeography, cytochrome *b*, Queen Charlotte Islands, *Ursus americanus*.

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One of the largest and most remote of north temperate archipelagos is Haida Gwaii (previously the Queen Charlotte Islands), found 80 km off the western coast of Canada. The assemblage of endemic taxa from these islands, which include mammals (Foster 1965; Cowan 1989), birds (Foster 1965; Cowan 1989), fish (Moodie and Reimchen 1973), beetles (Kavanaugh 1992), angiosperms (Calder and Taylor 1968), and bryophytes (Schofield 1989), have cumulatively provided support for a Pleistocene glacial refugium on or near these islands (summary in Scudder and Gessler 1989). Palynological (Warner et al. 1984) and molecular data (O'Reilly et al. 1993) have further implicated Haida Gwaii as a potential refugium. However, with the exception of nunataks, geological data suggest that Haida Gwaii, Vancouver Island, and the adjacent mainland were inundated by ice during the last (Wisconsin) glacial advance (Sutherland-Brown 1968). As a consequence, this entire region is currently believed to have been postglacially colonized from two refugia, one in Alaska/Yukon and the other in southern Washington (summary in Heusser 1989). If so, the endemism that characterizes Haida Gwaii's flora and fauna could be a consequence of postglacial differentiation (e.g., see Moodie and Reimchen 1976).

Seven subspecies of black bear (*Ursus americanus*) occur in western North America, of which the Haida Gwaii black bear (ssp. *carlottae*) is the most distinct. In addition to being the largest black bear in the world, *carlottae* has a unique skull structure (a massive cranium and elongate rostrum) and proportionately large molars (Foster 1965). So substantial are these differences that *carlottae* was originally described as a distinct species by Osgood (1901) and the unique characteristics were surmised by Foster (1965) to have developed over prolonged periods of time. Such differentiation would require isolation from other subspecies, arguing that the features characterizing the Haida Gwaii black bear developed in a glacial refugium on or near this archipelago. If true, this refugium may have been an important source area for postglacial recolonization of northwestern North America.

Urged by recent data that showed the presence of a possibly relict mtDNA lineage in morphologically distinct freshwater stickleback populations near the northeastern corner of Haida Gwaii (O'Reilly et al. 1993), we have undertaken an analysis of mtDNA divergence between populations of black bear from Haida Gwaii and conspecifics on Vancouver Island and several continental regions. Close mtDNA similarity between populations on the archipelago and those on the continent would be consistent with recent separation and postglacial morphological differentiation. In contrast, marked dissimilarity would suggest a pre-Wisconsin separation from continental lineages and survival in a coastal refugium during the last glacial advance.

MATERIALS AND METHODS

Samples

DNA from black bear was obtained primarily from muscle tissue. However, blood samples for three black bear from Vancouver Island, and a hide preserved with salt for the white phase *kermodei* were also used. Using the preserved hide was necessary because fresh tissue was unavailable. Sample details are given in Table 1.

DNA Isolation

DNA was obtained from tissue according to a modification of the protocol originally developed by Doyle and Doyle (1987). Small pieces of frozen muscle, skin, and/or organs were immersed in enough cetyltrimethylammoniumbromide (CTAB) buffer to saturate the tissue. The mixture was vigorously shaken for 1 min. and incubated at 60°C for 45 min.

TABLE 1. Subspecies, geographical location, and sample sizes for black bear.

Subspecies	Location	Sample size	Source
carlottae	Haida Gwaii	11	authors
vancouveri	Vancouver Island	5*	authors
kermodei	Terrace, BC	3	authors
kermodei	Prince Rupert, BC	3	authors
kermodei	Lakeisle, BC	1*	authors
kermodei	Moricetown, BC	1	authors
kermodei	Rosswood, BC	1	authors
ssp?	Khutzymateen	1	authors
cinnamomum	Williston Lake, BC	1	authors
ssp?	Jasper, AB	1	authors
americanus	Yukon	1	authors
americanus	Alaska	2*	authors, Shields and Kocher 1991
ssp?	Montana	1*	Zhang and Ryder 1995
americanus	New Jersey/Pennsylvania	1*	Vrana et al. 1994
altifrontalis	Olympic Peninsula	1	authors
ssp?	Tweedsmuir, BC	2*	authors

* Indicates that only partial sequences were used. The region H15149 to L14841 was used for one individual from Vancouver Island, one individual from Tweedsmuir, one individual from Alaska (Shields and Kocher 1991), and the individuals from Montana (Zhang and Ryder 1995) and Pennsylvania (Vrana et al. 1994). The region L15086 to H15560 was used for the individual from Lakeisle.

DNA was extracted by chloroform:isoamyl alcohol (96:4), and precipitated with 70% ethanol and 3M sodium acetate pH 5.2. The pellet was washed with 70% ethanol. DNA was resuspended in 0.1 × TE (1mM Tris, 0.01mM EDTA) buffer, pH 7.5, and stored at −20°C. DNA from whole blood was obtained by using the DTAB/CTAB protocol from Gustincich et al. (1992).

Amplification

A 719-bp fragment was amplified from cytochrome *b*. First, a 307-bp region from the cytochrome *b* gene was amplified using primers H15149 and L14841 as described by Kocher et al. (1989). The following PCR conditions were used: 0.2 mM dNTPs, 0.1 mM of each primer, 10× PCR buffer, and 0.5 units of taq polymerase. PCR was carried out for 30–35 cycles using the following steps: denaturation 94°C for 1 min, annealing for 2 min at 50°C, and extension at 72°C for 2 min. Each reaction was initially denatured for 2 min at 94°C and hot started. A second region from the cytochrome *b* gene was amplified using H15560 (Kocher et al. 1989) and a primer designed by the authors (L15086 TAC TAT GGC TCA TAC CTA CTC) using the same PCR conditions described above. PCR products were cut out of a 2% Nusieve (FMC Bioproducts) gel stained with 0.25 μg/mL ethidium bromide and then purified through commercial columns (Promega Wizard® PCR Purification system). These purified products were subsequently cloned (Invitrogen TA® Cloning/One Shot® Kit).

DNA Sequencing

Manual cycle sequencing (Sanger et al. 1977) was performed using the Promega *fmol*® Cycle Sequencing System on DNA directly obtained from PCR. We used the reaction conditions suggested by Promega (Madison, Wisconsin). Directly incorporated ^{35}S bands were run on 8% acrylamide gels and then exposed for 72 hours. Automated cycle sequencing of cloned PCR fragments was performed using the 21 M13 primer kit (PRISM) on the ABI 373A according to conditions suggested by Applied Biosystems, Inc., Foster City, California (ABI).

Phylogenetic Analyses

Sequence consensuses from ABI and sequence alignments were generated using Lasergene Navigator (DNASTAR, Madison, Wisconsin). At least three sequences were used to generate the consensus. Weighted and unweighted parsimony analyses (Eck and Dayoff 1966; Fitch 1977) were performed using PAUP, vers. 3.1 (Swofford 1993). Weights were determined by the frequency of substitutions at each of the three-codon base positions and the ratio of transitions and transversions in our dataset. Majority rule trees were produced by a heuristic search of character state matrices using brown bear (*Ursus arctos*) and Asiatic black bear (*U. thibetanus*) as outgroups for all analyses. Data were resampled using 100 heuristic bootstrap replicates.

Distance trees were generated using the neighbor-joining (Saitou and Nei 1987) and UPGMA (Sokal and Michener 1958) methods on Phylip 3.2 (Felsenstein 1989). Distance matrices were generated using the one-parameter (Jukes and Cantor 1969) and two-parameter (Kimura 1980) models. Resulting matrices were used in neighbor-joining and UPGMA analyses.

Synonymous (*ps*) and nonsynonymous (*pa*) variation was calculated using an in-house program based upon methods by Nei and Gojobori (1986). Neighbor-joining and UPGMA analyses were done using corrected (Jukes and Cantor 1969) *ps* and *pa*.

RESULTS

A total of 719 bp from 30 individuals and 307 bp from three individuals were sequenced (see appendix for GenBank accession numbers). Phylogenetic analyses of these 33 cytochrome *b* sequences and three additional black bear sequences from GenBank revealed the existence of two geographically structured, monophyletic black bear lineages on the northwestern coast of North America (Fig. 1). A continental lineage grouped samples from British Columbia, Yukon, Alberta, Montana (Zhang and Ryder 1995), Pennsylvania (Vrana et al. 1994), and Alaska (Shields and Kocher

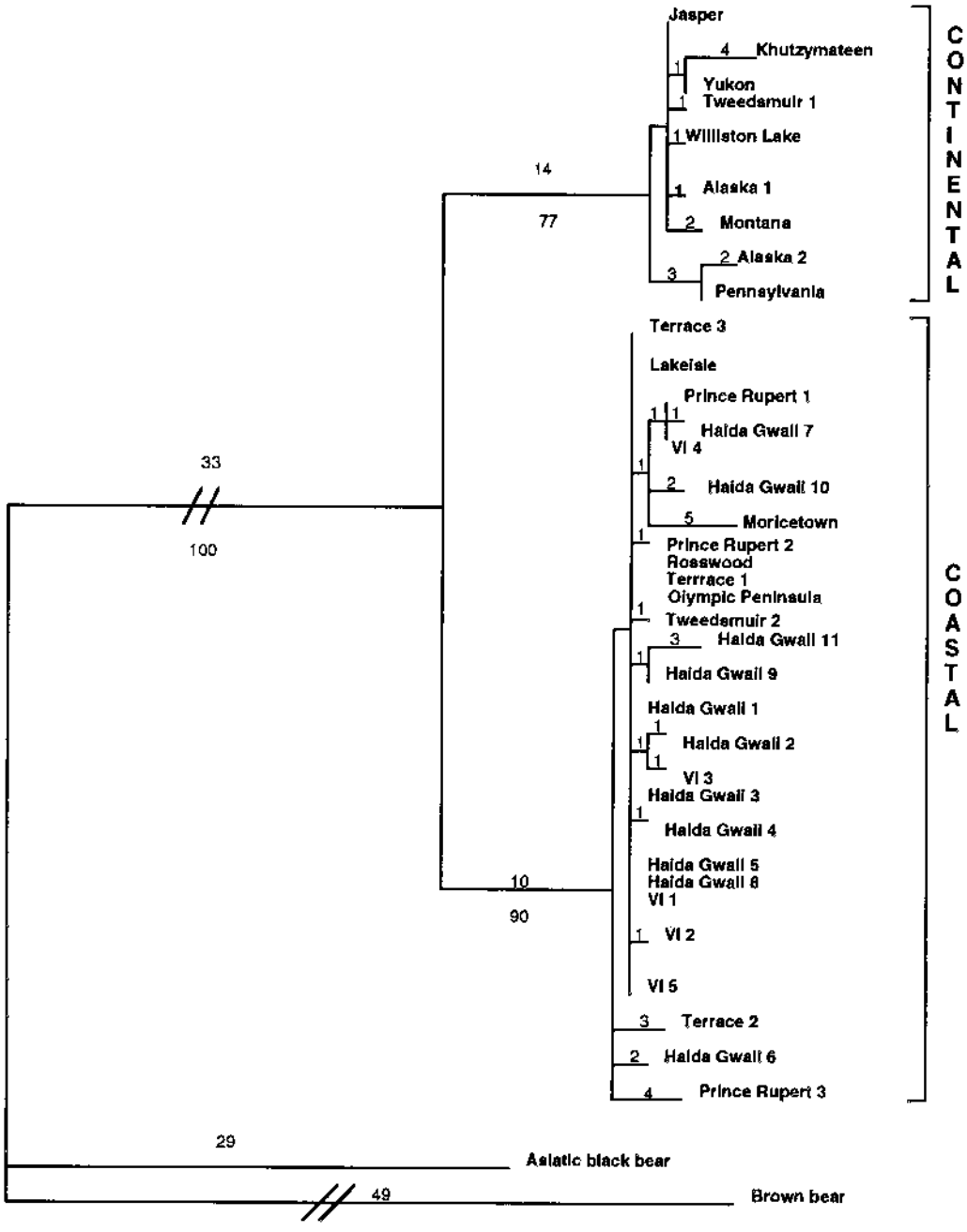

FIG. 1. All methods of phylogenetic analyses produced trees with the same general topology. We present the 50% majority rule tree from parsimony analysis as an example with Asiatic black bear and brown bear as outgroups. We designate the clades as coastal and continental to reflect the general areas in which they were found in this study. Numbers above branches indicate branch lengths from PAUP and numbers below branches indicate bootstrap values (100 replicates). The total tree length is 176 and the consistency index is 0.88.

1991) and encompassed the distribution of two subspecies—*americanus* and *cinnamomum*. The second coastal lineage was found exclusively in bears from Haida Gwaii (ssp. *carlottae*), Vancouver Island (ssp. *vancouveri*), the Olympic Peninsula (ssp. *altifrontalis*) and most bears from the coastal fringe of British Columbia (ssp. *kermodei*) (Fig. 2).

Continental and coastal lineages differ by 24 synapomorphies. All 24 synapomorphies were transitions, 79.2% occurring at the third-base, 12.5% occurring at the first-base, and 8.3% occurring at the second-base position, a substitution rate consistent with previous observations (Brown 1985). The substitutions were observed in the transmembrane, outer (Qo-redox center), and inner (Qi) segments in the ratio 9:14:1 respectively. Although about 3.5% of the unambiguous changes occurred in the Qo-redox reaction center, none of these occurred in the most conserved region of the center. Most of the substitutions (71%) were C-T transitions. The amount of intraspecific variability for this region was low;

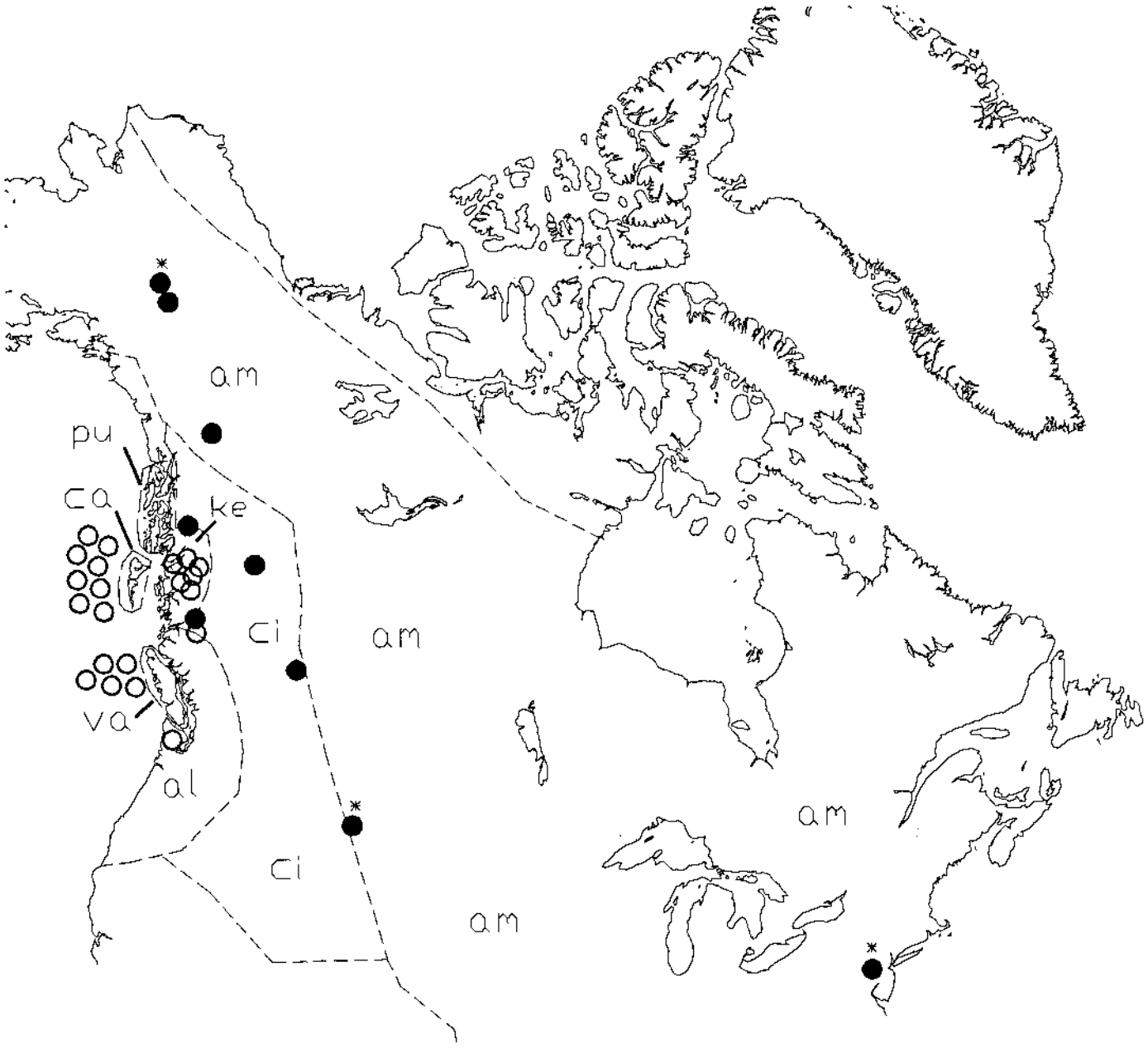

FIG. 2. Geographic distribution of coastal (○) and continental mtDNA lineages (●) in black bear. Note that the coastal lineage is restricted to Haida Gwaii, Vancouver Island, and the adjacent northern coast of British Columbia. Subspecies abbreviations are as follows: *americanus* (am); *kermodei* (ke); *cinnamomum* (ci); *altifrontalis* (al); *carlottae* (ca); *vancouveri* (va); *pugnax* (pu). Distributions are after Hall (1981). * indicates those locations from which sequences were taken from other studies (see Table 1 for more details).

3.3% of the sites were variable, 25% of which were transversions. 62.5% of variable sites were third-base changes, 20.8% were first-base changes and 16.7% were second-base changes.

Ps between continental and coastal black bear clades was 13% whereas within clades it was between 1.5% to 1.7%. *Pa* between continental and coastal bear clades was 0.7%, and 1.6% within clades. Average sequence divergence as determined by UPGMA between coastal and continental clades was calculated to be 3.6%, whereas the average divergence within each of these clades was 0.1%. Application of a divergence rate of 10% per million years for silent changes (Brown et al. 1982; Irwin et al. 1991) resulted in a putative separation time ranging from 360,000 years (average sequence divergence) to one million (synonymous) for coastal and continental mtDNA clades. Divergence within each clade was estimated at 10,000 years (average divergence) and 150,000 (synonymous).

DISCUSSION

Presence of divergent intraspecific lineages in the absence of current physical and/or reproductive barriers typically imply historical substructuring of existing populations (Avise

1994). This has been observed in a diversity of taxa including the sharp-tailed sparrow (*Ammodramus caudacutus*; Rising and Avise 1993), spotted salamander (*Ambystoma maculatum*; Phillips 1994), and dunlins (*Calidris alpina*; Wenink et al. 1996). Highly divergent lineages have also been detected within Ursidae, most notably within the brown bear where divergences of about 3% (Talbot and Shields 1996) and 7.13% (Taberlet and Bouvet 1994) have been reported.

Previous studies of North American black bear (Cronin et al. 1991; Paetkau and Strobeck 1996) from localities east of the Rocky Mountains showed that the majority of bears belonged to the same lineage with several outliers. Our molecular data however show that in coastal black bears of the Pacific Northwest, a second highly divergent lineage predominates (Fig. 1). Recent concerns about nuclear insertions of mtDNA genes have raised questions about the reliability of substantial mtDNA phylogenetic breaks, but our evidence of nonrandom lineage distribution, consistent PCR with regards to the number and types of amplification products as well as reasonable divergences based on estimated times of black bear entry into North America, makes it unlikely that the divergent coastal lineage is an artifact of nuclear integration (Zhang and Hewitt 1996). Furthermore, our finding of a divergent lineage in the Pacific Northwest is consistent with other molecular studies of various species. These include the identification of a disjunct mtDNA haplotype in stickleback from Haida Gwaii (O'Reilly et al. 1993; Orti et al. 1994), a disjunct distribution of chloroplast DNA haplotype in a perennial herb (*Tellima*), (Soltis et al. 1991), an ancient molecular lineage in brown bear (*Ursus arctos*) in the Alexander Archipelago immediately north of Haida Gwaii (Talbot and Shields 1996), as well as preliminary indications of a distinct coastal lineage in marten (*Martes americana*) and short-tailed weasel (*Mustela erminea*), two other Haida Gwaii endemics (Byun et al., unpubl. data).

Our cytochrome *b* data allows us to comment on two controversial issues: (1) that the divergent morphological traits of the Haida Gwaii black bear are preglacially derived; and (2) that Haida Gwaii was a Wisconsin glacial refugium.

We show clear evidence for two mtDNA lineages among North American black bear, yet with the sequence data we were unable to resolve any separation of subspecies within each lineage. This was unexpected, particularly for coastal subspecies such as the Haida Gwaii bear, which exhibit substantive morphological differences from other coastal bear. Similar sequences among these subspecies suggests a recent common ancestor and that the morphological attributes that differentiate the coastal subspecies probably arose in postglacial periods.

One of the attributes of the Haida Gwaii black bear that distinguish it from other coastal subspecies is its large body size. Although Foster (1965) concluded that this was a functional adaptation for foraging on marine resources, it is unknown whether large size is ancestral or derived. Recent discovery of bear skeletal remains (ca. 10,000 yr BP) on northwestern Vancouver Island show that early postglacial black bear colonists were significantly larger than modern black bear on both Vancouver Island and adjacent continental regions (Nagorsen et al. 1995). As such, it is reasonable to suggest that the large body size of Haida Gwaii black bear is ancestral and that bear on Vancouver Island and the coastal mainland may have undergone a size reduction in postglacial periods. Rapid postglacial derivation of morphological traits has been reported in other studies of Haida Gwaii endemics (Moodie and Reimchen 1976; Reimchen et al. 1985; Deagle et al. 1996) as well as in a diversity of taxonomic groups from other geographical areas (summary in Gingerich 1983).

Our data suggest that the traits characterizing the Haida Gwaii bear originated postglacially. However, the existence and distribution of the two mtDNA lineages do support the presence of a coastal refugium. The occurrence of a divergent lineage restricted to Haida Gwaii, Vancouver Island, and coastal regions of mainland British Columbia suggest that these areas were colonized by the same source population. The two well established source areas for postglacial recolonization of the Pacific Northwest were Alaska/Yukon and southern Washington. Black bears are currently believed to have recolonized the region from the south, largely because the Alaska/Yukon refugium was still ice locked when southern areas were deglaciated and also because no fossil evidence of black bear has been found in northern areas (Kürten and Anderson 1980). Northern dispersal from Washington probably occurred during the early Holocene within continental areas. Movement up the coastal corridor was unlikely due to rising sea levels, which had already begun to submerge the continental shelf previously exposed by lower sea levels (100–150 m) during the glacial maxima. These rising sea levels would have also impeded dispersal to Haida Gwaii 80 km from the mainland.

Although two refugia in the Pacific Northwest are currently known (Pielou 1989), various lines of evidence suggest that a third midcoastal glacial refugium persisted on the now submerged continental shelf separating Haida Gwaii from the mainland. Remains of herbaceous and arboreal plants dated at 16,000 yr BP from the northeastern coast of Haida Gwaii (Cape Ball) strongly suggests the existence of a nearby refugium (Warner et al. 1982; Mathewes 1989). Cores taken midway between Haida Gwaii and the mainland indicate that large portions of the Hecate Strait were terrestrial and ice free (see Barrie et al. 1993; Josenhans et al. 1993, 1995). The coastal plain which was uncovered on the shelf would have connected Haida Gwaii and the coastal mainland with probable access to Vancouver Island. We suggest that black bear persisted in the Hecate refugium and during the early stages of deglaciation recolonized Haida Gwaii, the coastal mainland, Vancouver Island, and eventually the Olympic Peninsula. Movement into the interior of British Columbia would have been excluded by the Cordilleran ice sheet. The rapid rise in sea level in early postglacial periods would have isolated the mainland from Vancouver Island and Haida Gwaii resulting in the present lineage distribution. Black bear populations in the interior of British Columbia that contain both lineages may represent easterly dispersal of the coastal lineage and a westerly or northerly dispersal of the continental lineage. Although our data suggests a lineage separation largely congruent with the boundary between *kermodei* and *cinnamomum*, individuals with the coastal lineage may extend as far as the Rockies if the outliers reported by both Cronin et al. (1991) and Paetkau and Strobeck (1996) are equivalent to our coastal lineage.

The 3.6% average sequence divergence between black bear lineages indicate that they have persisted through multiple glacial and interglacial periods. This is surprising given the numerous population bottlenecks and opportunities for lineage sorting (see Avise 1993). These two lineages could have been maintained if they were reproductively isolated, but as of yet, there is no evidence to suggest that there are any reproductive barriers between subspecies. These lineages could have also persisted if they had been geographically isolated for the past 360,000 years. Given the cyclical nature of glacier formation, it is reasonable to suggest that during the last two glacial periods, the coastal lineage was segregated from the continental lineage by being restricted to the Hecate refugium, and during the last two interglacials by being isolated on Haida Gwaii.

As previous morphological analysis has not been able to identify the close biogeographic affinity of these regions, we conclude that morphological attributes in North American black bear do not reliably indicate refugia and that the traits differentiating black bear subspecies in the coastal region are likely to be postglacially derived. If phylogeographic structure exists in a taxon with such high dispersal, then we might expect to observe comparable structure in more sessile groups.

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APPENDIX

GenBank accession numbers for *Ursus americanus* sequences.

Locality	cytb
Haida Gwaii 1	AF007936
Haida Gwaii 2	AF007917
Haida Gwaii 3	AF007935
Haida Gwaii 4	AF007918
Haida Gwaii 5	AF007937
Haida Gwaii 6	AF007919
Haida Gwaii 7	AF007920
Haida Gwaii 8	AF007922
Haida Gwaii 9	AF007921
Haida Gwaii 10	AF007915
Haida Gwaii 11	AF007916
Vancouver Island 1	AF007929
Vancouver Island 2	AF007930
Vancouver Island 3	AF007931
Vancouver Island 4	AF007932
Vancouver Island 5	AF007928
Terrace 1	AF007924
Terrace 2	AF007925
Terrace 3	AF007906
Tweedsmuir 1	AF007926
Tweedsmuir 2	AF007927
Prince Rupert 1	AF007912
Prince Rupert 2	AF007913
Prince Rupert 3	AF007914
Khutzymateen	AF007908
Lakeisle	AF007909
Moricetown	AF007910
Williston Lake	AF007933
Yukon	AF007934
Alaska 1	AF007907
Olympic Peninsula	AF007911
Jasper	AF007809