MOLECULAR POPULATION GENETICS AND SYSTEMATICS OF ALASKA BROWN BEAR (*URSUS ARCTOS* L.)

A

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OF ALASKA BROWN BEAR (*URSUS ARCTOS* L.)

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ABSTRACT

Complete nucleotide sequences of the mitochondrial cytochrome *b*, $tRNA^{proline}$ and $tRNA^{threonine}$ genes of the eight extant species of ursids, as well as 166 brown bears (*Ursus arctos* L.) from 10 geographic regions of Alaska and elsewhere, are used to generate hypotheses about phylogenetic relationships among ursids and phylogeographic relationships among brown bears. Additional data were obtained from mitochondrial DNA control region from over 200 brown bears among 14 populations in Alaska, to assess structuring among brown bears. Phylogenetic analyses indicate the giant panda (*Ailuropoda melanoleuca*) and spectacled bear (*Tremarctos ornata*) represent basal extant taxa. Ursines, including the sun bear (*Helarctos malayanus*), sloth bear (*Melursus ursinus*), Asiatic and American black bears (*Ursus thibetanus* and *U. americanus*), brown bear, and polar bear (*U. maritimus*) apparently experienced rapid radiation during the mid-Pliocene to early Pleistocene. The two black bears appear to be sister taxa; brown and polar bear are the most recently derived of the ursines. Polar bears apparently arose during the Pleistocene from within a clade of brown bears ancestral to populations currently inhabiting islands of the Alexander Archipelago of southeastern Alaska. Thus, brown bears are paraphyletic with respect to polar bears. Parsimony and distance analyses suggest two distinct clades of mtDNA: one (Clade I) composed only of Alexander Archipelago bears, and the other clade comprised of bears inhabiting all other regions of Alaska (Clade II). This latter clade represents bears inhabiting eastern (Clade IIa) and western (Clade IIb) Alaska. Mismatch analysis uncovered a pattern suggestive of recent expansion among some populations comprising Clade IIb. Over 90% of populations in Alaska were significantly differentiated as measured by variance in haplotype frequencies, suggesting limited contemporary female-mediated gene flow and/or shifts in gene frequency through genetic drift. The degree of population genetic differentiation revealed using mtDNA, as well as limited information from comparisons of multilocus microsatellite genotypes from bears representing four Alaska populations, suggests many Alaskan populations are evolving independently. Analyses of molecular

variance gave little support for currently accepted subspecies hypotheses. This research has provided new perspectives on processes that drive population structure of brown bears of Alaska and worldwide.

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PREFACE

This thesis is written as three separate manuscripts for publication in peer-reviewed journals; these manuscripts are listed below. Each is in the format required for the specific target journal. The first two manuscripts have been published in the journal Molecular Phylogenetics and Evolution; minor differences in formatting of these two manuscripts have been made herein for consistency among chapters. The third manuscript will be submitted to the journal Molecular Ecology.

Although this is a single-authored dissertation, two of the three chapters are co-authored for publication to acknowledge significant contributions to the papers. The "we" in each manuscript refers to the authors listed on the title page of that chapter.

This research generated information used in collaboration with a number of other researchers studying similar issues in different geographic areas. These collaborative efforts resulted in three additional manuscripts intended for publication, for which I was not the primary author. However, because my contributions represented significant portions of these collaborative efforts, I list them below and include them as an appendix (Appendix A-C) of this thesis.

A. Manuscripts comprising chapters herein:

Talbot, S. L., and Shields, G. F. (1996). A phylogeny of the bears (Ursidae) inferred from complete sequences of three mitochondrial genes. *Molecular Phylogenetics and Evolution* **5**: 567-575.

Talbot, S. L., and Shields, G. F. (1996). Phylogeography of brown bears (*Ursus arctos*) of Alaska and paraphyly within the Ursidae. *Molecular Phylogenetics and Evolution* **5**: 477-494.

Talbot, S. L. (In prep). Population genetic structure and population history of *Ursus arctos* in Alaska: evidence for female philopatry and male-biased gene flow. Draft manuscript.

B. Manuscripts resulting from collaborative efforts (listed as Appendix A, B, and C):

Heaton, T. H., Talbot, S. L., and Shields, G. F. (1996). An ice age refugium for large mammals in the Alexander Archipelago. *Quaternary Research* **46**:186-192.

Waits, L. P., Talbot, S. L., Ward, R. H., and Shields, G. F. (1998). Mitochondrial DNA phylogeography of the North American brown bear and implications for conservation. *Conservation Biology* **12**:408-417.

Waits, L., P., Kohn, M., Talbot, S. L., Shields, G. F., Taberlet, P., Pääbo, S., and Ward, R. (In prep). Substantial phylogeographic structure in a world-wide survey of brown bear (*Ursus arctos*) mitochondrial DNA sequence variation. Draft manuscript.

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I would also like to thank some of the many people who have helped me in many ways: Judy Reed Gust provided excellent laboratory expertise and managment as well as unwavering friendship and readily available housing during long commutes to Fairbanks. I also thank Nella and Joe Cook and the rest of the Cook family, Tracy Martinson, and the Robert Dauenhauer family, for providing temporary housing, sustenance and good conversation. Joe Cook and Kim Scribner provided much guidance on DNA analysis procedures. Chris Conroy and John Demboski volunteered helpful information about laboratory techniques and analysis of results, and of course provided the much appreciated good conversation and comic relief. Kim Scribner, Kevin Sage, Bobbi Pearson, John Pearce and Rick Lanctot made me welcome in the USGS/BRD Molecular Ecology Laboratory at the Alaska Biological Science Center, where I completed many of the laboratory and statistical analyses for the population genetics portion of the thesis.

Finally, I would like to thank my family, particularly my husband Steve Talbot, for their unwavering support during the program.

INTRODUCTION

Background

Considerable concern has been voiced as we enter the 21st century about the probability of decreased genetic variation within populations of North American wildlife species (Allendorf and Servheen 1986; Schoenwald-Cox et al. 1983; USFWS 1990), particularly those species whose population numbers have been reduced significantly from historic levels and which have experienced extreme reduction or fragmentation of required habitat. Additionally, a number of investigations began to demonstrate that loss of genetic integrity of large mammalian species through introgressive hybridization (Cronin 1991; Lehman et al. 1991) may occur subsequent to reduction of habitat size and population numbers. These concerns were in response to discussions during the last two decades regarding the burgeoning role of population genetics and phylogeographic systematics in the management and conservation of threatened species (Soule and Wilcox 1980; Frankel and Soule 1981; Schoenwald-Cox et al. 1983; Avise 1989), coupled with the recognition that virtually all remaining natural wildlife habitats/reserves in the contiguous United States and elsewhere are destined to become small, isolated fragments of formerly continuous habitat (Harris 1984; Erwin 1991). Such "isolate or island populations" (Wilcox 1980) have definable physical and biological properties requiring special management; such management is proposed to include management of genetic diversity and detection of reduced genetic variability (USFWS 1990). Unfortunately, despite increased efforts during the past decade, baseline information about historic levels of genetic variability and population substructuring of many wildlife populations is still not available. Furthermore, as habitats decrease in size and continuity, our opportunity to test hypotheses dealing with evolutionary biology concepts is also diminished.

The object of my thesis research is to provide and interpret baseline genetic data from which to assess genetic consequences of fragmentation of a formerly large, continuous vertebrate population into smaller, completely or somewhat isolated

populations, and to examine the relationship between the effect of reduced gene flow and genetic variability. I also wanted to examine the relationship between morphological and genetic differentiation, particularly at the so-called subspecies level, and to provide a phylogenetic foundation from which to assess these parameters. This study was implemented to examine genetic relationships within and among populations of Alaskan brown bear (*Ursus arctos* Linne), and to assess levels of population substructuring and extent of gene flow between populations. Genetic predictions can, and should be, independently tested using both nuclear and cytoplasmic markers. For my research, I primarily employed analysis of cytoplasmic (mitochondrial) DNA to address these questions.

Choice of Model

The target organism, the grizzly or brown bear was selected for study because it displays a number of characteristics amenable to answering evolutionary and population genetics questions. As large, mobile carnivores, brown bears are highly vagile, with great dispersal abilities as demonstrated by numerous population studies (see below). The historical range of the brown bear in the Holarctic is widespread and well-documented in the fossil record, but has subsequently been greatly reduced. Still, brown bear populations in large areas of Alaska are thought to represent the historical range and numbers of brown bears, and support densities adequate for sampling. The reproductive biology, physiological ecology and demography of brown bear has been extensively investigated in Alaska as well as elsewhere (Dean 1976; Craighead et al 1974, 1976; Titus and Beier, 1992, 1993; Schoen and Beier 1990; Schoen et al. 1994; Miller 1993; Reynolds and Hechtel, 1980, 1984; Reynolds 1989, 1992; Sellers 1994; Sellers et al. 1993; Wielgus et al. 1994; and many more). Because of the species' value as an important wildlife resource, and in view of its threatened or endangered status in the coterminous United States as well as in Asia and Europe, there is considerable interest in

the investigation of the effects of habitat fragmentation on gene flow among isolated populations (Allendorf and Servheen 1986).

Since only brown bear populations in Alaska, western Canada and Siberia are considered to be representative of historic worldwide distributions (USFWS 1987, 1990; Servheen 1989), study of genetic components of Alaskan brown bears has the potential to indicate natural levels of variability which can then be compared to more fragmented isolates. Such comparisons can indicate the extent of genetic separation in isolated groups and provide the framework upon which management strategies might be based. From a management perspective, these types of genetic data are of value to those charged with the recovery and management of brown bear populations in other areas as well as the maintenance of currently viable populations in Alaska.

In addition to the management implications of resulting data, genetic data allow clarification of intraspecific phylogeographic relationships (Avise et al. 1987) between allopatric subspecies of brown bear. Postglacial isolation of populations in Europe and Asia from those in North America, and the likely occurrence of unglaciated refugia in areas along the coast of southeastern Alaska, as well as in interior Alaska and Kodiak Island, may have resulted in an unexpectedly high degree of genetic variation and divergence among brown bear populations. Possibly, this genetic variation has been reflected in the considerable morphological variation observed among subspecies of brown bear in North America as well as Asia and Europe (Rausch 1963; Kurtén 1973; Hall 1984). Assessment of genetic divergence, or extent of gene flow, among populations with different levels of morphological divergence may provide insights into genetic components of morphological evolution, or the extent of environmental variance.

Choice of Tool and Molecule

Over the last four decades, several "revolutions" have occurred in the evolution of molecular markers for use in assessing genetic diversity. During the mid 1960's, protein electrophoresis provided one of the first feasible methods (analysis of allozymes) to

measure genetic variabilty in natural populations. The subsequent bustle of inquiry --both empirical and theoretical--provided the foundation for the modern field of molecular population genetics. However, allozymes, while providing great ability to associate fitness gradients with specific alleles or suites of alleles, proved to be less than satisfactory in their ability to reconstruct phylogenies. The advent of technologies to study variation at the nucleotide level--first through the use of restriction enzymes and later through actual sequencing of the DNA itself -- was the next dramatic technological advance. Nevertheless, it was not until the revolutionary technological advances of the last decade that the determination of nuclear and cytoplasmic -- mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA)--gene sequences became a practical tool for reconstructing phylogenies as well as assessing population genetics parameters.

The development of the polymerase chain reaction (PCR) in 1987 (Mullis and Faloona 1987, Saiki et al. 1988) made it possible to generate sufficient DNA from numerous samples, and obtain, relatively easily, target sequences from a large number of individuals within a species. This allows direct comparison of nucleotide sequences of individuals within populations, and provides a mechanism whereby the "intraspecific phylogeography" (Avise et al. 1987) of a gene within a species might be reconstructed. The PCR is able to generate DNA from a variety of markers, both nuclear and cytoplasmic, and it often seems that new marker types (and their associated acronyms) appear daily.

Although a wide array of PCR-based genetic markers and techniques is currently available to conservation biologists, all PCR-based markers fall into two general categories. The first are those that provide a means of classifying individuals into nominal genotype categories, and include fragment-based markers, such as restriction- and amplified-fragment length polymorphisms (RFLPs and AFLPs, respectively), variable number of tandem repeats (VNTRs) (both nuclear and cytoplasmic), short- and long-interspersed elements (SINES and LINES, respectively), retrotransposons, SSRs and ISSRs (simple-sequence repeats and inter-simple-sequence repeats, respectively), and random amplified polymorphic DNA (RAPDs). The second category includes markers

that provide a means of classifying individuals into genotypic categories, which themselves may be grouped according to degree of relationship, and include haplotype-based markers as DNA sequences, and restriction site maps.

The two types of markers are distinct in the kinds of information they can yield, and the different analytical methods that make use of the information. These differences can be important for conservation biologists, particularly those concerned with applications such as inferring demographic properties from genetic markers in addition to relationships based on genealogical descent (phylogenies). Specifically, markers that provide only frequency information are less able to distinguish among alternative demographic properties of a population or set of populations. On the other hand, markers such as DNA sequences carry a record of their own history and are able to provide a means of assessing relationships among genotypic classes. Such relationships among classes can be assessed for genes within and between populations, as well as at higher hierarchical levels, and can be separated from relationships between individuals (Kingman 1982). As a result, genetic processes such as mutation can be examined independent of demographic factors that also influence the structure of the genealogy of the sampled genes. Such demographic factors include population size, mating and migration: classic factors of prime interest to most conservation biologists.

It is clear that all of these markers, regardless of membership in the above two categories, are useful in detecting genetic variation at some hierarchical level -- from within the individual to between kingdoms -- and each variously targets particular areas of the genome(s). Each marker or suite of markers is appropriate under certain circumstances and given certain questions. Nevertheless, each has a suite of unsavory qualities which preclude assessment of variation at some level. For example, the "hypervariable" multilocus markers known as VNTRs (variable number of tandem repeats) are so variable they are often called DNA fingerprints, as are the extremely popular hypervariable single-locus markers known as microsatellites (which are also called SSRs). Both marker types can be extremely useful in detecting variation within or perhaps even between many populations, and microsatellites in particular have become

the marker of choice among researchers who study variation at or below the population level. Unfortunately, due to their high frequency of homoplasy and widely varying and generally unknown rates of evolution among specific loci, certain microsatellite markers appear to be often inappropriate for studying variation at the supra-population level, and perhaps even at the "population" level for some organisms (Goldstein et al. 1996; Rubinsztein et al. 1996; Primmer et al. 1996; Palsbøll et al. 1999), and their performance can be misleading if used to assess "subspecies" or species-level relationships. In addition, these markers, like allozymes, provide a means of classifying individuals into nominal genotypic categories. Thus, their defining characteristics are such that frequency data are available for each genotypic category, but these categories cannot be ordered or grouped in any way.

Intra-and inter-specific phylogenies reconstructed using single copy nuclear genes or introns appear to be more reliable than those derived from fragment analyses of VNTRs or some of the randomly amplified DNA fragments such as AFLPs, ISSRs or RAPDs. This is because the former haplotype-based markers, like restriction site maps, fall into the class of markers which are able to classify individuals into genotypic categories that are themselves able to be grouped according to degree of relationship. However, tracing lineages using recombining molecules such as single-copy nuclear genes is difficult and often unreliable. On the other hand, mitochondrial DNA gene phylogenies have proved extremely useful in extrapolating phylogenies at this level, for a variety of reasons. First, because the vertebrate mtDNA is uniclonally (maternally) inherited, its analysis facilitates lineage tracing without the noise produced by recombining molecules such nuclear DNA (Brown 1981, 1985). Second, the rate of evolution of the mtDNA is, in general, considerably faster than that of single-copy nuclear DNA, and therefore is expected to show mutational changes among lineages at a much greater rate than those of single-copy nuclear genes (Brown et al. 1979, 1982; Brown 1985). This makes detection of nucleotide differences between very recently diverged mtDNA lineages more likely. Finally, vertebrate mtDNA is a well-known molecule, and thus rates of evolution of different mtDNA genes, and regions within

genes, can be more appropriately estimated when reconstructing phylogenies (Brown et al. 1982; Brown 1985).

The publication of the first set of "universal" or "versatile" mtDNA primers in 1989 (Kocher et al. 1989) --which targeted small (150-350nt) portions of the small ribosomal RNA gene, the major non-coding region, and the cytochrome *b* gene -- stimulated numerous investigations into the phylogenetic relationships of diverse vertebrate organisms, using these short, easy to obtain sequences. As a result, many initial hypothesized phylogenies were reconstructed using data gathered from only one complimentary strand of the mtDNA, and rarely from complete genes. In such instances, the goals of the systematist conflicted with those of the molecular evolutionist, were interested in different questions involving patterns across entire genes (Fickett and Burks 1989). Miyamoto and Cracraft (1991) considered this conflict regrettable, citing the importance of understanding functional constraints on character-state data used in phylogenetic analyses, as well as the importance of DNA sequences to fields other than molecular systematics.

Many ambiguous phylogenetic relationships have been better resolved using mtDNA molecular data. Nevertheless, due to rapid technical advancements and automation, coupled with availability of entire genomic data for representative model species, large amounts of comparative sequence information can be obtained rapidly and at a reasonable cost. Ultimately, however, the degree of resolution yielded by molecular lineages depends upon the level of diversity present, and how informative that diversity is. Often, due to funding constraints, the systematist must choose between collecting much molecular data from one or a few loci, and collecting limited information across multiple loci. Gene-specific peculiarities of a single locus may cause a signal found through an analysis of a single gene to be an artifact of convergent evolution, or result in an erroneous phylogenetic inference even though the gene tree has been correctly resolved-- due, for example, to sorting of lineages when the ancestral species is polymorphic for the gene. This concept in particular can be extended from a single mitochondrial gene to the entire mtDNA genome, since the mitochondrial genes are

inherited as a single linkage group and thus do not provide independent estimates of the species tree. Such errors may be avoided by using multiple, independent loci from the nuclear genome in conjunction with the mtDNA genome when attempting to resolve phylogenies (Pamilo and Nei 1988, Wu 1991), or by examining phylogenies of co-evolving species such as ecto- or endoparasites. Nevertheless, information relative to the secondary structure of RNA genes or functional properties of entire protein coding genes, whether nuclear or cytoplasmic, provides an important basis for assigning weight to different regions of the molecules in a phylogenetic analysis (Wheeler and Honeycutt 1988; Smith 1989). Indeed, several studies (Árnason and Gullberg 1994; Moore and DeFillipis 1997; Johns and Avise 1998; Mindell, et al. 1997), involving the use of used complete gene sequences or genomes to resolve within-family relationships, suggested that use of complete mtDNA gene sequences, rather than use of smaller, combined portions of several mtDNA genes, may allow for more accurate weighting schemes and therefore more accurate estimation of phylogeny.

Again, the nuclear genome can provide alternative independent tests of hypotheses of gene phylogenies with which to examine phylogenetic relationships (Pamilo and Nei 1988; Wu 1991). However, while studies comparing nuclear vs. mtDNA gene trees are becoming more common, development of single-copy nuclear gene markers still lags behind the development of organelle DNA markers. Such studies are still somewhat uncommon, and the relative efficiencies of mitochondrial-haplotype trees, nuclear-gene trees, and sets of multiple nuclear gene trees in reconstructing the species trees have not been determined for population-level investigations.

Moore (1995) explored one aspect of the gene tree vs. species tree problem which favors the use of mtDNA over nuclear DNA for inferring species trees. The probability that a gene tree will track a given three-species segment of the species tree depends upon the effective population size (N_e) of ancestral species between the first two bifurcations of a phylogenetic tree; the smaller the effective population size, the higher the probability a given gene tree will track a species tree. Because the effective population size (N_e) of the haploid, maternally-inherited mitochondrial gene is one-fourth as large as that of the

nuclear-autosomal gene, the mtDNA tree has a better chance of tracking the species tree than any single nuclear gene tree. When comparing phylogenies inferred from mtDNA variation vs. nuclear autosomal gene variation, Moore (1995) found that, when there is a probability of 0.95 that the mtDNA tree is congruent with the species tree, the required number of independent nuclear-gene trees is 40, of which 20 or more must be congruent to reject the null hypothesis of a trichotomy. In other words, with a population of 40 independent and correctly resolved nuclear-gene trees, one could reject the null hypothesis and correctly infer the species tree with a probability of 0.95, which is the same probability that the species tree inferred from the single mtDNA-haplotype tree is correct. Consequently, the sampling effort targeted toward the nuclear genome must be at least an order of magnitude higher than that toward the mtDNA genome, and the financial costs of this effort may prove restrictive in many cases.

Of mtDNA genes, the cytochrome *b* gene is the most widely used for phylogenetic investigations at the species level for vertebrates. This is due in part to the easily available versatile primers mentioned above, and in part to early suggestions by various workers that the cytochrome *b* gene is variable enough to answer population questions (Wenink et al. 1993) and conserved enough to answer "deeper" phylogenetic questions among distantly related organisms (Kocher et al. 1989; Edwards et al. 1991; Meyer and Wilson 1990). However, the utility of a "single-locus" mtDNA approach in general, as mentioned earlier, and specifically the cytochrome *b* gene as a molecular marker, has been challenged (Meyer 1994; Hillis and Huelsenbeck 1992), largely due to its inability to resolve very deep relationships (those higher than the family level), saturation of third codon positions, as well as the problems that occur throughout the entire mitochondrial genome (e.g., base compositional bias and rate variation between different lineages). Nevertheless, the cytochrome *b* gene has several characteristics which point to its continued use as a phylogenetic marker. Although its rate of evolution is slow in terms of amino acid substitutions, the rate of evolution for silent substitutions at some first and third codon positions is similar to that of other mitochondrial genes. Additionally, the cytochrome *b* gene is still the best-understood of the 13 functional mtDNA protein genes,

in terms of structure and function, and it has been demonstrated to be useful for resolving relationships of recently evolved groups, such as the relationships among the species comprising the family Ursidae (Johns and Avise 1998). Finally, entire cytochrome *b* sequences are available for a growing number of vertebrates (Irwin et al. 1991; Thomas and Martin 1993; Kornegay et al. 1993; Stanley et al. 1994; Johns and Avise 1998); comparative analysis of such sequence data provide an invaluable tool for the evolutionary biologist in further understanding evolutionary patterns and rates of evolution of specific genes (Johns and Avise 1998). Indeed, in addition to the sampling effort required for nuclear-gene phylogenies outlined above, it is likely that many of the same problems cited for the cytochrome *b* gene, as well as the entire mtDNA genome, such as base compositional bias and rate variation among different lineages, will be found in various genes, or intron regions, found within the nuclear genome as well.

Ultimately, congruence of many observations, whether from independent genetic (organelle and nuclear), fossil, morphological, behavioral or ecological sources, will manifest the presence of a pattern implying a need for a common explanation, and congruence of such evidence will allow preference of one hypothesized phylogeny over another. To this end and for the reasons outlined above, I focused on obtaining inter-and-intraspecific gene trees from the mitochondrial genome, directing the majority of my sampling effort toward the complete mitochondrial cytochrome *b* gene.

Specific Aims

For this study I sequenced the complete cytochrome *b* gene, and the adjacent transfer RNA proline ($tRNA^{pro}$) and transfer RNA threonine ($tRNA^{thr}$) genes, in their entirety, to examine phylogenetic relationships among the extant ursids (Chapter 1) as well as intraspecific phylogeography among brown bears of Alaska (Chapter 2). For population genetics analysis (Chapter 3), I added to these sequences information from the rapidly-evolving hypervariable region I of the non-coding control region (Vigilante 1990; Wakely 1993) of the brown bear mitochondrial genome, and compared these data to data

collected from microsatellite loci of individuals from many of the same populations (Craighead 1994; Paetkau et al. 1998; Scribner et al, unpublished manuscript). The hypervariable region I of the mtDNA control region is considered by some workers (Saccone et al. 1991; Meyer 1994) to provide less appropriate data than protein-coding genes for assessment of interspecific phylogenies, due to difficulties in establishing alignment and positional homology across species, and the increased probability that homoplastic characters may result in incorrect phylogenetic reconstructions. In fact, sequencing the major non-coding region has been compared to, on average, sequencing a protein-coding gene that is entirely composed of third positions (Meyer 1994). Nevertheless, this hypervariable region has been demonstrated to be able to adequately resolve within-species comparisons, such as comparisons among closely-related populations (Vigilante et al. 1991; Baker et al. 1993; Wenink et al. 1993; Meyer 1994).

Specifically, I employed PCR amplification and Sanger di-deoxy (Sanger et al. 1977) sequencing of these portions of the mtDNA to:

1. assess the genetic divergence and extent and gender bias of gene flow among spatially subdivided coastal, interior and insular populations of Alaskan brown bear;
2. assess the degree of genetic variability within Alaskan brown bear populations to document proposed postglacial founder events and subsequent genetic drift;
3. examine genetic differentiation within and among currently designated morphologically differentiated subspecies of Alaskan brown bear;
4. clarify phylogenetic relationships among brown bears inhabiting the Palearctic and those inhabiting Alaska;
5. clarify phylogenetic relationships among all extant bear species so to provide a phylogenetic framework for interpretation of intraspecific relationships among Alaskan brown bears.

These specific goals allow testing of a number of hypotheses dealing with evolutionary and population genetics theory, which are delineated below. A brief

description of ursid evolution and taxonomy is provided in Chapter 1, and a similar description of brown bear evolution, subspecies taxonomy of Alaskan brown bears, and distribution, is presented in Chapter 2. Information regarding brown bear population structure, based on numerous field observations and radiotelemetry studies, is given in Chapter 3.

Models, Hypotheses and Predictions

Insularization in Fragmented Populations

Hypothesis 1: The genetic consequences of creating several population isolates from a formerly single large unit are theoretically reasonably well understood for single locus characters (Varvio et al. 1986). Fragmentation of populations and subsequent decrease in population size could result in decreased genetic variability within each population due to loss of alleles (or haplotypes) by drift and founder effect, and an increase in the frequency of homozygous genotypes due to inbreeding (Wright 1969; Allendorf 1986). Loss of variability through founder effect reflects the number of founding individuals and their genetic composition; decrease due to drift reflects the intrinsic growth rate and the harmonic mean of the effective population size over time (Wright 1969; Nei et al. 1975). These effects will be manifested if habitat fragments are smaller than the area typically occupied by a single random-mating unit (roughly equivalent to a neighborhood area -- Wright 1943), and if gene flow between population fragments has been substantially reduced. These predictions are strongly supported by experimental studies in laboratory populations (Speiss 1977; Wright 1978; Falconer 1989), but not in natural populations.

Spatially subdivided populations are thought to become genetically differentiated due to decreased gene flow (Wright 1978; Endler 1977; Falconer 1989). Again, this may come about by genetic drift of an originally homogenous population or founder event, and may be accompanied by morphological differentiation (Fox 1975).

Specific predictions follow from the hypothesis of habitat fragmentation and geographic subdivision related to brown bear populations in Alaska:

A. Fragmentation of a large, continuously distributed population to smaller, isolated populations, such as those found on the Kodiak Island Archipelago in southern Alaska, and Alexander Archipelago in southeastern Alaska, will result in a loss of variability within subpopulations, through mechanisms such as founder and drift effect.

B. Elimination of gene flow between subpopulations small enough to be strongly influenced by drift will increase gene (haplotype) frequency differences and/or sequence variation between those subpopulations. Thus, for example, peninsular populations are predicted to display reduced genetic variability due to restricted movement (and hence gene flow) at the neck of the peninsula, as well as differentiation, in terms of sequence variation or haplotype frequency differences, from source populations, such as those found in adjacent mainland areas. Island populations are also predicted to display genetic differentiation or haplotype frequency differences relative to mainland populations, due to reduced gene flow as well founder effect and drift (see Prediction A above).

C. Smaller isolated populations of bears, such as those on Admiralty (est.1,800 bears on 4,306 km^2), Chichagof (est. 1,550 bears on 5,445 km^2) and Baranof Islands (est. 1,044 bears on 4,159 km^2) of the Alexander Archipelago (Titus and Beier 1993), will show lower levels of genetic variability than larger isolated populations, such as on Kodiak Island (est. 3,000 bears on 13, 890 km^2: ADF&G 2002) . This prediction is based on the assumption that current populations on each of the islands have been isolated from source populations for approximately the same period of time, and that the source populations were similarly heterogenous. While there is disagreement among researchers regarding the post-Pleistocene history of the Alexander and Kodiak Archipelagos and the presence of glacial refugia (Karlstrom 1960, Heusser 1960, Rausch 1969, Fladmark 1979, Dixon 1993, Busch 1994) on their component islands, it has generally been accepted that both Kodiak Island and the islands of the Alexander Archipelago of southeastern Alaska were mostly or fully covered with ice during the last Pleistocene glaciation, and the progenitors of current bears inhabiting the islands of both archipelagos recolonized after

the ice receded, approximately 10,000 years ago (Prest 1969; Clague 1989; Klein 1965; Rausch 1969).

D. Populations separated by geographical barriers, such as mountain ranges or large bodies of water, will show considerable divergence in rapidly evolving mtDNA nucleotide sequences from more panmictic interior Alaskan populations.

Alternative Hypothesis 1: Spatial subdivision is not accompanied by genetic subdivision. Observed spatial subdivision may be of recent origin, and high levels of gene flow, or colonization after local extinction, has kept the population genetically homogenous. This alternative is based on reports of large dispersal distances of brown bear, even across saltwater channels (Rausch 1969; C. Dau, USFWS, 1990, personal communication) which may result in levels of gene flow sufficient to maintain homogenous populations.

To test these predictions, variation within and among variable-sized isolated populations can be compared to each other and to populations in undisturbed habitat. The distance between populations sampled in the continuous habitat should include both geographically close localities, to control for population subdivision, and geographically distant localities, to control for isolation-by-distance (Jackson and Pounds 1979).

Congruence of Morphological and Genetic Differentiation

Several situations occur whereby morphological data alone are insufficient for defining taxon (species, subspecies or population) boundaries. Two taxa may be sympatric or parapatric, but be so similar morphologically that their specific status goes undetected (Donellan and Aplin 1989). Second, two allopatric populations may be morphologically different, but their status as species is questionable. Third, two parapatric populations may be morphologically distinct, but show clinal variation or broad hybridization (or introgression). Fourth, two morphologically distinct forms may represent polymorphisms within a single interbreeding population (Titus et al. 1989).

Morphological data and genetic data (Shields and Kocher 1991; G. F. Shields, UAF, unpublished data), suggest brown bears of Alaska fit within the third category described above, and various levels of introgression maintain clinal variations. However, isolated island populations, such as those in the Kodiak Archipelago, and mainland populations are thought to represent allopatric populations that are currently on separate evolutionary trajectories. Models, hypotheses and predictions are listed in Chapter 2, but are more specifically delineated below:

Hypothesis 2: Several subspecific taxonomic designations, based largely on skull morphology, have been proposed for Alaskan brown bears (Rausch 1963; Kurtén 1973; Hall 1984) and are described in Chapter 2. These taxonomic schemes suggest brown bears of Alaska can be assigned membership to two (Rausch 1963), three (Kurtén 1973), or five (Hall 1984) subspecies. Among all three schemes, there is concordance in only one element: the assignment of endemic subspecies status to brown bears of the Kodiak Archipelago. Kurtén (1973) alone compared Palearctic and Nearctic bear skulls and considered the post-glacial migration history of the species in his taxonomic descriptions, and thus presented a taxonomy based on a more comprehensive data set than either Rausch (1963) or Hall (1984). An acceptance of Kurtén's hypothesis assumes ultimate bear skull size is largely determined genetically, not environmentally, and that the post-glacial migration patterns proposed by Kurtén reflect the actual migration history of Nearctic brown bears. In considering Kurtén's taxonomic description of brown bears of Alaska and the Palearctic, I predict Kurtén's proposed taxonomy will be supported by an analysis of mtDNA gene sequences. Predictions generated from this hypothesis are:

A. Kodiak bears (*U. a. middendorffi*) will be more closely genetically allied to Kamchatkan brown bears (*U. a. piscator*) than to mainland and coastal Alaskan brown bears (*U. a. horribilis* and *U. a. dalli*, respectively). This prediction is based on the supposition that the Kodiak Archipelago was originally colonized by bears ancestral to those currently comprising populations in Kamchatka (Kurtén 1973). Kodiak bears possibly survived in a refugium on Kodiak Island during the last Pleistocene glaciation

and were postglacially isolated from the mainland, which was recolonized subsequent to glacial retreat (Rausch 1969, Kurtén 1973). Recolonization of mainland populations is proposed to have been by bears of the interior or southeastern Alaskan refugia, which may have shared affinities with the subspecies that inhabited eastern and northern Beringia (Kurtén 1973).

B. Brown bears of southeastern coastal Alaska (*U. a. dalli*) will be genetically more similar to Manchurian brown bears (*U. a. manchuricus*) than to Siberian or European brown bears.

C. Brown bears of Alaska will show high levels of differentiation from brown bears of Europe, Asia and Siberia, despite proposed affinities, due to mutation of nucleotide sequences subsequent to post-glacial separation.

Alternative Hypothesis 2: Analysis of mtDNA genes will not support subspecific designations as proposed above, because Kurtén's taxonomy does not reflect the true relationships among and within Nearctic and Palearctic brown bears. Alternatively, variation in morphology used to describe subspecies has no genetic basis, or has a genetic basis not detectable by the methodologies used.

Hypothesis 3: Morphologically differentiated subspecies represent genetically differentiated groups. However, because there is likely some gene flow across presently contiguous ranges, groups are expected to differ in mtDNA haplotype frequencies and not necessarily in fixation of alternate haplotypes. I therefore predict:

A. Populations of brown bears of Alaska will display increased genetic differentiation, in the form of haplotype frequency differences, on a cline from southeastern Alaskan islands to North Slope Alaskan populations, and from east to west along the arctic coast. This prediction is based on observed clinal morphological differentiation of Alaskan brown bear populations by all taxonomists (Rausch 1963; Kurtén 1973; Hall 1984), as well as preliminary observation of genetic differentiation between interior and Alexander Archipelago brown bears (Shields and Kocher 1991).

This trend in genetic differentiation is expected to parallel differentiation observed in skull size and morphology along this cline (Rausch 1963; Kurtén 1973; Hall 1984), and is in accordance with the isolation-by-distance model of population genetic substructuring (Wright 1943; Chesser 1983; Baverstock and Moritz 1990).

B. Alaska peninsular, coastal and interior brown bear populations, which may represent different subspecies but likely experience some gene flow (leakage) among populations, are expected to differ in shared haplotype frequencies. Kodiak Island brown bears, considered a unique subspecies by all taxonomists (Rausch 1963; Kurtén 1973; Hall 1984), however, should display fixation of alternate haplotypes relative to mainland populations.

Comparison Between Nuclear and MtDNA Differentiation: Dispersal

The tendency for female brown bears to remain on or near their natal ranges and for males to disperse more than females should result in increased subdivision of maternally inherited mtDNA compared to nuclear genes (Birky et al. 1983). Dispersing males will contribute mtDNA genes to a new population only transiently, until death. Thus, the representation in a population of foreign mtDNA types brought in by male immigrants will be negligible. Because mtDNA is haploid and maternally inherited (Gyllesten et al. 1985, Birky et al. 1983, 1989), it is predicted that population subdivision will be approximately 4 times greater for mtDNA than for nuclear genes, especially if dispersal is primarily by males. However, polygamy resulting in relatively low numbers of breeding males, and the increased rate of mtDNA mutation, will counter this trend. For example, restriction endonuclease analysis of mtDNA by Avise et al. (1979) and DeSalle et al. (1987) revealed genetic differentiation of pocket gophers (*Geomys*) and *Drosophila* populations, respectively, which were not apparent from concurrent allozyme analysis. Similarly, Shields and Wilson (1987) and Van Wagner and Baker (1990) showed differences in mtDNA between subspecies of Canada geese that were not reflected in nuclear genes (Van Wagner and Baker 1986), presumably based on dispersal differences between males and females.

Although this study did not address nuclear genetic variability, a study of microsatellite allele differences among three of the populations included in this study was performed from 1990-1994 by Lance Craighead, as part of his doctoral research at the Montana State University (Craighead 1994). Craighead performed interpopulational comparisons of eight microsatellite loci from Arctic Alaska (n = 15 bears), Alaska Range (n = 17 bears) and Brooks Range (n = 159 bears), as well as for brown bears in Montana. I used many of the same samples for my research. Craighead's results indicate that considerable gene flow occurs among these three Alaskan subpopulations, with little evidence of subdivision within or between populations. I was also able to obtain and analyze data obtained from the same eight microsatellite loci of 30 brown bears from Katmai National Park on the Alaska Peninsula (Scribner et al., unpublished manuscript). By sampling from the same populations, I am able compare the results of his nuclear DNA data for these three populations with mtDNA data to test the following hypothesis:

Hypothesis 4: Maternally-inherited mtDNA will show a greater degree of differentiation than nuclear encoded allozymes due to smaller female home ranges and philopatry, promiscuous mating system, and relatively lower rates of evolution of nuclear DNA. This is predicted for mtDNA given its smaller effective gene number due to haploidy and maternal inheritance.

Alternative Hypothesis 4: Maternally-inherited mtDNA and nuclear DNA will show similar levels of differentiation due to large dispersal distances of bears. If dispersal results in high levels of gene flow, mtDNA gene differentiation will be similar to that of nuclear gene markers, and genetic subdivision may be dampened.

Insular Bear Populations and Conservation Biology

Analytical studies performed during the last two decades have explored mechanisms of population extinction (Richter-Dyn and Goel 1972; Leigh 1981) and

fostered conceptualization of a "minimum viable population" (MVP) size necessary to establish a certain probability of persistence (usually $p < 0.05$ or 0.01) over a given time span (usually 100-1000 yr) (Shaffer 1981, 1987). The MVP concept led to the early development of several "rules of thumb" for the establishment of the minimum number of breeding individuals in captive propagation programs (usually given as 50, see Soule and Wilcox 1980; Soule and Simberloff 1986), or the minimum effective population size (N_e = 500; Franklin 1980; Lande and Barrowclough 1987), necessary for long-term survival of natural populations. These estimates are based on calculations of rates of loss of genetic heterozygosity under various breeding structures, and the presumed loss of fitness that could result either from inbreeding depression or erosion of genetic variability through drift (Conway 1980; Foose 1983; Frankel 1983). Several empirical studies do show a loss of fitness associated either with inbreeding depression (Senner 1980; Ralls and Ballou 1983; Ralls et al. 1986, Templeton and Read 1983; Ryder 1988), or with loss of overall genetic variability as estimated by allozyme heterozygosity levels (Beardmore 1983; Allendorf and Leary 1986; Hedrick et al. 1986). That loss of genetic variability has caused the extinction of any natural populations cannot currently be documented; however, inbreeding depression data from captive populations and the correlation between heterozygosity and several fitness parameters in wild populations have convinced many biologists that the genetic aspects of conservation are important management considerations, both for population viability (Allendorf and Servheen 1986) and perhaps the maintenance of stable species associations (Futuyma 1983).

Although the absolute minimum number of animals necessary to avoid serious loss of genetic diversity is not known, computer simulations (Harris 1985, Harris and Allendorf 1989) indicate that, to avoid degradation of genetic diversity, one animal should enter the breeding population each generation. Harris and Allendorf (1989) estimated generation time for brown bears to be 10 years. Thus, to maintain adequate genetic variability among and within the brown bear populations in the United States, levels of gene flow should be restored to isolated subpopulations at the rate of at least one successfully breeding individual approximately every 10 years (Allendorf and Servheen

1986; Harris and Allendorf 1989). Since current land use patterns will not likely revert to allow natural movement between these populations, even at these low levels, researchers suggested this gene flow to isolated brown bear populations in the contiguous United States be restored through augmentation from healthy but distant populations such as those in western Canada and Alaska. It has been argued, however, that augmentation of organisms from locally adapted subpopulations could cause hybridization between animals with different co-adapted gene complexes, resulting in outbreeding depression which would subsequently decrease viability of the target population, particularly in later generations (Templeton 1986). The notable morphological variability among brown bears of North America may indeed indicate a great deal of genetic differentiation between local brown bear populations in the past. Documentation of levels of genetic variation and differentiation within and among brown bear subpopulations, through studies such as the one described here, will presumably help to elucidate these problems, and allow for proper selection of source populations for any augmentation programs, should they become necessary. Estimates of effective population size, a population parameter required for assessing MVP, are extremely difficult to quantify in natural populations. With this research I hope to provide a rough index of the influence of insularization on genetic processes. In addition, confirmation of the predictions tested herein will provide a genetic basis for extending ongoing demographic studies to very small populations in other areas. Thus, in the light of conservation criteria developed by wildlife biologists in response to local, national and international concerns, this study relates directly to informed population management of brown bears in North America and to wildlife populations in general, by providing understanding of such biological parameters as genetic population structure, gene flow, and genetic variability characterize brown bear populations. It will also help elucidate issues that are currently under considerable debate worldwide among conservation biologists, described below.

Phylogenetics and Conservation Biology

Avise et al. (1987) described the usefulness of mtDNA in intraspecific phylogenetic reconstruction, citing significant divergence of mtDNA ("phylogenetic gaps") between allopatric populations of several vertebrate species. These researchers suggested that phylogenetic differences within species are qualitatively similar, although often smaller in magnitude, than higher-order phylogenetic reconstructions. While certainly debatable as a general conclusion, this view, coupled with work in the past decade differentiating gene trees from species trees (Pamilo and Nei 1988), indicates the importance of understanding intraspecific phylogeny in the study of evolution.

Patterns of genetic variation, particularly mtDNA variation, among populations may provide two perspectives on evolutionary processes (Wilson et al. 1985). First, phylogenetic inference about relationships and divergence times may be made. Second, genetic data may be used to describe population genetic structure and estimate the extent of gene flow among populations. Assessment of genetic divergence, or extent of gene flow, among populations with varying levels of morphological or behavioral divergence, provides perspective into rates at which morphological evolution may occur, or the extent of environmental variance.

Many studies in the last several decades have demonstrated the importance of understanding phylogenies when making decisions in conservation of biotic diversity. Avise (1989) discussed how mismanagement of endangered species has occurred when conservation programs are based on erroneous phylogenetic premises. Such mismanagement possibly endangers not only the target species, but other species as well, by diverting valuable financial and manpower resources away from some species and by weakening conservation programs. Furthermore, phylogenetic studies early in the last decade (Lehman et al. 1991; Wayne et al. 1991; Wayne and Jenks 1991) demonstrated a potential loss of genetic integrity of the endangered wolf (*Canis lupus*) of North America through introgression of coyote (*C. latrans*) DNA, and that the endangered red wolf (*C. rufus*) may not be a "distinct species" at all, but rather an intraspecific hybrid of wolves

and coyotes. The study resulted in an animated (but by no means new) discussion about the legitimacy of current species concepts and the value of these concepts for use in our nation's conservation programs (Cowen 1991; Gittleman and Pimm 1991; Mann 1991). Regardless of the varying viewpoints, these studies pointed out the importance of understanding the phylogenetic relationships of wildlife species, and how evolution has shaped these species and their distributions. Certainly the phylogenetic relationships of the brown bear, currently intensely managed in our nation and considered a threatened species in the coterminous United States and endangered in many parts of the world, require clarification in order to properly evaluate management strategies.

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CHAPTER 1

A PHYLOGENY OF THE BEARS (URSIDAE) INFERRED FROM COMPLETE SEQUENCES OF THREE MITOCHONDRIAL GENES[1]

1.1 ABSTRACT

Complete sequences of DNA are described for the cytochrome b, tRNA^{Thr} and tRNA^{Pro} genes of mitochondria of four extant species of ursids and compared to sequences of four other species of ursids previously studied by us. Phylogenetic analyses indicate that the giant panda and the spectacled bear are the basal taxa of the ursid radiation. The ursines, a group which includes the sun bear, sloth bear, American black bear, Asiatic black bear, brown bear and polar bear experienced a rapid radiation during the mid-Pliocene to early Pleistocene. The Asiatic black bear and American black bear are sister taxa. The brown bear and polar bear are the most recently derived of the ursines with the polar bear originating from within a clade of brown bears during the Pleistocene. This paraphyletic association suggests that the rate of morphological evolution may be accelerated relative to that of molecular evolution when a new ecological niche is occupied. Calibration of the corrected average number of nucleotide differences per site with the fossil record indicates that transitions at third positions of codons in the ursid cytochrome b gene occur at a rate of approximately 6% per million years, which is considerably slower than comparable values reported for other species of mammal.

[1]Talbot, S. L., and Shields, G. F. (1996). A phylogeny of the bears (Ursidae) inferred from complete sequences of three mitochondrial genes. *Molecular Phylogenetics and Evolution* **5**: 567-575.

1.2 INTRODUCTION

Phylogenetic relationships among the Ursidae are of considerable interest because of the value of bears as wildlife, their worldwide status as threatened and endangered species, and their suitability for investigation of patterns and processes of evolutionary change in large, mobile carnivores. Because the fossil record of the Ursidae is weak or absent for some lineages, most phylogenetic hypotheses have been inferred from comparative studies of extant taxa. General disagreement regarding the phylogeny of ursids is reflected in the several taxonomic revisions made for this relatively small group (Popock 1914, Simpson 1945, Erdbrink 1953, Thenius 1979a, b; Hendey 1980, Nowak and Paradiso 1983, Wozencraft 1989a, b) and the various generic names attributed to some of the species. Despite the large number of morphological and molecular studies of evolutionary relationships among the extant species of ursids, few have yielded congruent results.

The most uncertain relationships among phylogenies of ursids are: (1) whether the giant panda, *Ailuropoda melanoleuca,* should be included among the Ursidae; (2) the location of the spectacled bear, *Tremarctos ornata*, in relation to the ursines; (3) the hierarchical order of divergence among the six ursine bears and (4) whether the brown bear, *Ursus arctos*, is paraphyletic with respect to the polar bear, *U. maritimus*.

Modern methods of molecular genetics offer some hope of resolving these issues but several uncertainties remain. For example, Zhang and Ryder (1993) used partial sequence data of the mitochondrial cytochrome b, 12S rRNA, $tRNA^{Pro}$ and $tRNA^{Thr}$ genes to examine relationships of the Ursidae. Their analysis removed the polar bear from the subfamily Ursinae and placed it as the sister taxon to the spectacled bear of the subfamily Tremarctinae. These results are inconsistent with evidence from fossils (Kurtén 1964, 1968, 1971), proteins (O'Brien *et al.* 1985), DNA (Shields and Kocher 1991, Cronin *et al.* 1991) and morphology (Thenius 1959). Moreover, a subsequent analysis by Zhang and Ryder (1994) including a portion of the control region of mtDNA and additional sequences of polar bears (including those published by Shields and Kocher 1991) revealed two very divergent lineages of polar bears. Zhang and Ryder (1994) place one lineage of the polar bear in a close relationship with the spectacled bear, while they

place the other with the brown bear. They rule out contamination as a source of the disparity and propose a recent hybridization event to account for the polar bear/spectacled bear relationship.

Similarly, Vrana *et al.* (1994) examined 307 nucleotides of the cytochrome b gene and 394 nucleotides of the 12S rRNA as well as anatomy, to compare relationships of ursids to other carnivores based on the "total evidence" approach but were unable to resolve relationships among the five species of ursids included in their study.

In order to investigate these relationships further, we compared sequence information from the entire cytochrome b, tRNA^{Thr} and tRNA^{Pro} genes of four previously undescribed species of bears and compared these to homologous sequences that we had previously studied (Talbot and Shields, 1996). As our study was completed, we became aware of an independent study of mtDNA sequence evolution among the Ursidae (Waits, O'Brien and Ward, unpublished) based on partial sequences of six mitochondrial genes. By comparing our sequence data to those of Zhang and Ryder (1993, 1994), Vrana (1994) and Waits, O'Brien and Ward, (unpublished) we hope not only to improve our understanding of ursid phylogeny but also to determine whether congruent phylogenies can be derived from analysis of several complete genes (as is the case in our present study) and analysis of portions of larger numbers of genes (Waits, O'Brien and Ward, unpublished).

1.3 MATERIALS AND METHODS

DNA Sources. Purified mtDNAs from a single sloth bear *Melursus* {=*Ursus*} *ursinus* and a single spectacled bear were obtained from the St. Louis, Missouri Zoo (via John Patton). Whole blood from three additional sloth bears was obtained from the Lowry Zoo, Tampa, Florida. Purified mtDNAs of an Asiatic black bear, . *thibetanus*, and a giant panda were obtained from Steve O'Brien (via Lisette Waits and Ryk Ward). DNA sequences from a single sun bear *Helarctos* {= *Ursus*} *malayanus* , three American black bears, *U. americanus*, two polar bears and four brown bears were available from our previous studies (Shields and Kocher 1991; Talbot and Shields, 1996).

Amplification and Sequencing of mtDNA. Primers used in this study were described in Talbot and Shields (1996). Single-stranded (asymmetric) amplifications of DNA were performed in 50 µl total reaction volumes with 40 cycles of the polymerase chain reaction (PCR) using a cloned version of *Thermus aquaticus* DNA polymerase (Amplitaq, Perkin Elmer Cetus) and diluting one of the primers 50-100 fold. Parameters for amplification of specific DNAs and visualization and purification procedures followed those described in Talbot and Shields (1996). Sequencing was performed manually using a commercial kit (Sequenase 2.0, U.S. Biochemical), which follows the method of Sanger *et al.* (1977), using the primer that was limiting in the asymmetric PCR reaction. To check for scoring mistakes and to verify variation, both strands of DNA for each individual were sequenced. DNAs of an individual representing each species were also amplified and sequenced more than once.

Sequence Analyses. Sequences were manually aligned and analyzed using both parsimony and distance methods. Analyses based on weighted parsimony were performed using PAUP (Version 3.1.1, Swofford, 1993). Published harbor seal sequences (Árnason and Johnsson 1992) were used as an outgroup. The most parsimonious trees were obtained using the branch-and-bound option in PAUP. A majority rule consensus tree based on 1000 bootstrap replicates was produced using the bootstrap option of PAUP, which is based on the methods of Eck and Dayhoff (1966) and Kluge and Farris (1969) and on Fitch's (1971) method of counting the number of base changes needed on a given tree. Distance trees were produced using the neighbor-joining method of Saitou and Nei (1987) and the computer program MEGA (Version 1.01, Kumar et al. 1993). The Tamura-Nei (1993) model of DNA sequence evolution, as well as the Kimura 2-parameter model, was used to generate distance matrices. Sequences of cytochrome b were converted to amino acid sequences using the computer program MEGA and analyzed using the PROTPARS program of PHYLIP (Version 3.5, Felsenstein 1993) as well as PAUP. Base compositions of cytochrome b genes were analyzed using the computer program MEGA (Kumar *et al.* 1993), employing the method of relative synonymous codon usage (Sharp *et al.* 1986). All sequences have been deposited in

GENBANK (Accession numbers: U18870, U18878, U18888, U18897-18905, and U23552-U23563); users are kindly requested to refer to the present paper and not only to the accession numbers.

1.4 RESULTS AND DISCUSSION

Characterization of Cytochrome b Sequences. The cytochrome b gene in all eight species of bears is 1140 nucleotides long and corresponds to 379 amino acids; no length variation was observed. Like other genes of mitochondria of mammals, the base composition in cytochrome b genes of ursids is strongly biased. The strongest compositional feature is the lack of guanines (G) in the sense strand, particularly in third positions of codons (average bias = 0.094, Table 1.1). Mitochondrial genomes of animals evolve with a strong bias toward transitions (Brown *et al.* 1979, Irwin *et al.* 1991). This, coupled with the low number of guanines, and the correspondingly high number of adenines (A) observed in the ursid cytochrome b gene, indicates that the probability of G to A transitions must be an order of magnitude higher than A to G transitions. Thus, we employed the Tamura-Nei (1993) 5-parameter model in addition to the Kimura 2-parameter model for estimates of divergence rates as well as computations of distance.

Patterns of Nucleotide Variation. Seven hundred and forty-one of the 1140 nucleotides of the cytochrome b gene were identical among the ursids examined. One-hundred and eleven of the 399 variable sites were autapomorphies and were distributed as follows: giant panda, 43; spectacled bear, 23; sun bear, 9; sloth bear, 9; American black bear, 12; Asiatic black bear, 8; brown bear, 4 and polar bear, 3. Among the brown bear lineages, the sequences of GB01 contained two autapomorphies, and sequences of GB09 and GB19 each contained one. An additional 21 sites contained uninformative multistate characters. Thus, 225 of the variable sites (56.4%) were phylogenetically informative.

These 225 sites were partitioned as follows: 177 sites (78.6%) involved differences at third positions of codons, 36 (16%) at first positions, and 12 (5.3%) at second positions. Of the differences at third positions, 34 (19.2%) were transversions. Of the differences at first positions, 8 (22%) were transversions. All 12 differences at second

positions were C-T transitions. Thus, like other comparisons of closely related animals, (Quinn *et al.* 1991, Irwin *et al.* 1991) ursid cytochrome b sequences differ from one another predominantly by transition replacements. Brown *et al.* (1979) and Higuchi *et al.* 1984, 1987) noted transition bias in vertebrate mtDNA. Their work suggests that pairwise transition: transversion ratios should decrease with increasing distance between taxa as transition sites become saturated. As expected, a high ratio of transitions to transversions is evident among ursids (Table 1.2). The mean number of transversions among species of bears was 12.1 (range 1-28), with the lowest (1) being between brown bears of islands of southeastern Alaska (GB01) and polar bears, and the highest (28) being between the spectacled bear and giant panda. The highest transition/transversion ratio (95:1) was between the American black bear and the Asiatic black bear.

The largest average number of nucleotide differences per site among ursids is between giant panda and spectacled bear (17.6%, Table 1.2). That among ursines (11.2%) occurs between lineage GB19 of Alaskan brown bear and the American black bear. The smallest interspecific value (1.1%) occurs between brown bears of the islands of southeastern Alaska (GB01) and the polar bear.

Description of Sequence Variation in tRNA^{Pro} and tRNA^{Thr} Genes. The average number of nucleotide substitutions per site for the combined tRNAs of ursids is approximately four times lower (10%) than that for the cytochrome b gene (38%, Table 1.3). The average number of nucleotide differences per site for tRNA^{Pro} alone (10.4%) is similar to that for tRNA^{Thr} (9.1%). Intraspecific variation in tRNA^{Thr} was observed in brown bears (four lineages) and sloth bears (two lineages), and for tRNA^{Pro} among sloth bears (two lineages).

Parsimony Analyses. A strong phylogenetic signal was detected in the combined and weighted cytochrome b/tRNA data, as evidenced by the highly skewed distribution of trees obtained in an exhaustive search of over 500,000,000 possible trees (g1= -0.560044, $p<0.01$). Analysis of nucleotide changes in the cytochrome b, tRNA^{Thr} and

tRNA^{Pro} genes by weighted parsimony yielded a single most parsimonious tree (length =1300, CI=0.558). A tree based on weighted maximum parsimony (Figure 1.1) indicates that the ancestor of the spectacled bear was the first to emerge as the Ursidae radiated, followed closely by the giant panda and then by the sloth bear. The Asiatic black bear and American black bear occur as sister taxa, emerging from the ursine lineage subsequent to the divergence of the sloth bear but prior to the divergence of the sun bear. The sun bear is placed closest to the brown bear/polar bear clade, the most recently derived of the ursines; and the brown bear is paraphyletic relative to polar bear. We examined all sub-optimal trees within five steps of the single most parsimonious tree to test for confidence of branching patterns hypothesized in the tree with minimal length. All trees found within five steps of the optimum one supported these relationships; the only disagreement among suboptimal and optimal trees was in the relative placement of brown bear lineages GB09, GB19, and GB28. Additionally, all relationships among species were supported by bootstrap analysis (Figure 1.1). Thus, the proposal of a close relationship between the spectacled bear and the polar bear (Zhang and Ryder, 1993) is not supported by our analysis.

Distance Analyses. Trees based on analysis of distance by neighbor-joining were generated using both the Kimura 2-parameter model and the Tamura-Nei distance model, using only first and second positions of the cytochrome b gene, eliminating leucine codons, and including all tRNA sequences. Topologies of these trees based on both models are identical to the tree in Figure 1.2. The neighbor-joining tree has the giant panda as the earliest species to emerge from the ursine line, followed by the spectacled bear. The ursine bears are divided into two clusters, one containing sloth bear, sun bear, American black bear and Asiatic black bear, and the other containing the brown bear and the polar bear. The American black bear and Asiatic black bear are again placed as sister taxa. Like the parsimony analysis, the neighbor-joining tree places the brown bear in a paraphyletic relationship with the polar bear, with the GB01 lineage of brown bears from the islands of southeastern Alaska as the sister taxon to the polar bear.Analysis of Amino Acids. Inconsistencies between the two analyses, i.e., the branching order of spectacled

bear vs. giant panda and the placement of the brown/polar clade relative to the remaining ursines were also addressed in two PROTPARS comparisons (Version 3.5, Felsenstein 1993). A "deep" analysis (Figure 1.3A) indicates that the spectacled bear and giant panda share an ancestor which diverged from the ursid lineage prior to the emergence of sloth bear and the remaining ursines. The tree generated by bootstrap analysis (500 repetitions) of the amino acid sequences and based on the 50% majority rule joined spectacled bear with the giant panda 92% of the time (Figure 1.3A).

A second analysis of amino acids using parsimony examined "shallow" relationships and included all the ursine species with the sloth bear as the outgroup. Figure 3B presents one of two trees produced by this analysis; the other (not shown) differs in the placement of the black bear clade relative to sun bear. Both trees place the brown/polar bear clade at the terminus of the radiation of the Ursinae. The tree based on the 50% majority rule and evaluated by bootstrap analysis (500 repetitions) placed this clade as the terminal one 95% of the time (Figure 1.3B).

Times of Divergence. Rates of DNA change were estimated by calculating the average number of nucleotide differences per site for all third positions of codons and calibrating this with the date of the earliest fossil record for the giant panda lineage (12 million years ago, Thenius 1979b, 1982) and using the Tamura-Nei corrections. The predicted divergence at 12 million years before the present is a minimum estimate, making the rate estimate a maximum one. The average overall probability of substitution was 6% per million years of evolution for third positions of codons for the cytochrome b gene of ursids. This is slower than the 10% per million year estimate for third positions of codons in other mammalian species (Irwin *et al.* 1991).

Based on these rates, we place the beginning of the divergence of the ancestral cytochrome b gene of the spectacled bear at approximately 12-13 million years ago, close to the divergence time of the ancestral giant panda cytochrome b gene. The origin of the Ursinae (the emergence of sloth bear) is placed in the mid-Miocene, approximately 7 million years ago, followed within approximately 1 million years by the rapid radiation of the remaining ursines. The lineage leading to Asiatic black bears and to American black

bears diverged from the ursine lineage approximately 6 million years ago, with the species of black bears diverging from each other within another million years. At about the same time (5 million years ago), the sun bear diverged from the lineage leading to the terminal ursine taxa (brown bears and polar bears). Finally, we estimate that the brown bear mtDNA lineages (GB09, 19 and 28) diverged from the ancestral lineage of the clade containing brown bear lineage GB01 and polar bears approximately 1-2 million years ago, toward the end of the early Pleistocene epoch. Brown bear lineage GB01 and the polar bear lineage diverged during the mid-Pleistocene, approximately 300-400,000 years ago (also see, Talbot and Shields 1996 and Waits, O'Brien and Ward, unpublished).

Because of the disparate evolutionary rates detected among the older ursids, we have less confidence in our time estimates for these taxa than for the ursines. If the cytochrome b/tRNA lineages examined herein are reflective of relationships of species of ursids, they push back the origin of the Tremarctinae, represented by the spectacled bear, some 3-4 million years, or suggest a more recent divergence for the Ailuropodinae, represented by the giant panda, regardless of the relationship between the hypothesized and actual time of divergence. A possible affinity between the spectacled bear and panda bear is suggested by the fact that both species are herbivorous foragers and possess "unbearlike" behavioral patterns (i.e., neither hibernates, nor "roars"; O'Brien *et al*. 1985).

The Ursine Radiation. Our suggested date of 5 to 7 million years ago for the ursine radiation is in relatively good agreement with estimates of from 4.4 to 9.3 million years ago made by Sarich (1973), O'Brien (1985) and Goldman *et al.* (1989) based on nuclear markers as well as those based on the fossil record (Kurtén 1964, 1968, 1976). A consensus tree based on nuclear markers (Goldman *et al.* 1989) did not elucidate relationships among the ursines, however. Our analysis places the Asiatic black bear and American black bear as sister taxa, diverging during the Astian (late Pliocene). This relationship has been suggested by several workers (Allen 1938, Kurtén 1964) based on morphological characters and the fossil record, although divergence times between these two species are generally considered much more recent due to the appearance of black

bear fossils in North America approximately 500,000 years ago during the 2-Mindel (Pleistocene).

Our calculated divergence rates among the ursines are based largely on transition substitutions at third positions of codons. These apparently saturated prior to the divergence and radiation of the ursine bears. A test for rate constancy based on maximum likelihood (Felsenstein 1993) indicated no significant difference ($\chi^2 = 13.95$, $p > 0.05$) in rates of evolution among the ursine species. Thus, we are confident that the analysis of ursines based on maximum parsimony is not misleading. Nevertheless, although our parsimony analysis appears to resolve the relationships among the Ursinae, estimated branch lengths among them were very short, indicating rapid radiation and the potential for error in estimating branching order. Because sequences of ursines, particularly those of the black bear, still differ primarily by transition substitutions, we suggest that this group of bears may have diverged over a relatively short period. If so, it is possible that a single locus such as the mitochondrial genome may not represent the actual evolutionary history of all genes. Therefore, analysis of nuclear genes may be required to develop a clearer understanding of the evolutionary history of ursids.

Comparisons With Other Studies of mtDNA. Vrana *et al.* (1994) presented partial sequences of cytochrome b genes of several carnivores, including four species of ursines and the giant panda. There is a 23% difference between our giant panda data and the partial cytochrome b sequence presented by Vrana *et al.* (1994). Árnason *et al.* (1995) also report disparities between the data of Vrana *et al.* (1994) and their own when walrus (11%)
and domestic cats (>20%) were compared. It is unlikely that values larger than 11% can be explained by intraspecific variation. We compared the Vrana sequences with those deposited in Genbank and conclude that their sequences are incorrect and possibly represent those of another organism; they appear closest to goat and dog. We also compared our cytochrome b sequences from the panda with those reported by Zhang and Ryder (1993, Gen Bank accession # L21877). Our sequences for giant panda are more than 98% similar to theirs, and with the exception of one difference, all were synonymous

changes at third positions of codons. Such a small divergence value (1.5%) could be due to intraspecific variation.

Zhang and Ryder (1993, 1994) present a phylogeny for ursids which places one of their lineages of polar bears as the sister taxon to the spectacled bear. Their analyses which were based on maximum parsimony and maximum likelihood included portions of the cytochrome b gene (397nt), the control region (368nt), the 12S rRNA (349nt) and the $tRNA^{Pro}$ and $tRNA^{Thr}$ genes (71nt) and used giant panda as an outgroup. They rule out contamination as a source of the disparity between their polar bear sequences, and propose a hybridization event, presumably recent, between the polar bear and the spectacled bear. However, the numbers of chromosomes differ substantially between the spectacled bear (2n=52) and the polar bear (2n=74, O'Brien *et al.* 1985, Hsu and Benirschke 1977) and therefore, we consider it unlikely that a successful hybridization between these two species could have occurred.

A phylogeny for the Ursidae based on partial sequence information from six regions of mtDNA has been proposed by Waits, O'Brien and Ward, unpublished. This study included the slowly evolving 16s rRNA gene (480nt), the rapidly evolving control region (260nt), and portions of four additional genes believed to have intermediate rates of evolution: NADH subunit 5 (243nt), NADH subunit 4 (223nt), cytochrome b (257nt) and cytochrome oxidase II (453nt). As with our study, the harbor seal constituted the outgroup. Their proposed phylogeny based on weighted maximum parsimony and maximum likelihood is largely congruent with our proposed phylogeny, and places giant panda, then spectacled bear, as the initial species to emerge from the ursid radiation. In their phylogeny, the sloth bear is the most ancient ursine lineage while brown bear and polar bear are the most recent. They also identified a paraphyletic relationship among the brown bears. Their analysis was equivocal in its resolution of the relationships among the two species of black bears and the sun bear; whereas our study resolved this relationship by placing the two black bears as sister taxa. This indicates that congruent phylogenies do not necessarily result when analyses based on partial sequences of many genes are compared to analyses based on complete sequences of fewer genes.

Comparisons With Other Studies. Our interpretation of relationships based on sequences of mitochondrial genes differs from those based on nuclear markers and chromosomes. The mitochondrial data place the emergence of Ailuropodinae at or very near the emergence of the Tremarctinae. Studies based on nuclear markers place the giant panda as the oldest of the ursid taxa, diverging approximately 15-25 million years ago prior to the emergence of spectacled bear approximately 3 million years later (Sarich 1973, O'Brien 1985, Goldman *et al.* 1989). The amino acid phylogeny reported herein places giant panda in the same divergent clade with spectacled bear and indicates some affinity between Tremarctinae and Ailuropodinae. Unfortunately, this inconsistency cannot currently be supported or refuted using fossil evidence since that evidence is weak for early Tremarctinea.

Paraphyly Within the Brown Bear. Twenty nucleotide substitutions are shared by polar bears and brown bears of the islands of southeastern Alaska. Our sequences for ursids thus, support the close phylogenetic relationship of polar bears and brown bears predicted by Shields and Kocher (1991) who analyzed restriction fragment length polymorphisms of mtDNA and a more restricted set of sequences of the cytochrome b gene and the control region. Our observations of paraphyly are also supported by Cronin et al. (1991), Taberlet and Bouvet (1992), Waits, O'Brien and Ward, unpublished and Waits et al., unpublished. In our previous study (Talbot and Shields, 1996) we noted little evidence for monophyly among brown bears, whereas paraphyly was indicated in all of our trees, having at least 90% bootstrap support in all analyses. The present analysis which includes a different data set and different OTUs also suggests paraphyly. In the present study, use of different OTUs including more divergent organisms and, therefore, more divergent outgroups affects the branching order in phylogenetic trees.

Our phylogeographic observations suggest that polar bears originated from an ancestral stock that also resulted in the brown bears which now reside on the ABC Islands of southeastern Alaska. This molecular observation supports hypotheses by Kurtén (1964,1968) based on fossils that polar bears arose from coastal brown bears possibly in Siberia. We place the divergence of polar bears from brown bears at about 300,000-

400,000 years ago. It is not unlikely that the separation of the polar bear/ABC brown bear clades occurred somewhere in Siberia about that time. The clade of polar bears may have expanded to their current circumpolar distribution while the ancestors of the ABC brown bears of Alaska expanded eastward across Beringia, became isolated in southeastern Alaska by latter glacial advances but were subsequently eliminated from the rest of their original range. It is also possible that the two taxa could have diverged in southeastern Alaska with the subsequent movement of polar bears across coastal ice and into the circumarctic region. Fossils of brown bears on the islands of southeastern Alaska date at least to the middle of the Wisconsin period (35,365 yr B.P; Heaton, 1995a) and at least some of those bears may have subsisted on a diet that included marine fauna (Heaton, 1995b). The suggestion by Klein (1965) that all species of the islands of the Alexander Archipelago are post-glacial invaders is not true for bears. O'Reilly *et al.* (1993) have suggested glacial refugia on the Queen Charlotte islands of British Columbia based on genetic analysis of sticklebacks (*Gasterosteus*).

It is also possible that the close affinity between polar bears and brown bears of the ABC islands could be the result of "capture" of polar bear mitochondrial DNA through hybridization between polar bears and brown bears. While hybridization between polar bears and brown bears has been observed in captivity (Kowalska 1965), the two species have not been observed to hybridize under natural conditions. We follow Stanley's suggestion (1979) that the morphological features distinguishing polar bears from brown bears have evolved rapidly in response to selective pressures of adapting to a new environment, prior to the emergence of distinguishing molecular features. Such patterns are also paralleled in other closely related mammals, such as in the true seals (Árnason *et al.* 1995) and the baleen whales (Árnason and Gullberg 1994). In these marine mammals, rates of morphological evolution may be accelerated relative to that of molecular evolution when a new ecological niche is occupied.

Classification. Our data, coupled with data from other mtDNA phylogenies discussed above, support the inclusion of the giant panda in the Ursidae as proposed by several investigators using molecular and morphological characters (O'Brien *et al.* 1985,

Goldman *et al.* 1989; Sarich, 1973). While a close affinity between Tremarctinae and Ailuropodinae is indicated, we cannot suggest changing subfamilial relationships until additional analysis of nuclear markers is complete. We do, however, suggest that Tremarctinae be retained separately from Ursinae, and that Ursinae be comprised of the six species which have undergone rapid radiation during the period from the mid-Pliocene to early Pleistocene.

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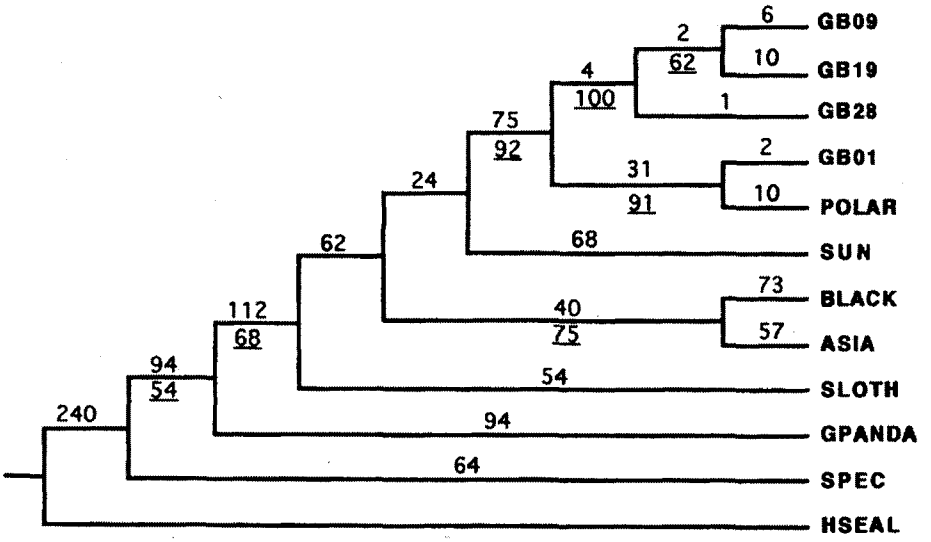

Figure 1.1. Single most parsimonious phylogenetic tree inferred by weighted parsimony methods based on the combined cytochrome b/tRNA^{pro} and tRNA^{thr} genes of eight species of ursids and using harbor seal as an outgroup. First, second and third positions of codons of the cytochrome b gene were given the weight of 4, 15 and 1, respectively. These weights were based on differences at all codon positions in the cytochrome b gene as well as differences in domains of the tRNA genes.Transversions at third positions of codons were given the weight of 6 and those at first positions 26. First positions of leucine codons were given the weight of 1. Values not underlined indicate branch lengths, values underlined indicate bootstrap values (50% majority, 1000 replications). Labels are as follows: H. SEAL = harbor seal, GPANDA = giant panda, SPEC =spectacled bear, SUN= sun bear, SLOTH = sloth bear, ASIA = Asiatic black bear, BLACK = American black bear, BROWN = brown bear, and POLAR = polar bear. Lineages of brown bears are as follows: GB 01 = islands of southeastern Alaska, GB 09 = coast of southeastern Alaska, GB 19 = western Alaska, GB 28 = Turkey. Sequences of the harbor seal are published (Árnason and Johnsson 1992).

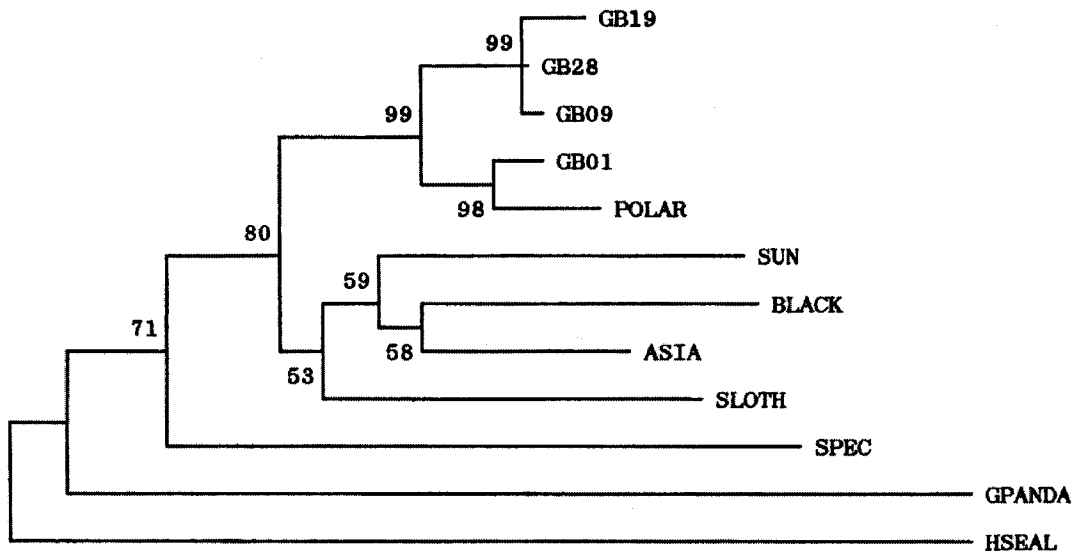

Figure 1.2. Phylogenetic relationships among sequences of the cytochrome b and tRNA genes of eight species of ursids. The tree was constructed by the neighbor-joining method (Saitou and Nei 1987) and was rooted using sequences of the cytochrome b gene of the harbor seal (Árnason and Johnsson, 1992). Distance values were derived from first and second positions of codons of the cytochrome b gene combined with complete sequences of the $tRNA^{pro}$ and $tRNA^{thr}$ genes and then subjected to the Tamura-Nei (1993) distance algorithm. The branching order was supported by a majority rule consensus of neighbor-joining trees generated by 1000 bootstrap simulations (values given at nodes). Species labels are as in Figure 1.1.

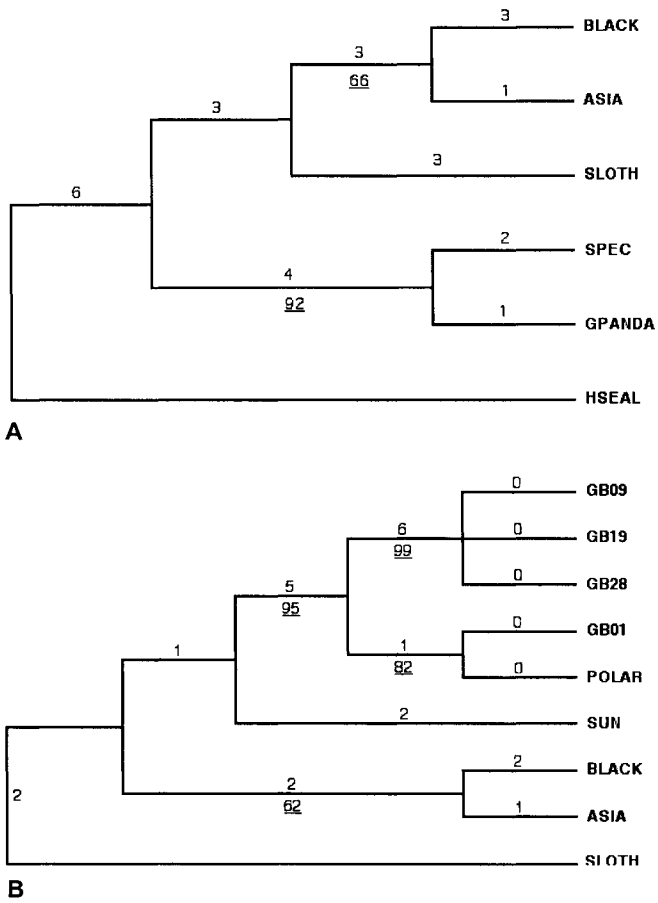

Figure 1.3. Parsimony trees based on analysis of amino acids of the cytochrome b gene. A) shows the single most parsimonious tree generated from an analysis of "deep" (Tremarctinae-Ailuropodinae) relationships of ursids, rooted by using sequences of the harbor seal. B) shows one of the most parsimonious trees generated from an analysis of "shallow" ursine relationships. Values not underlined show branch lengths; those underlined are bootstrap values (50% majority rule, 500 replications).

Table 1.1. Base composition of ursid cytochrome b genes at first, second, and third positions of codons. Species labels are as given in Figure 1.3.

	First				Second				Third			
Species	G	A	T	C	G	A	T	C	G	A	T	C
GB01	23.2	27.1	22.4	27.4	13.7	19.7	40.5	25.9	7.1	38.7	18.2	36.1
GB09	22.6	27.9	22.1	27.4	13.9	19.7	40.5	25.8	6.6	38.7	19.5	35.3
GB19	22.6	27.9	22.2	27.4	13.9	19.7	40.5	25.9	7.1	38.4	18.9	35.5
GB28	22.6	27.9	22.1	27.4	13.9	19.7	40.5	25.8	7.4	38.2	18.4	36.1
POLAR	23.2	27.1	22.6	27.1	13.9	19.7	40.8	25.5	7.6	38.4	18.7	35.3
SUN	22.4	28.4	23.4	25.8	13.6	19.7	40.5	25.8	6.8	38.4	16.0	38.2
BLACK	23.2	27.4	21.8	27.6	13.9	19.7	40.5	25.8	6.3	38.9	16.3	38.4
ASIA	23.2	27.4	22.1	27.4	13.9	19.7	40.3	26.1	7.1	38.4	14.5	40.0
SLOTH	22.1	28.7	22.4	26.8	13.9	19.7	40.8	25.5	7.1	38.7	15.0	39.2
SPEC	22.9	28.2	22.9	26.1	14.2	19.5	41.3	25.0	8.7	36.8	18.2	36.3
GPANDA	20.8	29.7	24.5	25.0	14.2	19.5	41.3	25.0	8.4	37.6	25.0	28.9
Average	22.4	28.03	22.6	26.8	13.9	19.73	40.7	25.5	6.9	38.7	17.52	36.7
Bias[a]		0.002				0.053				0. 094		

[a]Bias is the extent to which the observed frequencies of each base differ from 0.25 and is calculated as $B = (4/3) \text{ S } (b_i - 0.25)^2$, where B is the bias and b_i is the frequency of the i^{th} base.

Table 1.2. Observed pairwise differences (in percentage) among ursid mitochondrial cytochrome b genes (above diagonal). Values below the diagonal are ratios of transitions to transversions. Zero values are designated with an asterisk (*). Harbor seal data are from Árnason and Johnsson (1992).

Species	1	2	3	4	5	6	7	8	9	10	11	12
1. GB09	-	0.8	0.9	2.8	2.8	9.2	11.0	10.2	9.0	13.7	14.4	17.5
2. GB19	8.0	-	0.6	2.7	2.7	9.5	11.2	10.3	9.4	13.5	14.8	17.6
3. GB28	9.0	*	-	2.5	2.6	9.2	10.8	10.2	9.1	13.6	14.4	17.3
4. GB01	9.7	14.5	13.0	-	1.1	8.0	10.4	9.7	8.2	14.4	14.3	16.7
5. POLAR	4.0	9.3	9.0	11.0	-	8.5	10.5	9.6	8.3	14.4	14.9	17.1
6. SUN	20.0	26.0	25.3	21.8	18.4	-	9.7	9.7	9.3	15.7	14.8	17.0
7. BLACK	30.3	41.7	40.0	10.0	29.0	36.0	-	8.4	9.7	17.2	14.7	17.6
8. ASIA	37.7	57.2	57.0	54.0	35.3	54.0	95.0	-	9.4	16.5	14.9	17.1
9. SLOTH	19.5	25.8	25.0	22.3	17.8	16.6	21.2	25.8	-	15.4	13.4	16.8
10. GPANDA	6.4	6.7	6.8	7.0	6.8	8.0	9.3	8.4	7.0	-	17.6	20.2
11. SPEC	5.6	6.0	5.8	5.8	6.4	6.0	5.7	6.1	5.4	6.2	-	17.5
12. H. SEAL	2.0	2.1	2.0	1.9	2.0	2.0	2.0	2.0	2.0	2.2	1.9	-

Table 1.3. Observed pairwise differences (in percentage) among ursid mitochondrial $tRNA^{thr}$ (above diagonal) and $tRNA^{pro}$ genes (below diagonal). Zero values are designated with an asterisk (*). Harbor seal data are from Árnason and Johnsson (1992).

Species	1	2	3	4	5	6	7	8	9	10	11	12
1. GB09	-	2.9	1.4	4.3	4.3	10.0	4.3	5.7	4.3	12.9	11.4	11.6
2. GB19	*	-	1.4	4.3	4.3	10.0	4.3	5.7	4.3	12.9	11.4	14.5
3. GB28	*	*	-	2.9	2.9	8.6	2.9	4.3	2.9	14.3	10.0	13.0
4. GB01	*	*	*	-	*	8.6	5.7	4.3	2.9	12.9	10.0	14.5
5. POLAR	4.6	4.6	4.6	4.6	-	8.6	5.7	4.3	2.9	12.9	10.0	14.5
6. SUN	6.1	6.1	6.1	6.1	10.6	-	5.7	4.3	8.6	14.3	7.1	14.5
7. BLACK	6.1	6.1	6.1	6.1	10.6	6.1	-	1.4	5.7	14.3	7.1	14.5
8. ASIA	6.1	6.1	6.1	6.1	10.6	3.0	6.1	-	4.3	12.9	8.6	16.0
9. SLOTH	6.1	6.1	6.1	6.1	10.6	6.1	6.1	6.1	-	14.3	12.9	16.0
10. GPANDA	10.6	10.6	10.6	10.6	12.1	10.6	13.7	10.6	13.7	-	17.1	16.0
11. SPEC	12.1	12.1	12.1	12.1	13.7	12.1	12.1	9.1	15.2	19.7	-	11.6
12. H. SEAL	13.7	13.7	13.7	13.7	15.2	13.7	16.7	10.6	16.7	13.6	13.6	-

CHAPTER 2

PHYLOGEOGRAPHY OF BROWN BEARS (*URSUS ARCTOS*) OF ALASKA AND PARAPHYLY WITHIN THE URSIDAE[1]

2.1 ABSTRACT

Complete nucleotide sequences of the mitochondrial cytochrome *b*, $tRNA^{proline}$ and $tRNA^{threonine}$ genes were described for 166 brown bears (*Ursus arctos*) from ten geographic regions of Alaska to describe natural genetic variation, construct a molecular phylogeny and evaluate classical taxonomies. DNA sequences of brown bears were compared to homologous sequences of the polar bear (*U. maritimus*) and of the sun bear (*Helarctos malayanus*) which was used as an outgroup. Parsimony and neighbor-joining methods each produced essentially identical phylogenetic trees that suggest two distinct clades of mtDNA for brown bears in Alaska: one composed only of bears that now reside on some of the islands of southeastern Alaska and the other which includes bears from all other regions of Alaska. The very close relationship of the polar bear to brown bears of the islands of southeastern Alaska as previously reported by us and the paraphyletic association of polar bears to brown bears reported by others have been reaffirmed with this much larger data set. A weak correlation is suggested between types of mtDNA and habitat preference by brown bears in Alaska. Our mtDNA data support some, but not all, of the currently designated subspecies of brown bears whose descriptions have been based essentially on morphology.

[1]Talbot, S.L., and Shields, G. F. (1996). Phylogeography of brown bears (*Ursus arctos*) of Alaska and paraphyly within the Ursidae. *Molecular Phylogenetics and Evolution* **5**: 477-494.

2.2 INTRODUCTION

Elucidation of natural genetic variation among relatively undisturbed populations of brown bears (*Ursus arctos*) in Alaska will enhance the understanding of the phylogenetic history of this species as well as provide insights into genetic processes. Because of its threatened or endangered status in some regions of the coterminous United States as well as in parts of Asia and in Europe, there is considerable interest in the elucidation of genetic relationships within and between naturally occurring populations of *U. arctos*. Understanding of processes affecting population genetics could help wildlife biologists maintain genetic diversity of brown bears in Alaska, and provide relevant information to help preserve threatened and endangered populations of bears elsewhere. Of the six species of bears that are declining in number, more is known about the historic numbers and distribution of the brown bear than of any of the other species (USFWS 1987; 1990; Servheen 1989). The distribution of brown bears in Alaska has undergone little alteration from its historic range with development encroaching on original habitat only in localized areas surrounding centers of human habitation. Thus, in Alaska, we have the opportunity to describe natural genetic variation and consequently to understand the genetic parameters that characterize this large, free-ranging carnivore.

Brown bears occur throughout Alaska with the following exceptions: the southern islands of the Alexander Archipelago in southeastern Alaska and most islands of Prince William Sound; the Aleutian islands west of Unimak Pass and the outer part of the Yukon-Kuskokwim Delta. Densities vary considerably, with southeastern Alaska, the Kodiak Archipelago and the Alaskan Peninsula having the highest concentrations, whereas the Arctic regions have the lowest (Miller and Schoen, in press).

The proposal of over 90 subspecies of brown bears in North America (Merriam 1918) and 271 subspecies in Eurasia (Ognev 1931), once recognized by various workers and based largely on cranial and dental characteristics, is now considered a classic case of taxonomic over-splitting (Kurtén 1973; Hall 1984). There are, however, marked morphological differences between the large brown bears of southern Alaska and western British Columbia (mean length of condylobasal process = 341-399 mm.; weight > 250 kg; Rausch 1953; 1963; Kurtén 1973; Spraker et al. 1981) and the somewhat smaller grizzly

bears of the interior of Alaska (mean length of condylobasal process = 296-341 mm.; weight < 180 kg; Reynolds 1981) such that these bears were once considered different species (Merriam 1918). Thus, the considerable amount of morphological variation occurring within Palearctic and Nearctic *U. arctos* suggests the potential for genetic differentiation among local populations.

Based on length of condylobasal processes of 357 skulls, Rausch (1963) considered all mainland brown bears in North America to be the same subspecies (Fig. 2.1a) and believed geographic variation in brown bears of North America was essentially clinal. This cline is paralleled in the overall size of bears which tends to decrease to the northeast and south from a maximum on the Alaskan Peninsula and Kodiak and Afognak Islands. Rausch (1963), however, considered only a series of skulls of bears from certain islands (Kodiak, Afognak and Shuyak) to represent populations isolated by geographic and reproductive barriers and applied the subspecies epithet *U. a. middendorffi,* (Merriam) to these populations. His classification proposed that *U. a. horribilis* (Ord) be used for brown bears over the greater part of the range of the species in North America, including Alaska.

Kurtén (1973) used size and allometry of width-of-skull data from Rausch (1963) for bears of North America and from Ognev (1931) for Eurasian bears to study subspecific relationships of brown bears of Beringia, the land mass that connected the two continents at an earlier time. He explained morphological variation in skulls, overall size of bears, and color variation in pelage as inheritance from ancestral populations of bears in eastern Asia and western North America and proposed affinities between: (1) *U. a. piscator* of Kamchatka and *U. a. middendorffi* of Kodiak; (2) *U. a. manchuricus* of Manchuria and *U. a. dalli* of the coastal mainland of Alaska and (3) among *U. a. jeniseensis, kolymensis* and *berigianus* of Siberia and the "grizzly, "*U. a. horribilis* (Fig. 2.1b).

Finally, Hall (1984) used selected cranial dimensions as well as dentition to review the *U. arctos* group in North America and proposed nine subspecies of which five (*U. a. alascensis, U. a. middendorffi, U. a. gyas, U. a. dalli* and *U. a. sitkensis)* are described from Alaska (Fig. 2.1c). Thus, because of considerable morphological variation within the species, there is marked disagreement regarding taxonomic descriptions of *U. arctos* from Alaska.

To describe natural genetic variation and to assess subspecific relationships among brown bears of Alaska we compared variation among the entire nucleotide sequences of: the mitochondrial cytochrome b *(cyt b),* tRNA ^{proline} ($tRNA^{pro}$) and $tRNA^{threonine}$ ($tRNA^{thr}$) genes of brown bears from ten geographic regions of Alaska and ostensively the five subspecies of *U. arctos* in Alaska (Rausch 1963, Kurtén 1973, and Hall 1984). We also compared sequences of: brown bears from eastern Siberia and Turkey, polar bear (*U. maritimus)* and sun bear *(Helarctos malayanus),* which we have determined to be phylogenetically closest to the brown bear/polar bear clade among all other ursids (Talbot and Shields, unpublished data). For general reviews of the usefulness of mtDNA in evolutionary studies, see Wilson et al. (1985), Avise (1986), Avise et al. (1987), Moritz et al. (1987) and Harrison (1989).

Although Shields and Kocher (1991) proposed a close phylogenetic relationship between *U. arctos* and the polar bear and Cronin et al. (1991) and Taberlet and Bouvet (1992) reported that brown bears are paraphyletic with respect to polar bears in their maternal (mtDNA) phylogeny, no detailed survey of the molecular population genetics of brown bears exists. Here we examine genetic relationships within and between Alaskan populations of brown bears to assess levels of population sub- structuring and extent of gene flow, and to clarify intraspecific phylogeographic relationships (Avise et al. 1987) among putative subspecies of brown bears. We examine three major issues concerning the genetic relationships of these bears: (1) The number of migrations of brown bears into the New World from Asia might be elucidated by molecular data. (2) The brown bear is paraphyletic with respect to the polar bear; and (3) Genetic diversity among mtDNAs might resolve differences among currently proposed taxonomic schemes. Because of our unusually large data set (223,300 nucleotides compared), we also attempt to describe phylogeographic patterns within *U. arctos* in Alaska (Smouse et al. 1991). A subset of the *cyt b* data reported here has been used together with sequences of control regions of brown bears to construct a "world-wide" phylogeny for the species as part of a separate study (Waits et al. 1999; Appendix III).

2.3 MATERIALS AND METHODS

Sample Sources. DNAs from 166 brown bears from ten geographic regions of Alaska were compared in this study. Regions represented are: southcoastal, southcentral, Arctic (Arctic National Wildlife Refuge, ANWR), interior Alaska, Seward Peninsula, Kodiak Island, Alaska Peninsula (Katmai National Park and Izembek National Wildlife Refuge), Brooks Range, Alaska Range, and the islands of southeastern Alaska (Fig. 2.2). Also, small numbers of tissues of brown bears from Eastern Siberia (n = 5) and Turkey (n = 2) became available during the course of this study, and, we analyzed mtDNA from them. Blood, skin or muscle tissue of brown bears from across much of the range of distribution in Alaska were collected by state (Alaska Department of Fish and Game) and federal (U. S. Fish and Wildlife Service) agency employees. Dried skin biopsies of brown bears from Russia and Turkey were collected respectively, by M. Kretchmar (Institute of Biological Problems of the North, Magadan, Russia) and by J. Fuller and E. Holmes (U. S. Fish and Wildlife Service, Anchorage) from confiscatedhides. Purified DNAs of sun bears were obtained from the St. Louis, Missouri Zoo (via Dr. J. Patton). Purified DNAs from polar bears were available from a previous study (Shields and Kocher 1991). Blood was collected in tubes containing 15 mg potassium EDTA, heparin or without preservatives, from immobilized brown bears. All other samples consisted of skeletal or heart muscle or skin taken from hunter-killed bears. Tissues and blood were kept frozen at -20°C until analyzed.

Extraction of DNA. DNA was extracted from approximately 0.2 g of frozen muscle tissue by digestion in 550 µl of buffer composed of 50 mM Tris HCL (pH 8.0), 50 mM EDTA (pH 8.0), 1% sodium dodecyl sulfate (SDS), 100 mM *b*-mercaptoethanol and 10 mg/ml Proteinase K. Tissue was dissolved in the extraction buffer at 56°C for approximately two hours. RNase A (10 mg/ml) was added one hour prior to the end of incubation. DNA was purified using sodium chloride and ethanol precipitations and was isolated from whole blood by differential lysing of red and white cells, using a lysis buffer composed of 150 mM NH_4Cl, 2 mM $KHCO_3$ and 100 mM EDTA. This mixture was centrifuged at 2000 rpm for 8 minutes, after which the pellet was resuspended in lysis

buffer to which phosphate buffered saline (PBS, pH 7.4) was added. The samples were then centrifuged; and the pellet was resuspended and centrifuged twice, first with lysis buffer and finally with PBS. The pellet was then incubated at 56° C from two hours to over night in 1 ml PBS, 5 μM EDTA, 50 μl 20% SDS, 2 ml TE and 11 μl Proteinase K (10 mg/ml). RNase A (10 mg/ml) was added one hour prior to the end of incubation. The solution was then purified using sodium chloride and ethanol precipitations. To eliminate interference from co-extracted heme pigments, some blood samples were further purified using the chelating resin, Chelex® (Walsh et al. 1991).

Because some sequences of mitochondrial DNA have been observed among nuclear DNAs (Gellissen et al. 1983; Quinn and White 1987; Smith et al. 1992) we subjected a subset of samples (a polar bear, brown bears from: the southeastern islands of Alaska (n = 2), southcoastal Alaska (n = 1) and Kodiak Island (n = 1)) to CsCI-PDI density gradient centrifugation using procedures described by Shields and Wilson (1987) to assure that initial sequences described were the result of amplifications of mitochondrial DNAs and not of nuclear origin.

Amplification and Sequencing of mtDNA. Primers used in this study are shown in Table 2.1 and included "universal" primers designed by Kocher et al. (1989) and Irwin et al. (1991) to recognize conserved areas of human, mouse and cow (Anderson et al. 1981) mtDNA sequences. The bear *cyt b* gene was amplified initially and sequenced using three sets of species-specific primers that recognize sites flanking the $tRNA^{glutamine}$ gene: (L14702) and the *cyt b* gene (H15273), *cyt b* (L15168) and (H15590), and *cyt b* (L15513) and $tRNA^{thr}$ (H15915). Additional nested primers were used as necessary to augment sequence information when only partial sequences were obtainable using the above primers. The $tRNA^{thr}$ and $tRNA^{pro}$ genes were amplified and sequenced using the "universal " primer (L15774), which recognizes a site on the downstream portion of the *cyt b* gene, as well as an ursid-specific primer (H00019) which recognizes a site in the control region.

Single-stranded (asymmetric) amplifications were performed in 50 µl total reaction volumes with 40 cycles of the polymerase chain reaction (PCR) using a cloned version of *Thermus aquaticus* DNA polymerase (Amplitaq®, Perkin Elmer-Cetus) and diluting one of the primers 50-100 fold. Amplification was performed using a Perkin Elmer-Cetus thermal cycler, with denaturation at 93° C for 45-60 sec., annealing at 51-54° C for 45-60 sec. and extension at 72° C for 60-90 sec. Electrophoresis of 5 µl of the single-stranded product was performed on a 1.5% agarose minigel containing ethidium bromide in Tris-borate-ethylenediaminetetraacetate (TBE). Amplified products were visualized by fluorescence under ultraviolet radiation. The remaining product was subjected to three cycles of millipore purification at 6000 rpm for eight minutes. Single-stranded DNA was then diluted in 21 µl sddH20, 1/3 of which was subjected to single-stranded sequencing.

Sequencing was performed with a commercial kit (Sequenase 2.0®, U. S. Biochemical), which follows the Sanger et al. (1977) method. The sequencing primer used was the one primer that was limiting in the asymmetric PCR reaction. Both strands of DNA were amplified for each individual to score sequences close to both primers. To check for possible scoring mistakes, DNAs from at least one individual representing each population were amplified and sequenced more than once.

Sequence Analyses. Sequences were manually aligned and sequence data were analyzed using both parsimony and distance methods. Parsimony analysis was performed using PAUP (Version 3.0s, Swofford 1991). Cladistic analyses of character state matrices using parsimony methods were performed using the branch-and-bound or exhaustive search options, which produce majority rule consensus trees. 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), and on Fitch's (1971) method of counting the number of base changes needed on a particular tree. This method assumes independent evolution of sites and lineages, and provides confidence values for internal lineages. DNA of the sun bear was used as an outgroup for all analyses of maximum parsimony.

Distance trees using the neighbor-joining method of Saitou and Nei (1987) were generated using the computer program MEGA (Version 1.01, Kumar et al. 1993). The Tamura-Nei (1993) model of DNA sequence evolution, as well as the two-parameter model (Kimura 1980) was used to generate distance matrices. The Tamura-Nei model accounts for differences in rates of transition substitutions, as well as accounting for differences between transitions and transversions, whereas Kimura's two-parameter model accounts only for differences between transition and transversion substitutions and assumes that nucleotide frequencies are represented in equal proportions, i. e. 25%. The resulting matrices were later subjected to the neighbor-joining option. The neighbor-joining distance method constructs a tree by successively clustering lineages, setting branch lengths as lineages join, with no further rearrangement and the method does not assume an evolutionary clock. Base compositions of *cyt b* genes were analyzed using Sharp et al.'s (1986) relative synonymous codon usage.

2.4 RESULTS

Verification of Mitochondrial Origin of Sequences. Although copies of mitochondrial genes occur in the nuclei of other animals (Gellissen et al. 1983; Quinn and White 1987; Smith et al. 1992), we conclude that sequences analyzed here are of mitochondrial origin because: 1) They differ predominantly by silent transitions, which is consistent with the evolution of functional, coding, mitochondrial genes (Wilson et al. 1985, Quinn and White 1987). If any or all of these sequences were of nuclear origin, they would be the result of very recent events. 2) Each sequence is the result of at least two overlapping initial amplifications and in all instances, the overlapping sequences were identical. If the sequences were of nuclear origin, each of the several amplifications would have selectively resulted in only a nuclear copy; an unlikely event, and 3) DNA from at least one representative of each major clade of brown bear in Alaska was purified by cesium chloride-PDI density gradient centrifugation (Shields and Wilson, 1987) and sequences from these samples were compared to sequences from the same individual and from individuals of the same population from which DNAs were extracted using different

procedures (salt, chelex). In the former instance the sequences were identical; in the latter, they were identical to at least one individual from the same population.

Cytochrome b Sequences. Brown, polar and sun bear *cyt b* sequences have been deposited in GENBANK (Accession nos. U18870 to U18899); users are expected to refer to the present paper and not only to the accession numbers. The *cyt b* gene is 1140 bp long in all individuals of this study. The corresponding *cyt b* protein is 379 amino acids in length. Twenty-eight separate lineages were identified among sequences of brown bears: one from Turkey, three from eastern Siberia and the remaining 24 from Alaska (Fig. 2.3). Lineages defined by their *cyt b* sequences are identified as to collection location in Table 2.2. All *cyt b* sequences begin with a conserved methionine initiation-codon and terminate with an AGA stop codon. Thus, *cyt b* sequences differ from the translational termination signals produced by the polyadenylation of processed mRNA occurring in human and mouse sequences, but are similar to products of 17 ungulate and dolphin *cyt b* genes (Irwin et al. 1991) as well as for harbor seal and gray seal (Árnason and Johnsson 1992, Árnason et al. 1993). The inferred amino acid sequences for ursids include areas of high overall amino-acid identity with other mammalian homologs and the overall amino-acid identity with the harbor seal *cyt b* protein ranges from 88 to 92% (data not shown).

Base Composition of Cytochrome b. Like mitochondrial genes of other mammals, the base composition in ursid *cyt b* genes is strongly biased. The strongest bias is the lack of guanine nucleotides in the sense strand. Guanines are most strongly underrepresented in the third positions of codons, where the average bias is 0.089. The low number of guanine nucleotides and the high number of adenine nucleotides, coupled with the observation that animal mitochondrial genomes evolve with a strong bias toward transitions (Brown et al. 1982, Irwin et al. 1991), indicate that the probability of a G to A transition must be an order of magnitude higher than the probability of an A to G transition. Thus, for estimates of rates of divergence, we employed the Tamura - Nei (1993) model for analysis in addition to the Kimura two-parameter model.

Patterns of Variation. One thousand four of the 1140 bases of the *cyt b* gene were identical in the DNAs of the sun, polar and brown bears examined. Seventy-six sites distinguished the sun bear from the brown-polar bear grouping. Five of the variable sites were autapomorphies in the polar bear, and 14 of the variable sites contained autapomorphies in the brown bear. Lineages GB1, GB 2, GB 3, GB 6, GB 7, GB13, GB 15, GB 18, GB 22, GB 25, and GB 27 each had one, whereas lineage GB 28 had two. Thus, 41 sites (3.6%) were phylogenetically informative for the entire group. These sites are partitioned as follows: 31 (75.6%) involved differences in third positions of codons, 8 (19.5%) involved differences in first positions, and 2 (4.8%) involved differences in second positions. Of the 31 informative third position changes, 2 (6.4%) involved transversions, and of the eight informative first position changes, one involved a transversion. Thus, like other comparisons of closely related animals (Quinn et al. 1991; Irwin, et al. 1991) *cyt b* sequences of brown bears differ from one another predominantly by transition replacements. Brown et al. (1982) and Higuchi et al. (1984, 1987) noted transitional bias in vertebrate mtDNA suggesting that pairwise transition:transversion ratios should decrease with increasing distance between taxa as transition sites become saturated. A mean number of 0.99 transversions (range 0 - 3) characterizes brown bears of different lineages in Alaska (Table 2.3).

Of the 136 variable sites in the sun bear and brown-polar bear complexes, 102 (75%) were variable at third positions of codons, 28 (20.6%) were variable at first positions and 6 (4.4%) involve second position changes. This pattern of variation at different codon positions conforms to that in functional mitochondrial protein-coding genes, in that most of the variation occurs at third position sites, and that the least is observed at second position sites. The highest transition:transversion ratio (104: 4) was found between brown bear lineages GB 6, GB 7, GB 13, GB 15, GB 16 and the sun bear indicating that the time since divergence of the brown bear lineages has not been sufficient to accumulate many transversional changes. A deeper comparison involving published harbor seal *cyt b* sequences (Árnason and Johnsson 1992), as well as *cyt b* sequences from other ursid species (Talbot and Shields 1996a) shows a decrease to very little transition bias (data not shown here, see Chapter 1, Table 1.2). Lineages of Alaskan brown bears are < 3%

divergent (table 3). The highest values of divergence (0.028-0.029) are between bears of the islands of southeastern Alaska (GB 1-GB 5) and those from either Kodiak Island (GB 22, GB 23) or eastern Alaska (GB 10). Sequence divergence between brown bears and polar bears is between 1.0 and 2.9%, the lowest value being between the polar bear and lineage GB 5 (Admiralty and Chichagof Island) and the highest value being between lineages on Kodiak Island (GB 22 and 23) and the polar bear. Differences in sequences of *cyt b* between sun bear and brown bears are 7.7 - 9.6%, with the lowest value being between GB5 and the sun bear and the highest value being between GB 6, GB 7, GB 15, GB 16 and the sun bear (Table 3).

Variability among the Cyt b Genes. Although variation is distributed throughout the *cyt b* gene of bears, of the 136 variable sites 62 (45%) occur in the transmembrane regions. Forty-two (31%) occur in the outer membrane region, and 32 (23%) occur in the inner membrane region. These observations are similar to those of Howell (1989). Overall, the highest variability occurs at the carboxylic end of the gene (positions 1050-1140), and in the middle portion (positions 400-600). Of the 28 variable amino acids observed among bears, five (53.6%) occurred in transmembrane regions, eight (28.6%) occurred in the outer membrane region and five (17.8%) occurred in the inner surface region. Almost all changes in the transmembrane portion are changes between non-polar (hydrophobic) residues (i.e., leucine, isoleucine, methionine, valine, alanine). Although a greater number of amino acid substitutions occurred in transmembrane areas, chi-square analysis (χ^2= 1.269) indicates no significant difference in rate of evolution among the three regions of the membrane and, therefore, the three regions were weighted equally in the weighted parsimony analyses.

Variation at Codon Positions. Because there is variation in evolutionary rates and significant differences in base composition among codon positions of ursids, we have estimated divergences separately for each position using the evolutionary model of Tamura and Nei (1993) and the two-parameter model of Kimura (1980). Estimates of sequence divergence based on these two models are nearly identical at both first and second positions in all pairwise comparisons. Nonetheless, estimates of sequence divergence at third

positions of codons between ursid species (with the exception of polar and brown bear) are consistently higher for the Tamura-Nei estimates than for the corresponding two-parameter corrections. Third positions of codons have the strongest bias in base composition, and the Kimura two-parameter model does not account for such biases, thus the differences are not surprising. Differences in divergence estimates between the two models for most lineages of brown bears, however, were not appreciable. On the other hand, values of divergence between some lineages of brown bears (GB 6-GB 28) and polar bear, between brown bear and sun bear and between polar bear and sun bear were appreciably higher for the Tamura-Nei corrections than for the Kimura two-parameter corrections. Estimates of divergence among third positions of codons among brown bears range from 0.0% to 6.9%. Substitutions at second positions, which usually result in amino acid replacements, show little divergence (0.0%-0.53%) among brown bears. Estimates of divergence for changes at first positions of codons in brown bears range from 0.0% to 2.15%. Estimates of divergence for first positions are almost always slightly higher than those for second positions; this is consistent with the expectation that second positions of codons are more conservative.

Transfer RNAs. Variation among tRNAs is shown in Figure 2.4. Overall the mean rate of substitution for the combined tRNAs of the ursids is slightly higher (112%) than that for the *cyt b* gene. Among all bears examined, the rate of substitution of the $tRNA^{thr}$ gene alone is on average higher (160% overall, 190% among brown bears) than that of the *cyt b* gene, whereas the rate of substitution for $tRNA^{pro}$ is much lower, with no variation detected among sequences of brown bears (Fig. 2.4). The rate of substitution among $tRNA^{thr}$ genes of brown bears and sun bears is approximately 98% that of the *cyt b* gene; while the rate of substitution among $tRNA^{pro}$ genes of brown bears and sun bears is about 68% that of the *cyt b* gene.

We determined the secondary structure of ursid $tRNA^{thr}$ and $tRNA^{pro}$ based on the models of Cantatore et al. (1982) and Gadaleta et al. (1989) to determine the distribution of variation. Among the nine variable $tRNA^{thr}$ positions for the taxa examined, five (1 transversion and four transitions, 55%) occurred in the T-C loop. Two (both transitions,

22%) occurred in the dHU loop. One transition occurred both in the anticodon stem (11%) and in the amino acid stem (11%). Among $tRNA^{thr}$ genes of brown bears, variation occurred only in the dHU loop (one transition) and in the T-C loop (one transversion and two transitions).

Seven variable sites, all transitions, occurred in the $tRNA^{pro}$ genes of all taxa examined. Three (43%) occurred in the anticodon stem, two (29%) in the T-C stem and one each in the variable loop (14%) and the T-C loop (14%). No variation occurred in the $tRNA^{pro}$ genes of brown bears. $TRNA^{pro}$ genes of the polar bear differed at three sites from those of the brown bear and at seven sites from those of the sun bear. Those of the sun bear differed from those of the brown bear at four sites.

Lineages of tRNAs. Transfer RNA^{thr} and $tRNA^{pro}$ sequences have been deposited in GENBANK (Accession nos. U18900 to U18905); again, users are expected to refer to the present paper and not only to the accession numbers. One lineage for $tRNA^{thr}$ is fixed in populations of brown bears of the islands of southeastern Alaska and is identical to that in polar bears (Fig. 2.4). A second lineage occurs in brown bears of "eastern" Alaska (*cyt b* lineages GB 8, GB 9, GB 10, GB 12 and GB 18) in regions of southcoastal and southeastern coastal Alaska, ANWR (n = 12, 71%), and in low frequency in: the Alaska Range (n = 4, 26%), the Brooks Range (n = 8, 13%) and in the interior (n = 1, 20%) of Alaska. A third $tRNA^{thr}$ lineage is fixed or nearly so in populations of "western" Alaska, which comprise the remainder of the Alaska lineages (GB 6, GB 7, GB 11, GB 13, GB 14, GB 16, GB 17, GB 19-24) and in the lineages of eastern Siberia (GB 25 -27). Geographically, this third $tRNA^{thr}$ lineage is fixed on Kodiak Island (n = 22), Katmai Nat'l Park (n = 23), Izembek Nat'l Wildlife Refuge (n = 14), Seward Peninsula (n = 19) and southcentral Alaska (n = 5). It occurs in high frequency in the Brooks Range (n = 16, 84%), interior Alaska (n = 4, 80%) and the Alaska Range (n = 11, 73%) and in lower frequency in brown bears of the ANWR (n = 5, 29%). Thus, three different $tRNA^{thr}$ lineages are described among brown bears of Alaska and eastern Siberia; another is observed in brown

bears from Turkey. The three Alaskan lineages are broadly correlated with different geographic regions. Sequence divergence values for tRNA genes are shown in Tables 2.4a and 2.4b.

Phylogenetic Estimates: Distance Analysis. Because only four variable sites were identified among the two tRNAs of brown bears examined, we performed phylogenetic analyses using the combined $tRNA^{pro}$, $tRNA^{thr}$ and *cyt b* gene sequences. The neighbor-joining distance method was used to examine relationships among brown bear lineages and to identify closely related lineages for selection as representatives for the subsequent parsimony analysis. The topology of the tree generated using the Tamura-Nei distance algorithm (a biased estimator) was identical to that generated using the Kimura two-parameter model (Fig. 2.5). These analyses indicate that lineages of brown bears from Alaska belong to two major clusters. One cluster, comprised solely of lineages GB 1-5, and found exclusively on the ABC islands (Admiralty, Baranof and Chichagof Islands) of southeastern Alaska, is a sister clade to the polar bear. This clade is remarkably separable not only from the remainder of the lineages of brown bears in Alaska but also from lineages of brown bears from eastern Siberia (GB 25, GB 26 and GB 27) and Turkey (GB 28).

A second major clade is composed of two smaller, distinct clusters; one comprised of bears mostly from populations of eastern Alaska, ANWR, Alaska Range, the coasts of south and southeast mainland, and to a lesser degree in the Brooks Range and the eastern portion of the interior (Fig. 2.5). The second small cluster includes bears predominantly from areas of western Alaska including Kodiak Island, Seward Peninsula, the Alaska Peninsula (Katmai and Izembek), and southcentral and interior Alaska. This latter cluster also includes lineages from Turkey and eastern Siberia.

Phylogenetic Estimates: Parsimony Analysis. Eleven lineages of brown bears (GB 2, GB 3 and GB 5 (joined to GB 1); GB 6, GB 7, GB 13, GB 15 and GB 16 (joined to GB 19); GB25 and GB 27 (joined to GB 26); and GB 22 (joined to GB 21)) defined by autapomorphies were collapsed onto other lineages for analyses using maximum parsimony. Unweighted branch-and-bound analysis of maximum parsimony produced 72

trees (CI=0.68, length=66, g1= -0.727297, Hillis and Huelsenbeck 1992). A consensus tree based on 50% majority rule was produced (Fig. 2.6a). Using the sun bear as an outgroup, all trees placed the polar bear in the same clade with brown bears from the islands of southeastern Alaska and separate from the clade containing all remaining lineages of brown bears. As with the distance analysis, Alaskan brown bears are paraphyletic with respect to polar bears (Fig. 2.6a). The remainder of Alaskan brown bears is placed in a second clade, which is comprised of two subclades, one contains lineages of bears generally occurring in eastern Alaska. Another clade is composed largely of lineages of bears occurring in western Alaska as well as in eastern Siberia (GB 26). This latter clade contains an unresolved polytomy consisting of lineages of brown bears from Seward Peninsula, Kodiak Island and arctic Alaska (ANWR), as well as GB 19, which occurs in seven of ten Alaskan regions we examined. Turkish bears cluster outside the second and third clades, but diverge subsequent to the lineage leading to the polar bear and to the brown bears of the ABC islands. Five-hundred resamples using bootstrapping with replacement of characters support these relationships (Fig. 2.6a).

Because the rate of nucleotide substitutions at first and second positions is approximately 34% and 12.5%, respectively, and the rate of third position replacements in the brown bear *cyt b* gene is 53.5% (Table 2.3), we performed a parsimony analysis that weighted second positions of codons eight times that of third position replacements, and first positions three times that of third position replacements. Additionally, since transitions occur approximately 13 times more frequently than transversions, we weighed transversions 13 times as heavily as transitions. This weighted analysis resulted in 36 "most-parsimonious" trees (CI=0.83, length= 140). The majority rule consensus tree generated from these 36 trees (Fig. 2.6b) agrees in topology with the consensus tree generated in the unweighted analysis of parsimony, with the exception of a resolution of the placement of GB 14 (Brooks Range) relative to GB 17 (Alaska Range) and GB 26 (E. Siberia). The brown bears of the ABC islands are again paraphyletic in relation to polar bears, here grouped with GB 1 (Admiralty Island) and diverging from the remainder of the brown bears, which form a separate clade.

Because transitions accumulate more rapidly than transversions, particularly at third positions of codons (Brown et al. 1982), parsimony analysis may be more accurate if some or all transition sites are ignored. Nonetheless, analyses using transversions alone as well as amino acid replacements did not resolve these relationships as too few of these changes existed among taxa examined. Consequently, inference regarding the branching order relies on the rapidly evolving transitions at third positions of codons.

Comparison of Distance (Neighbor-Joining) and Parsimony Trees. The distance and maximum parsimony analyses resulted in essentially the same tree topologies, with each placing Alaskan brown bears into two major clusters, one orienting bears of the islands of southeastern islands of Alaska paraphyletically with polar bears and the other containing all other brown bears. Distance analysis resulted in the same configuration except it placed GB 18 into one clade while parsimony placed it into another.

2.5 DISCUSSION

Uniqueness of Brown Bears of Southeast Alaska. Our analyses suggest at least two major clades of mtDNA of brown bears in Alaska: brown bears of Admiralty, Baranof and Chichagof Islands of southeastern Alaska (lineages GB 1-GB 5) and all other brown bears regardless of their geographic location. Lineages of brown bears of the islands of southeastern Alaska are fixed for 23 nucleotide substitutions, two of which are transversions. When included in a data set from another study which includes partial sequences of the *cyt b* gene as well as portions of the control regions of brown bears from a more extensive sampling across the present range of the distribution of the species (Waits et al. manuscript), lineages GB 1-GB 5 again appear unique and in a paraphyletic orientation with respect to polar bears. Consequently, they appear to be the oldest lineages of brown bears in the New World.

Irwin et al. (1991) used divergence in mtDNA sequences to date times of taxonomic separation of mammals. Assuming a rate of about 10% per million years for substitutions at third positions of codons of mitochondrial genes and applying this rate to the data on cytochrome b reported here, mtDNA lineages GB 1-GB 5 separated from other mtDNA

lineages of brown bears approximately 550,000 to 700,000 years ago. Thus, brown bears on the ABC islands of southeastern Alaska are unique in this study because of their apparent antiquity, homogeneity of their mtDNA sequences (Fig. 2.3) and because of their close relationship to polar bears.

Genetic uniqueness of the brown bears of the ABC islands may be correlated with morphological and behavioral traits. Brown bears on these islands have significantly smaller skulls than brown bears from: the adjacent coastal plain, the Alaska Range and Kodiak Island (K. Titus, pers. comm.). Some of these bears have jet-black pelage and are often misidentified as "huge" black bears by casual observers (J. Schoen, pers. comm.) Finally, there is evidence from radio-telemetry of bears on Admiralty Island that indicates essentially no recent flow of genes between Admiralty and other islands of the archipelago or between Admiralty and the coastal mainland, despite speculation by early investigators that significant exchange of animals occurred in that region (Merriam 1918; Rausch 1953). From 1982 to the present, more than 300 bears have been radio-collared on Admiralty Island and none of these bears has dispersed from the island (Titus, Pers. comm.). Strong oceanic currents in the Chatham Strait and other marine waters of the region may prevent gene flow between the islands and the coastal mainland.

The uniqueness of brown bears inhabiting the ABC islands of southeastern Alaska has implications for interpretation of the Pleistocene history of the area. Geological evidence suggests that much of the Pacific coast of North America was under the Cordillerian ice sheet during the last glaciation (Flint 1971, Clague 1989). However, numerous authors suggest that, during the last glacial maximum, ice-free refugia were scattered along the shores of what is now the Alexander Archipelago of southeast Alaska and British Columbia (Heusser 1972, Fladmark 1979, Warner et al. 1982, Rogers et al. 1991). These refugia are thought to have provided safe haven and to have served as stepping stones in the migrations of organisms at a time when other routes between Beringia and ice-free North America were blocked by ice. The glacial history of the region is complicated, however, and most evidence supporting lowland refugia in this area consists of a suite of unique biological features on Queen Charlotte and Vancouver Islands of British Columbia (Calder and Taylor 1968). These include endemism in birds and

mammals (McCabe and Cowan 1945, Foster 1965), crustaceans (Bousfield 1958) and insects (Kavanaugh 19800), as well as disjunct distribution and endemism of vascular plants (Ogilvie 1989) and bryophytes (Schofield 1969, 1989). Patterns of mtDNA diversity in freshwater sticklebacks of the Queen Charlotte Islands are also indicative of a Pleistocene refugium in the area (O'Reilly et al. 1993).

The existence of an ancient, endemic mitochondrial DNA lineage in brown bears from the ABC islands in the Alexander Archipelago of southeastern Alaska is consistent with the hypothesis of a northwestern coastal refugium. It is possible that, prior to deglaciation and associated rise in sea level, the outer coasts of southeastern Alaska and British Columbia were physiographically comparable to the present mainland coastline of the area. As the sea level rose and habitats, food resources and competitive interactions changed, brown bear populations surviving the expanding ice in coastal refugia may have become locally extinct, surviving in the region only on the ABC islands. Additional support for the northwestern coastal refugium hypothesis comes from a recent find of a fossil femur of a brown bear on the Prince of Wales Island, dated to over 35,000 years B.P. (Heaton, pers. comm., 1995). Brown bears no longer occur on this island and the most recent fossil and subfossil remains suggest they disappeared from the island after approximately 7,000 years B.P. Thus, the oldest fossil remains of brown bear in the area (35,000 years B.P) places them in the Alexander Archipelago well before the Wisconsin glacial maximum (ca. 18,000 years B.P.) but still within the Wisconsin glacial period (Heaton, pers. comm. 1995). We hypothesize that, subsequent to deglaciation, the southeastern coastal mainland may have been recolonized by brown bears radiating from a more recently derived clade (the "eastern" lineages herein) which were previously isolated in the Beringian refugium.

The proposed antiquity and genetic uniqueness of brown bears of Admiralty, Baranof and Chichagof islands may have ramifications for ongoing and proposed timber harvests in that region. Harvests of timber can lead to habitat fragmentation and increased bear-human confrontations. In such situations the ultimate result is usually a reduction in the numbers of bears (Schoen 1990, Schoen et al. 1995).

Paraphyly of Brown Bears with Respect to Polar Bears. Of the 23 substitutions that are fixed in brown bears of the islands of southeastern Alaska, 20 are identical to those of polar bears. Moreover, in all analyses polar bears are a sister taxon to the brown bears of the ABC Islands. This association holds true as well for the *cytb*/control region data set which includes bears from across the range of distribution (Waits et al. manuscript). Again using the procedures of Irwin et al. (1991) and employing the Tamura-Nei correction to divergence levels, we date the separation of mtDNAs of polar bears from those of brown bears of the islands of southeastern Alaska at about 200,000-250,000 years ago.

Thus, our sequence data support the close phylogenetic relationship for brown bears and polar bears predicted by Shields and Kocher (1991) using restriction fragment length polymorphisms of mtDNA and a more restricted set of sequences of the *cyt b* gene and the control region. Our data also support the paraphyletic association of brown bears with respect to polar bears proposed by Cronin et al. (1991) and by Taberlet and Bouvet (1992). Mt DNA evidence for paraphyletic relationships among closely related species is not uncommon (Avise et al. 1983, 1990; Lehman et al. 1991, Wayne and Jenks 1991) and may be the result of introgressive hybridization or random sorting of ancestral lineages during speciation (Avise 1986, Neigel and Avise 1986).

Brown bears on islands in southeastern Alaska may represent descendants of ancient ursids that diverged from other lineages of brown bears and subsequently founded the polar bear lineage. It is possible that this association is based on shared common ancestry rather than introgressive hybridization because Kurtén (1964, 1968) cites fossil evidence that polar bears arose from coastal brown bears. Polar bears have many derived morphological characters which have been used to identify them as a monophyletic group and these bears have been cited as an example of quantum speciation (Stanley 1979). Shields and Kocher (1991) point out that the similarity of mtDNA of brown and polar bear is not surprising considering the ability of the two species to produce fertile offspring in captivity (Kowalska 1965). Morphological divergence of polar bears has apparently occurred within the last 20,000-40,000 years. Although the fossil record is incomplete, polar bears likely diverged from the brown bear lineage less than 300,000 years ago (Kurtén 1968). Pamilo and Nei (1988) caution that phylogenetic trees based on mtDNA data may not reflect true species

trees and resolution of this paraphyletic association should be tested by analysis of nuclear genes.

The other major cluster of lineages of brown bears is shallow in time depth and includes bears from all other regions of this study including Turkey, eastern Siberia and all regions of Alaska, except the southeast islands. This suggests that bears in Alaska represented by these lineages probably entered the New World somewhat more recently than bears of the ABC islands and that the former share a most immediate common ancestry with bears from Eastern Siberia and Turkey. A similar pattern of relationships emerges from the *cyt b*/control region data set (Waits et al. manuscript). Bears from the mainland coast of southeast Alaska and some bears from ANWR, the Brooks Range, the interior, the Alaska Range and south coastal regions form a subclade within this larger second clade. Although the generalization is a broad one, the presence of a mtDNA clade in western Alaska and a mtDNA clade in northeastern Alaska (Fig. 2.7) may reflect a habitat preference for tundra in the west and for forested or taiga areas in the northeast.

The earliest fossils of the *U. arctos* line are recognized from China during the middle Pleistocene (Kurtén 1968). Nonetheless, fossil records indicate the brown bear group did not pass into North America until 4 Würm (late Pleistocene), and then it was supposedly confined to Alaska by the continental ice sheet, spreading southward only during postglacial times (Craighead and Mitchell 1982). After the continental ice flows retreated, *U. arctos* was able to expand its range and fossil evidence and historic accounts suggest that brown bears subsequently occurred throughout most of western North America and south to Mexico with concentrations in the Rocky Mountains and major river valleys (Craighead and Craighead 1967, Dood et al. 1986). Subsequent to the westward expansion of Europeans into North America, however, populations of brown bears were greatly reduced and by the early 1900s were virtually eliminated throughout most of the conterminous United States.

Variation Among tRNAs. We have observed a relatively rapid rate of change among the $tRNA^{thr}$ genes of this study. Brown et al. (1982) and Thomas et al. (1989) observed that tRNA genes of mitochondria evolve more slowly than most protein-coding

genes of mitochondria. In this study, mean pairwise sequence divergence among $tRNA^{thr}$ genes (.0396) is high when compared to that of *cyt b* genes (.0332). Alternatively, the rate among $tRNA^{pro}$ genes (.0216) is low. Rate of change among $tRNA^{thr}$ genes is among the most variable of all tRNAs of the mitochondrian (Gadaleta et al. 1989). Cantatore et al. (1982) and Gadaleta et al. (1989) compared secondary structure of homologous tRNAs of the mitochondria of various mammals and found an overall increase in variation in dHU loops, T-C loops, and in "extra" variable arms. The lowest variation was found in the A-C loops. Specifically, for $tRNA^{thr}$, Gadaleta et al. (1989) found that for vertebrate $tRNA^{thr}$, 75-99% of the variation occurred in the T-C loop, 55-74% in the amino acid stem and 30-54% in the dHU loop. For $tRNA^{pro}$, they determined that most of the variation was found in the T-C loop (75-99%), with the remaining variation being distributed generally among the anticodon, amino acid and T-C stems (55-74%). Thus, both tRNA genes studied here conform to their proposed model. All individuals of the present study use the $tRNA^{thr}$ anticodon TGT (UGU) and the $tRNA^{pro}$ anticodon GGT (GGU).

Subspecific Relationships. Hall (1984), Kurtén (1973) and Rausch (1963) proposed different designations for subspecies of Alaskan brown bears based on a phenotypic approach with the underlying assumption that phenotypic differences reflected, to varying degrees, the genotypic and, therefore genetic differences between the populations examined. Here we have presented evidence for phylogenetic distinction of several populations of brown bears in Alaska based on analysis of mtDNA. We can detect a relationship between phylogeny and geographic distribution in some, but not all, putative subspecies of brown bears in Alaska. Therefore, our mtDNA support some, but not all, of the currently designated subspecies.

Bears of southeastern Alaska - Only Hall (1984) designates subspecific status (*U. a. sitkensis)* to the bears of southeastern Alaska. However, he includes brown bears of the mainland of this region within this subspecies. On the contrary, sequences of mtDNA obtained from brown bears of the coastal regions of the mainland of southeastern Alaska

(lineage GB 9) indicate these bears belong to a separate clade (Fig. 2.5) and not with the clade composed of bears from the ABC islands of southeast Alaska. Thus, the *U. a. sitkensis* designation of Hall (1984) is not uniformly supported by our molecular data.

Southcoastal Alaskan brown bears - Similarly, our data indicate that bears of southcoastal Alaska, represented here by GB 8, are genetically divergent from most of the remainder of Alaskan bears; however, this lineage occurs in bears from the Alaska Range as well as in a single bear from the Brooks Range, so the subspecies designation (*U. a. dalli)* for brown bears of southcoastal Alaska alone (Hall 1984, Kurtén 1973) is not fully supported.

Bears of the mainland-noncoastal regions - Populations of bears from the Brooks Range, Alaska Range, the Arctic, Interior, and southcentral Alaska have been designated as *U. a. horribilis* by Kurtén (1973) and by Rausch (1963) and as *U. a. alascensis* by Hall (1984). Our molecular data show that brown bears from this region belong to at least 13 different lineages, one of which (GB 19) occurs on Kodiak Island and on the Alaska Peninsula, while another (GB 8) occurs in southcoastal Alaska. Many of these lineages are unique to a certain geographic region, but differ from other lineages in the same population by only one or two nucleotide substitutions.

Brown bears of the Alaska Peninsula - The designation of *U. a. gyas* (Hall 1984) to populations of brown bears on the Alaska Peninsula is not fully supported by our data. Populations on Izembek National Wildlife Refuge appear to be fixed for the GB 19 *cyt b* lineage. On the other hand, most (n = 23, 87%) bears sampled from Katmai National Park belong to a unique lineage that differs from GB 19 by one change in a base pair; the remaining 13% of bears from Katmai share the GB 19 lineage with most of Alaskan bears. Information from the hypervariable segment of the control region of the mtDNA (data not shown, manuscript in prep.), however, indicate that bears of the Alaska Peninsula, Katmai NP and Izembek NWR, along with bears from southcentral Alaska can be distinguished

from the Kodiak Island lineage (GB 19) at from 1-2 nucleotide sites. This indicates at least the possibility of some gene flow among these populations.

Brown bears of Kodiak Island - Three taxonomic schemes give subspecies status (*U. a. middendorffi)* to bears of Kodiak Island. While at least four unique lineages of mtDNA occur on Kodiak Island (GB 20-23), our sequence data indicate that the most prevalent lineage (GB 19, 9/22 bears = 41%) there also occurs in varying proportions in all other regions of mainland and coastal Alaska we examined, except in southcoastal, southeast coastal and southeast island populations. This indicates that some gene flow occurs, or has recently occurred, between Kodiak Island and the mainland, or that Kodiak Island may have been colonized by bears from mainland Alaska. Four additional unique lineages on Kodiak differ from lineage GB 19 by one or two substitutions. Because these lineages have not been observed outside of Kodiak, reduced gene flow between Kodiak and the adjacent mainland is suggested. Our *cyt b* and tRNA data do not support the hypothesis that bears of Kodiak Island are completely differentiated genetically from bears on the mainland of Alaska. However, we cannot reject the hypothesis that populations of bears on Kodiak Island were recently colonized by bears from the mainland and are now experiencing very limited gene flow. Kodiak Island was glaciated during the Wisconsin period, although Kurtén (1973) cites evidence that a refugium existed there and was at least large enough to support plants. It is likely that brown bears colonized Kodiak after glacial retreat.

If we adhere to the concept that designations of subspecies should be based on phylogenetic differentiation from other groups (Avise and Ball 1990), we should reject the hypothesis that Kodiak Island bears constitute a separate subspecies distinct from the remainder of Alaskan bears. Correspondingly, because of their considerable genetic differentiation from other brown bears in Alaska, it seems reasonable to designate the bears of the ABC islands of southeastern Alaska as a separate subspecies.

Overall, our data indicate that some populations of brown bears of Alaska are geographically substructured and are experiencing reduced, or in some cases, no gene flow. Diversity in mtDNA sequences appears to be lowest within peninsular populations of

brown bears, with the least variation occurring among brown bears inhabiting Izembek NWR and Katmai NP (Alaska Peninsula), and the Seward Peninsula. This pattern supports the hypothesis that peninsular populations may demonstrate reduced genetic variation due to reduced immigration and therefore gene flow .

Alaskan brown bears constitute at least two major clades and a third subclade: one (ABC islands) is clearly divergent from the rest while the other two comprise an "eastern" Alaskan clade with bears largely from: ANWR, Alaska Range, southcoastal and southeast coastal areas of Alaska and a "western" Alaskan clade, comprised largely of bears from the Seward Peninsula, Kodiak Island, the Alaska Peninsula, Brooks Range, interior and southcentral. The presence of many different lineages in the interior, Brooks Range and southcoastal regions and the presence of lineages from the two mainland clades in single populations (Brooks Range, Alaska Range, Interior and ANWR) suggest that a significant level of gene flow has occurred among these areas. Our molecular data suggest that brown bears in Alaska may have undergone periodic episodes of dispersal accompanied by gene flow followed by periods of stable population structure accompanied by restricted gene flow. Analysis of additional genes, both nuclear and mitochondrial, may allow a more detailed description of these processes.

Brown bears of Asia - We have shown that 23 lineages of brown bears, including those from Turkey and Siberia, diverged after the split of brown bears on the ABC islands and polar bears. Thus, there is considerable genetic diversity among brown bears of Alaska. The *cytb*/control region data set strongly suggests that both old and new lineages of brown bears occur in Europe (Waits et al. manuscript) and that at least three major clades occur in the New World. Thus, the considerable divergence between European brown bears and the progenitor of Alaskan brown bears and polar bears hypothesized by Taberlet and Bouvet (1992) is supported by the larger data set. Additional analysis of European and Siberian brown bears as well as those from Canada may provide further clarification of the complexity of migratory patterns into the New World from Asia.

The proposal by Kurtén (1973) that brown bears of mainland Alaska show affinities to bears of eastern Siberia is supported by our mtDNA data. Siberian bears from Chukotka

and from the area around the Sea of Okhotsk are closely related to the brown bears of western Alaska; all cluster into the third clade of our analysis. Kurtén's hypothesis (1973) that bears of Kodiak Island share affinities with bears of the Kamchatka Peninsula could not be directly tested in our study. However, our mtDNA data place bears of Kodiak Island in the same clade with bears of eastern Siberia.

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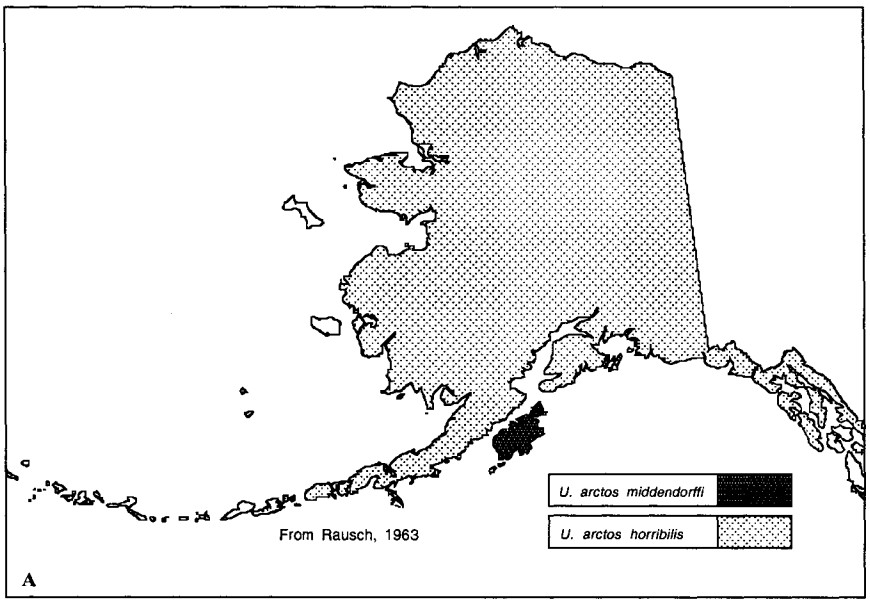

Figure 2.1a. Geographic distributions of taxa of brown bear in Alaska according to Rausch (1963).

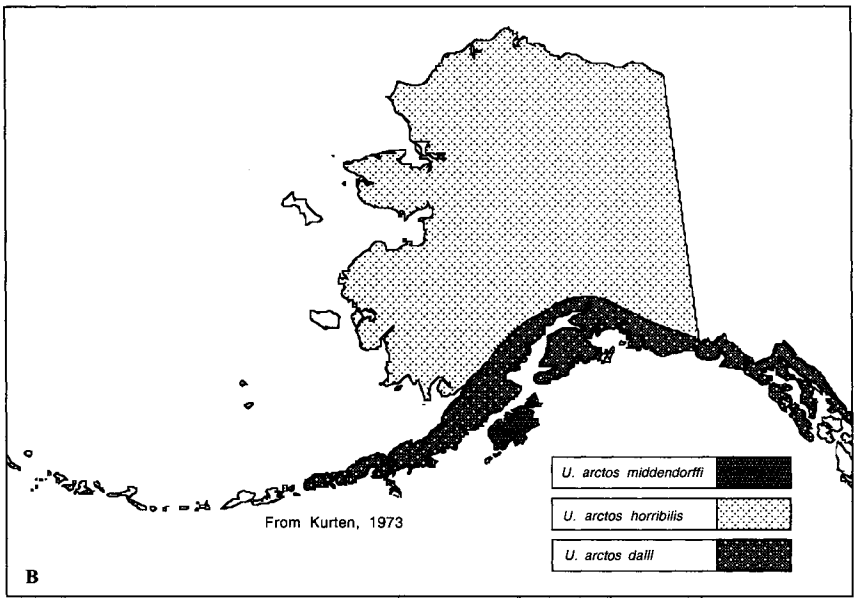

Figure 2.1b. Geographic distributions of taxa of brown bear in Alaska according to Kurtén (1973).

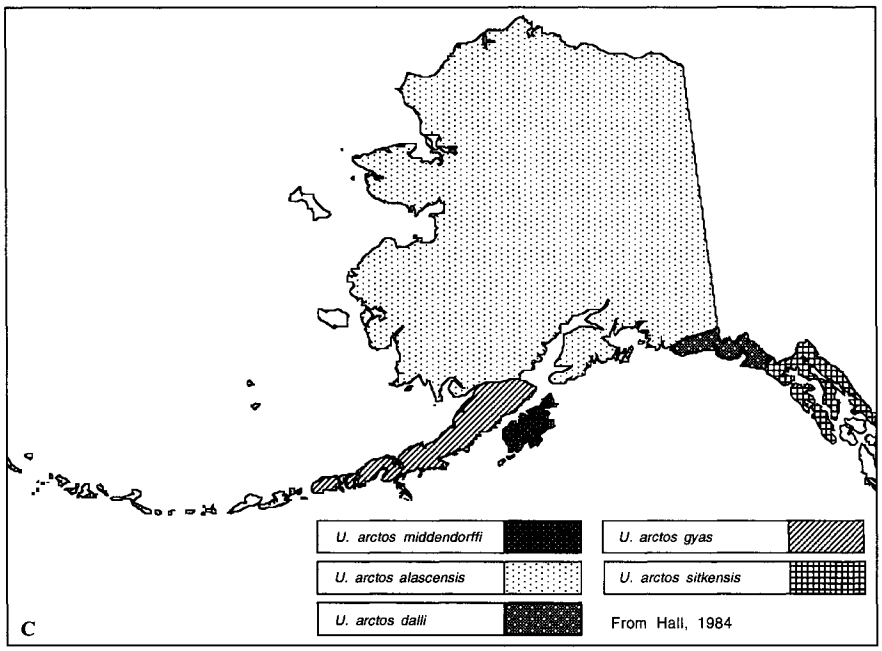

Figure 2.1c. Geographic distributions of taxa of brown bear in Alaska according to Hall (1984).

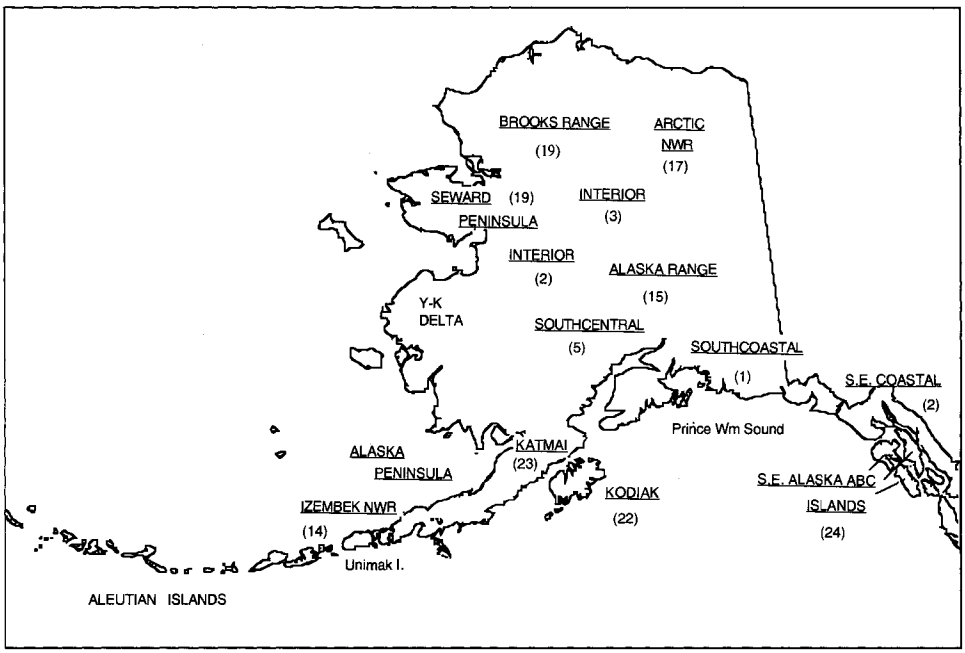

Figure 2.2. Sample sizes and collection locations in Alaska for the 166 brown bears analyzed in this study.

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       1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
       4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5
       8 8 8 9 9 9 9 9 0 0 0 0 1 1 1 1 1 1 2 2 2 2 2 3 3 3 4 4 4 4 4 5 5 5 5 5 5 5 6 6 6 6 7 7 7 7 7 8 8 8 8 8 8 8 8
       0 6 9 1 3 3 4 7 1 3 7 9 1 2 2 5 7 9 0 1 3 4 6 3 4 6 0 1 2 5 6 0 1 3 5 6 6 9 2 5 6 8 0 2 8 9 9 0 2 3 4 5 6 7 8
       4 4 7 6 3 6 2 5 4 2 5 5 4 4 6 2 6 1 4 2 3 5 9 5 1 2 5 1 9 4 0 9 7 9 7 0 3 6 8 5 3 1 2 3 3 5 8 7 8 5 9 1 4 2 5
GB01   T G T T T T A G G C T C C G T A A T G T A G T C A C A C G A G C T C C G G C C C T A G T C T C A A C C C T C G
GB02   . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . .
G803   . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . .
GB04   . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . A
GB05   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . .
GB06   . A C C . C G . C T C . . A . G G C A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB07   . A C C . . G . C T . . . A . G G C A C G . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB08   . A C C . . G A C T . . . A . G G . A C . . C T G T C T A G A T C T . A . T . A . . A . T . . . . . T T . . .
GB09   . A C C . . G A C T . . . A . G G . A C . . C T G T C T A G A T C T . A . T . A C . A . T . . . . . T T . . .
GB10   . A C C C . G A C T . . . A . G G . A C . . C T G T C T A G A T C T . A . T . A . . A . T . . . . . T T . G .
GB11   . A A C C . G . C T C . . A . G G . A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB12   . A C C C . . A C T . . . A . G G C A C . . C T G T C T A G A T C T . A . T . A . . A . T . . . . . T T . G .
GB13   . A C C . . G . C T C . . A . G G . A C . . C T G T . T . G A T C T . A . . . A . . A . T C . . . T T T C . .
GB14   C A C C . . G . C T . . . A . G G C A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB15   . A C C . . G . C T C . . A . G G C A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB16   . A C C . . G . C T C . . A . G G C A C . . C T G T . T . G A T C T . A . T T A . . A . T . . . . T T T C . .
GB17   . A C C . . G A C T . . . A . G G . A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
G818   . A C C . . G . C T C . . A . G G C A C . . C T G T . T . G A T C T . A . . . A . . A . T . T . . . T T . . .
GB19   . A C C . . G . C T C . . A . G G C A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB20   . A C C . . G . C T C . . A . G G C A C . . C T G T . T . G A T C T . A . . . A . G A . T . . . . T T T C . .
GB21   . A C C . . G . C T C . . A . G G C A C . A C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB22   . A C C . . G . C T C . . A . G G C A C . A C T G T . T . G A T C T . A A . . A . G A . T . . . . T T T C . .
GB23   . A C C . . G . C T C . . A . G G C A C . A C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB24   . A C C . . G . C T C T . A . G G C A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB25   . A C C . . G . C T C . . A . G G . A C . . C T G T . T . G A T C T T A . . . A . . A . T . . . . T T T C . .
GB26   . A C C . . G . C T C . . A . G G . A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . . T T T C . .
GB27   . A C C . . G . C T C . . A . G G . A C . . C T G T . T . G A T C T . A . . . A . . A . T . . . G T T T C . .
GB28   . A C C . . G . C T . . . A . G G . A C . . C T G T . T . G A . C T . A . . . A . . A C T . . G . . . T C . .
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Figure 2.3. DNA sequence variation among cytochrome b genes for the 28 lineages of brown bears in this study.

	1 1 1 1 1 1 1 1 1...	...1 1 1 1 1 1 1
	5 5 5 5 5 5 5 5 5...	...5 5 5 5 5 6 6
	9 9 9 9 9 9 9 9 9...	...9 9 9 9 9 0 0
	0 0 2 3 4 4 4 4 5....	..7 7 7 8 8 0 0
	4 5 5 9 2 3 4 7 3...	...1 4 8 1 5 0 1
TAXA	tRNA-thr	tRNA-pro
GB01	A C G A T C T C T	C C T A C A A
GB02	. . . .	. . . .
GB03	. . . .	. . . .
GB04	. . . .	. . . .
GB05	. . . .	. . . .
GB06	G . . T C	. . . .
GB07	G . . T C	. . . .
GB08	G . . . C . . T .	. . . .
GB09	G . . . C . . T .	. . . .
GB10	G . . . C . . T .	. . . .
Gb11	G . . T C	. . . .
GB12	G . . . C . . T .	. . . .
GB13	G . . T C	. . . .
GB14	G . . T C	. . . .
GB15	G . . T C	. . . .
GB16	G . . T C	. . . .
GB17	G . . T C	. . . .
GB18	G . . . C . . T .	. . . .
GB19	G . . T C	. . . .
GB20	G . . T C	. . . .
GB21	G . . T C	. . . .
GB22	G . . T C	. . . .
GB23	G . . T C	. . . .
GB24	G . . T C	. . . .
GB25	G . . T C	. . . .
GB26	G . . T C	. . . .
GB27	G . . T C	. . . .
GB28	G . . . C	. . . .
POLAR	. . . .	T T . . . . G
SUN	G T A . . T C . C	. . C G T G .

Figure 2.4. Sequence variation in $tRNA^{thr}$ and $tRNA^{Pr0}$ among 28 lineages of brown bears, a polar bear and a sun bear. Lineage designations for brown bears follow those described for the cyt b gene (Figure 2.3). Numbers refer to the nucleotide positions described for the human consensus sequence (Anderson et al. 1981).

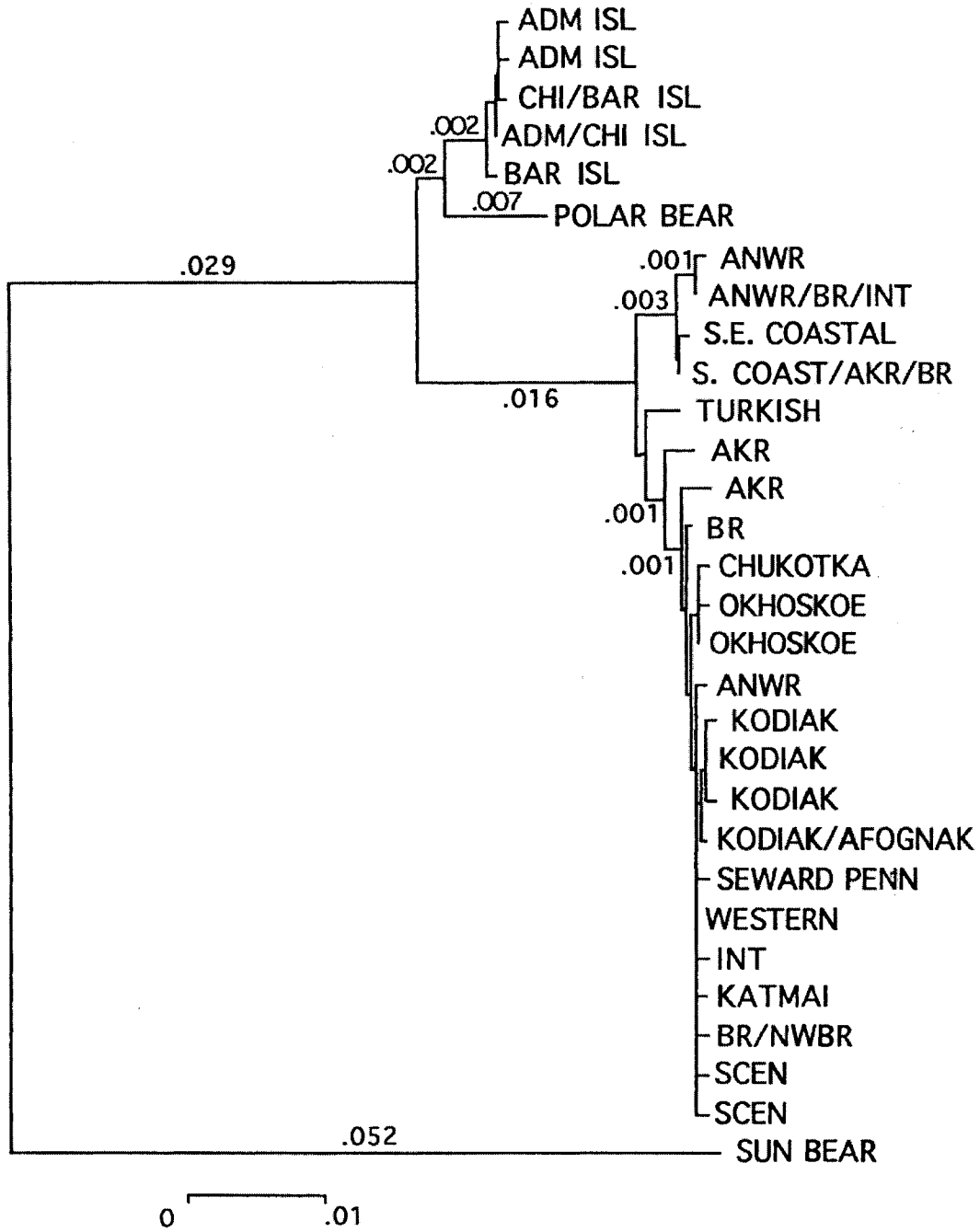

Figure 2.5. Neighbor-joining distance tree for the 28 lineages of brown bears, a polar bear, and a sun bear based on the two-parameter model of Kimura (1981).

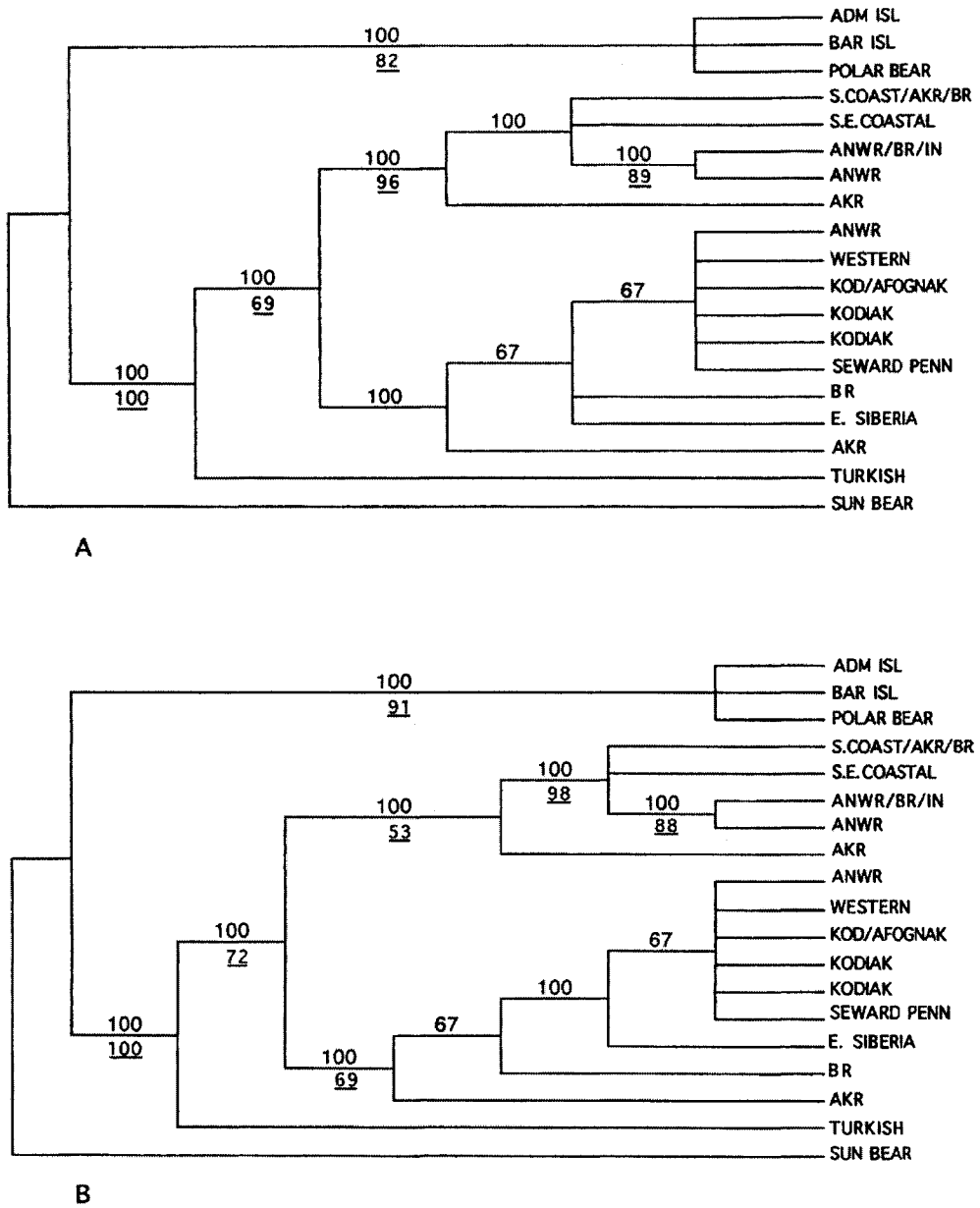

Figure 2.6. a) Majority-rule consensus tree based on maximum parsimony and using an unweighted branch-and-bound analysis. Underlined values denote bootstrap values for branches tested. b) Weighted majority-rule consensus tree based on maximum parsimony and weighted branch-and-bound analysis. Cytochrome b positions were weighted 3:8:1; transversions were weighted 13 times the transitions. Underlined values denote bootstrap values for branches tested. The "western" lineages listed here refers to lineage GB19.

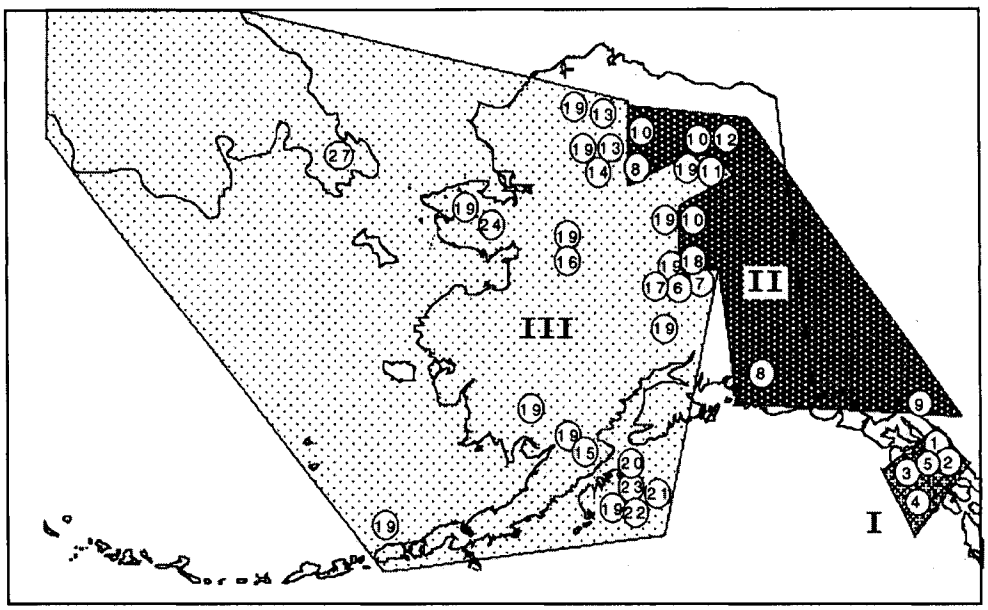

Figure 2.7. Geographic distribution of the three clades among 28 lineages of brown bears.

Table 2.1. Primers for amplification and sequencing of cytochrome b, $tRNA^{thr}$ and $tRNA^{pro}$ genes of mitochondrial DNA of bears.

Name of primer	Sequence
L14702	5'-CACATGGAATCTAACCATGACCA-3'
L14841[1]	5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'
H15149[1]	5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'
H15273	5'-AAGTGGAAAGCAAAGAATCGTG-3'
L15168	5'-TGAGGACAAATATCGTTCTG-3'
H15590	5'-CTCCTAGTTTATTAGGGATGGATCG-3'
L15513[2]	5'-CTAGGAGACCCTGACAACTA-3'
H15915	5'-CTCCGTTTTTGGCTTACAAG-3'
L15774[1]	5'-GTAAACGACGGCCAGTACATGAATT GGAGGACAACCAGTC-3'
H00019	5'-CCACAGTTATGTGTGATCATG-3'

Names identify the DNA strand (H or L) and the position of the 3'end of the oligonucleotide according the the numbering system for the human sequence (Anderson et al. 1981)

[1]Primers obtained from T. Kocher

[2]Primer described by Irwin et al. (1991)

Table 2.2. Geographic locations of brown bear lineages.

Lineage	(N)	Locality
Southeast Alaskan Islands n = (24)		
	GB01	Admiralty Island
	GB02	Admiralty Island
	GB03	Baranof Island
		Chichagof Island
	GB04	Baranof Island
	GB05	Admiralty Island
		Chichagof Island
Southcentral Alaska (5)		
	GB06	Nelchina
	GB07	Upper Susitna
	GB19	Lower Susitna
		Wasilla
		Bristol Bay
Southcoastal (1)		
	GB08	Cordova
Southeast coastal (2)		
	GB09	Bradfield Canal
Brooks Range (19)		
	GB08	Brooks Range
	GB10	Brooks Range
	GB13	Brooks Range
		Northwest Brooks Range
	GB14	Brooks Range
	GB19	Brooks Range
		Northwest Brooks Range
Interior (5)		
	GB19	Healy
		McGrath
		Tanana Flats
	GB10	Healy
	GB16	McGrath

Table 2.2. continued

Lineage	(N)	Locality
Alaska Range (15)		
	GB08	Alaska Range
	GB17	Alaska Range
	GB18	Alaska Range
	GB19	Alaska Range
Kodiak Island (22)		
	GB19	Kodiak Island
	GB20	Kodiak Island
		Afognak Island
	GB21	Kodiak Island
	GB22	Kodiak Island
	GB23	Kodiak Island
Seward Peninsula (19)		
	GB24	Seward Peninsula
	GB19	Seward Peninsula
Alaska Peninsula (37)		
	GB19	Katmai National Park
		Izembek NWR
	GB15	Katmai National Park
Arctic Alaska (17)		
	GB10	Arctic NWR
	GB11	Arctic NWR
	GB12	Arctic NWR
	GB19	Arctic NWR
Eastern Siberia (4)		
	GB25	Sea of Okhotsk
	GB26	Sea of Okhotsk
	GB27	Chukotka Peninsula
Turkey (2)		
	GB28	Artvin,Turkey

Table 2.3. Pairwise differences in transitions (above diagonal) and transversions (below diagonal) among 30 lineages of bears.

Lineage	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
1. GB01			2	2	2	1	30	30	28	29	30	30	29	30	28	30	30	29	28	29	30	30	31	31	30	29	28	29	26	11	86
2. GB02	0			2	2	1	30	30	28	29	30	30	29	30	28	30	30	29	28	29	30	30	31	31	30	29	28	29	26	11	86
3. GB03	0	0			2	1	30	30	28	29	30	30	29	30	28	30	30	29	28	29	30	30	31	31	30	29	28	29	26	11	86
4. GB04	0	0	0			1	28	28	26	27	28	28	27	28	26	28	28	27	26	27	28	28	29	29	27	26	27	27	24	9	84
5. GB05	0	0	0	0			29	29	27	28	29	29	28	29	27	29	29	28	27	28	29	29	30	30	29	28	27	28	25	10	85
6. GB06	2	2	2	2	2			2	8	9	10	2	11	2	2	2	2	5	4	1	2	2	3	3	2	3	2	3	8	29	104
7. GB07	2	2	2	2	2	0			8	9	10	2	11	2	2	2	2	5	4	1	2	2	3	3	2	3	2	3	8	29	100
8. GB08	3	3	3	3	3	1	1			1	2	8	3	8	6	8	8	3	6	7	8	8	9	9	8	7	6	7	8	27	100
9. GB09	3	3	3	3	3	1	1	0			3	9	4	9	7	9	9	4	7	8	9	9	10	10	9	8	7	8	9	28	99
10. GB10	3	3	3	3	3	1	1	0	0			8	1	10	8	10	10	5	8	9	10	10	11	11	10	9	8	9	10	29	100
11. GB11	2	2	2	2	2	0	0	1	1	1			9	2	2	2	2	5	4	1	2	2	2	3	3	2	3	2	8	29	102
12. GB12	3	3	3	3	3	1	1	0	0	0	1			11	9	11	11	6	9	10	11	11	12	12	11	10	9	10	11	28	99
13. GB13	2	2	2	2	2	0	0	1	1	1	0	1			2	2	2	5	4	1	2	2	3	3	2	3	2	3	8	29	104
14. GB14	2	2	2	2	2	0	0	1	1	1	0	1	0			2	2	3	4	1	2	2	3	3	2	3	2	3	6	27	102
15. GB15	2	2	2	2	2	0	0	1	1	1	0	1	0	0			2	5	4	1	2	2	3	3	2	3	2	3	8	29	104
16. GB16	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0			5	4	1	2	2	3	3	2	3	2	3	8	29	104
17. GB17	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0			7	4	5	5	6	6	5	4	3	4	7	28	101
18. GB18	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0			3	4	4	5	5	4	5	4	5	8	27	102
19. GB19	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0			1	1	2	2	1	2	1	2	7	28	103
20. GB20	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0			2	3	1	2	3	2	3	8	29	102
21. GB21	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0			1	1	2	3	2	3	8	29	102
22. GB22	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0			2	3	4	3	4	9	30	103
23. GB23	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0			3	4	3	4	9	30	101
24. GB24	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0			3	2	3	8	29	102
25. GB25	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0			1	2	7	28	103
26. GB26	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			1	6	27	102
27. GB27	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0			7	28	103
28. GB28	2	2	2	2	2	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			27	100
29. Polar	1	1	1	1	1	3	3	4	4	4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3			91
30. Sun 4	4	4	4	4	4	4	5	5	5	4	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4			

Table 2.4a. Observed pairwise differences among selected brown bear $tRNA^{thr}$ and $tRNA^{pro}$ genes. Divergence values above the diagonal are from $tRNA^{thr}$ substitutions; values below the diagonal are from $tRNA^{pro}$ substitutions. Species and lineage abbreviations are as listed in Table 2.2.

SPECIES		1	2	3	4	5	6	7	8	9
	BROWN BEAR									
1	GB01	-	0.000	0.040	0.040	0.040	0.040	0.027	0.000	0.080
2.	GB04	0.000	-	0.040	0.040	0.040	0.040	0.027	0.000	0.080
3.	GB08	0.000	0.000	-	0.000	0.027	0.027	0.013	0.040	0.093
4.	GB10	0.000	0.000	0.000	-	0.027	0.027	0.013	0.040	0.093
5.	GB19	0.000	0.000	0.000	0.000	-	0.000	0.013	0.040	0.093
6.	GB26	0.000	0.000	0.000	0.000	0.000	-	0.013	0.040	0.093
7.	GB28	0.000	0.000	0.000	0.000	0.000	0.000	-	0.027	0.080
8.	POLAR	0.045	0.045	0.045	0.045	0.045	0.045	0.045	-	0.080
9.	SUN	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	-

Table 2.4b. Observed pairwise differences among selected brown bear tRNA genes. Divergence values are based on all substitutions found in $tRNA^{thr}$ and $tRNA^{pro}$ sequences. Species and lineage abbreviations are as listed in Table 2.2.

SPECIES	1	2	3	4	5	6	7	8	9
BROWN BEAR									
1 GB01	-	0.000	0.021	0.021	0.021	0.021	0.014	0.021	0.071
2. GB04		-	0.021	0.021	0.021	0.021	0.014	0.021	0.071
3. GB08			-	0.000	0.014	0.014	0.007	0.043	0.078
4. GB10				-	0.014	0.014	0.007	0.043	0.078
5. GB19					-	0.000	0.007	0.043	0.078
6. GB26						-	0.007	0.043	0.078
7. GB28							-	0.035	0.071
8. POLAR								-	0.092
9. SUN									-

CHAPTER 3

POPULATION GENETIC STRUCTURE AND POPULATION HISTORY OF BROWN BEARS (*URSUS ARCTOS*) IN ALASKA: EVIDENCE FOR FEMALE PHILOPATRY AND MALE-BIASED GENE FLOW

3.1 ABSTRACT

Patterns of gene flow and genetic structuring were examined in 200 brown bears (*Ursus arctos*) sampled from 14 populations in Alaska, using mtDNA sequence information from the complete cytochrome b gene, 2 transfer RNAs and a 371 basepair portion of the hypervariable portion I of the of the mitochondrial DNA (mtDNA) control region. Sixty-one haplotypes were found among the 200 brown bears. Hierarchical analyses of molecular variance were performed on populations ascribed previously to phylogeographic regions and hypothesized subspecies ranges. Results from AMOVA reveal that 75-90% of the recovered genetic variation resulted from differences in the apportionment of genetic variation between subdivisions of the primary phylogeographic regions. AMOVA gave no statistical support for hypothesized subspecies designations. Over 90% of pairwise population comparisons were significantly differentiated based on variance of haplotype frequency ($p < 0.05$). Gene flow estimates based on the coancestry coefficient Φ_{ST} reveal moderate gene flow occurs among some populations within phylogeographic regions, but little or no gene flow between phylogeographic regions, suggesting historical boundaries between these regions may have been important in the formation of phylogeographic structure of brown bears in Alaska. This structure may be maintained by strong female philopatry. Analyses of levels of genetic variation, phylogenetic networks and mismatch distributions indicates populations within one phylogeographic region (Clade IIb, western Alaska) show evidence of more recent expansion, while another region exhibits characteristics of a more demographically stable population with relatively high levels of genetic diversity but very limited genetic

exchange with other phylogeographic regions (Clade I, found only on the islands of the Alexander Archipelago in southcoastal Alaska). Estimates of contemporary gene flow, based on nuclear microsatellite data, obtained for populations from two distinct phylogeographic regions that may be demonstrating secondary contact, were higher than for mtDNA, but still not sufficient to prevent population differentiation. Differences in estimates between nuclear and mtDNA can be explained in terms of the effective population sizes of the two genomes, and male-biased gene flow.

3.2 INTRODUCTION

The brown bear (*Ursus arctos*) is threatened or endangered throughout much of its worldwide range, due in large part to the fragmentation or loss of essential habitat (Servheen 1990). However, the brown bear survives in healthy populations in Alaska and is distributed in varying densities throughout most of the Alaskan mainland as well as on several outlying island and archipelago systems, at levels thought to be representative of historic levels. Proper management of brown bear populations in Alaska and elsewhere requires an understanding of the structure of these populations.

Mark-recapture and radio-telemetry programs have described contemporary movements of individual brown bears within and between study areas, and tentatively identified populations of Alaskan brown bear for management purposes. Because "ecological dispersal" does not necessarily measure "genetic dispersal" (the movement of genes from one population to another), such studies cannot reveal the relative degree of gene flow among populations, nor do they reveal components of genetic structuring of these populations or past evidence of episodes of bottlenecking or population expansion. By quantifying the relationship between dispersal ability and the level and spatial scale of population structuring, genetic studies can establish a link between the ecology and the evolution of a species. Studies assessing population genetic differentiation are of particular value in this regard, since they permit inferences about how microevolutionary forces have interacted throughout the history of the species. As such, they can contribute substantially to the understanding and management of species, particularly those with

fragmented remnant distributions like that of the brown bear throughout most of its historical range.

In particular, studies of genetic variation can provide information about levels of current and historical gene flow among populations, and about the distribution of a species' genetic diversity. Nevertheless, patterns of genetic variability resulting from current levels of gene flow are difficult to distinguish from patterns imposed by past population structuring and movement. Analyses of the mitochondrial DNA (mtDNA) have been particularly powerful in this respect (Avise 1994, Moritz 1994a), because they generate information on the phylogeny as well as the frequency of alleles, and thus provide perspectives on population processes across both ecological space and evolutionary time (Avise 1989; Hudson 1990). Furthermore, due to its uniclonal inheritance, the mtDNA is expected to be more sensitive to population subdivision than are single-copy nuclear genes, particularly for species where females are philopatric, or more so than males (Birky et al. 1989). This has been demonstrated for a variety of species, such as humpback whales (*Megaptera novaeangliae*: Baker et al. 1993), marine turtles (*Chelonia mydas* and *Caretta caretta*: Bowen et al. 1992, 1993; Norman et al. 1994), and Canada geese (*Branta canadensis*: Shields and Wilson 1987; Van Wagner and Baker 1986, 1990; Pearce et al. 2000; Talbot et al. 2003).

Nevertheless, levels of polymorphism within even the fastest mutating regions of the mtDNA are thought to be lower than for nuclearly-encoded microsatellite loci, which have become a tool of choice for assessing population-level relationships (see Goldstein and Schlotterer 1999, and references therein). Concurrent use of both classes of markers to describe population subdivision within and among populations have often yielded incongruent results, particularly for organisms that demonstrate gender-biased dispersal, or that have gone through recent population bottlenecks and/or expansion (Pearce et al. 2000; Talbot et al. 2003; Scribner et al. 2001, 2003). Indeed, investigations of population genetics characteristics of brown bears in Alaska, using biparentally-inherited microsatellite markers, yielded results that were incongruent with similar studies based on maternally-inherited mtDNA sequence information (Paetkau et al. 1998a; Waits et al.

2000, Jackson et al. 2006, Talbot et al. 2006). Because population genetic surveys using sequence data provide information on the evolutionary relationships of the genetic variation being scored as well as frequency information, insight into the alleles' existence through evolutionary time as well as geographical space can be gleaned. Assessment of both short- and long-term evolutionary behavior of alleles gives insight into the evolutionary forces acting on populations and species over time, and can thus be of great value to conservation biologists seeking to better understand and apply population genetic and phylogeography information to the management of wildlife species.

A previous study of mitochondrial DNA variation in Alaskan brown bear (Talbot and Shields 1996a, Chapter 2), which focused on intraspecific phylogeography of the brown bears of Alaska using information from three mtDNA genes, is expanded herein so that I may compare results with theoretical expectations derived from conservation biology literature, and with information from studies describing brown bear population genetics based on nuclear markers considered to be neutral or nearly-so (Craighead 1994; Paetkau et al. 1998a,b). Besides extracting a more detailed assessment of population structuring and genetic variation among populations uncovered using the more conserved mtDNA cytochrome b sequence information, I investigate whether: i) female-mediated gene flow is restricted among populations of Alaskan brown bear; ii) mtDNA genetic information provides a similar picture of population structuring of brown bears of Alaska to that painted by studies of allelic and genotype frequency variation derived from microsatellite data; iii) population structure determined by studying mtDNA genetic characteristics among Alaskan brown bear populations reflects theoretical expectations derived from models of island biogeography theory, and iv) a signature of ancient bottlenecks and population expansions can be detected.

Herein I examine nucleotide variation across more than 1,600 nucleotides (nt) of the mitochondrial DNA, including the entire cytochrome b gene, 2 tRNA genes, and a portion of the hypervariable region I of the control region (Vigilante 1990; Wakely 1993). Samples from 200 brown bears from throughout Alaska were analyzed to assess levels of genetic variation, gene flow and population structuring, and explore possible episodes of

past population bottlenecking and expansion. The brown bear is a good choice for estimation of total genotypic variation for a variety of reasons: it possesses great dispersal capabilities, there is a great deal of fossil, historical and current information elucidating past and present distribution, and large numbers of samples can be obtained from brown bears representing different populations in Alaska.

3.3 MATERIALS AND METHODS

MtDNA Data. MtDNA from 200 brown bears from populations throughout their geographic range in Alaska was compared in this study. Regions represented are southcoastal (SCOA), southcentral (SCEN), Arctic (ARC), interior Alaska (INT), Seward Peninsula (SPEN), the Kodiak Archipelago [KODARC: Kodiak Island (KOD) and Afognak Island (AFOG)], Alaska Peninsula [AKP: Katmai National Park (KAT) and Izembek National Wildlife Refuge (IZE)], Brooks Range (BRNG), Alaska Range (AKR) and the islands of the Alexander Archipelago of southeastern Alaska [AARCH: Admiralty (ADM), Chichagof (CHI) and Baranof (BAR) islands)] (Figure 3.1a,b).

Tissue collection procedures, extraction of DNA from frozen tissues, asymmetrical amplification of target mtDNA using the polymerase chain reaction (PCR), and sequencing were performed essentially as described by Talbot and Shields (1996a). Some DNA sequences were obtained with the use of fluorescent dye-terminator chemistry and an Applied Biosystems 373A Automated DNA Sequencer. In addition to primers targeting the cytochrome *b* gene, described in Talbot and Shields (1996a), I used as amplification and sequencing primers a pair of species-specific oligonucleotide primers (L15962 and H00019) that targets and amplifies a 533-570 nt portion of the mtDNA of the brown bear, including 456-563 nt from a portion of the brown bear mtDNA control region homologous to the hypervariable portion 1 of the human mtDNA (Vigilante 1990; Wakely 1993). The nucleotide sequence for the primer L15962 is 5' - CTCCCTAAGACTCAAGGA -3' and anneals to sites found on the $tRNA^{pro}$ and $tRNA^{thr}$ genes; the nucleotide sequence for H00019 is listed in Talbot and Shields (1996a) and anneals to a site in the Conserved Sequence Block C of the control region. From the

initial 485 nt sequence consistently amplified and sequenced from this region, I identified a variable segment spanning approximately 370 nt, found between the region coding for the $tRNA^{pro}$ and the Conserved Sequence Block F of the control region. This variable region, along with sequences from the cytochrome b gene and the two tRNAs, was used in subsequent analyses.

Sequences were aligned using the multiple biosequence alignment program CLUSTALV (clustalw-big'n'FAT, version 1.4; Higgins et al. 1991), using a Power Macintosh (6100/60); results were examined visually. A transition/transversion ratio of 2:1 and a gap-weighting of 10 were used. .

I used MODELTEST (Posada and Crandall 1998) to determine the simplest model of evolution for the data set that did not significantly differ from more complex models. I conducted phylogenetic analyses using maximum parsimony methods employed by the computer program PAUP (Version 4.0b8*, Swofford 2000), and minimum evolution (neighbor-joining; Saitou and Nei 1987) methods. Minimum-spanning trees (MSTs) were manually constructed following the recommendations of Excoffier et al. (1992), using the graphic matrix obtained by the algorithm found in the NTSYS package (Rohlf 1990).

The sequences were tested for consistency with neutrality using Tajima's *D* (Tajima 1989). Tajima (1989) suggested that difference in the estimate of $\theta = 4N\mu$ derived from the number of polymorphic (segregating) sites (s) and the average number of nucleotide differences (k) could be used to detect deviations from neutral evolution (here N is the effective population size and μ is the substitution rate per genome per generation). Tajima's *D* evaluates the differences in these two estimates. A large absolute value of *D* indicates a deviation from neutrality. We also assessed neutrality using Fu's F_S (Fu 1997).

I used three measures of intrapopulational genetic diversity (for samples where $n \geq 4$), the haplotype diversity index (*h*; equations 8.4 and 10.4 of Nei 1987), and two nucleotide diversity indices (π: Nei and Tajima 1981, and $E(\upsilon)$: Watterson 1975), to examine levels of genetic variation among populations and regions. The haplotype

diversity (h) index is approximately equivalent to the probability that two randomly chosen individuals will have different genotypes. The maximum value of h is 1.0 if all members of a population possess different mtDNA haplotypes, and the minimum value is 0.0 if all members share the same haplotypes. The nucleotide diversity index π takes levels of divergence among clones into consideration to estimate the probability that randomly chosen haplotypes in a population differ at the nucleotide level. Thus, the measurement π (Nei and Tajima 1981) corrects h for the size of the nucleon examined (Nei 1987). Both h and π were calculated using the program REAP (McElroy et al. 1991) from pairwise haplotype divergence values generated by the MEGA program (Kumar et al. 1993).

Both nucleotide diversity indices π (Nei and Tajima 1981) and E(υ) (Watterson 1975) measure the average pairwise nucleotide difference between individuals within samples. However, Watterson's E(υ) is considered to be more appropriate for detecting long-term levels of nucleotide diversity (Excoffier and Langaney 1989), since it is independent of haplotype frequencies and measures a long-term average rather than a value estimated from the current generation. Watterson's E(υ) was calculated using the formula

$$E(\upsilon) = K/[0.577 + \log_e(n-1)]$$

where K is the number of polymorphic sites and n is the sample size.

I estimated genetic distance among the 13 populations using the coancestry coefficient [$\ln(1- F_{ST})$: Reynolds et al. 1983], where F_{ST} is represented by pairwise variance in haplotype frequency between populations (Φ_{ST}; see below). This distance measure assumes divergence among populations is strictly a function of genetic drift. Graphic representations of inter-populational distances, in the form of a tree displaying relationships among populations, were made using the NEIGHBOR program in PHYLIP

(Version 3.52c, Felsenstein 1997). Significance of each inter-populational distance estimate was based on significance of pairwise estimates in interpopulational variance of haplotype frequency, tested using random permutation tests.

Genetic differentiation based on the distribution of mtDNA haplotype frequency differences, or the heterogeneity of haplotype distribution, among the brown bears of Alaska was tested with the Monte-Carlo χ^2 test of Roff and Bentzen (1989), using the REAP program. This approach is suitable for genetic data matrices in which many or most elements are very small (< 5) or zero. A total of 5,000 resamplings of the data matrix was used.

I tested for differences in haplotype frequency at two levels: 1) between bears from different regions, and (2) among specific populations. I assessed significance of spatial variation in gene frequency for mtDNA using F-statistics (Cockerham 1969; Weir and Cockerham 1984). These measures can be viewed simply as variance components, and they describe the apportionment of allelic variance among individuals within populations (F_{IS}) and among populations (F_{ST}). Values of F_{ST}, which are summary statistics describing the extent of spatial variation among populations or population groupings, range from 0.0 to 1.0. A value of 1.0 for a specific locus (i.e., mtDNA) would imply that all populations are "fixed" for different alleles (i.e., the total variance in mtDNA frequency is segregating among populations). On the other extreme, a value of 0.0 implies all populations share the same alleles, in equal frequency. For haploid genetic systems, such as mtDNA, the coancestry coefficient (Φ_{ST}) is equivalent to the more familiar F_{ST} of Wright (1951) used for diploid nuclear genes; however, Φ_{ST} takes into account molecular differences or differences in allele size between pairs of genes from different populations (Michalakis and Excoffier 1996).

Estimates of interpopulational variance (pairwise Φ_{ST}) were derived using ARLEQUIN (Schneider et al. 1997). Significance of pairwise Φ_{ST} values was based on random permutation tests (N = 1000), whereby alleles were randomly permuted among individual populations. A significant Φ_{ST} implies a significant portion of the total

genomic variation of the specific locus (mtDNA) is partitioned among the populations compared.

I examined regional as well as population differentiation using a hierarchical analysis of molecular variance (AMOVA, in ARLEQUIN; Excoffier et al. 1992; Schneider et al. 1997). AMOVA estimates the proportion of total genetic variation attributable to different hierarchical levels based on the geographical distribution of haplotypes and the pairwise distances between them. Correlations among haplotypes are measured in terms of "Φ" statistics, which are similar to F-statistics (Excoffier et al. 1992; see above) such that Φ was used to partition variance in haplotype frequency among individuals within populations (Φ_{ST}), among populations within regions (Φ_{SC}) and between regions (Φ_{CT}). Molecular distances for these hierarchical analyses were estimated using the model of evolution that best fit the data, as determined under the Akaike Information Criterion (AIC) in MODELTEST (see above).

This hierarchical analysis of genetic variance partitioning requires that *a priori* considerations be used to group sets of populations together to form hierarchical levels. I used results of phylogenetic analyses of brown bear mtDNA relative to other ursids and within brown bear clades in Alaska (Talbot and Shields 1996a; Chapter 2) and worldwide (Waits et al. 1998, Appendix B, C) to justify population groupings into Alexander Archipelago [AARCH: ADM, CHI and BAR, Clade I; Talbot and Shields 1996a], eastern [Clade IIa populations, SCOA, ARC, AKR, INT, BRNG] and western [Clade IIb; comprised of AKR, SCEN, BRNG, ARC, KAT, IZE, KOD, AFOG, SPEN, INT; Talbot and Shields 1996a] sets of populations before performing nested analyses of variance. In addition, I explored results of other groupings of populations suggested by the analysis of DNA sequence and population trees as well as potential for geographic isolation. I also used AMOVA to partition variance observed among bears within hypothesized subspecies' ranges (Rausch 1963, Kurtén 1973, Hall 1984; see Talbot and Shields 1996a, Chapter 2). I assumed the groupings that maximized values of Φ_{CT} and

were significantly different from random distributions of individuals were the most probable geographical subdivisions.

Microsatellite Data. To examine differences in genetic variation assessed using nuclear markers, and assess levels population structuring and gender-bias in gene flow among brown bear populations in Alaska, I analyzed data from eight microsatellite loci (G10A, G10B, G10C, G1D, G10M, G10P, G10X; Paetkau 1997, Paetkau et al. 1998a, b) reported in Appendix 1 of Craighead (1994) for bears from BRNG (n = 95), AKR (n = 17), and ARC (n = 15), and Scribner et al. (unpublished manuscript) for KAT (n=29). Three of these populations (BRNG, AKR, and ARC) are located in interior Alaska, in an area where phylogeographic regions abut or overlap (a clade "contact zone"; see Talbot and Shields 1996a, Chapter 2, and Waits et al. 1998, Appendix B). Individuals from these populations belong to one or the other of the two clades found in interior Alaska (Chapter 2). On the other hand, KAT is located more remotely from the rest, on the Alaska Peninsula (Figure 3.1a), and individuals sampled from KAT represent only one clade (CladeIIb).

Because these microsatellite data were generated in different laboratories using different PCR protocols and genotyping platforms, allele sizes generated for the same individual likely varied by arbitrary differences due to type of label used, spectral reflectance and gel matrix conditions. Since I shared subaliquots of DNA from individuals genotyped by Craighead (1994; Appendix 1), I was able to use them to calibrate allele sizes for the KAT dataset (Scribner et al., unpublished manuscript). Amplification conditions for each of the eight loci were similar to those reported in Craighead (1994) and Paetkau (1997), modified for labeling using a radioactive probe ($\gamma^{32}P$) or infrared probe (IRD700 or 800; Jackson et al. 2006).

Subsequent to calibration, I conducted standard population genetics statistical analyses of the microsatellite data using GENEPOP Version 3.2 (Raymond and Rousset 1995a). Genetic polymorphism was measured and is reported as the number of alleles per locus (A), observed heterozygosity (H_O) and Nei's unbiased expected heterozygosity (H_E;

Nei 1978). All microsatellite loci were tested for deviation from Hardy-Weinberg equilibrium (HWE), using Fisher's Exact Test for loci/populations with combinations of four or fewer alleles (Raymond and Rousset 1995b, Guo and Thompson 1992). For loci/populations with five or more alleles, a Markov chain was set to 100,000 steps with 1,000 steps of dememorization. Global tests of HWE across loci and populations were performed using Fisher's exact method, as implemented by GENEPOP. I also conducted pairwise tests for linkage disequilibrium, again using Fisher's Exact Tests of data in contingency tables, implemented using GENEPOP.

Tests for differences in distribution of allelic and genotypic frequencies between and across all four populations were conducted using a test of homogeneity computed as an exact test, for which the null hypothesis of identical allelic and genotypic frequency distributions were rejected when the probability value of the exact test is < 0.05. This test was implemented using the program GENEPOP (version 3.2). Significance in distribution of allelic variation among and within populations was tested over all populations, using θ_{ST} values (Cockerham and Weir 1993) generated by the program FSTAT (Goudet 1994), and pairwise population θ_{ST} values generated using ARLEQUIN (Schneider et al. 1997). All pairwise multilocus values were corrected for multiple experiment-wise error using Bonferroni procedures (Rice 1989; Weir 1996).

Pairwise comparisons of the unbiased multilocus R_{ST} statistic (Goodman 1997) were also calculated for microsatellite data, using ARLEQUIN. The R_{ST} statistic is based on the stepwise mutation model (SMM: Ohta and Kimura 1973; Shriver et al. 1993), which considers that mutations at microsatellite loci occur at relatively high rates and involve addition or subtraction of a single or small number of repeat units for a given allele with equal probability. Significance of pairwise R_{ST} values was obtained using 1,000 Monte Carlo simulations.

I initially quantified genetic divergence for the biparentally-inherited microsatellite loci among the four populations using two distance matrices: $(\delta\mu)^2$ (Goldstein et al. 1995) and Cavalli-Sforza and Edward's chord distance (D_{CE}, Cavalli-

Sforza and Edwards 1967). Delta-mu^2 takes into account deviations of allelic size variance from theoretical expectations under SMM. It is considered to be independent of population size under mutation-drift equilibrium, and allows a direct estimate of time since population divergence when mutation rates can be estimated. It is also appropriate for approximating real branch lengths (Takezaki and Nei 1996). Conversely, D_{CE} does not assume a constant population size, or make assumptions about mutation rates among loci. Distance measures derived using $(\delta\mu)^2$ and D_{CE} were used independently to generate consensus neighbor-joining trees using a program written by J. Cornuet (INRA, Laboratoire de Neurobiologie Comparee des Invertebres, Bures-surYvette, France). I tested statistical significance of branch topology by bootstrapping across loci, using the same program. I then generated a final population tree using a combined method whereby topology and branch-length were estimated from D_{CE} and $(\delta\mu)^2$, respectively, using the user-tree option in FITCH/PHYLIP. Cavalli-Sforza and Edward's (1967) least-squares method was used to estimate branch lengths from $(\delta\mu)^2$.

Levels of Gene Flow. Evaluation of long-term effective size of the female population, $N_{e(f)}$, was conducted from the frequency distribution of genetic distances among individuals. Average effective number of females (N_{fm}) exchanged per generation was determined from the mtDNA F_{ST}-analog (Φ_{ST}) values according to the approximation

$$F_{ST}=1/(1+2N_{fm})$$

(Wright 1951; Slatkin 1987, 1993) as implemented by ARLEQUIN (Schneider et al. 1997).

The accuracy of these estimates of gene flow based on these models depends upon several assumptions. Among these assumptions is that the populations examined are in

equilibrium with respect to genetic drift and migration, in accordance with the island model of population structure (Wright 1951; Slatkin 1987, 1993); this assumption may not be met in all populations. Nevertheless, despite the limitation placed on the applicability of the actual values (see Bossart and Prowell 1998), these gene flow estimates provide a basis for comparison of gene flow estimates across populations, as well as relative estimates generated from data gathered from different loci.

Population Demography: *MtDNA*. Coalescent theory (Hudson 1990) makes predictions of the pattern of segregating sites that can be informative about the evolutionary history of a sample of alleles. Past episodes in population growth and decline leave signatures in the distribution of nucleotide site differences between pairs of individuals. The frequency distribution of the observed number of differences between pairs of haplotypes, or "mismatch distribution" (Hartl and Clark 1989; Harpending et al. 1993), can be used to detect ancient bottlenecks and population expansions by examining the shape of waves in distribution of pairwise genetic differences (Rogers and Harpending 1992; Rogers 1995). The distributions are expected to be multimodal, or "ragged" in samples drawn from populations that are at demographic equilibrium, since they reflect the highly stochastic shape of gene trees. On the other hand, the distribution is usually smooth and unimodal and approaches a Poisson distribution in populations that have passed through a recent demographic expansion (Rogers and Harpending 1992; Slatkin and Hudson 1991). The latter pattern reflects an underlying starlike geneology in which all of the coalescent events occurred in a narrow window of time. Ancient signatures of bottlenecks and expansions can obscure the effects of later expansions and minor bottlenecks for a long time (Rogers 1995).

I used the method of Rogers and Harpending (1992) to detect signatures of ancient historical bottlenecks and expansions among the populations examined, using the mtDNA sequence data. I obtained mismatch distributions within different population groups and the raggedness index (*rg*) of the observed distribution (Harpending 1994) using ARLEQUIN (Schneider et al. 1997). The raggedness index takes larger values for

multimodal distributions commonly found in stationary populations than for unimodal and smoother distributions typically characterizing expanding populations (Harpending 1994). Significance of *rg* was tested using the sums of squared deviations method implemented by ARLEQUIN.

Values of Tajima's *D* and Fu's F_S, used to assess neutrality of sequences (see above), were also used to infer patterns in population size because they are sensitive to departures from demographic equilibrium (Aris-Brosou and Excoffier 1996, Fu 1997). I applied critical significance values of 5%. This significance value requires a *p*-value below 0.02 for Fu's F_S (Fu 1997). Significantly large negative F_S values can be interpreted as evidence for population expansion.

Microsatellites. Populations that have experienced a recent reduction of effective population size, such as during a founder event, are expected to show a reduction in both number of alleles and levels of heterozygosity at polymorphic loci (Watterson 1984), such as at microsatellite loci. However, allelic diversity is reduced much more rapidly than levels of heterozygosity (Nei et al. 1975; Denniston 1978; Maruyama and Fuerst 1985), observed heterozygosity being larger than expected if the population was at mutation-drift equilibrium. I used two statistical tests, the sign test and the Wilcoxon test, to detect excess heterozygosity (relative to number of alleles) for polymorphic microsatellite loci as an indicator of recent bottlenecks (Cornuet and Luikart 1996) in each of the four populations (BRNG, ARC, AKR and KAT) examined for population genetic structuring. The sign test determines if the proportion of loci with heterozygosity excess is significantly larger than expected at equilibrium, and the Wilcoxon test determines if the average of standardized differences between observed and expected heterozygosities is significantly different from zero. Unlike the mtDNA mismatch distributions discussed earlier, these two statistical tests detect very recent bottlenecks -- that is, bottlenecks occurring within the past several generations (Luikart 1997) -- using heterozygosity and allele frequency data for each of several loci. The tests require no data on historical population sizes or levels of genetic variation. Tests were conducted using the program BOTTLENECK (Cornuet and Luikart 1996; Luikart and Cornuet 1998), under three

models thought to represent the range of possible mutation modes generating polymorphism at microsatellite loci. These include the infinite-alleles model (IAM; Maruyama and Fuerst 1985), the stepwise mutation model (SMM; Ohta and Kimura 1973, Freimer and Slatkin 1996; Jarne and Lagoda 1996), and the two-phase model (TPM; see Di Rienzo et al. 1994) of microsatellite mutation. One thousand simulations were performed for each population.

3.4 RESULTS

mtDNA Sequence Data. One hundred nucleotides (nt) were variable among the 1,629 nt examined for 200 individual brown bears, defining 61 different mtDNA types (Figure 3.2, 3.3). Additional variable positions describing additional genotypes were observed among the hypervariable portion I of the control region of the mtDNA from 19 brown bears from CHI of the Alexander Archipelago of southeastern Alaska, for which cytochrome b, $tRNA^{thr}$ and $tRNA^{pro}$ data were not obtained. For many of the subsequent analyses, these haplotypes were collapsed into sister lineages (data not shown), so some measures of genetic diversity for CHI reported herein underestimate the true sample diversity.

All variable positions in the cytochrome *b* and tRNA genes, and the majority (74%) of variable positions in the control region, occurred as substitutions. The remainder of variable sites in the control region occurred as insertions/deletions (indels) at a site near the 5' end of the control region that contains a series of thymine repeats (T_n) (described below, see Figure Appendix 3.0). This site alone defines 23 of the 61 distinct sequence haplotypes detected among Alaskan brown bears. These haplotypes differ at between one and nine nucleotide substitutions or indels (Appendix 3.0). Haplotype prefixes 1-24 have been reported previously and correspond to haplotypes named from cytochrome *b*, $tRNA^{thr}$ and $tRNA^{pro}$ genotypes only (Talbot and Shields 1996a; Chapter 2); haplotypes with alphabetical suffixes are sequences corresponding to haplotypes published in Talbot and Shields (1996a), augmented by the addition of control region sequence data. Some sequences have been submitted to GenBank (see Talbot and Shields

1996a, b; Waits et al. 1998, Appendix B); novel haplotypes will be submitted. Variation among the cytochrome *b* and two tRNA genes is described elsewhere (Talbot and Shields 1996a, Chapter 2); description of variation in the hypervariable I portion of the control region follows.

Sequence Variation in the mtDNA Control Region Hypervariable I. Nucleotide site information from 371 to 485 nt of the hypervariable control region was obtained for each of the 200 Alaskan brown bear samples; a subset of aligned sequences comprising 371 nt are described herein. Thirty-seven substitutions occurred among all bears, with 36 (97%) occurring as transitions (C-T, 56%; A-G, 44%) and one as a transversion (C-A). Four of these substitutions occurred in a single haplotype and were thus not phylogenetically informative.

Additional variation occurred in the form of indels, located near position L16475 (Árnason and Johnsson 1992; Anderson et al. 1981). This indel region (Table 3.3) is characterized by a short string (12-17 nt) of tandem mononucleotides (pyrimidines). Its gene geography is the locale identified by Shields and Kocher (1991) as a "deletion" site evident only in brown and polar bears (*U. maritmus*) relative to black bears (*U. americanus*). The region appears to be an area of high frequency of nucleotide site change, and includes several informative substitution sites as well as indels. Most change occurs in the form of indels of thymine repeats (T_n). Twenty-three of the 61 lineages (37%) listed herein are differentiated from other lineages by indels in this region only, and these haplotypes are not included in all analyses.

Absolute values of Tajima's *D* within all populations, and for all populations pooled with or without an outgroup (sun bear, *Helarctos melayanus*; see Talbot and Shields 1996b) were not significant ($p > 0.1$), and ranged from 0.37 to 1.72 (Table 3.1). Tajima's *D* was not significant over all sequences (2.071, $p = 0.97$). Although negative Fu's F_S were observed in KOD, AFOG, KAT and SCEN, values were not significantly different from zero in any population (Table 3.2) or overall (data not shown).

Statistical and Phylogenetic Analysis. MODELTEST identified TN+I (Tamura and Nei 1993) as the simplest evolutionary model fit to the combined dataset. Parameters of this model were used in subsequent analyses implementing distance models.

Phylogenetic analysis of the entire cytochrome b gene, the 2 genes encoding $tRNA^{pro}$ and $tRNA^{thr}$, and 371 nt of the hypervariable I portion of the control region, using maximum parsimony, indicates mtDNA haplotypes found in populations of Alaskan brown bear belong to two major clades (Figures 3.4a, b, c), supporting the results reported in Talbot and Shields (1996a). Analysis of control region sequences alone gives similar results (data not shown). Figure 3.4d shows one of the maximum parsimony trees produced using the combined data set. As before, one clade (Clade I) is comprised of mtDNA found only in bears inhabiting the ADM, CHI and BAR of the Alexander Archipelago (Figures 2.7); the second clade is comprised of bears found throughout the remainder of Alaska. This latter clade is again further subdivided into two clades, one comprised of bears inhabiting eastern Alaska (Clade IIa), as well as northern Canada (Waits et al. 1998; see Appendix B), and the other comprised of bears inhabiting the western portion of Alaska (Clade IIb) (Clades II and III, respectively, in Figure 2.7), as well as parts of Asia (Talbot and Shields 1996a; Waits et al. 2006, Appendix C). Genotypes 6 (GB03B), 20 (GB10A) and 39 (GB19A) (Figure 3.2) are the most widespread genotypes found among samples representing Clades I, IIa and IIb, respectively. Results of phylogenetic analyses using parsimony were supported in general by neighbor-joining of Tamura-Nei distances among 38 haplotypes, with differences in the topology hypothesized among clades with bootstrap support of > 99% (Figure 3.5).

The MST-network illustrated in Figure 3.6 provides graphical summary of the relationship among the 61 mtDNA Alaskan haplotypes assayed in the three clades, and is consistent with the principal features of the parsimony and neighbor-joining analyses. The MST shown, however, represents only one of many possible MST networks for this data set. A principal feature derived from the MST analysis was the presence of deep phylogeographic structuring in the mtDNA haplotype data. In general, there were few examples of identical haplotypes shared by more than one group, or dissimilar haplotypes

observed within the same group. There were six exceptions, and these were limited to within-clade sharing. These included haplotype 6 (GB03B: found on both BAR and CHI) within Clade 1, haplotype 20 (GB10A: found in low frequency in the INT and BRNG) within Clade IIa, and Clade IIb haplotypes 48 (KOD, INT, BRNG and SPEN), 49 (GB19L: KOD, AFOG and AKR), 39 (GB19A: AKR, ARC, KOD and SPEN) and 41 (GB19C: SCEN and INT). The MST demonstrates the star-like phylogeny that characterizes Clade IIb, the "western Beringian" clade. Such starburst patterns are highly characteristic of a population expansion, which could apply equally as well to a demographic expansion of the brown bears themselves, or to a selective sweep of their mtDNA.

Levels of Genetic Variability: *MtDNA*. The nucleon (haplotype) diversity (h) and the nucleotide diversity (π) for each of 14 populations are given in Table 3.1. Haplotype diversity (h) was highly variable among populations, and ranged from zero (IZE) to 1.000 ± 0.30 (SCOA), with overall haplotype diversity 0.935 ± 0.008 (Table 3.1). For populations with $n > 5$, the highest nucleotide diversity was found on ADM (0.728 ± 0.06).

Among the groups examined, the sample representing the Alexander Archipelago populations has the highest haplotype diversity ($h = 0.855$) and is also the largest sample size ($n = 44$). Populations on mainland Alaska demonstrated moderate levels of haplotype diversity as well ($h = 0.381 - 0.712$) for which $n \geq 5$. Correcting h for size of nucleon, however, suggests populations from mainland, non-peninsular region of Alaska are more genetically diverse than those inhabiting the Alexander Archipelago (see π values, Table 3.1).

By contrast, the lowest levels of haplotype diversity among Alaskan brown bears are found within peninsular populations (SPEN and AKP). Moderate levels of haplotypic diversity were found on the Kodiak Archipelago ($h = 0.654$), but after correction, diversity values were among the lowest examined for populations with $n > 5$ ($\pi = 0.00071$). Similarly, nucleotide diversity is lowest within the peninsular populations

(SPEN, AKP), and also within several island populations (KOD, AFOG, CHI and ADM; Table 3-2). Watterson's nucleotide index (Eυ) also suggests populations on the AKP, SPEN and KOD have had the lowest levels of historic nucleotide diversity among samples with n > 5, and mainland Alaskan interior populations (AKR, ARC, BRNG) the highest (Table 3.1)

Microsatellites. From four to 11 alleles were found across all four populations (ARC, AKR, BRNG, KAT) analyzed at each of eight dinucleotide microsatellite loci (Craighead 1994). Genotype proportions at autosomal microsatellite loci accorded well with Hardy-Weinberg expectations. Twenty-nine of 32 individual comparisons (91%) revealed no significant departure from HWE equilibrium proportions. Combining probabilities over populations, using Fisher's exact method, shows that one locus (10L) displayed significant departure from HWE in a single population (BRNG).

Tests for genotypic linkage disequilibrium rejected the null hypothesis of independence in 9 of 112 locus-by-locus population comparisons (8 %). Fisher's exact tests for each locus pair across all populations revealed significant overall association between G10B and G10C, and between G10B and G10P. Because eight of the nine significant associations were found among samples from one population only (BRNG), observed deviations from overall genotypic equilibrium appear to be due mostly to disequilibrium between these locus pairs within this population only. Although linkage disequilibrium may occur between loci located in close proximity on chromosomes, admixture or migration can also result in non-random association of neutral loci (Lewontin and Hartl 1991). Since gametic-phase disequilibrium was detected in only one of these populations, and has been variously observed in other brown bear populations (Paetkau et al. 1995, 1998a, b; Waits et al. 2000), the significant result detected within the BRNG population is most likely due to the latter (demographic) rather than the former (gene locality) explanation.

Levels of overall heterozygosity among eight microsatellites for each of the populations, plus additional populations for which published data are available, are given in Table 3.1. Expected levels of heterozygosity for the population on the three islands of

the Alexander Archipelago (Nei's [1987] unbiased expected heterozygosity, $H_E = 0.496$ to 0.628) are low in contrast to values reported for mainland populations ($H_E = 0.737$ to 0.797), but higher than those reported from KODARCH ($H_E = 0.265$). By contrast, levels of heterozygosity expected for populations on the three islands of the Alexander Archipelago were similar to those reported for the Alaska Peninsula ($H_E = 0.53 - 0.69$) (Table 3.1). Allelic diversity, in terms of number of alleles, was highest for AKR and again lowest for KOD populations. These results contrast with results presented earlier for genetic variability in mtDNA in terms of haplotypic and nucleotide diversity, where high levels of diversity are found both across the Alexander Archipelago and mainland (non-peninsular) populations; and lowest values are found within peninsular (SPEN, AKP) populations. AFOG of the Kodiak Archipelago also was characterized by low levels of haplotype and nucleotide diversity at levels similar to those found in peninsular populations, but this population was represented by only seven individuals.

Fisher's combined test of independence across all eight loci showed significant differentiation in frequency of alleles across all four populations analysed (χ^2 = infinity; df = 16, $p < 0.0000$), between all populations across loci ($\chi^2 = 44.52$ - infinity, $p < 0.0001$) and for 75% of total pairwise locus-by-locus comparisons (36/48, $p < 0.05$). Non-significant pairwise comparisons occurred most often between ARC and AKR (4/8 loci). Distribution of genotype frequencies across populations was also significantly different overall (χ^2 = infinity; df = 16, $p < 0.0000$), and for 73% of locus-by-locus pairwise comparisons (35/48, $p < 0.05$).

Population Differentiation. Ninety-three percent of all population pairwise Φ_{ST} comparisons for mtDNA were significantly different from zero ($p < 0.05$), indicating substantial spatial differences in the variance in mtDNA haplotype frequency (Figure 3.7). Non-significant pairwise comparisons all involved those between mainland Alaskan populations represented by low sample size (SCEN – INT) with other mainland interior Alaskan populations (BRNG - AKR) or the Kodiak Archipelago (SCEN - KOD).

Population pairwise differences in the distribution of haplotype frequencies were also significantly different for all but eight of the 91 pairwise comparisons (χ^2 = infinity; $p <$ 0.05).

Comparison of overall θ_{ST} values among four mainland Alaskan populations (BRNG, ARC, AKR, KAT; Craighead 1994; Scribner et al., unpublished manuscript) indicates that while both markers indicate significant levels of population structuring overall, a much higher level of population structuring ($\Phi_{ST} = 0.456$) is revealed from the mtDNA data than from microsatellite data (overall $\theta = 0.069, p < 0.002$). All population pairwise θ_{ST} comparisons for eight microsatellite loci were significant ($p < 0.0063$), indicating substantial spatial differences in allele frequency between the four populations (Figure 3.8). However, only two of six population pairwise R_{ST} comparisons were significantly different from zero (between KAT and ARC or BRNG).

Relationships among populations is illustrated by the neighbor-joining phenogram built from a matrix of coancestry coefficients [$\ln(1- \Phi_{ST})$: Reynolds et al. 1983], estimated from mtDNA haplotype frequency data (Figure 3.9a). Not surprisingly, the populations comprising Clade I are clustered together and away from the other populations in Alaska (Clade II). The populations represented in Clade IIa tended to cluster together, as did those representing Clade IIb; however, there was no clear delineation of this cluster, as populations representing both Clades IIa and IIb (AKR, INT, BR) were sometimes placed in the same terminal dichotomy as populations characterizing only Clade IIb. Nevertheless, populations representing Clade IIb were, in general, clustered together and represented terminal units for the neighbor-joining phenogram.

Only four populations were represented in the neighbor-joining phenogram displaying population relationships based on distances among eight microsatellite loci (Figure 3-9b). This phenogram clusters BRNG and AKR populations together. This contrasts with the mtDNA population tree, which clusters BRNG together with KAT, and

more distantly with AKR and ARC. Nodes were supported by bootstrap values greater than those for the majority rule criterion of 50% (data not shown).

Results from AMOVA reveal that significant interpopulational variance occurs among Alaskan brown bear populations and regions as measured by mtDNA nucleotide sequence data (Table 3.3). The overall coancestry coefficient (Φ_{ST}) is significantly different from zero, (0.847, $p < 0.002$), indicating substantial genotypic differentiation attributable to subdivision among samples. AMOVA revealed that, regardless of grouping, a significant portion of the overall mtDNA variation is partitioned both within ($\Phi_{ST} = 0.859 - 0.961$, $p < 0.000$) and among populations within regions ($\Phi_{SC} = 0.399$ to 0.862, $p < 0.000$) and, in general, between regions (island, peninsular and mainland Alaska ($\Phi_{CT} = 0.756$ to 0.910, $p < 0.000$), when analyses were weighted by estimates of haplotype sequence divergence. Exceptions involve Φ_{CT} values associated with two of the three subspecies hypotheses (Rausch 1963, Kurtén 1973; Table 3.3). Thus, aside from these two tests, approximately 75-90% of the variance observed was partitioned among regions, and the remaining 10-25 % among populations within regions (4 - 6%), and within populations (5 - 11%). Not surprisingly, among population groupings examined hierarchically, Φ_{CT} was maximized when populations comprising each major mtDNA clade (Clades I, IIa and IIb) were grouped ($\Phi_{CT} = 0.91$, $p < 0.000$; Table 3.3); followed by a population grouping for which populations of the Alexander Archipelago were grouped away from the remainder of populations in Alaska ($\Phi_{CT} = 0.857$, $p < 0.000$). Nevertheless, Φ_{CT} values were still large and significant when populations were partitioned into five groups comprising the AARCH, the Kodiak Archipelago, mainland Alaska, the Alaska Peninsula and the Seward Peninsula (Table 3.3). Among the three subspecies hypotheses tested, Hall's (1984) scheme gave the maximum Φ_{CT} value (0.75) and was the only significant value ($p < 0.004$) observed among the three hypothesized subspecies distributions.

Levels of Gene Flow. Estimates of gene flow inferred from Φ_{ST} (Figure 3.7) for mtDNA suggest extremely weak female effective migration rates overall, with very low levels of mtDNA gene flow (x = 0.004 - 0.069, s = 0.026 for N_{mf}, Wright 1951; Slatkin 1987, 1993; Figure 3.7) between brown bear populations inhabiting the islands of the Alexander Archipelago and those occupying other areas of Alaska. Taken absolutely, N_{mf} values translate to movement of only ~1-6 females every 100 generations.

Levels of gene flow estimated using θ_{ST} values derived from eight microsatellite data for four populations are indicated in Table 3.8. Levels of gene flow estimated from microsatellite data among these populations are much higher than those estimated from Φ_{ST} for the same populations, with the highest levels of gene flow estimated between BRNG and ARC (Figures 3.7 and 3.8).

Population Demography: *mtDNA.* Mismatch distributions, or the pairwise count of nucleotide differences within a population or taxon, were calculated for all sequences combined (Figure 3.10a), the three clades (Figure 3.10b-d) and for all population pairs found within clades (not shown). An expansion rate was calculated based on the estimate of 9,300 years/unit of mutational time as calculated after Harpending et al. (1993), and a divergence rate for mtDNA control region I of 30% per million years determined using a Clade I/polar bear divergence date of 0.50-0.56 mya (based on combined cytochrome b/control region data, Talbot and Shields 1996a; Appendix C). Although these values are based on calibration of pairwise distances between brown bear haplotypes worldwide with the earliest fossil date for ursine bears (4.8 million years ago) and using maximum likelihood scaled branch lengths (Appendix C), the divergence rate values reported herein are not intended to be absolute but rather serve as a relative measure of timing of past evolutionary events.

Pairwise mismatch analysis indicates variation within and among the three clades (Figure 3.10b-d). The overall frequency or mismatch distribution of pairwise sequence divergences among all the samples generated a multimodal curve, with one peak near the ordinate reflecting the close similarity among haplotypes within each clade, and two other

peaks reflecting more distant comparisons among haplotypes from different clades (Figure 3.10a). This type of pattern can reflect a history of three bottlenecks (or expansions) in population size, or geographic structure (Rogers and Harpending 1992), and the lack of a smooth, unimodal curve is inconsistent with any hypotheses of population expansion. If the observed trimodal pattern reflects a history of bottlenecks and subsequent expansion, then the series of expansions include expansions at from 57--69, 21-- 27 and 1 -- 13 mutational units ago. Given the calculation of 9,300 years/mutational unit of time, this would correspond to an ancient expansion at about 560,000 years before present (ybp), one at about 250,000 ybp and a more recent one at about 75,000 ypb. The mean pairwise number of distances within the brown bear populations sampled was 26.16 ± 25.54 (= 1 SD) (Table 3.2).

Pairwise analyses of each of the three clades suggested each differ significantly from expectations according to the sudden expansion model ($\chi^2 = 296.7$ to 493.318, $p < 0$ 0001; Figure 3.10b-c, Table 3.2) of Rogers (1995), although examination of the curves indicates that, in the absence of the observed peak at 6 - 7 mutational units, Clade IIb would appear indistinguishable from an expansion model.

Populations occupying the islands of the Alexander Archipelago (Clade I brown bears) individually display a recent peak at between 2 and 9 units of mutational time, suggesting a recent expansion at roughly 18,000 to 84,000 ybp (Figure 3.10b, individual population data not shown). However, early contributions to this peak are mostly due to mismatches in the ADM and CHI populations at from 2-7 units of mutational time. Populations comprising BAR showed the more ancient peak at approximately 9-11 mutational units of time (or roughly 84,000 ybp; data not shown).

Populations among Clades IIa and IIb (Figure 3.10c,d) share a recent peak in their mismatch distributions at approximately 7 units of mutational time, suggesting a common expansion from about 65,000 ybp (at 9,300 years/mutational unit). In addition Clade IIa shows a peak in mismatch distribution at between 22 and 27 units of mutational time, suggesting a more ancient expansion at 205,000 to 250,000 ypb (Figure 3.10c). Mean pairwise distances within Clade IIa (6.2 ± 39.02) are higher than within Clade IIb (3.4 ±

8.57), as is the raggedness index ($rg = 0.109$, Table 3.2), suggesting populations comprising Clade IIa are more stationary than populations comprising Clade IIb. Among all three clades, mean pairwise differences and observed variance were highest within Clade IIa, and lowest within Clade IIb. This is consistent with the star-like phylogeny estimated for Clade IIb and illustrated by the MST tree (Figure 3.5).

No smooth, unimodal mismatch distribution was observed among any of the 14 populations examined, although six populations did not deviate significantly from expectations under Rogers' (1995) model of rapid expansion (Table 3.2). These six included four populations for which sample sizes were 5 or fewer (BAR, SCOA, SCEN, INT), IZE and AGFN. Population IZE ($n = 14$) is characterized by a single haplotype (when T_n repeats are not included), and AFOG by only two haplotypes ($n = 7$).

Mismatch distributions calculated for four of the five populations characterizing Clade IIa (AKR, ARC, INT, BRNG; data not shown) were bimodal with peaks observed again between 21 to 35 units of mutational time (about 213,000-310,000 ybp), and more recently at between two and three mutational units of time (18,000-30,000 ybp). The latter peak also characterized SCOA, the single exception to the bimodal mismatch distribution characterizing Clade IIa overall (data not shown). Again, all populations for which $n > 5$ demonstrated a multimodal distribution, suggesting either substructuring in the populations or long-term equilibrium conditions within the populations (data not shown).

Individual populations representing Clade IIb also deviate from the sudden expansion model of Rogers (1995) but do not demonstrate a clear wave either (data not shown). The mismatch distributions characterizing these populations are similar to those expected for a theoretical distribution of a population at equilibrium between mutation and drift. Among Clade IIb populations, only BRNG displays a bimodal distribution similar to that found among Clade IIa populations (data not shown).

Table 3.2 displays statistics associated with the calculation of mismatch distributions. Harpending's (1994) raggedness values within clades range from 0.020 to 0.109, with values lowest for Clade I and highest for Clade IIa. High raggedness indices

are expected for populations or groups that have been stationary for long, whereas low values are expected for expanded populations (Harpending 1994).

Microsatellites. I detected signatures of significant recent bottlenecks in three of the four mainland Alaskan populations sampled, using the most appropriate test and underlying model of evolution for the marker type used (TPM, Wilcoxon one-tailed test for heterozygosity excess; Table 3.4). No significant recent bottleneck was detected for ARC, but observed heterozygosity exceeded the average of the corresponding distribution of heterozygosities expected at equilibrium for these tests in samples from the AKR, BRNG and KAT populations. This result suggests a recent bottleneck in all three populations. Under an IAM model, observed heterozygosity exceeded the average expected under equilibrium in all four populations. Under the most conservative model of evolution (SMM), however, no population sampled demonstrated a signature of a recent bottleneck.

None of the populations showed a significant deficiency of heterozygosity under the most appropriate model of mutation (TPM; Table 3.4), using the sign test. This implies that none of these populations have experienced a recent expansion (i.e., within 2-20 generations; G. Luikart, 1998; pers. comm.) in population size or recent influx of rare alleles from genetically distinct immigrants (Luikart and Cornuet 1998).

3.5 DISCUSSION

The existence of divergent clades within contemporary populations of brown bears in Alaska can be explained by two factors: 1) recurrent population contraction and expansion and 2) limited female dispersal. The fragmentation of populations, coupled with long term isolation of subpopulations as a result of glacial-interglacial cycles (Appendix C), would allow haplotypes to diverge between the isolated subpopulations. If subpopulations then expand in size, multiple *de novo* haplotypes could be maintained in the population, eventually giving rise to a distinctive clade.

The extreme climatic fluctuations associated with multiple Pleistocene cycles could have driven such a series of fragmentation and expansion episodes (Berger 1984,

DeChaine & Martin 2004, Hewitt 2000, 2004,). In North America, full glacial advance separated the continent into at least two unglaciated regions, including the high latitude sub-continent of Beringia which connected North America with Asia, and the continent south of the glacial ice. Additional refugia may have occurred along the north Pacific coast (Moodie and Reimchen 1976; Heaton et al. 1996, Appendix A; Byun et al. 1997, 1999), along the eastern coast of North America or in other areas to the south (Rand 1954). With the cyclic change of temperature, glacial ice sheets expanded and contracted to produce dramatic changes in available brown bear habitat. The fragmentation of brown bear populations into isolated refugia would provide ample opportunity for molecular divergence between subpopulations. Eventual glacial retreat would allow isolated brown bear populations to expand into previously unoccupied habitat. Since population fragmentation in Pleistocene refugia appears to have dictated the geographic structuring of mtDNA haplotypes in other highly vagile vertebrates (*Calidris alpina*; Wenink et al. 1993; *Canis lupus*: Weckworth et al. 2005), similar ecological pressures may also have caused mtDNA substructuring in the highly mobile brown bear.

Strong female philopatry will also drive, as well as maintain, a pattern of differentation among populations and regions at mtDNA loci. High levels of male-biased gene flow should lead to homogenization of nuclear markers while maternally-inherited markers would remain restricted to individual localities. A lack of congruence has been reported between distribution of variation in the maternally inherited mtDNA and biparentally-inherited nuclear microsatellite loci for brown bears of the Alexander Archipelago (Paetkau et al. 1998a), although F_{ST}-based analyses suggest there is significant differentiation among Alaskan brown bear populations at nuclear microsatellite loci as well. Differences in modes of inheritance, rates and modes of mutation, effective population size and gender-bias in gene flow are among the explanations given for lack of congruence among marker systems. Such a pattern has also been uncovered in a number of studies across wide taxonomic breadth, including, for example, brook charr (*Salvelinus fontinalis*) (Fergusen et al. 1991), Atlantic trout (*Salmo sp.)* (Bernatchez and Osinov 1995), the *Ensatina eschscholtzii* (salamander) complex

(Moritz et al. 1992), African elephant (*Loxodonta africana*) (Nyakaana and Arctander 1999), Bechstein's bats (*Myotis bechsteinii*) (Kerth et al. 2000), and Canada geese (*Branta canadensis*) (Shields and Wilson 1987; Van Wagner and Baker 1990; Talbot et al. 2003, Scribner et al. 2003).

Because mtDNA is haploid and maternally inherited, Birky et al. (1983, 1989) predicted that population subdivision will be approximately four times greater for mtDNA than for nuclear genes, and even more so, if dispersal is primarily by males. Given these assumptions, maternally inherited mtDNA should show a greater degree of differentiation, and concomitantly estimates of lower rates of gene flow, than autosomally-encoded microsatellite loci, due to smaller female home ranges and philopatry, promiscuous mating system, and smaller effective gene number due to haploidy and maternal inheritance. Comparison of Φ_{ST} values generated from mtDNA with F_{ST}-analogs (θ_{ST}) with those generated for the same populations in Alaska using eight biparentally-inherited microsatellite loci demonstrates, on average, an eight-fold increase in levels of substructuring at mtDNA and concomitant decreased levels of gene flow. This corroborates theoretical predictions of more restricted gene flow (N_{mf}) and increased genetic drift at mtDNA loci, resulting from smaller effective population size imposed by haploidy and maternal transmission (Takahata and Slatkin 1984), augmented by greatly higher levels of male-mediated gene flow.

Levels of Genetic Diversity and Population Structuring. High levels of polymorphism were detected in the mtDNA of brown bears of Alaska; one hundred variable sites were found among sequence information from over 1,600 nt of the mitochondrial genome among 200 individuals MtDNA appears to be a very discriminating molecule for analyzing genetic differentiation among populations of brown bears, as reflected by its high and significant overall and population pairwise Φ_{ST} values. Despite lower (though still significant) levels of spatial differentiation observed at microsatellite loci relative to mtDNA, high levels of polymorphism were also observed at eight microsatellite loci for four populations of brown bears on mainland Alaska and

are shown, in general, to characterize brown bear populations throughout their North American range (Paetkau et al. 1998b). Notable exceptions include populations on KOD (see below). Levels of differentiation, measured by θ statistics, were significant among all four populations but at lower levels than for mtDNA. R_{ST} values were significant at only 33% of population pairwise comparisons, however.

Comparison of θ_{ST} and R_{ST} analyses allow insight into forces shaping subdivision among these four populations. Since θ_{ST} analysis of genetic diversity proceeds under an IAM, each mutation observed is considered to be a completely new state, and thus any memory of a prior state is erased. Therefore, any genetic similarity between populations is attributable to migration or recent divergence from a common ancestor. However, the mutational processes of microsatellites do not erase all information about the ancestral allelic state, since mutations at these loci tend to proceed, at least approximately, under a SMM. Thus, when microsatellites are analyzed, θ_{ST} values tend to overestimate coalescence times.

Slatkin (1995) used SMM to calculate R_{ST} values and demonstrated that they generally provided a less-biased estimate for demographic parameters than did F_{ST} (and therefore θ_{ST}) values, given sufficient coalescence times. However, for recently diverged populations, genetic drift is the dominant process creating local differentiation, and mutational events are less important. For this reason, in recently diverged populations, or those still connected via gene flow (such as BRNG, AKR and ARC) the performance of θ_{ST} improves over R_{ST}. In this study, R_{ST} values are lower than θ_{ST} values for comparisons between mainland Alaskan populations of BRNG, AKR, ARC and KAT, suggesting these populations are of relatively recent evolutionary origin, and that drift and migration are still predominating over mutation in shaping the observed pattern of genetic differentiation.

Levels of Gene Flow. When gene flow is inferred from F-statistics, an assumption of equilibrium between drift and migration can be reasonable for some

populations, since migration is a relatively fast homogenizing force. However, genetic signatures of population expansions can remain for a long time, obscuring current ecological population structure (Rogers and Harpending 1992). In addition, since the rate of approach to equilibrium is dependent upon effective population size (Nei and Feldman 1992), it would take an extremely long period of time for large population sizes of brown bears to reach migration-drift equilibrium. Thus, it may be misleading to infer absolute migration rates from F_{ST} values. Therefore, to avoid biases due to possible violations of underlying assumptions associated with models of gene flow (Wright 1951), the estimates of levels of gene flow (N_{ef}, N_m) should not be considered absolute. They nevertheless provide a basis to quantify gender bias in gene flow and uncover differences in demographic processes among populations.

While absolute values of gene flow estimated may not reflect actual levels of gene flow, the low gene flow values generated using mtDNA data for AARCH relative to mainland populations stand in stark contrast to some of the values observed between other populations assessed using mtDNA, and for values observed using microsatellite loci. For example, the highest levels of female-mediated gene flow, estimated from mtDNA sequence data from brown bears across Alaska, appear to be between populations in ARC, AKR, INT and BRNG (Figure 3.7). Phylogeographic analysis demonstrates these populations share (albeit at different frequencies) a number of closely related mtDNA haplotypes which place individuals sampled from these populations into either the eastern or western clades (Clades IIa and IIb, respectively; Talbot and Shields 1996a). Again, this area of Alaska appears to represent an area where two phylogeographic regions abut (Talbot and Shields 1996a; Waits et al. 1998; Appendix B), or form a "clade hybrid zone," and may represent an area of secondary contact between Clades IIa and IIb. Among Clade IIb populations with larger sample sizes ($n > 5$), lower estimates of gene female-mediated gene flow are associated with peninsular (IZE, KAT and SPEN), and island (AFOG) populations.

The occurrence of "private alleles," or alleles that occur only in a single population, is an important indication of reduced gene flow among populations (Slatkin

1985). Thus, all of the populations examined herein contain individuals characterized by at least one autapomorphy that does little to resolve phylogenetic analyses, but does provide an indication of reduced gene flow characterizing a population. The question of whether a particular allele is "private" becomes statistically problematic in the presence of low within-sample genotype diversity. Models used to estimate gene flow, including Slatkin and Maddisons' (1989) algorithm, do not take into consideration the frequency distribution of rare "private" alleles: genotypes observed only once must by definition be private, yet are not convincing evidence of limited gene flow. Intuitively, the observation that rare genotypes are not observed outside of regions suggests that genotypes are not currently rapidly exchanged among brown bear populations. It is possible that additional population genetics studies with larger sample sizes may indicate that rare alleles are in fact more widely distributed geographically than this study indicates.

Comparison to Field Data. The pattern of disparity in levels of population structuring revealed using the different marker classes, as revealed by comparisons of F_{ST}-analog values among populations, suggests male-bias in gene flow among populations of brown bears in Alaska. These data support interpretation of field observations.

Brown bear populations in Alaska are managed by the Alaska Department of Fish and Game in 26 units that are grouped into six major subdivisions. Long-term radiotelemetry and marking studies within these subdivisions suggest that brown bears in Alaska generally comprise spatially subdivided populations (Glenn et al. 1976; Berns et al. 1980; Glenn and Miller 1980; Reynolds 1976, 1978, 1980; Schoen et al. 1986; Schoen and Beier 1990). Distinct seasonal ranges characterize each subpopulation (Mundy and Flook 1973; Darling 1987; Schoen et al. 1986; Schoen and Beier 1990). Individual bears tend to occupy the same ranges for several years, and there is limited movement among subpopulations; however, exceptions do occur (Reynolds and Garner 1986). Males, particularly subadults, tend to disperse more often and for greater distances than females, who usually remain on or near the maternal home range (Pearson 1975, but see Pearson

1976). Females in some areas show greater year-round fidelity to specific areas than do males (Murie 1981; Nagy et al. 1983 a,b; Smith and Van Daele 1984).

Although exceptions occur (Reynolds 1978, Ballard et al. 1982), home ranges of female brown bears are generally smaller and less variable in size than home ranges of males, with adult females with cubs usually having the smallest home ranges among age and sex classes (Glenn and Miller 1980; Murie 1981; Ballard et al. 1982; Miller and Ballard 1982; Dau 1990a). Home ranges tend to overlap, with greater overlap between intersexual vs. intrasexual ranges. Generally, the finding of larger male home ranges (from two to ten times greater than the average female home range, USFWS 1987), and movements are thought to be the result of early summer breeding behavior. Female dispersal is evidently rare in many bear populations; nevertheless, dispersal studies tend to underestimate dispersal levels and distances (Koenig et al. 1996). Significant movements by female bears, with or without cubs, have been documented (Glenn and Miller 1980). Without genetic data, however, it is difficult to determine how evolutionarily effective such dispersal is.

Radiotelemetry and other marking studies, which typically assess data from females due to technical difficulties in collaring male bears, are generally performed inclusively within pre-defined management units or within boundaries of state or federal parks or reserves. Thus, evidence of spatial subdivision of populations may have been an artifact of sampling as well as female behavior rather than a true reflection of brown bear population structuring overall. Nevertheless, these genetic data suggest that Alaskan bear populations do represent genetic demes with varying potential for gene flow among them. The tendency for female brown bears to remain on or near their natal ranges and for males to disperse more than females has apparently increased subdivision of maternally inherited mtDNA compared to nuclear genes.

Dispersing males will contribute mtDNA haplotypes to a new population only transiently, until death. Unless male dispersal rates are very high, the representation in a population of foreign mtDNA types brought in by immigrants will be negligible. This would facilitate mtDNA genetic structuring within a species and limit mixing of mtDNA

haplotypes from different geographic regions. However, it may also explain the existence of the widely dispersed Clade IIb if females dispersed into new habitats after deglaciation at the end of the Pleistocene.

Alexander Archipelago Brown Bears. A pattern of low genetic exchange is apparently demonstrated for mtDNA on the islands of the Alexander Archipelago relative to coastal mainland Alaska. I have not uncovered any mtDNA haplotypes on the islands that are characterized in any mainland population (i.e., Clade IIa or IIb haplotypes). Sample sizes (n = 3) are small for the southcoastal mainland populations included herein; however, additional work by others (Shields et al. 2000), in which cytochrome b sequence information was obtained from 13 additional individual brown bears from southcoastal Alaska, have recovered only Clade IIb, and no Clade I haplotypes there; neither was the reciprocal found.

Because analysis of microsatellite data using allele-sharing suggests homogenization of nuclear genes between bears of the islands of the Alexander Archipelago and bears of mainland, southcoastal Alaska (presumably Clade IIb bears) Paetkau et al. (1998a) suggested bears of the Alexander Archipelago are not genetically distinct from mainland bears. This is in direct contrast to mtDNA data reported in Cronin et al. (1991); Talbot and Shields (1996a, b); Shields et al. (2000), Waits et al. (1998, 2000) and Appendix B, C). Incongruence of these results may be due to extremely high levels of male-biased gene flow, incompatibility or mosaic incongruence (Farris 1971; Brower et al. 1996) due to high mutation rate and homoplasy observed among microsatellite loci (see Goldstein and Schlotterer 1999), or both. Although homoplasy at microsatellite loci is less likely to mislead interpretation of data compared between very closely related populations, it may do so for populations less connected via gene flow.

The extreme differences (i. e., complete fixation of AARCH populations for mtDNA haplotypes that contain ~40 nucleotide site differences) are difficult to explain in terms of male-bias in gene flow only. To explain the observed pattern, levels of male emigration from AARCH populations would need to be extremely high, over many

generations, concomitant with severely restricted male immigration. In addition, virtually complete philopatry of both Alexander Archipelago islands and southcoastal females would be required if the observed pattern of mtDNA diversity reflects the true situation. If the observed pattern is due to this extreme gender bias in philopatry and gene flow, it also not clear why bears in populations on AARCH and southeastern mainland Alaska would differ behaviorally from bears in mainland interior populations such as BRNG, ARC and AKR, which share mtDNA haplotypes and alleles at microsatellite loci, and yet are still significantly differentiated from each other. Analyses of juxtaposed microsatellite systems (JMS's; Estoup et al. 1999) and single-copy nuclear genes, as well as sequence information from regions flanking the microsatellite loci used by Paetkau et al. (1998a, b) and comparison of R_{ST}- vs. F_{ST}-based analyses (see above) can help resolve whether size homoplasy plays a role in the extreme noncongruence of molecular evidence based on different marker classes, or whether the high levels of gene flow hypothesized based on analysis of microsatellite loci, has resulted in homogenization of the nuclear genome of brown bears (Paetkau et al. 1998a).

A similar, though less dramatic, pattern of substantial differences in levels of population differentiation, as assayed by markers with different modes of inheritance, has been found in other island populations of highly vagile vertebrates in Alaska. In one example, Canada geese nesting on Middleton Island in the Gulf of Alaska (Pearce et al. 2000, Talbot et al. 2003, Scribner et al. 2003) demonstrate a genetic signal of strong female philopatry and weak male-mediated gene flow. These large, dark-bodied Canada geese were managed as dusky Canada geese (*Branta canadensis occidentalis*), a subspecies subject to considerable management, since its numbers decreased after an earthquake modified the habitat on the breeding area. However, Canada geese nesting on Middleton Island possess a unique haplotype that is not found in female geese nesting on the Copper River Delta, the type locale for the dusky Canada goose. The unique haplotype shared by the Middleton Island geese has been found elsewhere only in geese inhabiting other islands of Prince William Sound, and in high frequency (from 94% to 100%). The haplotype has not been found among mainland coastal nesting female

Canada geese. Nevertheless, only weak to moderate subdivision between these island populations of Canada geese and mainland coastal geese has been observed in a multi-year, multilocus analysis of biparentally-inherited microsatellites. Analyses of these data suggest male-bias in gene flow coupled with strong philopatry of females nesting on the islands of Prince William Sound (Talbot et al. 2003), a pattern that may be paralleled in brown bears inhabiting islands of the Alexander Archipelago.

Both female Canada geese and female brown bears show high levels of philopatry (Anderson et al. 1992; see citations above for brown bears). Female brown bears, like female Canada geese, are doubtless as capable as males of dispersing, and dispersal of male brown bears (but not females) over large bodies of water has been observed (C. Dau, USFWS,1990b, pers. comm.; K. Titus, ADF&G, 2000, pers. comm.). Thus, the genetic distinctness, as observed in the mtDNA in populations inhabiting the islands of the Alexander Archipelago, is remarkable, given the high vagility of the species and apparent absence of physical dispersal barriers. Such exceptional cohesiveness has been found among females in other species, such as Bechtein's bats (*Myotis bechsteinii*) (Kerth et al. 2000), loggerhead turtles *(Caretta caretta*) (Bowen et al. 1993), and Sri Lanka macaques (*Macaca senica*) (Hoelzer et al. 1994). Comparison of genetic patterns among species with gender bias in dispersal can help determine if social behavior is responsible for the observed disparity. Fred Dean (UAF, 2005, pers. comm.) suggests that a significant behavioral barrier – that is, an aversion of water by cubs less than two years of age – may contribute to the observed structuring. Regardless, the genetic data suggest contemporary demographic independence of female brown bears on the islands of the Alexander Archipelago, and this should be considered in any plan used to manage those populations, since island populations experiencing substantial loss of numbers cannot be expected to recruit females from neighboring mainland populations.

Insular and Peninsular Populations. Fragmentation of populations and subsequent decrease in population size are predicted to decrease genetic variability within each population due to loss of alleles (or haplotypes) by drift and founder effect. These effects

will be manifested if habitat fragments are smaller than the area typically occupied by a single random-mating unit (roughly equivalent to Wright's neighborhood area; Wright 1943), and if gene flow between population fragments has been substantially reduced (Wright 1978; Endler 1979; Falconer 1989). These effects may come about by genetic drift of an originally homogenous population, or a founder event.

Specific predictions follow from the hypothesis of habitat fragmentation and geographic subdivision (Introduction, pages 13-14). Insular and peninsular populations are predicted to display reduced genetic variability due to restricted movement (and hence gene flow), as well as differentiation, in terms of sequence variation or differences in haplotype frequencies, from source populations, such as those found in adjacent mainland areas. Island and peninsular populations also are predicted to display genetic differentiation or haplotype frequency differences relative to mainland populations, due to reduced gene flow. In addition, given equal time of isolation and similar heterogeneity of original populations, genetic variation should be lower in smaller, less dissected islands such as those on ADM (ca. 1,800 bears on 4,306 km^2), CHI (ca. 1,550 bears on 5,445 km^2) and BAR islands of the Alexander Archipelago (ca. 1,044 bears on 4,159 km^2), than on larger, dissected island such as on KOD (ca. 3,000 bears on 13,890 km^2) (see Introduction, page 13).

I found that levels of mtDNA haplotype and nucleotide diversity levels in peninsular and some island populations were low relative to mainland interior populations for which similar sample sizes were available. Relatively low levels of variability for peninsular and some island populations also have been observed at microsatellite loci (Table 3.1; Paetkau et al. 1998a, Talbot et al. 2006, , Jackson et al. 2006), consistent with the prediction of reduced genetic diversity in peninsular and island populations. The lowest levels of diversity among mtDNA measures were found within IZE. The brown bear populations from which this sample was drawn inhabits the end of the long, narrow Alaska Peninsula that is 140 km broad at its base, and 800 km long, and there are likely fewer opportunities for symmetrical exchange with other populations. Thus, IZE may represent the leading edge of a recent (post-Pleistocene) expansion from

populations at the base of the peninsula and mainland Alaska. Dau (1990a) concludes that relatively small home ranges determined for radiocollared bears on IZE are the result of compressed geographic characteristics of this area of the peninsula, and that critical habitat requirements for brown bears in the area are available within a relatively small area. Genetic diversity within KAT, at the base of the Alaska Peninsula, is higher than at the tip, but still moderate compared with other populations. This trend is paralleled for microsatellite loci obtained for KAT, and reported in the literature (Paetkau et al. 1998b).

The expectation of lower genetic variability found within island populations is upheld for KOD for microsatellite data (Paetkau et al. 1998b), but not for mtDNA data. Brown bears of the Kodiak Archipelago are characterized by moderate levels of mtDNA variability. Moderate levels of mtDNA variability observed on KOD may reflect heterogeneity of the founding population, which likely colonized KOD after the retreat of ice at the end of the Pleistocene (Van Daele 2003; Talbot et al. 2006). Talbot et al. (2006) found extremely low levels of variation at the DQB1 exon II gene of the ursid MHC gene, confirming that low levels of variation observed at nuclear microsatellite loci within populations on KODARCH reflect low genetic diversity at functional nuclear genes.

Conversely, expectation of lower genetic variability within island populations is not upheld for brown bears of the islands of the Alexander Archipelago. Populations on these islands are characterized by relatively high levels of neutral haplotype and nucleotide diversity reflected over the longer time in moderate E(υ) values. Moderate levels of microsatellite variability have been reported for the bears of the Alexander Archipelago, however (Table 3.1; Paetkau et al. 1998b).

Population Demography. Mismatch distributions suggest three episodes of population expansion among ancestors of brown bears currently inhabiting Alaska. Given the calculation of 9300 years/mutational unit of time, these expansions can be placed at 0.56 mya, 0.25 mya and 75,000 ybp. Though heuristic, these expansion times are concordant with existing paleontological data, which suggest that the earliest North

American fossils (found in Alaska) can be placed at 0.20 - 0.30 mya (Kurtén and Anderson 1980), just prior to the last glacial maxima. Until recently, the fossil record suggested that brown bears failed to reach mid-latitude North America until 11,000 to 13,000 ybp. However, Matheus et al. (2004) dated a specimen from southern Canada at 25,000 ybp, suggesting brown bears occupied southern Canada, and potentially the coterminous United States, prior to the recession of the ice sheets.

The oldest *U. arctos* fossils from Europe (France) are dated to 0.92 - 0.98 mya (Mazza and Rustioni 1994), whereas the oldest Asian specimens (China) date from 0.40 - 0.80 mya (Kurtén 1968; Nilsson 1983). Clade I bears, which may have diverged from the lineage leading to Clade II bears some 0.56 mya, may have increased in number subsequent to divergence and expanded into new areas. Talbot and Shields (1996b) suggested ancestors of the brown bears now inhabiting the islands of the Alexander Archipelago (Clade I) were once more widespread in Beringia but may have become eliminated from the majority of their range, or distributed to the south. This isolation in their current range resulted from changes subsequent to glacial advances and retreats. Chesser and Zink (1994) found that contemporary distributions of some organisms can be poor indicators of historical distributions, particularly given the times required for phylogeographic congruence to evolve.

Leonard et al. (2000) reported the recovery of mtDNA control region (168 nt) and cytochrome b (ca. 502 nt) sequence information from bones of seven individual brown bears preserved in permafrost in interior Alaska and the Yukon Territory and dated reliably to 14,000 to 42,000 ybp. These researchers determined that four of the bones, from a locale near Fairbanks, Alaska, dated to ca. 15,000 ybp (the end of the last glaciation) carried mtDNA haplotypes corresponding to Clade IIa herein. The other three bones were dated from 34,000 to 43,000 ybp to Fairbanks (n = 1, belonging again to Clade IIa herein), or the Yukon Territiories [n = 1, belonging to Clade I herein, and n = 1, belonging to Clade IV (southern Canada and Unites States, Waits et al. 1998, 2000: Appendix B, C)]. Leonard et al. (2000, Fig. 2A) suggest the distribution of Clade IIa bears had a broader Pleistocene distribution than currently. This is a misinterpretation of

the current distribution of Clade IIa members; bears with these haplotypes are found in BRNG and INT (Talbot and Shields 1996a; Waits et al. 1998, 1999, Appendix B, C) and the clade contact zone encompasses the Fairbanks area. Oddly, Leonard et al. (2000), like Barnes et al. (2002) elevate to population status the temporally and spatially disjunct samples from permafrost (comprising a total of seven individuals separated by thousands of years). It is undoubtedly as necessary to have adequate sample sizes (within the same time period) to evaluate population structure as is required in studies of contemporary populations. Nevertheless, these researchers' discovery of a Clade I (Alexander Archipelago) haplotype dated to around 36,000 ybp in the Yukon Territories, and their conclusion that the Beringian "population" was more genetically diverse than current North American populations, supports the suggestion of a broader distribution of Clade I bears (Talbot and Shields 1996a).

Leonard et al. (2000) suggest the most likely explanation for current geographic segregation of mtDNA haplotypes, including the highly restricted populations represented by extant Clade I (Alexander Archipelago) bears herein, is that founding populations of each "isolated" area contained representatives of a single clade. They also suggest that geographical partitioning of mtDNA in extant North American populations is a relatively recent event. A genetic signature of a population expansion subsequent to population bottlenecks and loss of genetic variability associated with such founder event [i.e., a unimodal mismatch distribution and concomitant low genetic variability (Rogers 1995; Marjoram and Donnelly 1994)], would lend support to such a hypothesis. My analysis of the distribution of pairwise mismatches does not support their hypothesis.

Pairwise mismatches are the distribution of the observed number of differences between pairs of haplotypes. The distribution of pairwise mismatches is usually multimodal in samples drawn from populations at demographic equilibrium, since it reflects the highly stochastic shape of gene trees. On the other hand, it is usually unimodal in populations that have passed through a recent demographic expansion (Rogers and Harpending 1992; Slatkin and Hudson 1991). Thus, the mismatch distribution within populations or clades may indicate whether these groups have

persisted over long periods of time at a relatively constant population size, or have undergone a bottleneck and/or a recent expansion into an area. Gene trees with a single major bifurcation are expected to demonstrate a bimodal distribution, whereas a unimodal distributional pattern is expected in populations that have undergone a recent expansion (Rogers 1995).

The distributional pattern observed within the bears populating the islands of the Alexander Archipelago of Alaska (Clade I, Talbot and Shields 1996a) -- i.e. moderate number of pairwise differences, with several peaks, coupled with relatively high levels of haplotypic and nucleotide diversity as well as generally non-negative Fu's F_S values and Tajima's *D* values and high *rg* values -- is inconsistent with a model of expansion (Tajima 1989, Marjoram and Donnelly 1994, Fu 1997, Avise 2000) subsequent to a bottleneck, as inferred by Leonard et al. (2000). A similar pattern of significant raggedness as well as bimodality in distribution (Clade II) is observed in populations representing both Clades IIa and IIb. However, the relatively low mtDNA divergence within the western Clade (IIb), exemplified by KOD, coupled with the observed starlike geneology of haplotypes (Figure 3.6), low *rg* values, and high haplotype diversity coupled with low nucleotide diversity, suggests recent expansion of some Clade IIb populations. Since Clade IIb includes brown bears from Asia and Europe (Talbot et al. 1996b, Appendix C), this expansion probably occurred since the last glacial maximum from Asia through Beringia.

MtDNA information has proven useful in uncovering ancient patterns of distribution and uncovering episodes of population expansion. Nuclear-inherited microsatellite loci, on the other hand, are widely used to uncover current demographic patterns of movement and examine effects of recent anthropogenic impacts on wildlife species (Pierson et al. 2000; Pearce et al. 2000; Talbot et al. 2003), i.e., within a timeframe of a two to twenty or so generations. Due to the relatively rapid rate of mutation of microsatellite loci, and concomitant high levels of polymorphism, these markers can be extremely valuable when used to examine contemporary relationships among closely related populations, particularly when those relationships are so close as to

be outside the focal range of most mitochondrial DNA sequences. Thus, microsatellite loci were used to search for a genetic signature of recent bottlenecks or expansions among four populations of brown bears considered now to be at demographic equilibrium (Craighead 1994; Luikart 1997). Although populations of brown bears inhabiting interior Alaska are considered to be stable both currently (Craighead 1994), and over longer evolutionary periods (Figure 3.10c, d), I detected signatures of significant recent bottlenecks (within the past 2-20 generations) in three of the four mainland Alaskan populations sampled (Table 3.4).

Concurrent analysis of nuclear data can lend insight. Unfortunately, levels of homoplasy among nuclear microsatellite loci may be so high that they preclude accurate assessment of more historical population structure, and are thus less able to track genetic response of organisms to longer-term impacts such as climate change and associated changes in distribution. In view of the potential for huge environmental changes in the global climate predicted for the near future, investigations into the history of populations are extremely relevant. Since micro- and macroevolutionary parameters are equally important in understanding the distribution of variation and structuring of populations, it would be prudent to employ as many markers as possible to uncover the historical and contemporary patterns characterizing extant populations. In addition, since a combination of several factors can be responsible for observed genetic differentiation among natural populations, it also would be prudent to develop analyses that allow researchers to discern the relative contribution of these complex factors to the observed pattern of genetic variation (Templeton et al.1992). Analyses of the data set herein would benefit by subjecting it to a nested clade analysis approach (Templeton 1998; Scribner et al. 2003), although this method does not distinguish statistically among alternative intepretations of population history (Knowles and Maddison 2002).

Implications for Conservation and Management: *Evolutionarily Significant Units and Management Units.* This research was conducted to (a) estimate levels of genetic diversity and population genetic substructuring among populations of brown bears in

Alaska and (b) to determine whether substantial differences in long-term levels of variation occur within island and peninsular populations relative to mainland populations. The research was also conducted to compare estimates of levels of gene flow among brown bear populations estimated from both maternally-inherited mtDNA, and biparentally-inherited nuclear microsatellite data, to thereby determine whether there is a molecular signal consistent with gender-bias in gene flow among brown bear populations. These three goals are conservation-oriented, and the discussion has focused on differences between the mtDNA, and microsatellite data reported herein, and elsewhere, for the same populations.

Substantial differences observed using the two classes of molecular markers are not uncommon (see Introduction), nor unexpected, since the two marker types lend resolution at different spatial and temporal scales. The disparate results serve to underline the importance of collecting molecular information from more than one marker class. Such disparities in results serve to enhance decision-making processes and analyses using more than one marker type should be performed before results of molecular studies are used to make recommendations for management and conservation of natural populations.

In the context of conservation, variation in populations can be assessed using many criteria, including the genetic markers used in this study. Vertebrate populations are often considered distinct for purposes of conservation if the population represents an Evolutionary Significant Unit (ESU) of the biological species. The genetic criteria proposed for identifying ESUs were promoted by Moritz (1994b), who suggests an ESU can be estimated as a set of populations distinguished by strong phylogenetic structuring of mtDNA, and significant divergence in frequencies of nuclear alleles. By this definition, designation as an ESU implies that the population is to some degree reproductively isolated, and additionally that it represents an important component of the evolutionary legacy of the species.

Management Units (MUs), on the other hand, are used to define an appropriate geographic scale for monitoring and setting management practices for current

populations. Unlike ESUs, MUs have been described as populations or sets of populations characterized by distinct allele or haplotype frequencies (Moritz 1994b). Moritz proposed the concept of ESUs and MUs to encourage debate and focus discussion about the relevence of taxonomic groupings to conservation (Moritz 1994b). There are differing views on what should genetically constitute an ESU or MU (Dizon et al. 1992; Moritz 1994a,b; Vogler and DeSalle 1994), and indeed, the ESU and MU concept has been heavily criticized recently as 1) failing to focus upon the process and potential, but rather on the result, of evolution, (Crandall et al. 2000), 2) by underestimating biodiversity (Dimmick et al. 1999), 3) by overestimating biodiversity or being severely limited in identifying intraspecific groupings (Paetkau 1999). Despite these arguments, all researchers insist upon some demonstrable level of uniqueness.

For neutral or nearly-neutral genetic markers, such as nuclear microsatellite loci and hypervariable portions of the mtDNA (i.e., third positions of codons for the cytochrome b and the hypervariable portion I or domain I of the mammalian or avian control region, respectively), divergence of populations is a function of time, degree of population separation, and evolutionary effective population size. Additional criteria, such as occupancy of unique and geographically separate habitats, variation in life history traits and morphology, coupled with considerations of the possibility that habitat modification may seriously impact an organism's fitness, must also be taken into consideration for targeting populations for conservation purposes. Despite perceived or real problems with the ESU concept, it provides a framework upon which to incorporate systematic research into conservation decisions. This is critical, since it is clear that a lack of understanding of systematic relationships of a number of organisms has rendered restoration attempts ineffective to disastrous (Avise and Nelson 1989; Daugherty et al. 1990; May 1990).

Alexander Archipelago. Alaback (1988) calculates that approximately one-quarter of the world's remaining temperate rain forests is found in southeastern Alaska and coastal British Columbia, and portions of these forests provide habitat for brown bears inhabiting the Alexander Archipelago. Extensive timber harvesting throughout the

archipelago are thought to impact wildlife populations and to be particularly detrimental to endemics inhabiting the area (Cook et al. 2001). Populations of brown bears on the islands of the Alexander Archipelago appear to form a monophyletic clade relative to brown bears elsewhere in Alaska, Canada, and worldwide (Talbot and Shields 1996a; Waits et al. 1998, Appendix B, C), and are differentiated from other such groups by substantial genetic differences (up to 40 sites for the entire data set examined herein). The patterns observed among brown bears in the Alexander Archipelago also have been observed among a number of other organisms in the area.

Cook et al. (2001) used mitochondrial DNA sequence information (cytochrome b) from eight mammalian species with different dispersal capabilities to provide a phylogeographic perspective on endemism in the Alexander Archipelago. Their interpretation of the pattern of genetic diversity observed among these species is that this region is a center of a dynamic faunal exchange, characterized mostly by Holocene colonizers (neoendemics: *Cleithronomys rutilus* and *Sorex cinereus*, Demboski 1999; *Glaucomys sabrinus griseifrons*, Demboski et al. 1998), but also by refugial persistors (paleoendemics: *Sorex monticolus, Microtus longicaudus, Martes americana, Mustela erminea, Ursus americanus*, Demboski et al. 1999; Conroy and Cook 2000; Stone 2000). The latter are species characterized by more than one reciprocally monophyletic lineage in southern Alaska, and demonstrate higher levels of differentiation than predicted under current taxonomies (Cook et al. 2001). Comparson of individual patterns for numerous species inhabiting the same area provides insight into understanding some of the same processes that may have shaped diversity in brown bears in that region. Cook et al. (2001) use these comparative data to suggest the possibility of persistent refugia along the Pacific Northwest mainland coast and islands, as found by other researchers (Heusser 1989; Heaton et al. 1996; Byun et al. 1997, Fleming and Cook 2002). Additional independent genetic markers and increased sampling are required to test these hypotheses, however (Cook et al. 2001).

Because island faunas demonstrate higher rates of extinction due to loss of habitat or introduction of competitive exotics (Diamond 1989), populations found on islands

represent considerable conservation concern. While the population of brown bears on the islands of the Alexander Archipelago appear healthy (Titus, ADF&G, 2000, pers. comm.), genetic data suggest the populations are characterized by contemporary and long-term reduced effective female gene exchange with mainland Alaskan populations, assessed using the maternally-inherited mtDNA. However, this pattern is not obvious using analyses of nuclearly-inherited microsatellite data alone (Paetkau et al. 1998a), although, as discussed earlier, the criteria for assessing isolation of the Alexander Archipelago populations using microsatellite data were different than for mtDNA.

The populations of brown bears inhabiting the Alexander Archipelago appear to constitute a monophyletic group with respect to mitochondrial DNA, characterized by extremely low or absent current and past gene flow among females relative to mainland populations. Thus, like many of the species inhabiting this region, they represent a segment of biological diversity that shares a common evolutionary lineage and inhabits a particular unique geographic region distinct from other regions inhabited by other members of the same species. Because any specified, distinct group of organisms can be claimed to have a unique evolutionary future, any single method may be inadequate to identify an evolutionary unit (EU: NRC 1995). If the brown bears of the Alexander Archipelago are significantly differentiated from other populations in terms of nuclear DNA, preferably for which allelic phylogenies can be determined (Dimmick et al. 1999), then by the genetic criteria proposed by Moritz (1994a), they deserve to be more closely examined as candidates as significant units of diversity, or evolutionary significant units.

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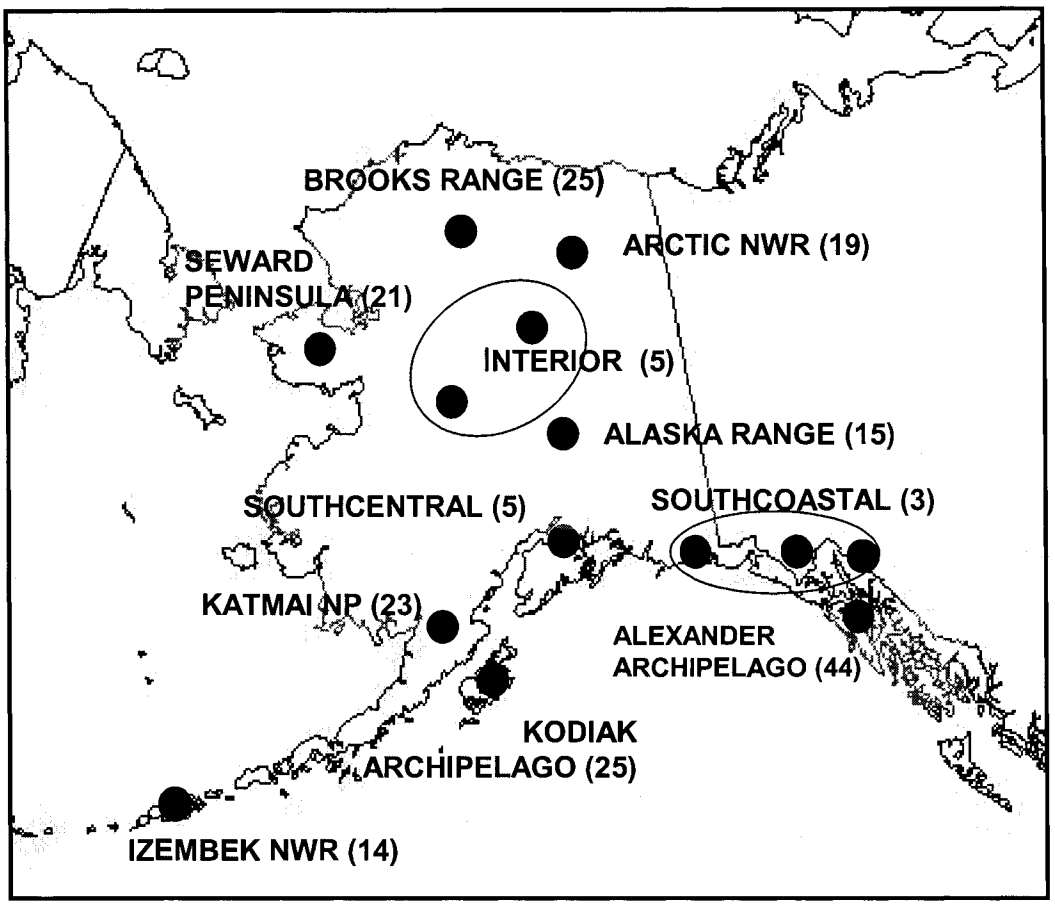

Figure 3.1a. Sample sizes and collection locations in Alaska for the brown bears analyzed in this study. Katmai and Izembek NWR comprise the Alaska Peninsula; Pooled samples from interior, and southcoastal Alaska, are encircled within ovals.

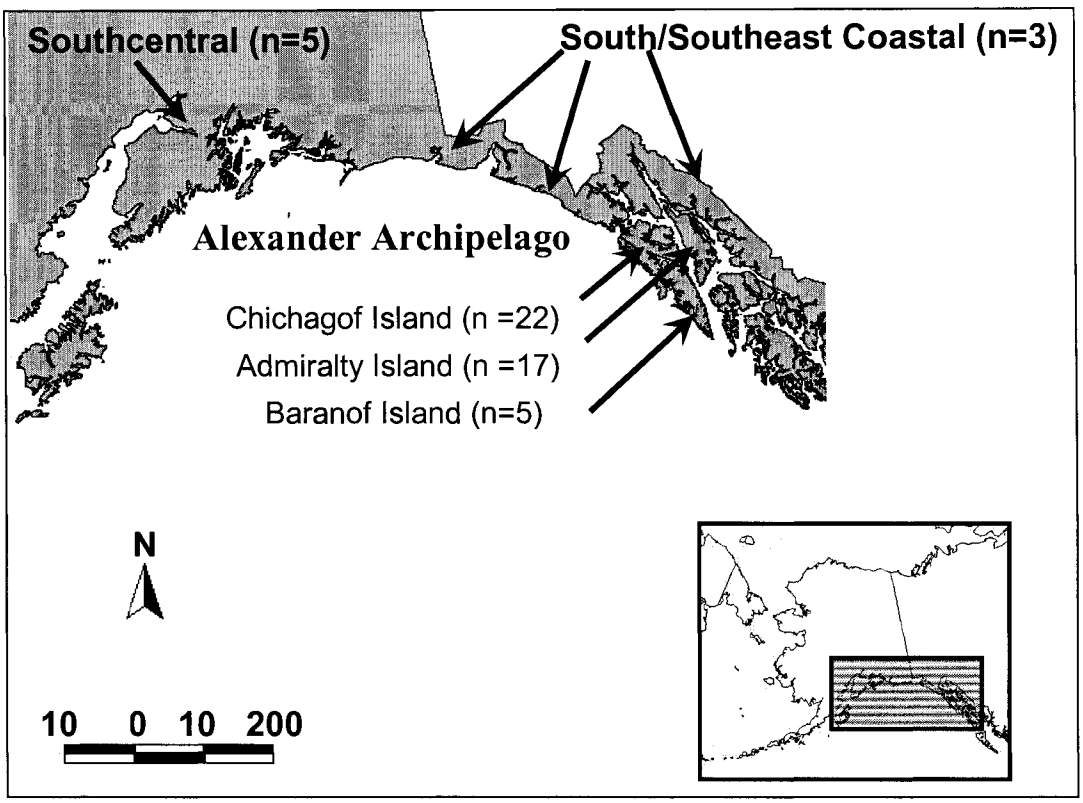

Figure 3.1b. Detail of Alexander Archipelago, south central and southeastern coastal Alaska showing sampling locations and sizes for bears analyzed in this study.

Haplotypes	*n* per locality													
	ADM	BAR	CHI	SCOA	AKR	ARC	INT	BRNG	SCEN	SPEN	KOD	AFOG	KAT	IZE
1. GB01	7	0	0	0	0	0	0	0	0	0	0	0	0	0
2. GB02A	2	0	0	0	0	0	0	0	0	0	0	0	0	0
3. GB02B	2	0	0	0	0	0	0	0	0	0	0	0	0	0
4. GB02C	1	0	0	0	0	0	0	0	0	0	0	0	0	0
5. GB03A	0	2	0	0	0	0	0	0	0	0	0	0	0	0
6. GB03B	0	1	5	0	0	0	0	0	0	0	0	0	0	0
7. GB04	0	2	0	0	0	0	0	0	0	0	0	0	0	0
8. GB05A	1	0	0	0	0	0	0	0	0	0	0	0	0	0
9. GB05B	4	0	0	0	0	0	0	0	0	0	0	0	0	0
10. GB05C	0	0	2	0	0	0	0	0	0	0	0	0	0	0
11. GB05D	0	0	2	0	0	0	0	0	0	0	0	0	0	0
12. GB05E	0	0	2	0	0	0	0	0	0	0	0	0	0	0
13. GB06	0	0	0	0	0	0	0	0	1	0	0	0	0	0
14. GB07	0	0	0	0	0	0	0	0	1	0	0	0	0	0
15. GB08A	0	0	0	1	0	0	0	0	0	0	0	0	0	0
16. GB08B	0	0	0	0	3	0	0	0	0	0	0	0	0	0
17. GB08C	0	0	0	0	1	0	0	0	0	0	0	0	0	0
18. GB09A	0	0	0	1	0	0	0	0	0	0	0	0	0	0
19. GB09B	0	0	0	1	0	0	0	0	0	0	0	0	0	0
20. GB10A	0	0	0	0	0	0	1	2	0	0	0	0	0	0
21. GB10B	0	0	0	0	0	7	0	0	0	0	0	0	0	0
22. GB10C	0	0	0	0	0	2	0	0	0	0	0	0	0	0
23. GB11	0	0	0	0	0	3	0	0	0	0	0	0	0	0

Figure 3.2. Distribution of 61 haplotypes found among brown bears from 13 populations in Alaska.

Haplotypes	*n* per locality													
	ADM	BAR	CHI	SCOA	AKR	ARC	INT	BRNG	SCEN	SPEN	KOD	AFOG	KAT	IZE
24. GB12	0	0	0	0	0	2	0	0	0	0	0	0	0	0
25. GB13A	0	0	0	0	0	0	0	4	0	0	0	0	0	0
26. GB13B	0	0	0	0	0	0	0	1	0	0	0	0	0	0
27. GB13C	0	0	0	0	0	0	0	1	0	0	0	0	0	0
28. GB13D	0	0	0	0	0	0	0	1	0	0	0	0	0	0
29. GB13E	0	0	0	0	0	0	0	1	0	0	0	0	0	0
30. GB14A	0	0	0	0	0	0	0	10	0	0	0	0	0	0
31. GB14B	0	0	0	0	0	0	0	2	0	0	0	0	0	0
32. GB15A	0	0	0	0	0	0	0	0	0	0	0	0	16	0
33. GB15B	0	0	0	0	0	0	0	0	0	0	0	0	2	0
34. GB15C	0	0	0	0	0	0	0	0	0	0	0	0	1	0
35. GB15D	0	0	0	0	0	0	0	0	0	0	0	0	1	0
36. GB15E	0	0	0	0	0	0	0	0	0	0	0	0	2	0
37. GB16	0	0	0	0	0	0	1	0	0	0	0	0	0	0
38. GB17	0	0	0	0	1	0	0	0	0	0	0	0	0	0
39. GB18	0	0	0	0	1	0	0	0	0	0	0	0	0	0
40. GB19A	0	0	0	0	8	2	0	0	0	3	7	0	0	0
41. GB19B	0	0	0	0	0	0	1	0	1	0	0	0	0	0
42. GB19C	0	0	0	0	1	0	1	0	1	0	0	0	0	0
43. GB19D	0	0	0	0	0	0	0	0	0	0	0	0	2	0
44. GB19E	0	0	0	0	0	0	0	0	0	0	0	0	0	8
45. GB19F	0	0	0	0	0	0	0	0	0	0	0	0	0	1
46. GB19G	0	0	0	0	0	0	0	0	0	0	0	0	0	4
47. GB19H	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Figure 3.2. continued

Haplotypes	*n* per locality													
	ADM	BAR	CHI	SCOA	AKR	ARC	INT	BRNG	SCEN	SPEN	KOD	AFOG	KAT	IZE
48. GB19J	0	0	0	0	0	0	0	4	0	0	0	0	0	0
49. GB19K	0	0	0	0	0	0	1	0	0	2	2	0	0	0
50. GB19L	0	0	0	0	1	0	0	0	0	0	1	0	0	0
51. GB19M	0	0	0	0	0	1	0	0	0	0	0	0	0	0
52. GB19N	0	0	0	0	0	0	0	0	0	0	1	0	0	0
53. GB19T	0	0	0	0	0	0	0	0	1	0	0	0	0	0
54. GB20	0	0	0	0	0	0	0	0	0	0	3	6	0	0
55. GB21A	0	0	0	0	0	0	0	0	0	0	1	0	0	0
56. GB21B	0	0	0	0	0	0	0	0	0	0	1	0	0	0
57. GB22	0	0	0	0	0	0	0	0	0	0	2	0	0	0
58. GB23	0	0	0	0	0	0	0	0	0	0	0	1	0	0
59. GB24A	0	0	0	0	0	0	0	0	0	14	0	0	0	0
60. GB24B	0	0	0	0	0	0	0	0	0	1	0	0	0	0
61. GB24C	0	0	0	0	0	0	0	0	0	1	0	0	0	0
GBCHIA	0	0	6	0	0	0	0	0	0	0	0	0	0	0
GBCHIB	0	0	1	0	0	0	0	0	0	0	0	0	0	0
GBCHIC	0	0	1	0	0	0	0	0	0	0	0	0	0	0
GBCHID	0	0	3	0	0	0	0	0	0	0	0	0	0	0
TOTAL	17	5	22	3	17	17	5	26	5	21	18	7	24	14

Figure 3.2. continued

Haplotypes	sequence position
	1111111111111111111111111111111111111111111111111111111
	1111112223333344444445556666777778888999900001111111233333333333333444444444444555555555666
	5156889268245660245689289156801679114801353458001235990000112344558990225556677890011134457111
	7709695875887795947568284573162202369075345707136475583246058627397453154673828623804565622345
1. GB01	TGTTTTAGGCTCCGTAATGTAGTCACACGAGCTCGGCCCTAGCTCCCCTAGAATCTCCCCCCAAATT--TACCGTAGGCTTCATCACAGATAAG
2. GB02A	.............C.....................................T.....
3. GB02B	.............C.....................................T.....
4. GB02C	.............C.....................................T.....
5. GB03A	...........T.....................................T.....................C...A.............A.....
6. GB03B	...........T.....................................T.....................C...A.............A.....
7. GB04	................A.....................................T..A...........G.....................A.....
8. GB05A	....T.....................C...A.............A.....
9. GB05B	....T.....
10. GB05C	....T.....................A.....
11. GB05D	....T.....................C.....................A.....
12. GB05E	....T...........T.........C.....................A.....
13. GB06	.ACC.CG.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
14. GB07	.ACC..G.CTC..A.GGCACG.CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
15. GB08A	.ACC..GACT...A.GG.AC..CTGTCTAGATCTA.T.A..AT...TT...G.CTC.T...TGG..C..C.TTACGAATCCTG.T.A.A.CGG.
16. GB08B	.ACC..GACT...A.GG.AC..CTGTCTAGATCTA.T.A..AT...TT...G.CTC.T....GGG.C....TTACGAA.CCTG.T.A.AGCGG.
17. GB08C	.ACC..GACT...A.GG.AC..CTGTCTAGATCTA.T.A..AT...TT...G.CTC.T....GGG.C....TTACGAA.CCTG.T.A.AGCGG.
18. GB09A	.ACC..GACT...A.GG.AC..CTGTCTAGATCTA.T.AC.AT...TT...G.CTC.T...TGG..C...GTTACGAATCC.G.T.A.AGCGG.
19. GB09B	.ACC..GACT...A.GG.AC..CTGTCTAGATCTA.T.AC.AT...TT...G.CTC.T...TGG..C...GTTACGAATCC.GCT.A.AGCGG.
20. GB10A	.ACCC.GACT...A.GG.AC..CTGTCTAGATCTA.T.A..AT...TT.G.G.CTC.T...TGGG.C..C.TTACGAATCC.G.TGA.AGCGG.
21. GB10B	.ACCC.GACT...A.GG.AC..CTGTCTAGATCTA.T.A..AT...TT.G.G.CTC.T...TGGG.C....TTACGAATCCTG.TGA.AGCGG.
22. GB10C	.ACCC.GACT...A.GG.AC..CTGTCTAGATCTA.T.A..AT...TT.G.G.CTC.TT..TGGG.C....TTACGAATCCTG.TGA.AGCGG.
23. GB11	.ACCC.G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
24. GB12	.ACCC..ACT...A.GG.AC..CTGTCTAGATCTA.T.A..AT...TT.G.G.CTC.T...TGGG.C....TTACGAATCCTG.TGA.AGCGG.

Figure 3.3. Variability among 61 haplotypes found among brown bears throughout Alaska at mtDNA cytochrome b, $tRNA^{pro}$ and $tRNA^{thr}$ and control region genes. Four additional haplotypes (GBCHIA – GBCHID) are describe based on control region sequences only. Dots indicate sites that are identical to reference sequence (GB01). Variable sites listed below do not include a thymine repeat "indel" region (between positions 1387 and 1403 below) consisting of mononucleotide (thymine) repeat motifs and which describe 23 of the 61 haplotypes listed below (see Appendix 3.0).

Haplotypes	sequence position
	1111111111111111111111111111111111111111111111111111
	1111112223333344444445556666777778888999900001111111233333333333333444444444444555555555666
	5156889268245660245689289156801679114801353458001235990000112344558990225556677890011134457111
	7709695875887795947568284573162202369075345707136475583246058627397453154673828623804565622345
25. GB13A	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..ATC.TTTC..GTC.C.TT..T..G.CT....TACGAATCC.G.T..GA.CGGA
26. GB13B	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..ATC.TTTC..GTC...TT..T..G.CTT...TACGAATCC.G.T..GA.CGGA
27. GB13C	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..ATC.TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
28. GB13D	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..ATC.TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
29. GB13E	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..ATC.TTTC..GTC.C.TT..T..G.CT....TACGAATCC.G.T..GA.CGGA
30. GB14A	.ACC..G.CT...A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
31. GB14B	.ACC..G.CT...A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
32. GB15A	CACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT.TT..G.C.....TACGAATCC.G.T..G..CGGA
33. GB15B	CACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.CTTT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
34. GB15C	CACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..G..CGGA
35. GB15D	CACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT.TT..GCC.....TACGAATCC.G.T..G..CGGA
36. GB15E	CACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT.TT..G.C.....TACGAATCC.G.T..G..CGGA
37. GB16	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA..TA..AT..TTTC..GTC.CTTT.TT..G.CTT...TACGAATCC.G.T..GA.CGGA
37. GB17	.ACC..GACT...A.GG.AC..CTGT.T.GATCTA.T.A..AT..TTTC..GTC.C.T....GGG.C....TTACGAA.CCTG.T.A.AGCGG.
39. GB18	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT.T.TT...G.CTC.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
40. GB19A	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
41. GB19B	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
42. GB19C	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.CTTT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
43. GB19D	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.CTTT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
44. GB19E	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
45. GB19F	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
46. GB19G	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
47. GB19H	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
48. GB19J	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.CT....TACGAATCC.G.T..GA.CGGA
49. GB19K	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
50. GB19L	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.CT....TACGAATCC.G.T..GA.CGGA
51. GB19M	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
52. GB19N	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA
53. GB19T	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A..AT..TTTC..GTC.CTTT.TT..G.C.....TACGAATCC.G.T..GA.CGGA
54. GB20	.ACC..G.CTC..A.GGCAC..CTGT.T.GATCTA...A.GAT..TTTC..GTC.C.TT..T..G.C.....TACGAATCC.G.T..GA.CGGA

Figure 3.3. continued

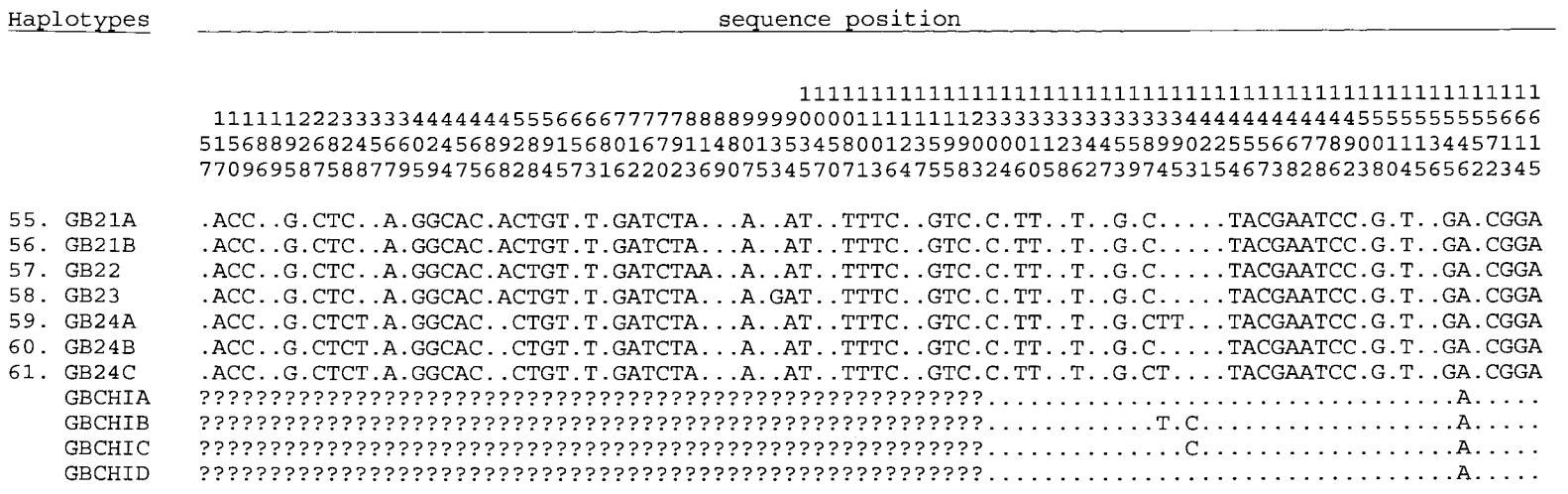

Figure 3.3. continued

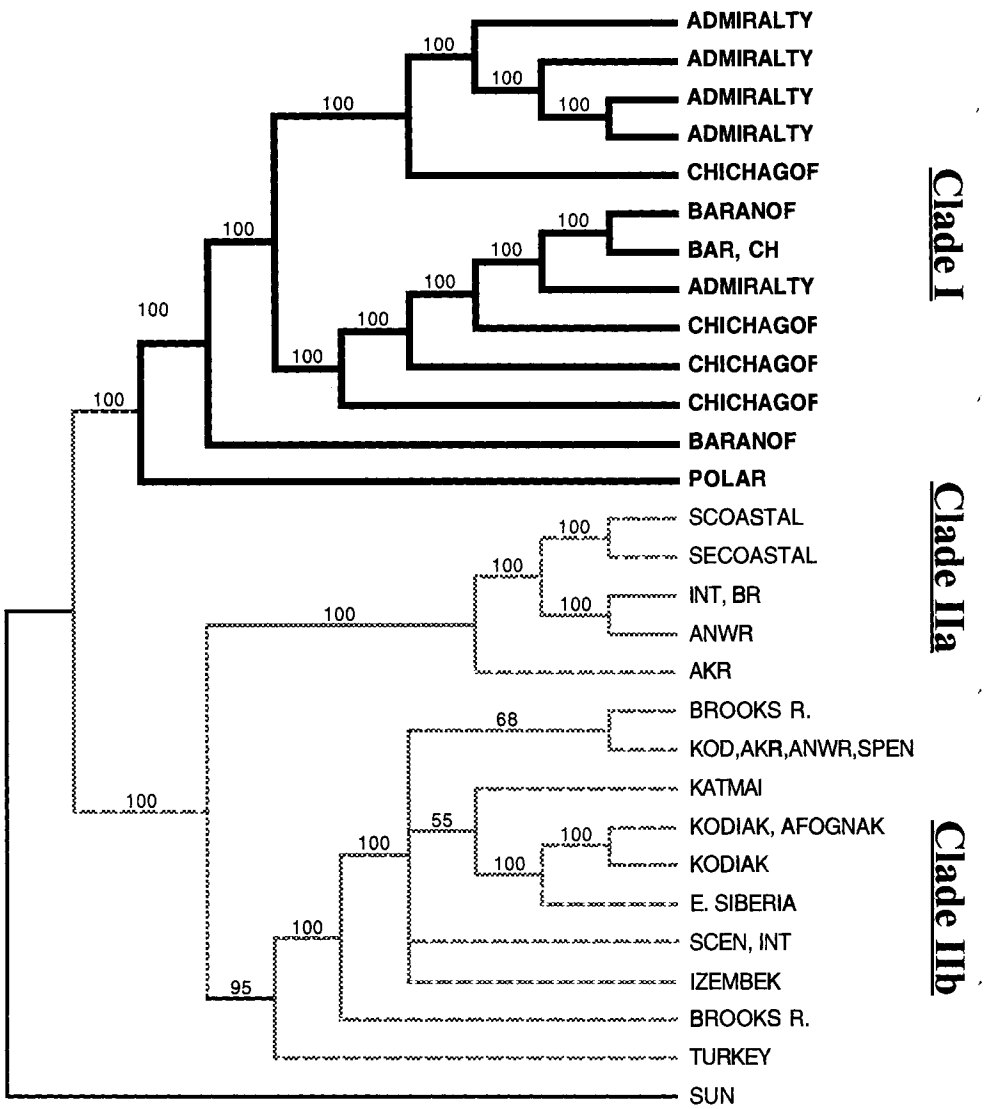

Figure 3.4. Majority rule consensus tree of a subset of haplotypes by population representing the three clades (Clade I, IIa and IIb), based on complete cytochrome b sequences, $tRNA^{pro}$ and $tRNA^{thr}$, and control region of the mitochondrial DNA. A sun bear (*Helarctos malayanus*, see Chapter 1) is used as an outgroup, and haplotypes from brown bears from eastern Siberia and Turkey are included for comparison (see Chapter 2). A haplotype from a polar bear (*Ursus maritimus*; see Chapter 1 and 2) is included in the analysis. Values at the nodes represent bootstrap support.

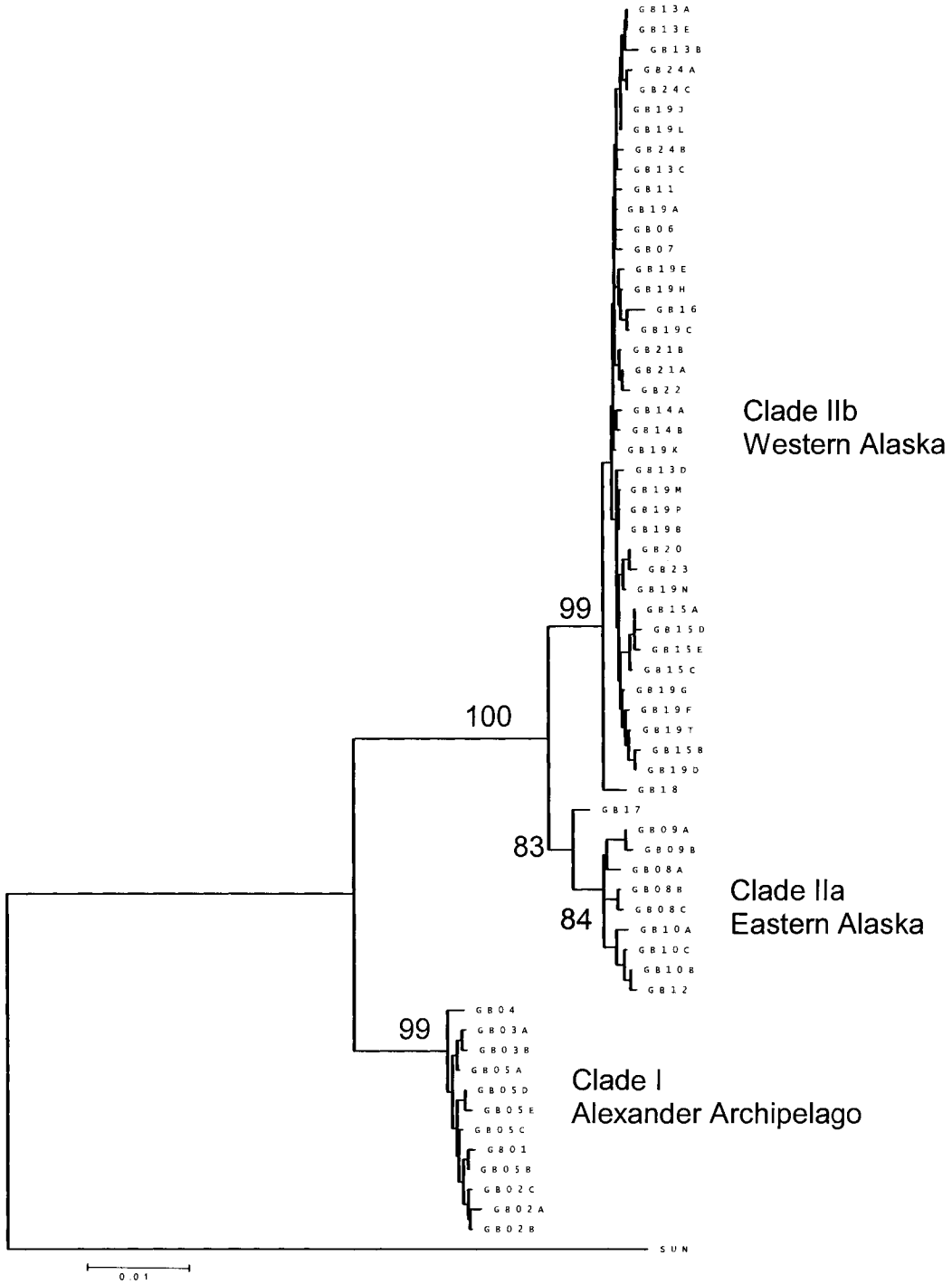

Figure 3.5. Neighbor-joining distance tree for the 61 lineages of brown bears observed in Alaska, based on the distance model of Tamura and Nei (1993). Values at major nodes are bootstrap values.

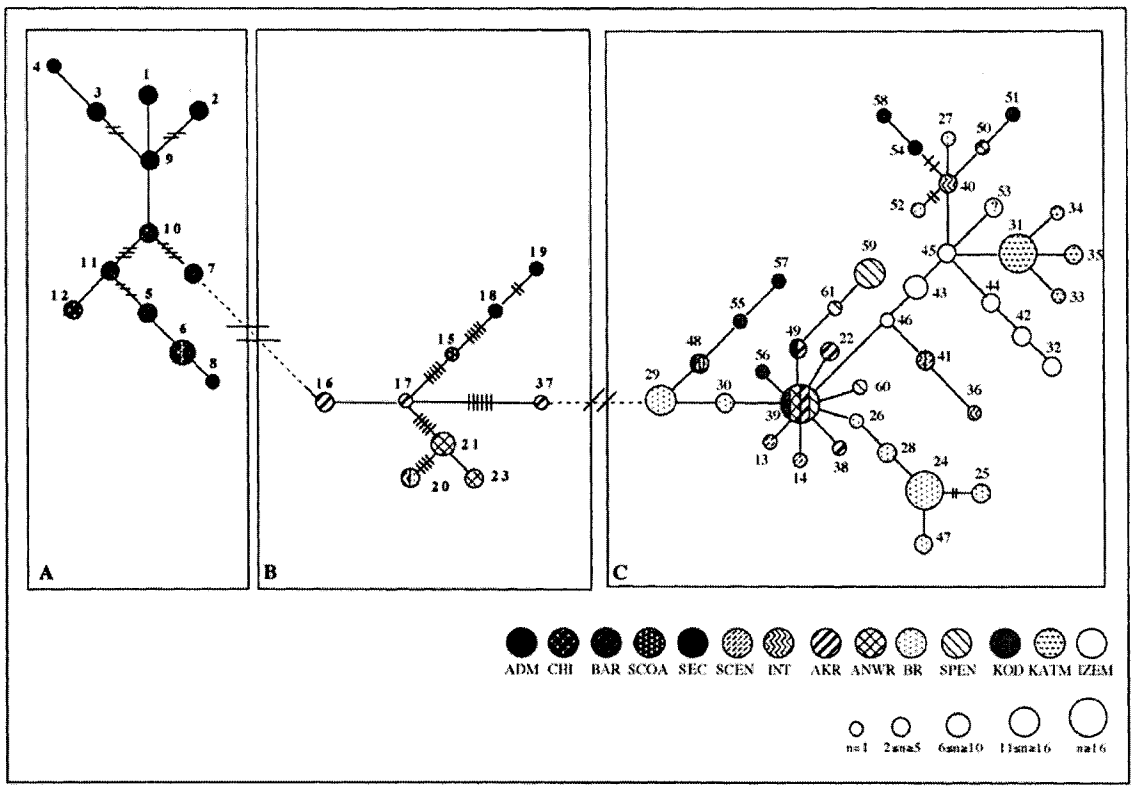

Figure 3.6. Minimum spanning tree showing relationships among 61 mtDNA haplotypes found among brown bears of Alaska. Subtrees within boxes represent members of (A) Clade I, (B) Clade IIa and (C) Clade IIb. The size of the circle reflects the frequency of haplotype; different fill patterns represent bears sampled from populations listed in the text. Each line connecting haplotypes represents a single mutation, except where small crosshatches enumerate mutations greater than one. Large cross-hatches between clades represent 55 mutations (when T_n repeats are included; see text) and 16 mutations between Clades I and IIa, and Clades IIa and IIb, respectively.

	Location	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	**ADM**		**-0.394**	**0.586**	**0.962**	**0.967**	**0.928**	**0.879**	**0.889**	**0.926**	**0.975**	**0.968**	**0.973**	**0.972**	**0.981**
2	**CHI**	0.769	-	**0.197**	**0.948**	**0.953**	**0.920**	**0.880**	**0.887**	**0.921**	**0.965**	**0.958**	**0.958**	**0.964**	**0.967**
3	**BAR**	0.353	2.035	-	**0.924**	**0.934**	**0.851**	**0.823**	**0.834**	**0.901**	**0.970**	**0.959**	**0.963**	**0.968**	**0.079**
4	**SCOA**	0.019	0.027	0.041	-	**0.913**	**0.594**	**0.248**	**0.532**	**0.768**	**0.951**	**0.922**	**0.951**	**0.941**	**0.979**
5	**SCEN**	0.016	0.025	0.030	0.048	-	*-0.016*	**0.488**	*0.095*	*0.090*	**0.589**	*0.139*	**0.833**	**0.724**	**0.819**
6	**INT**	0.039	0.004	0.087	0.341	inf	-	**0.268**	*-0.049*	**0.036**	**0.416**	**0.224**	**0.405**	**0.537**	**0.376**
7	**ARC**	0.069	0.068	0.018	1.517	0.524	1.364	-	**0.259**	**0.491**	**0.667**	**0.594**	**0.527**	**0.676**	**0.619**
8	**AKR**	0.063	0.064	0.099	0.439	4.756	inf	1.431	-	**0.131**	**0.380**	**0.213**	**0.318**	**0.461**	**0.319**
9	**BRNG**	0.040	0.043	0.055	0.151	5.028	13.525	0.518	3.305	-	**0.346**	**0.139**	**0.821**	**0.684**	**0.786**
10	**SPEN**	0.013	0.018	0.015	0.026	0.348	0.702	0.250	0.815	0.944	-	**-0.581**	**0.857**	**0.821**	**0.842**
11	**KOD**	0.017	0.022	0.021	0.042	3.082	1.732	0.341	1.839	3.100	0.360	-	**0.644**	**0.685**	**0.575**
12	**AFOG**	0.014	0.022	0.019	0.026	0.100	0.735	0.449	1.092	0.751	0.083	0.276	-	**0.786**	**0.978**
13	**KAT**	0.014	0.019	0.016	0.031	0.191	0.430	0.240	0.583	0.496	0.109	0.230	0.136	-	**0.728**
14	**IZE**	0.009	0.017	0.011	0.010	0.110	0.839	0.308	1.067	1.166	0.094	0.369	0.011	0.187	-

Figure 3.7. Population pairwise Φ_{ST} comparisons for mtDNA (above diagonal), and estimated number of female migrants (below diagonal), among 14 populations of brown bears in Alaska. Values of Φ_{ST} in bold are significant at $p < 0.05$.

Location	AKR	ARC	BRNG	KAT
AKR	-	0.0270*	0.0218*	0.0968*
		(0.041)	(0.031)	(0.018)
ARC	11.8285	-	0.0293*	0.1187*
			(-0.005)	(0.077)*
BRNG	15.4717	inf	-	0.1049*
				(0.056)*
KAT	26.8053	5.9830	8.4385	-

*significant at $p < 0.0063$

Figure 3.8. Pairwise θ_{ST}, R_{ST} and $2N_m$ values, based on eight microsatellite loci, among four populations of brown bears in interior Alaska. Values above the diagonal are θ_{ST} (upper) and R_{ST} (lower, in parentheses); values below the diagonal are $2N_m$.

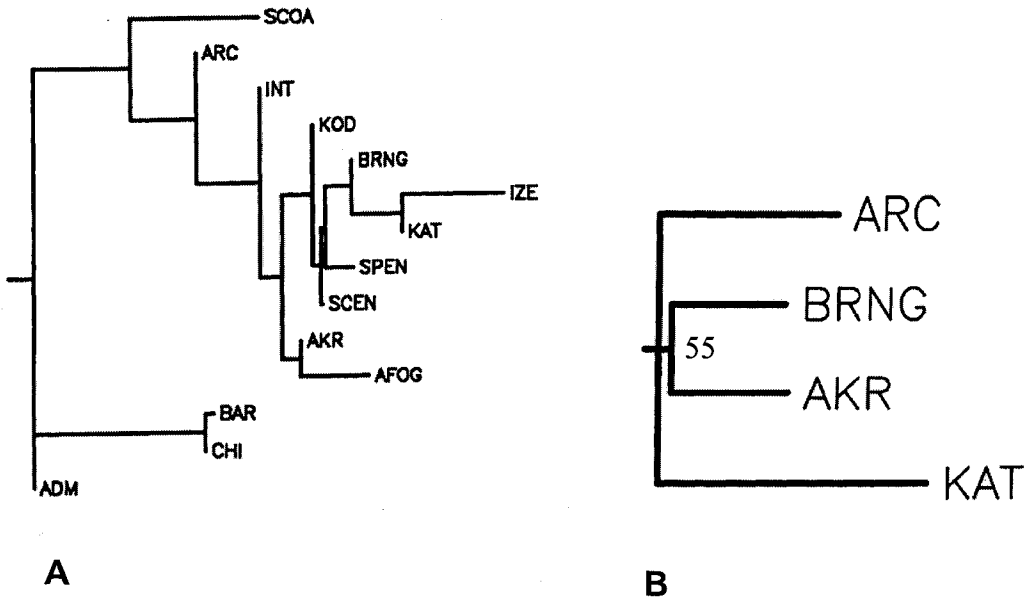

Figure 3.9. Neighbor-joining phenograms of genetic distances based on differences in allele frequency among (A) 14 populations of brown bear in Alaska at mitochondrial DNA, estimated using [-ln(1-Φ)], and B) four populations of brown bear in interior mainland Alaska at eight biparentally-inherited microsatellite loci (topology estimated using D_{CE}, branch lengths estimated using $\delta\mu^2$ (see text). Value in node for D_{CE} phenogram (B) indicates bootstrap support.

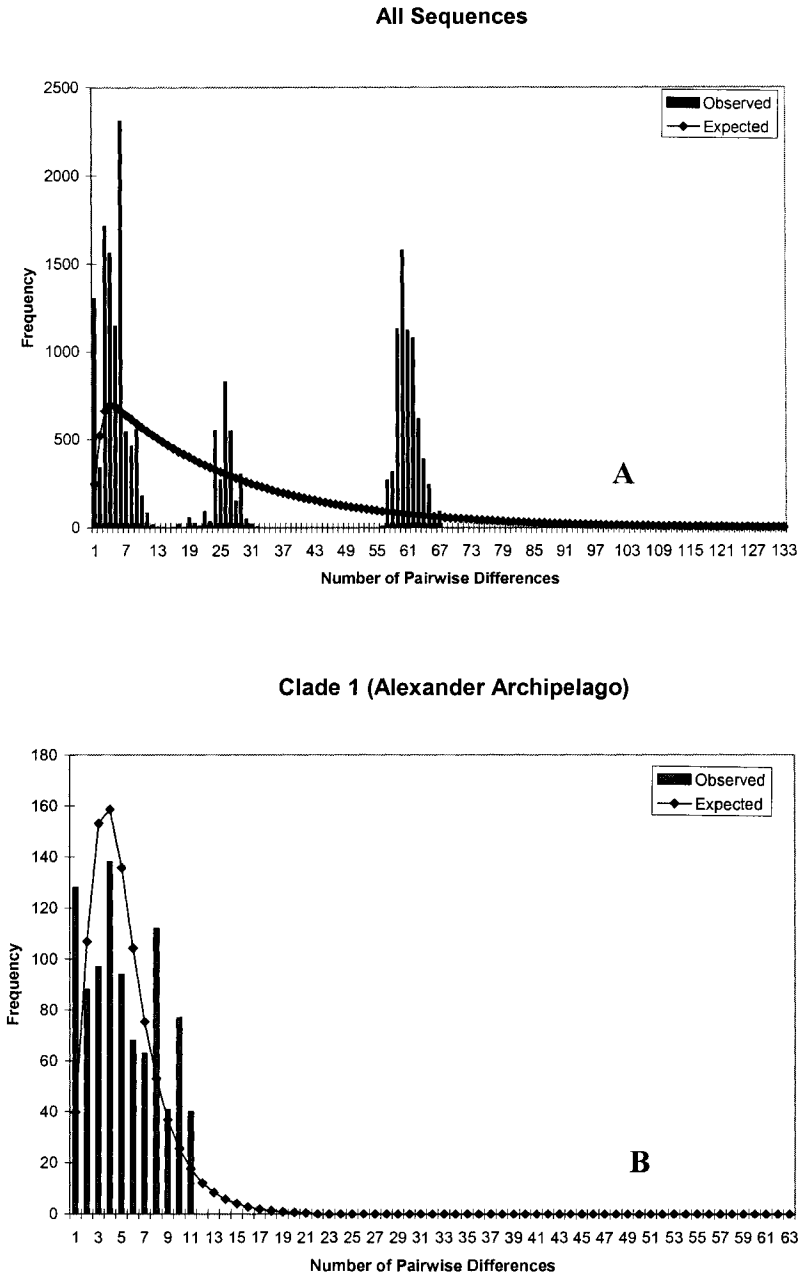

Figure 3.10. Charts of pairwise mismatch analyses for haplotypes found among brown bears of Alaska. The x-axis represents the number of nucleotide differences between any pair of sequences. The y-axis represents the frequency at which those differences were found in the set of sequences compared. Bars represent observed differences. Lines with squares indicate predicted frequencies under the expansion model of Rogers (1995). Chart A shows mismatch distributions for sequences analyzed from 200 brown bears throughout Alaska; Chart B shows mismatch distribution for Clade I bears (see text).

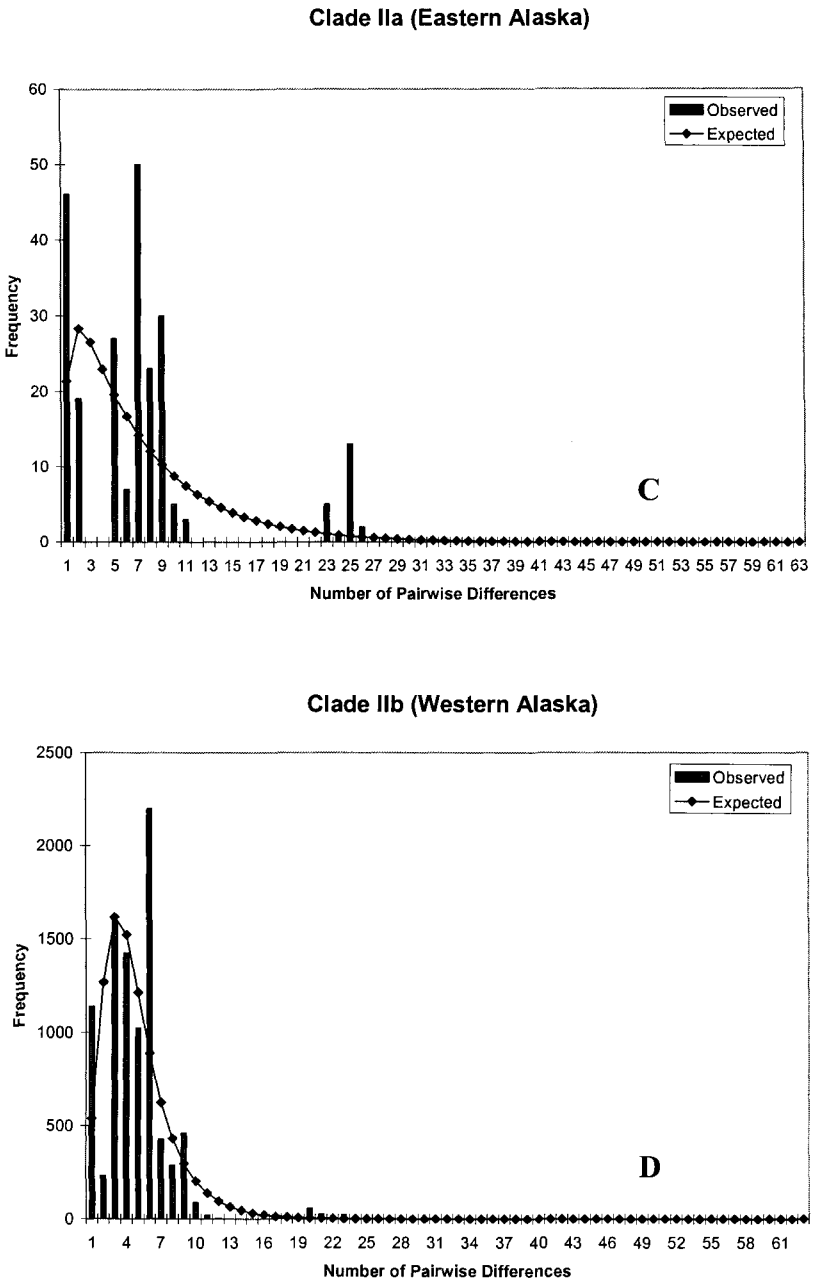

Figure 3.10 (continued). Chart C shows mismatch distribution for Clade IIa (eastern Alaska) bears, and Chart D shows mismatch distribution for Clade IIb (western Alaska) bears.

Table 3.1. Measures of genetic diversity estimated for brown bears among fourteen populations in Alaska.

		MITOCHONDRIAL DNA[1]							MICROSATELLITE DNA[2]			
Region/Population	N	*h*	(V_h)	π	(V_π)	Eυ	*D*	*k*	H_O	H_E	P	*F*
Alexander Archipelago	**44**	0.855	(0.03)	0.0015	(0.0009)	2.8	0.14	9	-	-	-	-
Admiralty Is. (ADM)	17	0.728	(0.06)	0.0008	(0.0005)	2.4	-0.37	4	0.646	0.628[c]	-	-0.029
Chichagof Is. (CHI)	22	0.688	(0.07)	0.0009	(0.0006)	1.9	0.86	4	0.493	0.496[c]	-	0.006
Baranof Is (BAR)	5	0.600	(0.17)	0.0022	(0.0016)	2.5	1.72	2	0.043	0.496[c]	-	0.006
South/SE Coasta (SCOA)	3	1.000	(0.03)	0.0025	(0.0021)	4.7	0.00	3	0.617	0.757	-	-
Arctic Alaska (ARC)	17	0.684	(0.09)	0.0067	(0.0036)	4.2	2.30*	4	0.750	0.737[a]	6.0	0.815
Alaska Range (AKR)	17	0.625	(0.11)	0.0059	(0.0030)	4.7	1.36	5	0.732	0.797[a]	6.0	0.082
Southcentral (SCEN)	5	0.900	(0.16)	0.0010	(0.0008)	2.5	-1.09	4	-	-	-	-
Interior (INT)	5	0.900	(0.16	0.0065	(0.0042)	9.2	-0.99	4	-	-	-	-
Brooks Range (BRNG)	26	0.712	(0.06)	0.0033	(0.0025)	4.9	-1.25	5	0.769	0.757[a]	7.5	-0.016
Seward Peninsula (SPEN)	21	0.381	(0.10)	0.0003	(0.0002)	1.1	0.65	2	-	-	-	-

Table 3.1. continued

Kodiak Archipelago	**25**	0.733	(0.05)	0.0007	(0.0005)	1.9	0.80	5	0.298	0.265[d]	2.1	-0.124
Kodiak Is. (KOD)	18	0.654	(0.10)	0.0006	(0.0004)	1.9	0.10	4	-	-	-	-
Afognak (AFOG)	7	0.286	(0.20)	0.0002	(0.0002)	0.4	-1.00	2	-	-	-	-
Alaska Peninsula (AKP)	**37**	0.635	(0.05)	0.0008	(0.0006	1.7	0.26	6	-	-	-	-
Katmai NP (KATM)	24	0.438	(0.01)	0.0006	(0.0005)	1.6	-0.94	5	0.680	0.692[c]	5.2	0.017
Izembek NWR (IZE)	14	0.000	(0.00)	0.0000	(0.0000)	0.9	0.00	1	0.536	0.532[d]	-	-0.007

[1]Values from mtDNA obtained from the entire cytochrome *b* gene, $tRNA^{pro}$, $tRNA^{thr}$ and the hypervariable 1 portion of the control region. MtDNA parameters: h = haplotype diversity (Nei 1897, eq. 8.4); π = nucleotide diversity (Nei 1987, eq.10); $E\upsilon$ = Watterson's nucleotide diversity (Watterson 1975); D = Tajima's D (1983); k = number of haplotypes (value does not include T_n repeat; see text and Figure 3.2 for actual number of haplotypes observed, including T_n repeat).

[2]Values from microsatellite loci from [a]Craighead (1994), [b]Scribner et al., unpublished manuscript); [c]Paetkau and Shields (1997); [d]Paetkau et al. 1998a. Microsatellite parameters: H_E = unbiased expected heterozygosity (Nei 1978); values assume Hardy-Weinberg equilibrium; H_O = observed heterozygosity; F = Wright's (1951) inbreeding coefficient.

*significant at $p < 0.05$.

Table 3.2. Summary of mismatch statistics associated with analyses of the distribution of pairwise comparisons of nucleotide sequences in Alaskan brown bear populations.

Location/population	Mismatch Obs.Mean.	Mismatch Obs.Var	θ	τ	rg^1	χ^2	Fu's F_S
Alexander Archipelago	4.22	9.17	2.23	2.000	0.020	486.77*	
Admiralty Island	2.22	5.94	1.93	0.293	0.089	53.74*	0.43
Chichagof Island	3.22	7.09	1.97	1.248	0.249	485.66	0.98
Baranof Island	5.40	21.60	4.03	1.375	0.880	0.35	3.97
South/Southeast Coastal	4.00	7.00	1.73	2.268	0.444	0.00	0.13
Arctic Alaska/Arctic NWR	13.63	187.81	13.20	0.435	0.136	1060.52*	9.74
Alaska Range/AKR	11.77	138.91	11.28	0.489	0.346	609.75	6.71
Southcentral	3.20	1.29	10.56	3.200	0.240	0.00	-1.41
Interior	13.60	197.16	13.55	0.052	0.270	0.17	1.67
Brooks Range	6.26	82.48	8.73	0.000	0.290	657.65*	3.93
Seward Peninsula	1.14	2.13	0.99	0.148	0.673	396.78*	0.94
Kodiak Archipelago							
Kodiak	1.14	2.52	0.83	0.997	0.175	73.67*	-3.99
Afognak	0.27	0.21	-	-	-	-	-0.95
Alaska Peninsula							
Katmai NP	1.38	3.66	1.51	0.000	0.252	170.51*	-1.14
Izembek NWR	-	-	-	-	-	-	-
TOTAL	26.16	652.77	25.03	1.132	0.025	100035.28*	-
Clade I	4.22	9.17	2.23	2.000	0.020	486.77*	-0.70
Clade IIa	6.20	39.02	5.73	0.475	0.109	296.70*	1.13
Clade IIb	3.84	8.57	2.18	1.664	0.093	4934.32*	-8.14

[1]rg = Harpending's Raggedness Index (Harpending 1994); [2]Fu (1977)
* $P < 0.0001$ that *rg* deviates from values expected under the sudden expansion model of Rogers (1995) or $P < 0.02$ for Fu's F_S (Fu 1997)

Table 3.3. Summary of Φ-statistics analyses of molecular variance (AMOVA) for various regional groupings of Alaskan brown bear populations. Fixation indices are indicated, along with the percentage of the total variance explained by the grouping, and its significance. Population and group names are given in the text.

Groups	Φ_{ST}	Φ_{SC}	Φ_{CT}	% among groups	*p*
[CLADE I][CLADE IIA][CLADE IIB]	0.961	0.565	0.909	90.90	<0.0001
[AARCH][SCOA, SCEN, AKR, BRNG, INT, KODISL, AFOG, AKP, SPEN]	0.929	0.509	0.857	85.7	<0.0001
[AARCH][SCOA,INT,ARC,AKR][SCEN,BRNG,SPEN,AKP][KODARCH]	0.877	0.423	0.787	78.60	<0.0001
[AARCH][KODARCH][SCOA,INT,ARC,AKR][SCEN,BRNG][SPEN][AKP]	0.859	0.399	0.767	76.69	<0.0001
[AARCH][SCOA,SCEN,AKR,BRNG,INT][KODARCH][AKP,SPEN]	0.868	0.458	0.756	75.60	<0.0001
[KODARCH][AARCH, SCOA, INT,ARC,AKR, SCEN,BRNG, SPEN, AKP][1]	0.866	0.885	-0.165	-16.45	0.6330
[KODARCH][AARCH, SCOA][INT,ARC,AKR, SCEN,BRNG,SPEN][AKP][2]	0.903	0.606	0.753	75.29	<0.0040
[KODARCH][AARCH, SCOA,SCEN,AKP][INT,ARC,AKR,BRNG,SPEN][3]	0.889	0.862	0.192	19.15	0.0155

[1]geographic distribution described for subspecies hypothesized by Rausch (1963)
[2]geographic distribution described for subspecies hypothesized by Hall (1984); *U. a. dalli* is not represented
[3]geographic distribution described for subspecies hypothesized by Kurtén (1973)

Table 3.4. Sign and Wilcoxon tests for heterozygosity excess at eight microsatellite loci in four populations of brown bears in Alaska.

	SIGN TEST			**WILCOXON TEST**					
	Mutational Model			Mutational Model					
Population (N)	**IAM**	**TPM**	**SMM**	**IAM**		**TPM**		**SMM**	
	e/d (p)	e/d (p)	e/d (p)	H_e	H_d	H_e	H_d	H_e	H_d
ARC (15)	7/1 (0.097)	6/2 (0.297)	5/2 (0.581)	0.010	0.994	0.125	0.902	0.473	0.578
AKR (17)	7/1 (0.107)	6/2 (0.311)	6/2 (0.304)	0.004*	0.998	0.014	0.990	0.320	0.727
BRNG (95)	8/0 (0.014)	8/0 (0.014)	4/4 (0.424)	0.002*	1.000	0.002*	1.000	0.769	0.273
KAT (29)	8/0 (0.012)	7/1 (0.099)	5/3 (0.574)	0.002*	1.000	0.004*	0.998	0.191	0.843

*significant deviation ($p < 0.006$) from equilibrium/non-bottleneck expectation (Bonferroni correction applied).

Appendix 3.0. Variable indel region found in the hypervariable I portion of the control region of the mtDNA of brown bears in Alaska. Sixty-one haplotypes are listed along with homologous sequences from sun bear (*Helarctos malayanus*), black bear (*Ursus americanus*) and polar bear (*U. maritimus*). Variable sites among haplotype found in Alaskan brownbears are denoted with an asterisk (*).

```
                                                ** ******          ****     *
SUN     TGTACCATCGTAGTATGTCCTCGATACTCCTTCTTTCATTTTTTTTT-----------CCCCCCTATGTAC
BLACK   TGTACCATGTA-GTATGTTTTTAAA---------TACTTTCCTCTTTTATTTTTTTCCTCCCCCTATGTAC
POLAR   TGTACCATACACGTATGTCCTCGAA---------TACTTTT---------------CCCCCCCTATGTAT

GB01    TGTACCATCACAGTATGTCCTCGAA---------TACTTTT---------------CCCCCCCTATGTAT
GB02A   TGTACCATCACAGTATGTCCTCGAA---------TACTTT----------------CCCCCCCTATGTAT
GB02B   TGTACCATCACAGTATGTCCTCGAA---------TACTTTTT--------------CCCCCCCTATGTAT
GB02C   TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTT-------------CCCCCCCTATGTAT
GB03A   TGTACCATCACAGTATGTCCTCGAA---------TACTTTTT----------------CCCCCCATGTAT
GB03B   TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTT---------------CCCCCCATGTAT
GB04    TGTGCCATCACAGTATGTCCTCGAA---------TACTTTTT--------------CCCCCCCTATGTAT
GB05A   TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTT---------------CCCCCCATGTAT
GB05B   TGTACCATCACAGTATGTCCTCGAA---------TACTTTT----------------CCCCCCCTATGTAT
GB05D   TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTC---------------CCCCCCATGTAT
GB05E   TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTC---------------CCCCCCATGTAT
GBCHID  TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTT--------------CCCCCCTATGTAT
GBCHIB  TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTT-------------TTCCCCCCATGTAT
GBCHIA  TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTT---------------CCCCCTATGTAT
GBCHIC  TGTACCATCACAGTATGTCCTCGAA---------TACTTTTTT---------------CCCCCCATGTAT
GB06    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC-------------CCCCCCTATGTAT
GB07    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC-------------CCCCCCTATGTAT
GB08A   TGTGCCATCACAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCCATGTAT
GB08B   TGTGCCATCGCAGTATGTCCTCGAA---------TACCTTTT--------------CCCCCCCTATGTAT
GB09A   TGTGCCATCACAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB09B   TGTGCCATCACAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB10A   TGTGCCATCGCAGTATGTCCTCGAA---------TACCTTT----------------TCCCCCCCATGTAT
GB10B   TGTGCCATCGCAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB10C   TGTGCCATCGCAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB11    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC-------------CCCCCCTATGTAT
GB12    TGTGCCATCGCAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB13A   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTT-------------CCCCCCTATGTAT
GB13B   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTTT------------CCCCCCTATGTAT
GB13C   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC-------------CCCCCCTATGTAT
GB13D   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT---------------CCCCCCCTATGTAT
GB13E   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTTC------------CCCCCCTATGTAT
GB14A   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTT--------------CCCCCCCTATGTAT
GB14B   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC-------------CCCCCCTATGTAT
GB15A   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT---------------CCCCCCCTATGTAT
GB15B   TGTACCATCGCAGTATGTCCTCGAA---------TACCTT----------------TCCCCCCCTATGTAT
GB15C   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT---------------CCCCCCCTATGTAT
GB15D   TGTACCATCGCAGTACGTCCTCGAA---------TACCTTTT---------------CCCCCCCTATGTAT
GB15E   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB16    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTTT-----------CCCCCCCTATGTAT
GB17    TGTGCCATCGCAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB18    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC-------------CCCCCCTATGTAT
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Appendix 3.0 continued.

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                                                ** ******          ****     *
SUN     TGTACCATCGTAGTATGTCCTCGATACTCCTTCTTTCATTTTTTTTT-----------CCCCCCTATGTAC
BLACK   TGTACCATGTA-GTATGTTTTTAAA---------TACTTTCCTCTTTTATTTTTTTCCTCCCCCTATGTAC
POLAR   TGTACCATACACGTATGTCCTCGAA---------TACTTTT----------------CCCCCCCTATGTAT

GB19A   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC-------------CCCCCCTATGTAT
GB19B   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT---------------CCCCCCCTATGTAT
GB19C   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT-------------TTCCCCCCCTATGTAT
GB19D   TGTACCATCGCAGTATGTCCTCGAA---------TACCTT----------------TCCCCCCCTATGTAT
GB19E   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT--------------TCCCCCCCTATGTAT
GB19F   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTT----------------CCCCCCCTATGTAT
GB19G   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT---------------CCCCCCCTATGTAT
GB19H   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT-------------TTCCCCCCCTATGTAT
GB19I   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTT--------------CCCCCCCTATGTAT
GB19J   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTT-------------CCCCCCTATGTAT
GB19K   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTT--------------CCCCCCCTATGTAT
GB19L   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTTC------------CCCCCCTATGTAT
GB19M   TGTACCATCGCAGTATGTCCTCGAA---------TACCCTTT----------------CCCCCCTATGTAT
GB19O   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTT--------------CCCCCCCTATGTAT
GB19P   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTT-------------CCCCCCCCCTATGTAT
GB19Q   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTTC------------CCCCCCTATGTAT
GB19T   TGTACCATCGCAGTATGTCCTCGAA---------TACCTT--------------TTCCCCCCCTATGTAT
GB20    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTC----------------CCCCCCTATGTAT
GB21A   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTT--------------CCCCCCCTATGTAT
GB21B   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC------------CCCCCCTATGTAT
GB22    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTT--------------CCCCCCCTATGTAT
GB23    TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTC----------------CCCCCCTATGTAT
GB24A   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTTT-----------CCCCCCCTATGTAT
GB24B   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTC------------CCCCCCTATGTAT
GB24C   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTTTTTC------------CCCCCCTATGTAT
GB26A   TGTACCATCGCAGTATGTCCTCGAA---------TACCTTTC----------------CCCCCCTATGTAT
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CONCLUSIONS

This thesis uses molecular genetic data to provide information and generate hypotheses about ancestral and contemporary relationships of brown bears at three hierarchical levels. Results of this research allowed me to 1) generate hypotheses of phylogenetic relationships among ursid species (Chapter 1; Talbot and Shields 1996a); 2) present a phylogeographic perspective to hypothesized relationships among brown bears in Alaska (Chapter 2; Talbot and Shields 1996b); and 3) describe within- and among-population characteristics uncovered in brown bear populations in Alaska using the maternally-inherited mtDNA, and compare some of these relationships with those hypothesized using biparentally-inherited microsatellite data (Chapter 3).

The mtDNA sequences reported herein support previous hypotheses of the close relationship between polar and brown bear, and between the two black bear species (Asiatic and American). These data also provide hypotheses that can be tested to further resolve relationships among other ursid species, preferably using multiple single-copy nuclear loci for which allelic phylogenies might be obtained.

Analyses of sequences of the mitochondrial DNA of brown bears of Alaska suggest brown bear populations in Alaska form two major clades, one (Clade I) comprising populations found only on the islands of the Alexander Archipelago, and (Clade II) one comprising the rest of the bears in Alaska. This latter clade can be further partitioned into two major subclades, one (Clade IIb) generally describing brown bear populations found in the western portion of their range in Alaska, and one in the eastern portion (Clade IIa). One weakness of this research was the small sample size for south coastal and southeast mainland coastal brown bears. However, subsequent investigations to increase the sample size of these populations (Shields et al. 2000) suggest the southeast coastal mainland Alaska bears belong, without exception, to Clade II, thus supporting my earlier findings. Control region sequence data obtained from 11 additional bears from south coastal Alaska also support this finding (Talbot, unpublished data). The vast genetic divergence between these populations, separated by only a deep, eight km wide

channel, suggests a complete lack of gene flow between island and mainland populations, despite hypotheses of high levels of gene flow, presumably due to male-bias in dispersal, between the island populations and the mainland as suggested by analyses of microsatellite loci (Paetkau et al. 1998).

In view of the increased likelihood of further habitat fragmentation in southeastern Alaskan, these data, when viewed with similar data from other species with different life histories (Cook et al. 2001), provide important baseline information that can be used to generate effective management strategies for large mammals in this region.

The data described, analyzed and interpreted herein provide one perspective on the phylogenetic, phylogeographic and population-level relationships of brown bears; the relationships proposed herein represent gene trees and not necessarily species trees (Pamilio and Nei 1988; Avise 1994). Some hypotheses generated using these data have been tested using bi-parentally inherited nuclear microsatellite loci. These markers provide additional resolution at the population level, as well as an important contrast to data collected using the maternally-inherited mtDNA, thus allowing inferences to be made about levels of gender-bias in gene flow (Palumbi and Baker 1994; Scribner et al. 2001, 2003). In addition, due to the slower rate of evolution of the mtDNA relative to hypervariable microsatellite loci, the latter can be more informative at the level of closely-related populations, particularly when assessing contemporary levels of gene flow. However, due to the real possibility of homoplasy at higher taxonomic levels, microsatellite markers may not be appropriate to make comparisons with populations that are distantly related, such as between populations occurring on the islands of the Alexander Archipelago relative to mainland populations, or at the level of divergence often found at the "subspecies" level. The mtDNA data at that level should be tested using single-copy nuclear loci, either at the intron level or third positions of codons for protein-coding genes. Preliminary analysis of two major histocompatibility complex loci (DQA and DQB, exon 2) of black bears, polar bears, and brown bears, including those from southeast Alaska, are ambiguous and do not appear to shed light on the phylogenetic

relationships of brown bears of the Alexander Archipelago and polar bears relative to the rest of the brown bears in Alaska (Cronin, pers. comm., 2000: unpublished data).

Although ursids are well-represented in the pre-Pleistocene fossil history (Chapter 1), there is a paucity of fossil evidence for Pleistocene and recent brown bears. This, coupled with the species' morphological plasticity, makes independent testing of the hypotheses presented herein difficult. Even with the recovery of DNA from fossil remains (Leonard et al. 2000), the interpretation of these data is based on extremely small sample sizes and should be considered preliminary until larger samples from larger areas across the range of the organism can be obtained, and until comparison of the data from other portions of the species' genome can be made.

Improvements in analytical techniques and the increasing volume of genetic information available will allow testing of many hypotheses generated by this thesis research. However, comparative phylogeographic analyses, in the Alexander Archipelago (Cook et al. 2001) and other localities across Beringia, may prove to be the most informative avenue for testing hypotheses relating to brown bear relationships in Alaska, and further examining the relationship between polar bears and brown bears. Additional future research may include nested clade analyses of mtDNA haplotype networks (Templeton 1998; Templeton et al. 1992) to test hypotheses about gene flow and population history generated herein, and to examine worldwide relationships of brown bears based on mtDNA sequence information, from the cytochrome b gene as well as the control region. Analyses of contact zones between populations predominantly characterized by haplotypes representing Clades IIa and IIb, would be particularly enlightening, as would information from a y-specific gene (Sundqvist et al. 2001), to corroborate gender bias in gene flow.

The data generated herein have been included within several other studies, including an investigation of a hypothesized glacial refugium in southeastern Alaska from a single-species perspective (Heaton et al. 1996; Appendix A), hypotheses of phylogeographic relationships among brown bear mitochondrial DNA across the species' entire North American range (Waits et al. 1998; Appendix B) as well as its world-wide

range (Waits et al. 2006; unpublished manuscript; Appendix C). Products generated as a result of this thesis have been included in an assessment of genus-level relationships among mammalian species (Johns and Avise 1998). More importantly, the data presented herein generated additional hypotheses, some of which have been subsequently indirectly or directly tested (Paetkau et al. 1998; Shields et al. 2000; Leonard et al. 2000, Cook et al. 2001). Thus, the material presented in this thesis has generated and tested hypotheses and provided incentive for subsequent studies. This thesis thereby fulfills one of the major roles of science: to develop laws and theories to explain, predict and understand natural phenomena (Hunt 1991).

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APPENDIX A

An Ice Age Refugium for Large Mammals in the Alexander Archipelago, Southeastern Alaska[1]

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ABSTRACT

Genetic and paleontological evidence are combining to provide a new and surprising picture of mammalian biogeography in southeastern Alaska. Prior to our study, the brown and black bears of the Alexander Archipelago were considered postglacial immigrants that never had overlapping ranges. Vertebrate fossils from caves on Prince of Wales Island now demonstrate that brown and black bears coexisted there (and even inhabited the same caves) both before and after the last glaciation. Differences in mtDNA sequences suggest that living brown bears of the Alexander Archipelago

[1]Heaton T. H., Talbot, S. L., and Shields, G. F. (1996). An ice age refugium for large mammals in the Alexander Archipelago, Southeastern Alaska. *Quaternary Research* **46**:186-192.

comprise a distinct clade and, and that the mtDNA of brown bears of the Alexander Archipelago is more closely related to the mtDNA of polar bears than to that of their mainland conspecifics. We conclude that brown bears, and perhaps other large mammals, have continuously inhabited the archipelago for at least 40,000 yr and that habitable refugia were therefore available throughout the last glaciation.

INTRODUCTION

Until recently, the prehistory of the Alexander Archipelago has been the subject of rough speculation but little study (Fig. A.1). Archaeologists have considered it a possible route for human travel to the New World--a route that would have circumvented the great ice sheets of Canada (Heusser 1960, Fladmark 1979, 1983, Dixon 1993, Gruhn 1994). But geologists and biologists have traditionally discounted the possibility of habitable areas in the archipelago during glacial maxima (Klein 1965, Prest 1969, Nasmith 1979, Cwynar 1990, Mann and Hamilton 1995). The controversy has centered around the question of whether nonglaciated refugia existed, with access to marine food sources, that were large enough to support populations of large mammals, including humans. Studies of modern and fossil bears from the Alexander Archipelago are providing the first conclusive evidence that such refugia existed.

David R. Klein (1965, p. 7) began his historic paper on the mammal distributions of the Alexander Archipelago with the following declaration:

"During the Wisconsin glaciation the present land areas of the coastal regions of Alaska bordering the Gulf of Alaska were virtually completely overridden by ice. The now existing flora and fauna of the region have presumably become established in the 10,000 years since the recession of the ice... The present distribution of mammals in these region, although complicated by the phenomenon of insularity, reflects the sequence of their arrival and their relationship to specific refugia."

A study of Kodiak Island mammals also discounted Ice Age refugia in favor of postglacial colonization (Rausch 1969), and a study of Queen Charlotte Island mammals left the issue unresolved (Banfield, 1969; Cowan and Guiget 1965, Cowan 1989). Klein (1965, p. 15) went on to explain the distribution of brown bears (*Ursus arctos*) and black bears (*U. americanus*) of the Alexander Archipelago in terms of postglacial invasions from the north and south, respectively, and competition between the two species:

"The failure of brown bears to occupy the islands south of Frederick Sound when access became available may be a result of prior occupancy by the black bear. A previously established species obviously has an advantage over a similar form attempting to occupy the same ecological niche."

Klein's conclusions, which represented the prevailing consensus prior to our work, entail two postulates that appear to be now unsupported, based on the evidence as discussed below: (1) that brown bears never inhabited Prince of Wales Island and other islands of the southern archipelago, and (2) that brown bears of the northern archipelago are closely related to their mainland counterparts.

POSTGLACIAL FOSSIL DEPOSITS

The extensive karstlands of Prince of Wales Island were largely ignored as a scientific resource until cavers Kevin and Carlene Allred began exploring and mapping the caves of Prince of Wales Island in 1987 (Heaton 1996a). A joint project between the National Speleological Society and Tongass National Forest emerged from their efforts, and in 1990 the first vertebrate fossil remains were discovered in a remote upper passage of El Capitan Cave, the largest know cave in Alaska. In 1992, a sealed entrance to the cave was reopened and a full excavation conducted (Heaton and Grady 1992a, 1992b, 1993). Early postglacial fossils, including the nearly complete skeleton of a black bear,

were found exposed on the cave floor and buried in shallow sediment. Along with four black bears, the remains of at least three brown bears were recovered, one of which was gigantic. The cave appears to have been a den site for both species of bears, showing that they one coexisted on the island.

A similar den site in Bumper Cave was excavated in 1994 and found to contain remains of at least nine brown bears, mostly females and juveniles, including one nearly complete skeleton (Heaton and Love 1995). Brown bear remains have also been found in two natural trap caves: Blowing in the Wind Cave on El Capitan Peak (2 juveniles) and Enigma Cave on Dall Island (a nearly complete skeleton). Table A.1 provides pertinent data about the cave sites, and Table 2 is a list of radiocarbon dates obtained thus far. The total minimum number of individuals (MNI) for brown bears from postglacial deposits of the southern archipelago is now 15, and they come from caves at all altitudes. Brown and black bear remains can be distinguished based on size and tooth morphology, although there is some overlap (Graham 1991; Heaton 1994).

The caves of Prince of Wales Island have preserved remains of two other extirpated mammalian species in addition to *U. arctos*. The jaw of a red fox (*Vulpes vulpes*) was found in El Capitan Cave, and the partial metacarpal of a caribou (*Rangifer tarandus*) was recovered from Bumper Cave. Neither species currently inhabits the Alexander Archipelago or the nearby Alaskan mainland (Klein 1965, Hall 1981). A dwarf form of caribou inhabited the Queen Charlotte Islands into historic times before going extinct (Banfield 1961, Cowan and Guiguet 1965, Cowan 1989). Early postglacial remains of the current large mammalian fauna of the southern archipelago, namely black bear, wolverines (*Gulo gulo*), river otter (*Lutra canadensis*), and mule deer (*Odocoileus hemionus*), have also been recovered in the cave deposits (Tables A.1 and A.2).

Postglacial extinctions suggest that species richness for large mammals in the Alexander Archipelago was one much greater than at present. There are two possible explanations for this higher diversity:

(1) Island colonization was easier in early postglacial times due to ice bridges and/or lower sea level.

(2) Some species survived in the archipelago during glaciation and thereafter had to endure changing climate and/or competition with invading species from the mainland.

Klein (1965) rejected the second explanation for large mammals but specifically invoked the first, especially for bears. New data now suggest that the second explanation may be more accurate.

MID-WISCONSIN FOSSIL DEPOSITS

Postglacial fossils are useful for establishing colonization history and dating local glacial retreat, but they alone cannot resolve the question of Ice Age refugia in the Alexander Archipelago. Fortunately, older fossil deposits have now been discovered.

On Your Knees Cave is located in the extreme northwest corner of Prince of Wales Island adjacent to Summer Strait (Heaton 1995a, 1995c, 1996a, 1996b, Fig. A.1). Seal (*Phoca* cf *hispida*) remains from the cave date near the peak of the Fraser (late Wisconsin) Glaciation, suggesting that the area was ice free (Table A.2). Remains of U. *arctos* and *U. americanus* from On Your Knees Cave date to the period preceding Fraser Glaciation, named the Olympia Nonglacial Interval in British Columbia (Fig. A.2). These bones were buried in shallow clay which may have aided their preservation. Since brown bears apparently did not reach the lower 48 United States until about 13,000 years ago (Kurten and Anderson 1980, Mustoe and Carstad 1995), their early presence in the Alexander Archipelago is surprising. This suggests that, at least for bears, the glaciated coastal islands were less inhibitive to travel than was mainland North America.

Stable carbon isotope values of fossil bones give an indication of an animal's diet. The brown bear from On Your Knees Cave has the highest value of any bear analyzed from the islands thus far (Table A.2), and this indicates an unusually large marine component to the animal's diet (Fry and Sherr 1984, Heaton 1995b). However, the black

bear from the same cave has a low isotopic value typical of black bears, and this indicates a primarily terrestrial plant diet (Bocherens et al. 1994). Marine feeders in the archipelago, such as brown bears and otters, would likely have a great advantage in surviving periods of glaciation, and this must be kept in mind when considering hypotheses of continuous habitation.

Devil's Canopy Cave on Prince of Wales Island has also produced a mammalian fauna predating Fraser Glaciation, although only rodents have been recovered thus far (Heaton 1995a). The most significant find is marmot (*Marmota* sp.), an animal not currently found on the island or in postglacial deposits. The only sample analyzed from the cave is beyond the range of radiocarbon dating (Table A.2), so unfortunately the glacial context is unknown. This cave suggests the possibility that fossil deposits in the archipelago could have survived multiple glaciations. Field work over the next several years will focus on these older deposits in hopes of obtaining a complete faunal record for the past 50,000 years.

GENETIC STUDIES

The fossil record of brown and black bears in the Alexander Archipelago still leaves ambiguous the question of whether these bears inhabited the islands throughout the period of Fraser Glaciation or merely colonized the archipelago more than once. We have compared the DNA sequences of three mitochondrial genes and associated noncoding segments of nearly 200 extant brown bears from all geographic regions of Alaskan and some regions of northeastern Siberia and Turkey, and these data, combined with the fossil evidence, suggest continuous habitation of the archipelago.

We studied mitochondrial structural genes such as the cytochrome b gene because its rate of DNA change is well known and because specific codons corresponding to specific amino acids can be aligned. We also included the noncoding control region since its rapid rate of change allows us to observe genetic divergence among populations. Like

many others (e.g., Avise 1986, Wilson et al. 1987), we assume that the relative rate of mutational change in the mtDNA is roughly correlated with the duration of time since two extant taxa last shared a common ancestor.

The DNA sequences suggest two very distinct clades for *U. arctos*, one composed exclusively of brown bears from Admiralty, Baranof and Chichagof islands (ABC islands) of the northern Alexander Archipelago (Fig. A.1) and another comprising all other populations (Talbot and Shields 1996, Fig. A.3). The brown bears of the ABC islands are fixed for a minimum of 27 point mutations, of which two are transversions, in the cytochrome b gene alone. This suggests ancient divergence of these mtDNA lineages (550,000 - 700,000 yr) from mtDNAs characterizing other populations of brown bears (Talbot and Shields 1996, Shields and Kocher 1991, Cronin et al. 1991). This ancient divergence in also apparent when the DNAs of the ABC bears are compared with those of over 300 brown bears from throughout their world-wide range (Waits et al. 1996). The most logical explanation for these results is that the brown bears of the ABC island are the relic of an invasion of *U. arctos* from the Asia into Alaska prior to Fraser Glaciation. This invasion may never have extended beyond Alaska, and this unique mtDNA lineage has apparently died out (except in bears of the Alexander Archipelago) prior to a more extensive postglacial invasion (Figs. 2 and 3).

The mitochondrial data also suggest that the bears of the ABC islands are the closest extant relative to the polar bear (Talbot and Shields 1996, Taberlet and Bouvet 1992). Kurten (1964, 1968) suggested from fossil evidence that polar bears arose from a coastal form of brown bear possibly in northeastern Siberia, and our data are consistent with this. However, our novel observation of paraphyly of brown bears with respect to polar bears will have to be tested using other genetic markers, including nuclear genes, before a full analysis can be made.

CONCLUSIONS

Because brown bears of the Alexander Archipelago were previously thought to be postglacial immigrants from the Alaskan mainland (Klein 1965), the distinct genetic makeup of the ABC islands bears and the recovery of pre-Fraser-age *U. arctos* remains from On Your Knees Cave came as complete surprises. The antiquity of the On Your Knees Cave specimen suggests that it is part of the modern ABC islands lineage and thereby supports a hypothesis this lineage inhabited the Alexander Archipelago at an early date. By far the most parsimonious explanation for all these data is continuous habitation of brown bears in the archipelago throughout Fraser Glaciation, as the Cordilleran Ice Sheet surrounding this are would have been a major barrier to travel. The ABC island bears may represent a living relic of that habitation, and postglacial fossil brown bears from Prince of Wales and Dall islands probably also belong to this clade. Future genetic studies on fossils may confirm this.

This combination of paleontological and genetic evidence offers strong documentation for habitable coastal refugia in the Alexander Archipelago during the last glaciation. The fossil record even demonstrates that brown bears had a more extensive range in the islands than at present, both before and after the last glacial maximum. This suggests that glaciation may actually have promoted, rather than inhibited, coastal range extension by brown bears. The combination of marine foods and ice bridges may have provided a coastal corridor for this species not available in interglacial or postglacial times. This suggests that at least some unglaciated land was available for denning and food supplement, and that other species of mammals my have survived glaciation in such refugia as well.

Figures 4 and 5 contrast two models of mammal invasion of new island habitat following deglaciation. The hypothesis of postglacial colonization of the Alexander Archipelago from the Alaska mainland, advocated by Klein (1965), can be termed the Inland Colonization Model (Fig A.4.). It may still be the best explanation for many island

mammals but it can no longer be applied exclusively. The hypothesis outlined in this paper for brown bears can be termed the Coastal Colonization Model (Fig. A.5; Heaton 1995c). It now provides the best explanation for brown bears, and it may also apply to black bears and other mammalian species.

As Calvin J. Heusser (1960) stated at the end of his book on Late Pleistocene environments of the Pacific Northwest, no discussion of this topic is complete without considering humans. Fladmark (1979) made a strong case that any refugia along the North Pacific coast would have been suitable for human occupation. Recent linguistic studies have reached conclusions strikingly similar to ours, namely that coastal southern Alaska housed an isolated refugium during the last glaciation (Rogers 1985; Rogers et al. 1990, 1991). While our study provides no direct evidence of human antiquity in the Alexander Archipelago, the evidence from brown bears furnishes a close analog. Humans and brown bears are both omnivores and have similar habitat requirements. If marine foods and coastal den sites were available for *U. arctos*, then the minimum requirements for seafaring *Homo sapiens* were almost certainly available.

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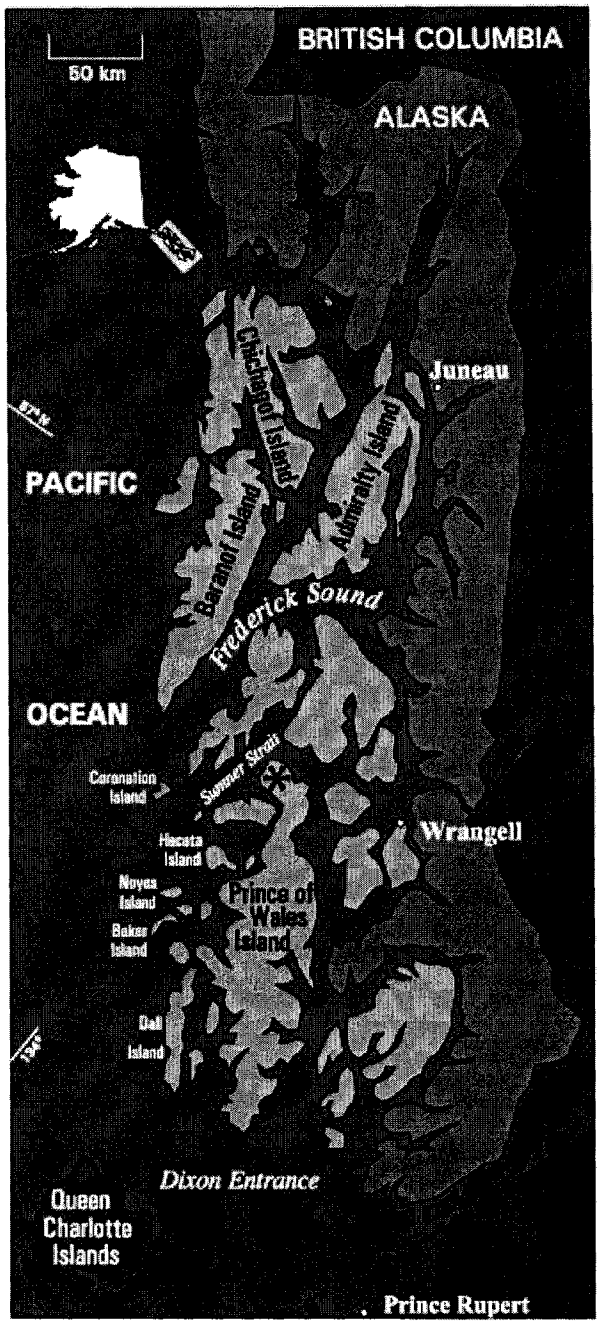

Fig. A.1. Index map of the Alexander Archipelago, southeast Alaska. Currently brown bears inhabit the northern (ABC) islands while black bears inhabit most islands south of Frederick Sound. Both species inhabit the mainland. The star shows the karst area of Prince of Wales Island where fossils of brown and black bears have been found in mid-glacial and postglacial cave deposits.

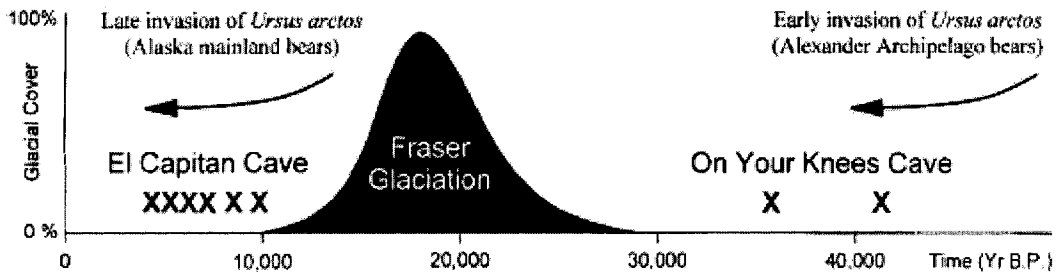

Fig. A.2. Time line of interpreted events in southeast Alaska from 50,000 years B.P. to the present. The early invasion of bears is represented by the fossils of On Your Knees Cave and the living brown bears of the ABC Islands. Mainland bears represent the later invasion. The affinity of the postglacial bears of the southern archipelago has not yet been determined, but their early fossil record at El Capitan Cave and other sites suggests that they may also represent the early invasion.

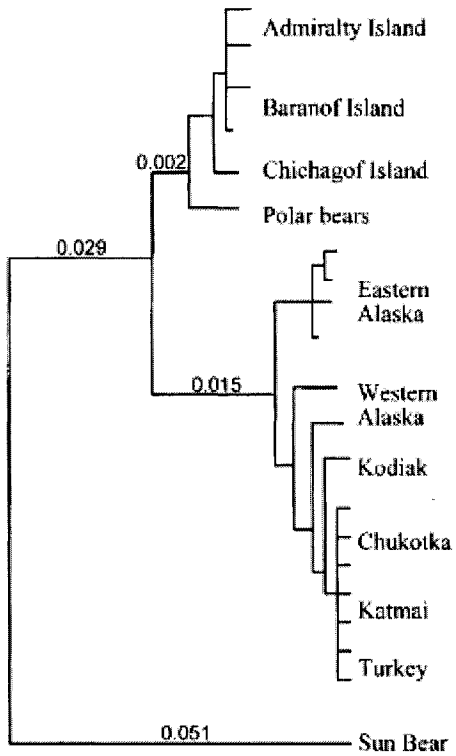

Fig. A.3. Neighbor-joining tree for the major lineages of brown bears and polar bears based on complete sequences of the mitochondrial cytochrome b gene and analyzed by the Kimura (1980) two parameter distance model. DNA sequence from the cytochrome b gene of a sun bear (*Helarctos* {= *Ursus*} *malayanus*) was used as an out-group reference

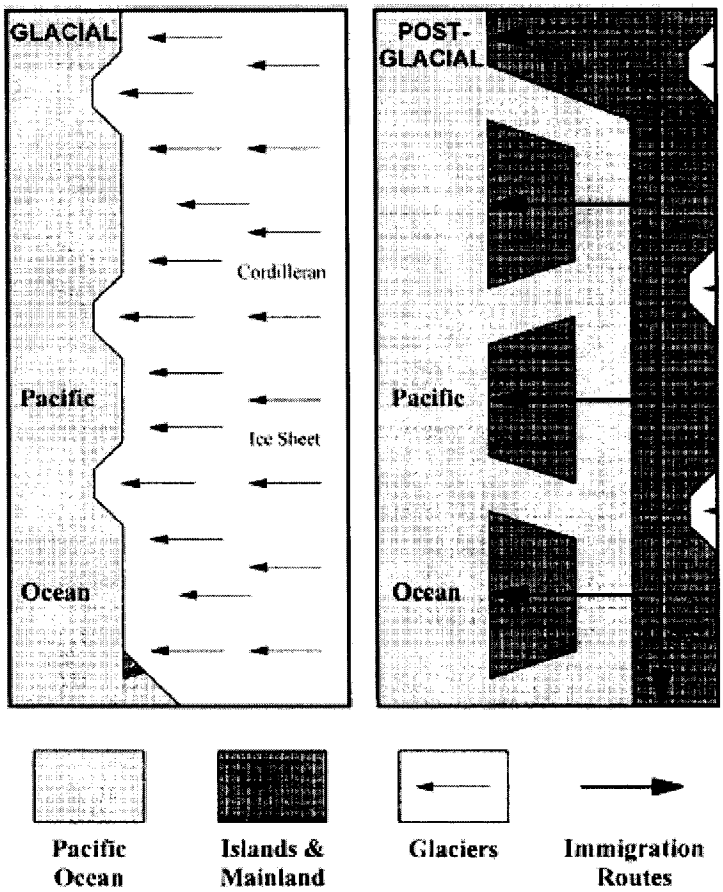

Fig. A.4. Idealized diagrams of southeast Alaska during and following Fraser Glaciation illustrating the Inland Colonization Model advocated by Klein (1965). Under this model there were no glacial survivors in the Alexander Archipelago, and the mainland and island populations have a postglacial common ancestry.

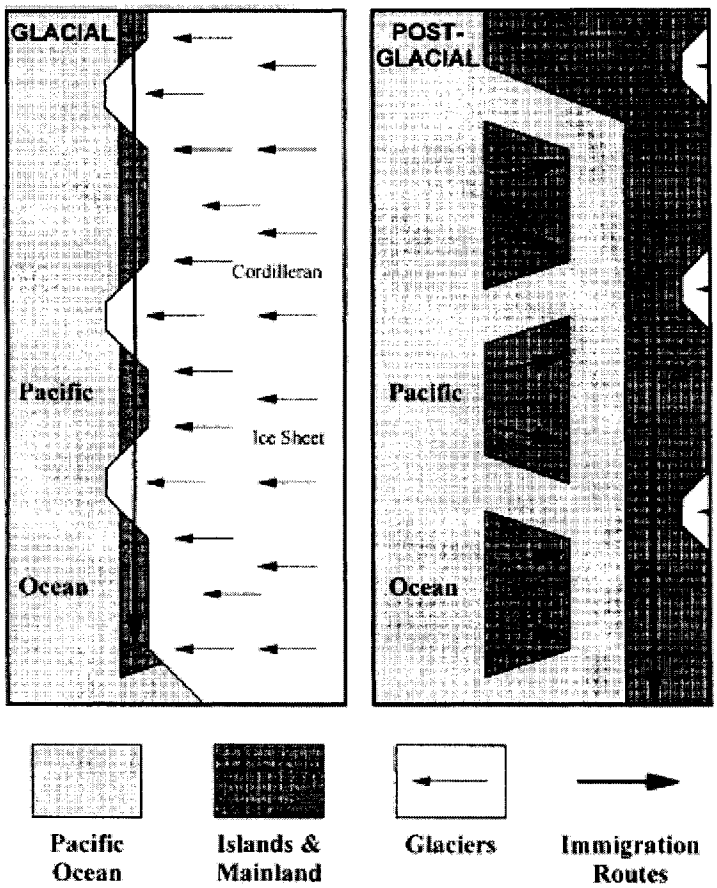

Fig. A.5. Idealized diagrams of southeast Alaska during and following Fraser Glaciation illustrating the Coastal Colonization Model presented for *Ursus arctos* in this paper. Under this model the mainland and island populations have a separate glacial and postglacial history and are therefore more distinct genetically.

Table A.1. List of limestone caves from which vertebrate remains have been recovered in the southern Alexander Archipelago indicating the minimum number of individuals (MNI) recovered of brown bears (*Ursus arctos*) and black bears (*U. americanus*).

Cave Name	Island	Altitude (m)	Cave type	Bears (MNI) brown	black	Other vertebrate remains recovered
El Capitan	Prince of Wales	130	Den	3	4	Fox, wolverine, river otter, fish
Bumper	Prince of Wales	520	Den	9	0	Caribou
Kushtaka	Prince of Wales	45	Den	0	2	Man, domestic dog, fish
Blowing in the Wind	Prince of Wales	670	Trap	2	0	
Enigma	Dall	150	Trap	1	0	Mule deer, river otter, fish
Nautilus	Heceta	170	Coastal	0	0	Mule deer
On Your Knees	Prince of Wales	85	Den	1	1	Mule deer, seal, river otter, fish
Devil's Canopy	Prince of Wales	180	Silt	0	0	Marmot

Note. Only El Capitan Cave and Bumper Cave have been thoroughly excavated thus far. The first six caves listed contain only postglacial remains. On Your Knees Cave contains bears that predate the Fraser Glaciation as well as other remains of glacial and postglacial age. The list of other vertebrate remains recovered is not exhaustive and excludes micromammals. Note that black bears, river otters, and fish (mostly tide pool species eaten by otters) are commonly associated in coastal caves (den sites) but are not found at higher elevations. Brown bear remains are found in caves in all altitudes.

Table A.2. Radiocarbon dates (AMS based on bone collagen) and associated stable carbon isotope values obtained thus far from vertebrate fossils in the southern Alexander Archipelago.

Laboratory number	^{14}C age (yr B.P.)	δ^{13}C	Description
	El Capitan Cave		
AA-10445	12,295 ± 120	−18.3	Brown bear (small)
AA-07794	9760 ± 75	−18.0	Brown bear (giant)
AA-10448	11,565 ± 115	−18.7	Black bear (juvenile cranium)
AA-10446	11,540 ± 110	−20.0	Black bear (complete skull)
AA-07793	10,745 ± 75	−21.1	Black bear (complete skeleton)
AA-10447	6415 ± 130	−22.1	Black bear (fused cranium)
AA-10449	6810 ± 65	−11.1	Fish bone (surface)
AA-11514	8535 ± 70	−13.2	Fish bone (deep)
AA-10450	3290 ± 60	−10.0	River otter (elsewhere in cave)
BT-55709	5770 ± 130	−16.1	Fish bone (elsewhere in cave)
	Bumper Cave		
AA-15222	11,640 ± 80	−17.8	Brown bear (complete skeleton)
AA-15223	11,225 ± 110	−16.8	Brown bear (large humerus)
AA-15225	10,970 ± 85	−19.5	Brown bear (giant molar)
AA-15224	7205 ± 65	−17.9	Brown bear (lower jaw)
AA-18449	10,515 ± 90	−19.1	Caribou (metacarpal)
	Blowing in the Wind Cave		
AA-10451	9995 ± 95	−18.5	Brown bear (juvenile skeleton)
	Kushtaka Cave		
AA-17451	8725 ± 70	−23.2	Black bear (femur)
CAMS-24967	8630 ± 60	−21.4	Black bear (rib)
CAMS-27263	2820 ± 60		Spear point (bone artifact)[a]
	Enigma Cave, Dall Island		
AA-15226	11,715 ± 120	−16.0	Black bear (complete skeleton)
	Nautilus Cave, Heceta Island		
AA-10574	8180 ± 70	−25.2	Mule deer (humerus)
	On Your Knees Cave		
AA-15227	35,365 ± 800	−15.9	Brown bear (large femur)
AA-16831	41,600 ± 1500	−20.7	Black bear (small tibia)
AA-18450	17,565 ± 160	−14.7	Ringed seal (ulna)
	Devil's Canopy Cave		
AA-8871A	44,500 +	−23.7	Marmot (incisor)

Note. The caves are located on northern Prince of Wales Island unless otherwise indicated (Fig. 1).
[a] The artifact from Kushtaka Cave is under study by E. James Dixon.

APPENDIX B

Mitochondrial DNA Phylogeography of the North American Brown Bear and Implications for Conservation[1]

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Abstract: *The historical distribution of the brown bear (*Ursus arctos*) in North America included Alaska, western Canada, the western and Midwestern states, plus northern Mexico. Currently, the brown bear is limited to Alaska, the Canadian provinces of the Yukon, Northwest Territories, British Columbia, and Alberta, and six threatened subpopulations in the lower 48 states. To examine the evolutionary history of* U. arctos *in North America and to assess the genetic divergence between individuals from different regions, we obtained 294 nucleotides of mitochondrial DNA sequence data from the control region for 317 free-ranging brown bears. Twenty-eight unique sequences, or mitochondrial DNA haplotypes were detected. The average sequence divergence between haplotypes was high (4.3%), and some haplotypes differed by as many as 23 nucleotides. Phylogenetic analyses using maximum parsimony revealed four major mitochondrial DNA phylogeographic groups, or clades. The significant phylogeographic structure*

[1]Waits, L. P., Talbot, S. L., Ward, R. H Ward, and Shields, G. F. (1998). Mitochondrial DNA phylogeography of the North American brown bear and implications for conservation. *Conservation Biology* **12**: 408-417.

detected in brown bears strongly contrasts with results obtained for other large carnivores and suggests limited female-mediated gene flow. The mitochondrial DNA phylogeographic clades do not correlate with taxonomic classifications for U. arctos, *and we hypothesize that the clades were formed prior to migration of this species into North America. We suggest evolutionarily significant units for conservation in three geographic regions: (1) the Alaskan islands of Admiralty, Baranof, and Chichagof· (2) mainland Alaska, Kodiak Island, and northern Canada; and (3) southern British Columbia, southern Alberta, and the states of Idaho, Montana, and Wyoming.*

Filogenia del ADN Mitochondrial del Oso Café de Norte América y sus Implicaciones en la Conservación

Resumen: *La districución histórica del oso café* (Ursus arctos) *en Norte América includía Alaska, oeste de Canada, oeste y medio oeste de los Estados Unidos y Norte de México. Actualmete, el oso café esta limitado a Alaska, las provincias Canadienses de Yukon, Northwest territories, British Columbia, Alberta y seis subpoblaciones amenazadas en 48 estados de los Estados Unidos. Obtuvimos datos de secuncias de 294 nucleótidos de ADN mitocondrial de la región control de 317 osos cafes para examinar la historica evolutiva de* U. arctos *en Norte América y evaluar la divergencia genética entre individuos de diferentes regions geográphicas. Fueron detectadas veintiocho secuencias únicas o haplotipos de ADN mitocondrial. El porcentaje de secuencia divergente entre haplotipos fue alto (4.3%) y algunos haplotipos difirieron por tantos como 23 nucleótidos. Análisis filogenéticos usando maxima parsimonia revelaron cuatro grupos mayores de ADN mitocondrial filogeográphico. La estructura filogeographica significantiva detectada en osos cafés contrasta fuertemente con resultados obtenidos de otros carnivoros mayores y sugiere flujo genético limitado, mediado por las hembras. Los grupos de ADN mitocondrial filogeográphico no se correlacionan con clasificaciones taxonómicas de* U. arctos *nosotros hipotetizamos que los grupos fueron*

formados antes de las migraciones de esta especie hacia Norte América. Sugerimos unidades evolutivamente significativas para su conservación en tres regions geográphicas: 1) las isles Admiralty, Baranof y Chichagof de Alaska; 2) el territorio continential de Alaska, la isla Kodiak y el norte de Canada, y 3) Sur de British Columbia, sur de Alberta y los estados de Idaho, Montana y Wyoming en los Estados Unidos.

Introduction

The brown bear (*Ursus arctos*) is one of the most widely distributed terrestrial mammals, with a current range spanning a variety of habitats in the lower-middle to high latitudes of Europe, Asia, and North America (Nowak 1991). A substantial portion of brown bear habitat has been destroyed worldwide, and previously contiguous populations have been divided into subpopulations. In North America the distribution of *U. arctos* in Alaska has changed little from historical distributions, with development encroaching on original habitat only in localized areas (Servheen 1989). In Canada the historical distribution of *U. arctos* has not been altered in the Yukon and Northwest Territories. Its historical range has been decreased substantially, however, by human development in southern British Columbia, and the brown bear has been eradicated from its historical range along the prairies of Alberta and Saskatchewan (Pasitschniak-Arts 1993). The species now occupies less than 1% of its original range in the lower 48 states and Mexico. Since the early 1800s the estimated population size in this area has plummeted from 100,000 to less than 1,000 (Allendorf & Servheen 1986). This reduction has resulted in the loss of corridors for gene flow, and six subpopulations are now isolated in the states of Idaho, Wyoming, and Washington (Servheen 1989). *U. arctos* was declared a threatened species in the lower 48 states in accordance with the U.S. Endangered Species Act of 1975 in response to the dwindling numbers of brown bears in this region (Allendorf & Servheen 1986).

Participants of a U.S. Fish and Wildlife Service-sponsored workshop on population genetics of the threatened brown bear recommended that gene flow should be

restored to isolated populations through population augmentation of one breeding individual per generation (Allendorf & Servheen 1986). To develop effective conservation strategies for *U. arctos* in N. America, it is essential to obtain a comprehensive understanding of the distribution of genetic variation within and between populations. Over 90 subspecies of brown bears have been proposed to describe the geographic variants of *U. arctos* in North America (Merriam 1918). This classification is now considered a classic example of taxonomic oversplitting, and current classifications group North American brown bears into two to seven subspecies (Rausch 1963; Kurtén 1973; Hall 1984). Despite taxonomic uncertainty, there are local variations in body size, skull structure, and pelage coloration, and this diversity may indicate underlying genetic differentiation. Thus, it is essential to characterize the distribution of genetic variation in *U. arctos* in order to make informed management decisions and to avoid the movement of bears between different locally adapted subpopulations, which may cause hybridization of animals with different coadapted gene complexes, possibly leading to outbreeding depression (Templeton 1986).

Molecular methods provide useful information for identifying genetic differentiation and reconstructing phylogenetic relationships among conspecific populations. Mitochondrial DNA (mtDNA) analysis has been a powerful tool for detecting population genetic structure at the intraspecific level (Avise et al. 1987), due primarily to its maternal mode of inheritance, lack of recombination, high mutation rate, and the process of lineage extinction within populations (Hutchinson et al. 1974; Brown 1983; Avise et al. 1984). Geographic patterns of genetic variation defined as "phylogeographic partitioning" by Avise et al. (1987) can provide important insights into evolutionary patterns of population fragmentation and gene flow. Previous studies of phylogeography in natural populations have demonstrated that small mammals with low mobility, such as pocket gophers (*Geomys pinetis;* Avise et al. 1979), generally exhibit significant mtDNA phylogeographic structure, and highly mobile mammals such as coyotes (*Canis latrans*; Lehman & Wayne 1991), moose (*Alces alces), caribou (Rangifer*

tarandus), and black bears (*U. americanus*) (Cronin et al. 1991; Cronin 1992) exhibit little or no mtDNA phylogeographic structure.

A mtDNA restriction enzyme analysis of North American brown bears revealed little genetic differentiation between geographic regions, suggesting considerable levels of female dispersal (Cronin et al 1991). But more informative mtDNA sequence analyses of *U. arctos* in Europe (Randi et al. 1994; Taberlet & Bouvet 1994; Kohn et al. 1995) and Alaska (Talbot & Shields 1996a) have revealed considerable genetic differentiation between geographic regions. In addition, mtDNA analyses have detected a close phylogentic relationship between the brown bear and the polar bear (*U. maritimus;* Shields & Kocher 1991), and some reported that the mtDNA phylogeny of the brown bear is paraphyletic with respect to the polar bear (Cronin et al. 1991; Taberlet & Bouvet 1992; Talbot & Shields 1996a; Talbot & Shields 1996b). We present the first comprehensive mtDNA sequence analysis of brown bears from across their current range in North America. These data are used to describe natural genetic variation, to infer evolutionary history and female gene flow, and to assess the phylogeographic structure of *U. arctos* in North America. Based on our results, we present recommendations for the conservation of the North American brown bear.

Methods

DNA Collection and mtDNA Sequencing

Blood and tissue samples of 317 free-ranging brown bears were collected from 22 geographic localities (Table B.1, Fig. B.1). Samples of four polar bears were also obtained from zoological parks with documented geographic origins; two from Norway, one from Russia, and one from Canada. All samples from endangered species were obtained with proper documentation from the Convention on International Trade in Endangered Species. Genomic DNA was isolated from these samples by means of previously described methods (Vardenplas et al. 1984; Talbot & Shields 1996a). The

mtDNA sequence data from the hypervariable 5' end of the control region (or D loop) were obtained from the products of polymerase chain reaction (PCR) amplification by use of previously described mammalian primers L15774 and H16498 (Shields and Kocher 1991) or L15997 and H16401 (Ward et al. 1991). The numbers of the primer designations identify the 3' ends according to the human reference sequence (Anderson et al. 1981), and L and H designate the light and heavy strands of the mtDNA molecule, respectively. The sequences analyzed were verified to be mtDNA sequences and not of nuclear origin (Talbot & Shields 1996a). Sequences for the outgroup species, the sun bear (*U. malayanus*) and the American black bear (*U. americanus*), were generated in earlier studies (Talbot & Shields 1996b; Waits 1996).

Phylogenetic Analyses

Sequences were aligned manually, and they corresponded to positions 16,381-16,711 of the harbor seal (*Phoca vitulina*) reference sequence (Arnason & Johnsson 1992). Unique DNA sequences were identified and are referred to hereafter as haplotypes. A 1-11 base pair insertion-deletion region of repeated thymine (T_a) nucleotides was identified at position 16,460 and was omitted from the data set because the number of repeated T nucleotides was often difficult to determine accurately. Mitochondrial DNA haplotypes of 294 nucleotides were analyzed by both parsimony and distance methods. Two different outgroups, the sun bear and the American black bear, were tested in all analyses.

Parsimony analyses were performed by PAUP 3.1 (Swofford 1993) and PHYLIP 3.5 (Felsenstein 1994). A consensus tree was generated from the minimal length topologies identified using the heuristic search option in PAUP. Parsimony bootstrap analysis (500 replicates) was performed with the SEQBOOT, DNAPARS, and CONSENSE programs of PHYLIP, using the bootstrap option based on the method of Felsenstein (1985). This method creates new data from the original data set under the assumption that characters evolve independently.

Sequence divergence among haplotypes was estimated by the two-parameter model of Kimura (1980) using DNADIST in PHYLIP. Distance trees were generated by the neighbor-joining method of Saitou and Nei (1987) in PHYLIP. Pairwise calculations of the number of nucleotide differences between haplotypes were obtained by use of the distance option in PAUP. To obtain the rate of sequence divergence for this portion of the mtDNA control region, we calibrated the average Kimura divergence between haplotypes of the Admiralty, Baranof, and Chichagof islands and all other brown bear haplotypes with a divergence date of 550,000-700,000 years (Talbot & Shields 1996a). This date was derived based on sequence divergence estimates from third-position codons in the cytochrome *b* gene of Alaskan brown bears and a mammalian divergence rate of 10% per million years (Irwin et al. 1991).

Results

Identification and Distribution of mtDNA Haplotypes

Examination of 294 nucleotides from the 5' end of the mtDNA control region in 317 brown bears and 4 polar bears identified 46 variable sites that defined 28 brown bear haplotypes and 4 polar bears identified 46 variable sites that defined 28 brown bear haplotypes and 4 polar bear haplotypes (Table B.2, Fig. B.2). The majority (44/46) of the variable sites represents brown bear sequence diversity because only two variable sites were unique to polar bear haplotypes (Fig. B.2). The average intraspecific sequence divergence among brown bear haplotypes was high (0.0425), whereas the average intraspecific divergence among polar bear haplotypes was much lower (0.0097). The average interspecific sequence divergence between brown bear and polar bear haplotypes was 0.0615.

The number of haplotypes observed in a single sampling locality was low, ranging from 1 to 5 with an average of 1.5 haplotypes per sampling locality (Table B.2). The majority of haplotypes (20/28) were observed in only one sampling location. The most

widespread haploptype (UA-29) was observed in six sampling locations in Alaska (Table B.2). It is difficult, however, to make direct comparisons between the number of haplotypes observed in different localities because the sample number varies widely, from 1 on the southern coast of Alaska to 53 on Kodiak Island (Table B.1).

Phylogenetic Analyses and Sequencing Divergence

Unweighted maximum parsimony analysis of the 28 brown bear and four polar bear haplotypes using the American black bear as an outgroup produced 75 minimal-length trees that have been summarized as a consensus tree based on 50% majority rule (Fig. B.3). Four major groups, or clades, of brown bears were detected. All trees placed four brown bear haplotypes from sampling location F, the Admiralty, Baranof, and Chichagof (ABC) islands of southeastern Alaska, in a group (Clade I) that clustered with the four polar bear haplotypes and excluded all other brown bear haplotypes. All trees grouped the eight haplotypes from southern Canada and the lower 48 states (Clade IV). Sixty-seven percent of the total topologies placed Clade IV as a sister clade to a group of nine haplotypes (Clade III) from northern Canada and extreme eastern Alaska. Clade III haplotypes clustered together in all 75 trees. The remaining group of brown bear haplotypes (Clade II) was clustered in 67% of the parsimony trees, and all seven haplotypes were found in Alaska. The robustness of the four clade groupings was examined by means of random bootstrap resampling (500 replicates). The bootstrap support for the clustering of haplotypes in Clades I, III, and IV was 90% or greater (Fig. B.3). Bootstrap support for the ABC Island brown bear and polar bear grouping was 75%. Clade II support was the lowest, with a bootstrap value of 43%. Bootstrap support for Clades II, III, and IV was 84%, but the branching order among these clades is unresolved (bootstrap value <50%). The topology obtained by using the sun bear as a an outgroup also detected the four major clades. The bootstrap support did not differ substantially, with the exception of the Clade I-polar bear grouping, which had an increased bootstrap value of 91%. The topology of the neighbor-joining distance tree

supported the groupings of the parsimony analysis with one exception: Clades II and III were merged into a single clade.

To obtain additional data to evaluate the support for four clades of North American brown bears, we calculated the number of pairwise nucleotide differences for all haplotypes and compared the average number of nucleotide differences within and between clades (Table B.3). The within-clade estimates ranged from a low of 1.5 differences among Clade III haplotypes to a high of 3.2 differences among Clade IV haplotypes. The average number of nucleotide differences between haplotypes of different clades was 3-15 times higher than the average number of differences among haplotypes within a single clade. The average number of nucleotide differences between clades ranged from a low of 9.2 differences between haplotypes from Clades II and II to a high of 22.4 differences between haplotypes from Clades I and IV. To estimate the divergence dates of the major clades, we divided the average Kimura sequence divergence (Table B.3) between Clade I haplotypes and those of all other clades by the divergence date of 550, 000 - 700,000 years, to obtain an estimate of 0.11-0.14% sequence divergence per 10,000 years. Using this divergence rate, we obtained the following divergence times: 146,000 - 185,000 years for polar bears and ABC Island brown bears; 245,000 - 310,000 years for Clades II and III; 280,000-356,000 for Clades II and IV; and 404,000 - 515,000 for Clades III and IV.

Discussion

Phylogeography

Our analyses of mtDNA genetic variation among North American brown bears revealed substantial sequence divergence between samples from different geographic regions. Phylogenetic analyses demonstrated that the genetic structure of mtDNA haplotypes or the North American brown bear is dominated by four major phylogenetic clusters of clades. When the haplotypes of each clade are mapped to their sampling locality, a

striking geographic pattern emerges (Fig. B.1). Clade 1 contains brown bears from the southeastern Alaskan islands of Admiralty, Baranof, and Chichagof. The four polar bear haplotypes are a sister group to the Clade I brown bears (Fig. B.3), supporting earlier studies that concluded that the brown bear was paraphyletic with respect to the polar bear (Cronin et al. 1991, Taberlet & Bouvet 1992; Talbot & Shields 1996a, 1996b). This clade of brown bears also appears to be the oldest group of brown bears in North America, as suggested by Talbot and Shields (1996a). Clade II includes brown bear haplotypes from throughout mainland Alaska and Kodiak Island. Clade III includes brown bear haplotypes from regions in extreme eastern Alaska and Canadian provinces of the Yukon and Northwest Territories. The final group, Clade IV, contains individuals from southern Alberta, and the states of Idaho, Montana and Wyoming.

The three Alaskan clades (I, II, and III) were also detected in an analysis of the complete nucleotide sequence of the cytochrome *b*, proline tRNA, and threonine tRNA mtDNA genes from 166 Alaskan brown bears (Talbot & Shields 1996a). Haplotypes from different clades do not coexist in a single locality, with one exception: haplotypes from Clades II and III are present in brown bears sampled from the Artic National Wildlife Refuge. This contact zone was also detected by Talbot and Shields (1996a). A contact zone between Clades III and IV may exist in Canada, but our current sampling did not detect one. Additional sampling from the geographic region between Clades III and IV and between Clades II and III will be necessary to obtain a more comprehesive description of clade contact zones.

The mtDNA phylogeographic structure observed in North American brown bears contrasts the pattern observed for three other large, mobile North American carnivores, the coyote, gray wolf, and polar bear. Extensive mtDNA restriction enzyme analyses of coyotes from across their range revealed high levels of genetic diversity but no genetic differentiation between coyote populations from different geographic regions (Lehman & Wayne 1991). An examination of mtDNA genetic diversity among gray wolf samples collected in Europe, Asia and North America revealed low levels of genetic variation and little to no phylogeographic structuring in North America. Genetic differentiation was

detected among haplotypes from different geographic regions in the Old World, but the sequence divergence between haplotypes was low, suggesting that the genetic differentiation reflected recent population decline and habitat fragmentation rather than a long-term history of genetic isolation. Population genetic structure has been examined for polar bears by means of allozyme markers (Allendorf et al. 1979; Larsen et al. 1983), mtDNA restriction enzyme analyses (Cronin et al. 1991), nuclear DNA restriction fragment analyses (Amstrup et al. 1993), and nuclear DNA microsatellite analyses (Paektau et al. 1995). All studies revealed low levels of genetic diversity; only the highly resolvative microsatellite analyses detected evidence for population structure. A mtDNA study of the American black bear using restriction enzyme analysis detected high levels of genetic diversity but no phylogeographic structure (Cronin et at. 1991). But a recent study using mtDNA sequence data from the control region detected two major phylogenetic clades that were estimated to have diverged 500,00 years ago (Woodling & Ward, in press). In contrast to the phylogeographic pattern observed in the brown bear, the two American black bear clades have contact zones in six sampling locations in the western United States and Canada.

Numerous field studies have documented that *U. arctos* is highly mobile and can disperse hundreds of kilometers (U.S. Fish and Wildlife Service 1987). Hence, the existence of substantial mtDNA phylogeographic structuring in North American brown bears is surprising and warrants an explanation. We propose that the presence of four mtDNA clades of brown bears in North America may be the result of a combination of the following: (1) separation and genetic divergence of brown bears populations in glacial refugia during the climatic fluctuations of the Pleistocene, (2) multiple migrations of brown bears into North America from Asia, and (3) low levels of female dispersal.

The earliest recognized of fossils of *U. arctos* are located in China and date to the middle Pleistocene (Kurtén 1968), but recent discoveries in France suggest that fossils with *U. arctos* characteristics date to the early Pleistocene (Mazza & Rustioni 1994). Fossil records suggest that *U. arctos* did not arrive in Alaska until the Wisconsin period (50,000-70,000 years ago; Kurtén 1968), and then the species is believed to have been

confined to Alaska by the continental ice sheet, spreading southward only during the postglacial period (Pasitschniak-Arts 1993). But recent paleontological work on Prince of Wales Island south of the ABC Islands has revealed an *U. arctos* fossil dating to 35,365 ± 800 years ago (Heaton & Grady 1993). This period precedes the peak of Wisconsin glaciation, suggesting that *U. arctos* may have survived the last glaciation south of the main ice sheet in refugia of the Alexander Archipelago of southeastern Alaska and British Columbia. Pleistocene climatic fluctuations may also have provided the opportunity for the development of divergent groups of brown bear mtDNA haplotypes. As the temperature fluctuated during this period, glacial ice sheets and sea levels expanded and contracted to produce dramatic changes in brown bear habitat. Population fragmentation with long-term isolation of subpopulations could allow divergent haplotypes to arise. If a subpopulation later expanded in size, perhaps as glacial retreat created new habitat, a number of derivative haplotypes would be maintained in a population, giving rise to a clade. The estimated time of divergence of different clades (245-000-700,00 years ago), combined with current fossil data, suggest that the divergence of haplotypes from the four major clades preceded the migration of *U. arctos* into North America.

Even if divergent clades were formed due to isolation, one might expect a greater mixing of clades after barriers to gene flow receded, yet we detected a mtDNA clade contact zone in only one locality. This may be explained by gender-biased dispersal in brown bears. A summary of studies of male and female home-ranged sizes in North American brown bears demonstrated that the average male home range is 2-10 times greater that the average female home range (U.S. Fish and Wildlife Service 1987). In addition, other studies have shown that young females tend to establish their home ranges within the home range of their mother (Pearson 1975). Such female philopatry would facilitate mtDNA genetic structuring within a species and would limit mixing of mtDNA haplotypes from different geographic regions. Additional studies of nuclear DNA and Y chromosomal genes are necessary to determine if male-mediated gene flow levels are hgher, as predicted by field studies.

MtDNA Phylogeny and Taxonomy

Based on the length of the condylobasal processes of North American brown bears skulls, Rausch (1963) classified all mainland brown bears as the same subspecies (*U. a. horribilis*) and all brown bears from Kodiak Island archipelago as subspecies *U. a. middendorffi*. Kurtén (1973) used skull measurements summarized by Rausch (1963) to propose three North American subspecies, *U. a. middendorffi* from the Kodiak Island archipelago, *U. a. dalli* of southern coastal regions of the Alaska panhandle, including the ABC islands, and *U. a. horribilis* for all other brown bears. Finally, Hall (1984) utilized cranial and dentition dimensions to propose seven North American subspecies. Five subspecies were restricted to Alaska: (1) *U. a. middendorffi* of the Kodiak Island archipelago, (2) *U. a. gyas* of the Kenai Peninsula, (3) *U. a. dalli* of the northwest panhandle of Alaska, (4) *U. a. sitkensis* of southeastern Alaska including the ABC Islands and the adjacent mainland, and (5) *U. a. alascensis* of the remaining mainland areas. The subspecies *U. a. sitkensis* was restricted to coastal British Columbia, Washington, and Oregon, and *U. a. horribilis* included all inland brown bears in Canada and the lower 48 states.

The mtDNA phylogeny does not uniformly support any of the taxonomic classifications, and no phylogeographic clade is supported by a subspecific taxonomic classification. The most striking example of discordance is brown bears from Kodiak Island. All three taxonomic classifications give subspecific status to these bears, but the mtDNA sequence observed in all individuals from Kodiak was identical to sequences observed in brown bears from many regions in mainland Alaska (Table B.2). In addition, identical mtDNA sequences for this portion of the control regions have been observed in brown bears from many regions in northern Asia and Europe (Taberlet & Bouvet 1994; Kohn et al. 1995; Waits 1996). Hall and Kurtén give subspecific status (*U. a. sitkensis* or *U. a. dalli*), but they also include bears from the adjacent mainland in these subspecific classifications. Two individuals were sampled from this mainland region (location G,

Table B.1), and both sequences cluster with Clade III rather than Clade I haplotypes. In addition, analyses of 34 new brown bears from the ABC Islands and 5 from the adjacent mainland support these findings (Williamson & Shields, unpublished data).

There is also no support for a subspecific group on the Kenai Peninsula as suggested by Hall (1984). All sequences from individuals sampled in this region (locations T and U, Table B.1) group with other mainland Alaska bears in Clade II. The subspecific group *U. a. horribilis* is used in all three classifications, but the mtDNA data do not support any of these geographic groupings. The classifications of Rausch and Kurtén include brown bears from all four mtDNA clades, and Hall's classification includes brown bears from Clades III and IV. In conclusion, there is little concordance between mtDNA phylogeny and morphological variation in brown bears. Discordant results were also obtained for subspecific classifications and mtDNA phylogeny in the gray wolf (Wayne et al. 1992), suggesting that the morphological differences used to defined subspecies in the brown bear and gray wolf may represent phenotypic plasticity in differing environments rather than long-term genetic isolation.

A revision of the taxonomy of North American brown bears in accordance with the phylogenetic species concept (Cracaft 1983) would result in drastic changes in the current classification. The most frequently reconginzed subspecies, *U a. middendorffi*, would be abolished, and four new subspecies distributions would be added. But it seems unreasonable to dramatically alter the current taxonomy based on the results from a single mtDNA region, especially with the knowledge that mtDNA gene trees may not always reflect true species trees (Pamilo & Nei 1988). Thus, we suggest that phylogenetic analyses of additional genes, particularly nuclear and Y chromosome genes, should be undertaken to provide additional molecular data.

Implications for Conservation

Genetically divergent populations are increasingly being recognized as appropriate units for conservation regardless of taxonomic status (Moritz 1994a). The degree of genetic

differentiation in the mtDNA gene tree of North American brown bears suggests a long-term matrilineal history of genetic isolation, and four clades may constitute evolutionary significant units (ESUs; Ryder 1986). But the mtDNA phylogeny may not accurately recflect the organismal phylogeny because of differences in male- and female-mediated gene flow (Avise 1995), lower effective number of genes, and random lineage sorting (Moritz 1994a). Such concerns have led to suggestions that evolutionary divergent groups defined by significant divergence of allele frequencies at nuclear DNA loci (Avise & Ball 1990; Moritz 1994b). There are currently no published reports of brown bear nuclear DNA diversity, but preliminary protein allozyme analyses of one locus have suggested substantial genetic divergence between Montana brown bears and Alaskan brown bears is in progress (Paektau et al., in press).

Until additional nuclear DNA and Y chromosomal data are available, we suggest that three phylogeographic groups of brown bears be defined as ESUs: (1) Clade I, (2) Clades II and III, and (3) Clade IV. Clade I bears from the Admiralty, Baranof, and Chichagof Islands warrant designation based on mtDNA genetic differentiation, antiquity, close phylogenetic relationships to polar bears, and apparent geographic isolation. Special efforts are necessary to preserve this unique group because these islands are suffering increased human pressure from ongoing and proposed timber harvests. These harvests should be limited because they lead to habitat fragmentation and increased human-bear conflict, ultimately resulting in a decrease in the numbers of bears (Schoen et al., in press). Because the neighbor-joining distance tree grouped haplotypes from Clades II and III, and because a contact zone was detected in the Artic National Wildlife Refuge, we suggest that the other brown bear populations in Alaska and Northern Canada constitute a single ESU. Additional sampling along the contact zone in eastern Alaska and examination of nuclear DNA and Y chromosome loci will be necessary to determine if this single ESU should be divided into two ESUs. The evolutionary distinctiveness of Clade IV brown bears from the lower 48 states and southern Canada is strongly supported by the mtDNA sequence data and preliminary allozyme analyses.

The designation of three ESUs for North American brown bears has numerous implications for conservation and management. To preserve the unique evolutionary history of the clades, we suggest that managers seek individuals from within the same ESU when augmentation of threatened populations is necessary. Specifically, these data demonstrate that maternal haplotypes of Alaskan brown bears diverged from haplotypes of brown bears of the lower 48 states over 250,000 years ago. Thus, any future augmentation of populations in the lower 48 states should include bears from more closely related southern Canadian populations. In addition, the genetic distinctiveness of Alaskan brown bears and brown bears of the lower 48 states indicated by mtDNA sequence analysis highlights the importance of listing *U. arctos* populations in the lower 48 states as threatened in accordance with the U.S. Endangered Species Act, despite the fact that brown bears are thriving in Alaska. The genetic differentiation of the four clades also suggests considerable demographic independence among populations as a result of limited female-mediated dispersal and gene flow. Thus, populations that are affected or extirpated by human or natural causes are unlikely to recover rapidly because recruitment of nonindigenous females is unnaturally low (Avise 1995).

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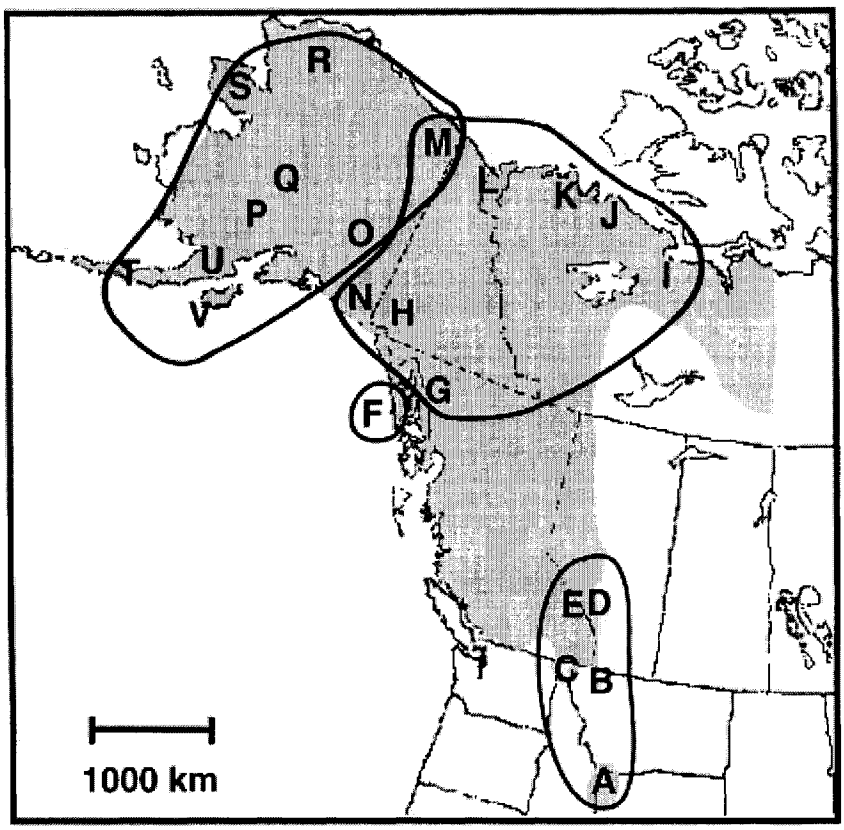

Figure B.1. Geographic sampling locations for all brown bears used in this mtDNA study. The name and sample size for each sampling location (A-V) are indicated in Table B.1. The current range of *U. arctos* is shaded. Four clades defined by mtDNA sequence analysis are circled.

```
                   11111111111111111111111111112222222
         111244555679901334444455677777888899990223335
        5468205167234560890128937312347068934590230177

UA-20   CCCTCCCAACGTTAACATTACGTAATCGAACAGCGGGTTAGGGAAC
UA-26   ..T.T.T.......................................
UA-27   ..T.TTT.......................................
UA-28    ...T.........................................
UA-29   ....T.T.......................................
UA-31   ....TTT....................................G..
UA-32   ....TTT.......................................
UA-21   ......TGG......T.......G.........T.......AA...
UA-22   ......TGG.....GT.........................AA.G.
UA-23   ......TGG......T.................T.....G.AA.G.
UA-53   ......TGG......T............G............AA.G.
UA-54   ......TGG......TG................T.......AA.G.
UA-56   ......TGG.....GT...G.................C...AA.G.
UA-57   ......TGG......T.................T...C...AA.G.
UA-58   ......TGG......T.................T.......AA.G.
UA-59   ......TGG.....GT.......G.............C...AA.G.
UA-37   TT..T.T..T..CG...................T......A.A..T
UA-38   TT..T.T..T..CG........C..........T........A..T
UA-39   TT..T.T.....CG........C.........ATA.......A..T
UA-40   TT..T.T..T..CG........C.........ATA.......A..T
UA-51   TT..T.T..T..CG........C..........T...........T
UA-52   TT..T.T..T.CCG........C.........ATA.......A..T
UA-55   TT..T.T..T..CG........C..........T..A.....A..T
UA-60   TT..T.T..T..CG................G..T..A.....A..T
UA-8    .T.C......A......C..TA.GGCT...T....A..C...A..T
UA-9    .T.C....G.A......C.GTA.GGCT...T....A..C...A..T
UA-10   .T.C......A......C.GTA.GGCT...T....A..C...A..T
UA-11   .T.C......A......C.GTA.GGCT...T....A..C...AG.T
UM-13   .T........A......CCGTA.GG.....T....A..C...A..T
UM-14   .T........A......CCGTA.GG..A..T....A..C...A..T
UM-15   .T........A......CCGTA.GG..A...GA..A..C...A..T
UM-16   .T........A......CCGTA.GG..A....A..A......A..T
```

Figure B.2. A compressed dot file of the 46 variable control-region positions that define 28 brown bear (UA) and four polar bear (UM) haplotypes. The position number is indicated at the top of the diagram; position 5 corresponds to position 16,385 of the harbor seal reference sequence. UA-20 is used as the reference haplotype, and a dot represents the same nucleotide as the reference haplotype, whereas all other letters represent nucleotide differences.

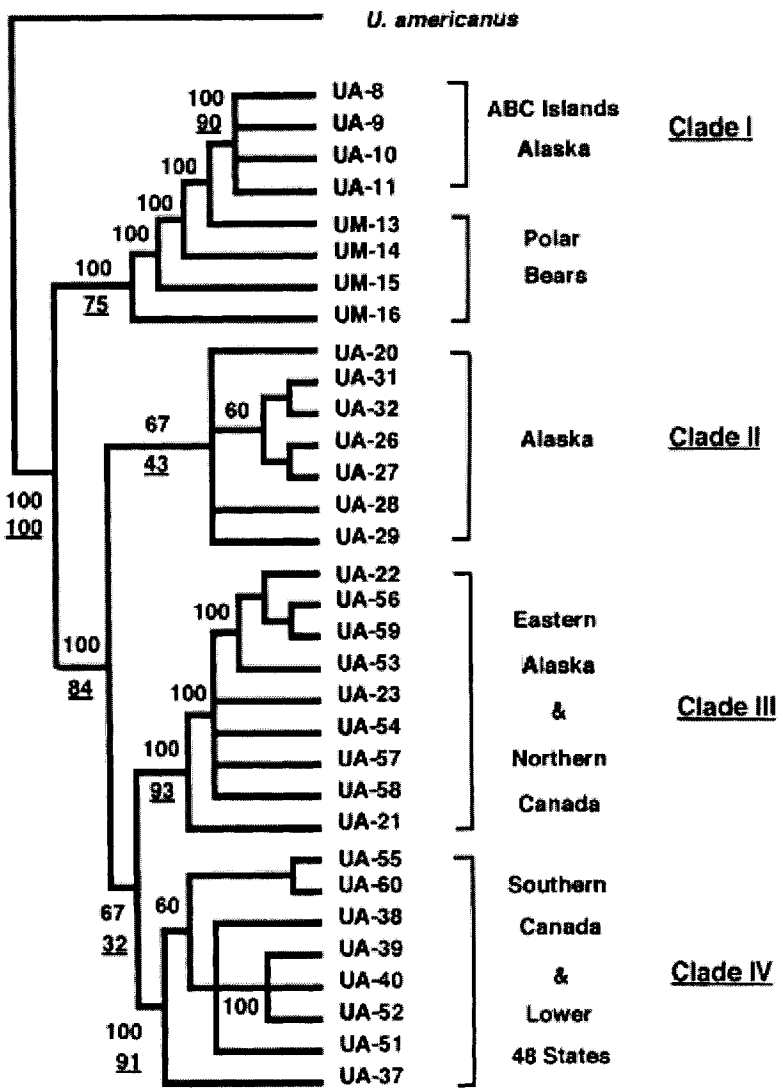

Figure B.3. Majority rule (50%) consensus tree for maximum parsimony analysis of 28 North American brown bear haplotypes (UA) and four polar bear haplotypes (UM). Consensus tree summarizes the 75 minimal length trees (length 88, consistency index 0.69) identified by a heuristic search. The support for this topology is indicated by the majority-rule percentages (bold numbers) and the underlined maximum parsimony bootstrap values (500 replications). The American black bear (*U. americanus*) was used as an outgroup.

Table B.1. Sampling location (Fig. B.1), locality, and sample size for brown bears used in the mtDNA analysis.

Sampling location	*Locality*	*Sample size*
A	Yellowstone ecosystem	46
B	Northern Continental Divide ecosystem	35
C	Cabinet-Yaak and Selkirk ecosystems	7
D	East Slope of the Canadian Rockies	30
E	West Slope of the Canadian Rockies	10
F	Admiralty, Baranof, and Chichagof Islands	21
G	Southeast Coast of Alaska	2
H	Kluane National Park	24
I	Coppermine	2
J	Paulatuk	2
K	Anderson	2
L	Richardson	2
M	Arctic National Wildlife Refuge	14
N	South Coast of Alaska	1
O	Alaska Range	5
P	Southcentral Alaska	2
Q	Interior Alaska	2
R	Brooks Range	9
S	Seward Peninsula	17
T	Izembek National Wildlife Refuge	10
U	Katmai	21
V	Kodiak Island	53
	Total	317

Table B.2. Distribution of mtDNA brown bear haplotypes by geographic region (A-V) as defined in Table B.1 and Figure B.1.

	Sampling location																				
Haplotype	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	T	U	V
UA-8						4															
UA-9						2															
UA-10						7															
UA-11						8															
UA-20															2						
UA-26																	1				
UA-27																1				2	
UA-28																	1				
UA-29													5		3	1		9	17		53
UA-31																				19	
UA-32																			10		
UA-21								2						1							
UA-22							2														
UA-23												2	7								
UA-53									2	2	1										
UA-54											1										
UA-56								3													
UA-57								12													
UA-58								6													
UA-59								1													
UA-37		18		1																	
UA-38	40	3	1	29	10																
UA-39		3																			
UA-40		11	4																		
UA-51	5																				
UA-52		1																			
UA-55	2																				
UA-60			2																		

Table B.3. Average number of pairwise sequence differences within and between the four brown bear mtDNA clades (above diagonal) and average Kimura sequence divergence between clades (below diagonal).

	Clade I	Clade II	Clade III	Clade IV
Clade I	1.5	18.6	21.9	22.4
Clade II	0.0674	2.1000	9.6	11.2
Clade III	0.0803	0.0343	3.1	15.8
Clade IV	0.0821	0.0392	0.0566	3.2

APPENDIX C

DRAFT -- IN REVISION

SUBSTANTIAL PHYLOGEOGRAPHIC STRUCTURE IN A WORLD-WIDE SURVEY OF BROWN BEAR (*URSUS ARCTOS*) MITOCHONDRIAL DNA SEQUENCE VARIATION

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Abstract

Among large contemporary carnivores, the brown bear (*Ursus arctos*) is second only to the gray wolf (*Canis lupus*) in the extent of its geographic range. To investigate the molecular phylogeny of brown bears, we analyzed mitochondrial DNA (mtDNA) sequence data for 455 free-ranging brown bears. Sequence variation in 558 nucleotides (nt) from the control region (294nt) and a segment of the cytochrome *b* gene (264nt) for 160 brown bears defined 37 mtDNA haplotypes, and control region sequences for 455 brown bears defined 48 haplotypes. The average sequence divergence in the mtDNA control region (4.4%) is substantially greater than intraspecific estimates for other widely dispersed mammalian species. Phylogenetic analyses of the combined data set, the Cyt*b* data set, and the control region data set, revealed the presence of four major clades. MtDNA haplotypes of clade 1 are present in European brown bears; clade II haplotypes are restricted to brown bears from Admiralty, Baranof and Chichagof islands of SE Alaska; haplotypes of the widespread third clade are present in brown bears from northern and eastern Europe, Asia, and North America; clade IV haplotypes are found only in bears from southern Canada and the US states of Montana, Wyoming and Idaho. We suggest that substantial phylogeographic structure in this highly mobile species can be explained by population fragmentation related to Pleistocene climatic fluctuations and by limited female dispersal.

Introduction

Brown bears (*Ursus arctos*) are one of the youngest members of the bear family, Ursidae (Nowak 1991). Fossil data (Kurtén 1964; Mazza & Rustioni 1994) and molecular analyses (Talbot & Shields; Waits 1996) date the origin of the brown bear to the Pleistocene, and molecular data suggest that polar bears (*U. maritimus*) emerged from within the brown bear clade during the mid-Pleistocene (Taberlet & Bouvet 1992; Talbot

& Shields 1996a, b; Waits 1996; Waits et al. 1998b. The close evolutionary relationships between brown bears and polar bears is supported by a wealth of fossil (Kurtén 1964; Kurtén 1968; Kurtén & Anderson 1980) and molecular (Kocher & Shields 1991; Cronin et al. 1991; Taberlet & Bouvet 1992; Talbot & Shields 1996a, b; Waits 1996; Waits et al. 1998b, b; Zhang & Ryder 1994) data, and by the existence of fertile F1 hybrids (Gray 1971). Brown bears are the most morphologically diverse of all bear species (Ursids) (Mazza & Rustioni 1994) and have been divided into as many as 232 subspecies (Erdbrink 1953). The contemporary brown bear is a generalist species that utilizes a variety of habitats ranging from the arctic tundra to alpine slopes, and spans the lower middle-to-high latitudes of Europe, Asia and North America. In the past 150 years, a substantial proportion of brown bear habitat has been destroyed, disrupting migration corridors, isolating subpopulations, and exterminating or endangering many local populations (Servheen 1990). Based simply on the nature of its current and historic range, the brown bear is an ideal model for investigating the relationship between intraspecific phylogenetic structure and geography. In addition, there is considerable interest in understanding the genetic relationships among brown bears from different geographic regions to provide information for the management and conservation of this imperiled carnivore species.

Mitochondrial DNA (mtDNA) analysis is a powerful tool for detecting the population genetic structure at the intraspecific level (Avise et al. 1979; Avise et al. 1987). The rapid mutation rate of the mitochondrial molecule (Brown et al. 1979) makes it an informative genetic marker over short evolutionary time periods. Its maternal, haploid mode of inheritance (Lansman et al. 1983) allows molecular phylogenies to be estimated without the ambiguity caused by recombination. And, the effective population size for mtDNA is one-fourth the effective population size for nuclear genes, making mtDNA analyses more sensitive to genetic drift (Birky 1991). The examination of geographic patterns of genetic variation is defined as phylogeography (Avise et al. 1987) and can provide important insights into evolutionary patterns of population fragmentation and gene flow. Previous studies of phylgeography in natural populations have

demonstrated that small mammals with low mobility, such as pocket gophers (*Geomys pinetis*) (Avise et al. 1979), generally exhibit significant mtDNA phylogeographic structure, and highly mobile mammals such as the coyote (*Canis latrans*) (Lehman & Wayne 1991) and the American black bear (*Ursus americanus*) (Cronin et al. 1991) exhibit little, or no, mtDNA phylogeographic structure.

The phylogeography of brown bears has recently been examined in North America (Talbot & Shields 1996a; Waits et al. 1998b) and Europe (Kohn et al. 1995; Randi et al. 1994, Taberlet & Bouvet 1994) using mitochondrial DNA sequence data. The North American studies have proposed two to four major phylogenetic groups, or clades, while the European data have suggested the existence of two major clades. Hence, current data suggest that the brown bear exhibits significant mtDNA phylogeographic structure although two biological characteristics of this species, recent evolutionary origin (Kurtén 1968; Kurtén & Anderson 1980, Mazza & Rustioni 1994) and high mobility (USFWS) 1987), are usually associated with species that display little or no mtDNA phylogeographic structure (Avise et al. 1987). To investigate the phylogeographic structure of the brown bear on a world-wide scale, we have combined mtDNA sequence data from earlier studies (Kohn et al. 1995; Taberlet & Bouvet 1994; Talbot & Shields 1996a, Waits et al. 1998b) and collected mtDNA sequence data from additional bears focusing on previously unsampled geographic regions. This worldwide data set includes 294 nucleotides (nt) of control region sequence plus 264 nt of the cytochrome *b* (Cyt*b*) gene for 160 brown bears and four polar bears, and 208-263 nt of control region data for an additional 294 brown bears. To our knowledge, this represents the most extensive survey of mtDNA sequence variation in any free-ranging mammal (excluding humans). Phylogenetic analyses identified four to five major mtDNA clades of brown bear haplotypes, which exhibit differing degrees of geographic clustering, and we discuss hypotheses to explain phylogeographic clustering in this wide-ranging, highly mobile species.

Materials and Methods

Population samples

DNA was collected from 455 free-ranging brown bears using blood, scat, hair, skin, or muscle tissues (Kohn *et al.* 1995; Taberlet & and Bouvet 1994; Talbot & Shields 1996a; Waits et al. 1998b). The sample set includes 127 brown bears from Europe, nine brown bears from Asia, and 319 brown bears from North America (Table C.1, Figs. C.1 a, b). Four polar bears were sampled from zoological parks: two originated in Europe, one in Asia, and one in North America. All samples were obtained with appropriate CITES documentation.

MtDNA sequencing

Sequences were obtained from the products of PCR amplification as described (Kohn *et al.* 1995; Taberlet & and Bouvet 1994; Talbot & Shields 1996a; Waits et al. 1998b). Sequences at the 5' end of the control region were generated using published primers (Kohn *et al.* 1995; Taberlet & and Bouvet 1994; Waits et al. 1998b). The assimilation of sequences generated in four different laboratories produced overlapping data sets with differing lengths: 209 nt for 455 brown bears. An additional 264 nt of Cyt*b* sequence data were obtained from four polar bears, ten European, seven Asian, and 143 North American brown bears using published primer sets (Kocher *et al.* 1998). Control region sequences were previously reported for 440 brown bears (Kohn *et al.* 1998). Control region sequences were previously reported for 440 brown bears (Kohn *et al.* 1995; Taberlet & and Bouvet 1994; Waits et al. 1998b), as were Cyt*b* sequences for 137 brown bears (Talbot & Shields 1996ba). Sequences for the outgroup taxa, the spectacled bear (*Tremarctos ornatus*), the sun bear (*U. malayanus*), and the American black bear (*U. americanus*), were generated in an earlier study (Waits 1996).

Phylogenetic analyses

Phylogenetic analyses were restricted to aligned sequences corresponding to positions 15,213 - 15,476 and 16,378 - 16,711 or the harbor seal (*Phoca vitulina*) reference sequence (Arnason & Johnsson 1992) for the Cyt*b* gene and control region, respectively. A 1 - 11 base pair insertion/deletion region of repeated thymine (T_n) nucleotides was identified at position 16,460, and it was omitted from the data set because the number of repeated T nucleotides was often difficult to accurately determine. Aligned sequences were compared, and mtDNA haplotypes, defined as unique sequences, were identified. Phylogenetic analyses of mtDNA haplotypes were performed using combined data set (558 nt) of control region data set (263 nt). Three different outgroups, the spectacled bear, the American black bear, and the sun bear were tested in all analyses. Phylogenies were evaluated using statistical criteria for maximum likelihood, neighbour-joining PHYLIP 3.5 (Felsenstein 1994) maximum parsimony, PHYLIP 3.5 and PAUP 3.1 (Swofford 1993), and by bootstrap analysis using a TS/TV ratio of 1:8 obtained by a maximum likelihood estimate. Trees were generated with and without weighing. For weighted phylogenetic analyses, the Cyt*b* data set was divided into domains corresponding to first, second, and third codon positions. In the maximum likelihood analyses, the categories option was used to specify relative variabilities: Cyt*b* codons – 1^{st} position = 6.8, 2^{nd} = 1.0, 3^{rd} = 22.5. Alternate topologies were tested for significant differences at the 95% confidence level using Kishino and Hasegawa's (1989) test for maximum likelihood and Templeton's test (1983) for maximum parsimony.

Results

Haplotype identification and sequence divergence

Homologous Cyt*b* sequences (264 nt) were obtained for 160 brown bears and four polar bears. Twenty-one mitochondrial haplotypes were defined based on 25 variable positions. Homologous control region sequences (209 - 294 nt) obtained for 455 brown bears and four polar bears contained 57 variable positions that defined 52 haplotypes.

Only two transversions were observed, one in control region and one in Cyt*b* sequences. The combined data set of Cyt*b* and control region sequences (294nt) for 164 bears includes 37 brown bear and four polar bear haplotypes defined by 77 variable nucleotides (Fig. C.2). Table C.2 summarizes the overlap between data sets: twenty-eight of the combined data haplotypes group in four control region haplotypes. Nineteen haplotypes (UA-42 – UA-60) were identified in 50 brown bears for which only control regions sequences were available (Fig. C.2). The other 244 brown bears for which only control region data were available had identical sequences to the control region segment of one of the 37 combined data haplotypes. We provisionally assigned these individuals to an appropriate combined data set haplotype (Fig. C.2), with the caveat that obtaining Cyt*b* sequence data for these individuals may reveal a small number of new haplotypes.

The 41 combined data haplotypes differed by an average of 19.4 (± 9.8) nucleotides, with brown bear haplotypes differing by 18.8 (± 1.6) nucleotides. Thus, the majority of the variation in the data set is due to brown bear intraspecific diversity and not brown bear/polar bear interspecific diversity or polar bear intraspecific diversity. The Cyt*b* sequence spanned 88 codons with 19 polymorphic third position sites and six polymorphic first position sites. All six first position variants, and one of the third position variants, resulted in amino acid substitutions. The mean number of pairwise differences among 19 brown bear Cyt*b* haplotypes was 6.9 (± 3.4), corresponding to 2.6% sequence divergence, and only a single difference was observed between the two polar bear haplotypes (0.4% sequence divergence). The average pairwise difference among the 48 brown bear control region haplotypes was 11.6 (± 4.9) or 4.4% sequence divergence. An average of 1.1% sequence divergence was observed among the four polar bear control region haplotypes.

Compared to sequence data from the 5' end of the mtDNA control region in the three other wide ranging mammals, the gray wolf (*Canis lupus*), humpback whales (*Megaptera novaeangliae*), and humans, brown bears exhibit a substantial level of intraspecific sequence divergence. Analyses of 261 nt of the control region in 162 wolves representing 27 worldwide populations revealed 17 haplotypes with an average

sequence divergence of 2.1% and a maximum of 4.0% (Vilà *et al.* 1997). Analyses of a homologous segment (283 nt) of the control region in humpback whales identified an average of 3.0% sequence divergence between 37 haplotypes (Baker *et al.*1993). Analyses of a homologous segment (360 nt) of the control region in humans revealed an average of 2.3% sequence divergence between 292 human haplotypes – 52 African; 38 Middle Eastern; 50 Caucasian; and 75 Amerindian (Ward, unpublished data).

Phylogenetic reconstruction

The best maximum likelihood topology from phylogenetic analysis of the combined data set is displayed in Fig. C.3. This topology is identical to the best weighted likelihood topology and the maximum parsimony bootstrap analysis (1,000 replicates) consensus tree. These analyses revealed four major phylogenetic groups of haplotypes which were defined as clades when a monophyletic group of haplotypes was supported by parsimony bootstrap values of 50% or higher. Neighbor-joining bootstrap analyses (500 replicates) also support the clade designations in Fig. C.3, with one exception. Neighbour-joining analyses support (83%) grouping haplotypes UA-1 and UA-2 in Clade I. Separate analyses of the Cyt*b* data set revealed a topology that was different from Fig. C.3; however, all major clades were preserved and the topology differed only in the placement of three isolated haplotypes.

Maximum parsimony phylogenetic analyses of the 52 bear haplotypes defined by a control region sequences from 455 brown bears and four polar bears identified 175 minimal length trees (169 steps). These results are summarized in a 50% majority rule consensus tree (Fig. C.4). The optimal maximum likelihood topology obtained from ten independent runs did not differ significantly from the parsimony consensus tree. The control region topology shown in Fig. C.4 preserves the branching order of combined data haplotypes UA-1 to UA-41 displayed in Fig. C.3, and nineteen new haplotypes (UA-42 to UA-60) are scattered throughout the tree. In all analyses, the branching pattern was unaltered by the choice of outgroup (spectacled bear, sun bear, or American black bear).

Both topologies (Figures 3 and 4) suggest that the distribution of mtDNA haplotypes in this comprehensive data set from free-ranging brown bears can be summarized by the divergence, and subsequent radiation, of four major clades. The oldest clade includes combined data haplotypes and control region haplotypes found in contemporary brown bear populations of Romania, Slovenia, Croatia, Bosnia, Bulgaria, Greece and Italy. The oldest mtDNA haplotypes (UA-1, UA-2, UA-43, UA-44) are found in brown bears from the French Pyrenees, Spain, southern Norway and Sweden, and do not cluster strongly in any of the major clades. However, a suboptimal control region topology (3 steps longer) gives 56% bootstrap support for including all the above haplotypes in a single clade (clade I).

Clade II includes haplotypes found in brown bears restricted to Admiralty, Baranof, and Chichagof (ABC) islands of Alaska, plus all of the polar bear haplotypes. There is a high bootstrap support (91% combined data; 83% control region data) for grouping polar bears and brown bears in clade II, and monophyletic brown bear topologies in which all polar bear haplotypes diverge either before UA-01, or after clade IV are significantly worse using the criteria of Kishino and Hasegawa (1989) and Templeton (1983). There is a strong bootstrap support (90% combined data; 86% control region data) for subclade (IIa) of ABC island bear haplotypes, but less support (58% combined data; 50% control region data) for a monophyletic polar bear subclade (IIb) because alternate topologies favor grouping UM-13 with subclade IIa. These data support previous mtDNA analyses that suggested the brown bear is paraphyletic with respect to the polar bear (Cronin *et al.* 1991; Randi *et al.* 1994; Taberlet & Bouvet 1992; Talbot and Shields 1996a; Talbot and Shields 1996b; Waits 1996; Waits et al. 1998b).

Clade III is a complex and extremely widespread clade that includes haplotypes found in brown bears from northern and eastern Europe, Asia, and North America. Although this clade contains haplotypes from geographically disparate populations, it has 79% bootstrap support in the combined data analyses and is supported by two shared Cyt*b* amino acid substitutions: a serine to proline substitution at position 57, and a valine to isoleucine substitution at position 123 (Fig. C.3). Clade III may also contain two

subclades. Subclade IIIa includes haplotypes found in brown bears of eastern Alaska and northern Canada and has greater than 95% bootstrap support in the combined data and control region analyses. Subclade IIIb includes widely dispersed haplotypes from northern and eastern Europe, northern Asia and Alaska. This subclade is defined by a shared amino acid substitution (serine to proline at position 110), but it has less than 50% bootstrap support in the combined data and control region analyses.

The strongly supported (<90%) clade IV is more geographically restricted and contains North American brown bear haplotypes from the southern Canadian Rockies, and the states of Idaho, Montana, and Wyoming. A small number of solitary haplotypes from Asian brown bears (UA-17, UA-49, UA-50) also diverged at approximately the same time as clades III and IV. Additional sampling from Asia will be necessary to improve the geographic coverage of the phylogeny and to determine if any additional clades are present. Figures 3 and 4 indicate that the mtDNA phylogeny of brown bears is dominated by four major clades, and these contribute to the high levels of intraspecific sequence divergence observed in brown bears. To assess the range of genetic divergence within and between clades, the average number of pairwise differences was estimated. In all comparisons, the average combined data sequence divergence among clades is three to ten times greater than the divergence within clades (Table C.3). The maximum sequence divergence within a clade was 1.1% (clade III) and the minimum was 0.5% (clades I and IV). In contrast, the minimum sequence divergence between clades was 3.5% (between clades I and II) and the maximum was 5.5% (between clades I and III and between clades I and IV).

Dating molecular divergences

The divergence date for the haplotype leading to clade II (Fig. C.3) has been estimated in two earlier studies (Talbot and Shields 1996a; Waits 1996). Using 1,064 nt of sequence data from four mtDNA coding regions (Waits 1996), an average pairwise Kimura distance of 0.290 ± 0.013 was obtained for third position synonymous sites between the oldest ursine species (*U. ursinus*) and all other ursine species. Calibration with the

earliest fossil date for ursine bears (4.8 million years ago) gave an average divergence rate of 6.0 ± 0.3% per million years, and a divergence date of 0.60 – 0.71 million years ago (mya) for the lineage leading to clade II. Using sequence divergence estimates from 3rd position codons for the complete Cyt*b* gene of Alaskan brown bears, Talbot and Shields (1996) obtained a divergence date estimate of 0.55 – 0.70 mya. Since molecular divergence will predate population divergences (Wilson *et al.* 1985), we chose the conservative upper bound of 0.70 mya, and the divergence dates of all other haplotypes were calibrated by maximum likelihood scaled branch lengths (Fig. C.3). These results suggest that the oldest brown bear haplotypes date to 0.91 mya; clade I diverged 0.79 mya; polar bears and clade IIa brown bears diverged 0.56 mya , and clades III and IV diverged 0.42 mya.

Discussion

Phylogeographic structure

The geographic distribution of brown bear haplotypes within each of the four major clades reveals a wide range of phylogeographic clustering: clade II haplotypes are restricted to three islands off the southeastern coast of Alaska; clade I haplotypes are restricted to western Europe; clade IV haplotypes are restricted to southern Canada and the states of Montana, Wyoming and Idaho; and clade III haplotypes span the northern latitudes of Europe, Asia, and North America. This phylogeographic structure is so pronounced that, in general, samples collected from a single population cluster in the same clade. Only two sampling localities, Sweden and Romania, contained bears that grouped into more than one phylogenetic clade. In the Romanian sample, bears with clade I and III haplotypes are found in the same population (Kohn *et al.* 1995), but in Sweden clade III haplotypes are found primarily in bears from three northern subpopulations and clade I haplotypes are found only in the southernmost subpopulation (Taberlet & Bouvet 1994). In Sweden, the contact zone has been examined more extensively using mtDNA restriction enzyme analysis of 127 bears, and only four

individuals (all male) were observed to cross the contact zone (Taberlet *et al.* 1995). A contact zone may exist between clades II and IV in Canada, but our current sampling did not detect one. Additional sampling from the geographic region between clades III and IV will be necessary to address this question.

The existence of highly divergent clades which dominate the mtDNA phylogeography of brown bears contrasts with the mtDNA phylogeographic pattern of other widely dispersed , mobile carnivores, such as the American black bear (Cronin *et al.* 1991) and the coyote (Lehman & Wayne 1991) that have little or no phylogeographical structuring. In contrast, two recent studies of mobile, wide-ranging carnivores, the leopard (*Panthera pardus*) (Miththapala *et al.* 1996) and the gray wolf (Vilà *et al.*1997), have also detected three to four phylogeographic clades suggesting that phylogeographical structuring in mobile carnivores may be more significant than previously believed. Numerous field studies have documented that *U. arctos* can disperse over large distances, ranging up to 800 km in a three month period ((USFWS) 1987). Hence, the existence of substantial phylogeographic structuring in brown bears warrants an explanation. One suggested hypothesis for the existence of clades I and III is that they were formed by the isolation of brown bear populations in Asia and southern Europe when the cave bear (*U. spelaeus*) was occupying the brown bear niche in Europe from the middle Pleistocene until 20,000 years ago (Hänni *et al.* 1994).

Phylogeography and climatic fluctuations

For another explanation, we propose that the existence of divergent clades within contemporary populations of brown bears can be explained by two factors: 1) recurrent population contraction and expansion, and 2) limited female dispersal. Population fragmentation, with long term isolation of subpopulations, would allow haplotypes to diverge between the isolated subpopulations. If subpopulations then expand in size, multiple derivative haplotypes could be maintained in the population, giving rise to a clade. The extreme climatic fluctuations of the Pleistocene could have driven such a series of population fragmentation and expansion. With the cyclic change of

temperature, glacial ice sheets expanded and contracted to produce dramatic changes in available brown bear habitat. Fragmentation of brown bear populations into isolated refugia would provide ample opportunity for molecular divergence between subpopulations. Eventual glacial retreat and rising temperatures would allow isolated brown bear populations to expand into previously unoccupied habitat. Since population fragmentation in Pleistocene refugia appears to have dictated the geographic structuring of mtDNA haplotypes in a migrant shore bird, *Calidris alpina*, (Wenink *et al.* 1993), similar ecological pressures may also have caused mtDNA substructuring in a mobile species like the brown bear.

Preliminary analyses of the dates for glacial advance and retreat defined by $d^{18}O$ analyses of deep sea cores (Crowley & North 1991; Raymo *et al.* 1990) and our estimated dates for the divergence and radiation of the mtDNA clades support the hypothesis that clades diverged during cold periods and expanded during warm periods (Fig. C.5). To extend these observations to the hypotheses about population isolation and subsequent range expansion, we need to consider the fact that molecular divergence will predate population divergence. One suggested method is to correct for polymorphisms that existed before populations sub-divided by subtracting the average within population sequence divergence (Nei 1987). Since the average sequence divergence within clades (Table C.3) is 6.5 times lower than the divergence between clades, we believe that the molecular divergence dates will not substantially predate the inferred population divergences. To more thoroughly address this issue, we have begun to collect a longer segment of mtDNA sequence data from the entire Cyt*b* gene using representatives of each of the four major phylogenetic clades.

Even if divergent clades were formed due to isolation in glacial refugia, one might expect a greater mixing of clades after barriers to gene flow receded, yet we detected a mtDNA clade hybrid zone in only two localities. This may be explained by low levels of female dispersal. A summary of studies of male and female home range sizes in North American brown bears demonstrated that the average male home range is

two ten times greater than the average female home range ((USFWS) 1987). In addition, other studies have shown that young females tend to establish their home ranges within the home range of their mother (Pearson 1975), particularly when bear density is high. However, when suitable unoccupied habitat is available, young females have been observed to disperse substantial distances from their mother's home range (J. Swenson, personal communication). This behavioral trait would facilitate mtDNA genetic structuring within a species and limit mixing of mtDNA haplotypes from different geographic regions. However, it may also explain the existence of the widely dispersed clade III if females dispersed to utilize a new habitat after the polar ice sheets receded following the last ice age.

In light of the above, it is significant that the gray wolf, a large carnivore whose evolution parallels that of the brown bear, has a similar phylogeographic structure. The gray wolf originated in Europe approximately 800,000 years ago (Nilsson 1983) and, like the brown bear, is highly mobile and extremely widespread. MtDNA restriction enzyme analysis of 350 wolves revealed distinct phylogeographic structuring in Eurasia, but none in North America. In addition, wolves have a single widespread mtDNA haplotype found throughout the northern latitudes of North America and Eurasia (Wayne *et al.* 1992). This suggests the evolution of the gray wolf may also have been influenced by the genetic differentiation of subpopulations in Pleistocene glacial refugia, coupled with range expansion during interglacial or postglacial periods. If so, it is likely that deep molecular divergences will be found in other mobile species that experienced population fragmentation and expansion due to the climatic fluctuations of the Pleistocene.

Concordance with fossil record

The molecular phylogeny and divergence dates are consistent with existing paleontological data. Fossil teeth with *U. arctos* characteristics found in Nihewan deposits of the late Pliocene to early Pleistocene (Qiu 1990) suggest that *U. arctos* became distinct from the ancestral Ursid lineage in the early Pleistocene, in agreement with the molecular data. The morphological differentiation leading to *U. arctos* was

initially believed to have occurred in Asia through the ancestral *U. etruscus* lineage (Kurtén 1968). However, a recent evaluation (Mazza & Rustioni 1994) of current fossil data has suggested that *U. arvernesis* is the Asian ancestor of brown bears and that the *U. arctos* lineage split into two divergent clades, one in Europe and one in Asia during the middle Pleistocene. The distribution of known *U. arctos* fossils exhibits a temporal gradient similar to that predicted by mtDNA phylogenetic analyses. The oldest putative *U. arctos* fossils from Europe are found in France and date to 0.92 – 0.98 mya (Mazza & Rustioni 1994), while the oldest Asian specimens, from China , date to 0.40 – 0.80 mya (Kurtén 1968; Nilsson 1983). A late entry into the Americas indicated by the earliest North American fossils (found in Alaska) which date to only 0.20 – 0.30 mya (Kurtén & Anderson 1980). Hence, the fossil data suggest brown bears had become established in Europe by the mid-Pleistocene and during the next 800,000 years radiated into Asia, and finally into North America.

Implications for conservation

The existence of highly divergent clades of brown bears has important implications for the conservation of this imperiled carnivore species. These four clades are divergent matrilineal phylogenetic groups and may constitute evolutionary significant units (ESUs), sets of populations with a common evolutionary history (Ryder 1986). While it is important to note that mtDNA sequence analysis only provides information about maternal haplotypes which may not accurately reflect the evolution of the species as a whole (Moritz 1994), we believe that the current geographic distribution of mtDNA clades should be preserved until additional analyses of nuclear and Y chromosome markers are available to provide a more comprehensive understanding of the genetic relationships of brown bears from different geographic regions. As brown bear numbers continue to shrink due to habitat destruction and high levels of anthropogenic mortality, there is no possibility of reintroducing bears to a large portion of their historic range, and if many current populations are to persist they will do so in physical isolation from larger populations. This situation raises the issue of potential

genetic threats to isolated populations to a loss of genetic diversity and increase in inbreeding.

When considering the 14 sampling locations with a sample size of greater than 12 individuals, an average of 2.8 control region haplotypes were observed per location, and the only one haplotype was observed in 36% of the sampling areas. The maximum number of haplotypes observed in a single location was seven in the Romanian population, where clades I and III meet. In general, the number of mtDNA haplotypes observed at a sampling location is not directly correlated with the degree of isolation or human impact. For example, only 1 and 2 haplotypes were detected for the non-threatened Alaskan populations at Seward Peninsula and the Arctic National Wildlife Refuge, respectively, while 3 and 5 haplotypes were detected for the endangered lower 48 state populations of the Yellowstone Ecosystem and Idaho/Montana, respectively. In contrast, recent studies of microsatellite DNA diversity for the North American brown bears (Paetkau *et al.* in press, Waits *et al.* 1998a) suggest that levels of nuclear DNA genetic diversity are associated with the degree of human impact and the amount of connectivity to other brown bear populations, and that low levels of genetic diversity are currently a potential problem for numerous populations. Unless gene flow corridors can be re-established between fragmented populations, and this is highly unlikely in most areas, genetic diversity levels will only be maintained or increased by population augmentation. We propose that the wildlife managers re-evaluate the status of the world's brown bears populations using morphological, behavioral, and genetic data to prioritize populations for conservation, to define management units, and to provide a guide for choosing source bears for population augmentation of endangered populations.

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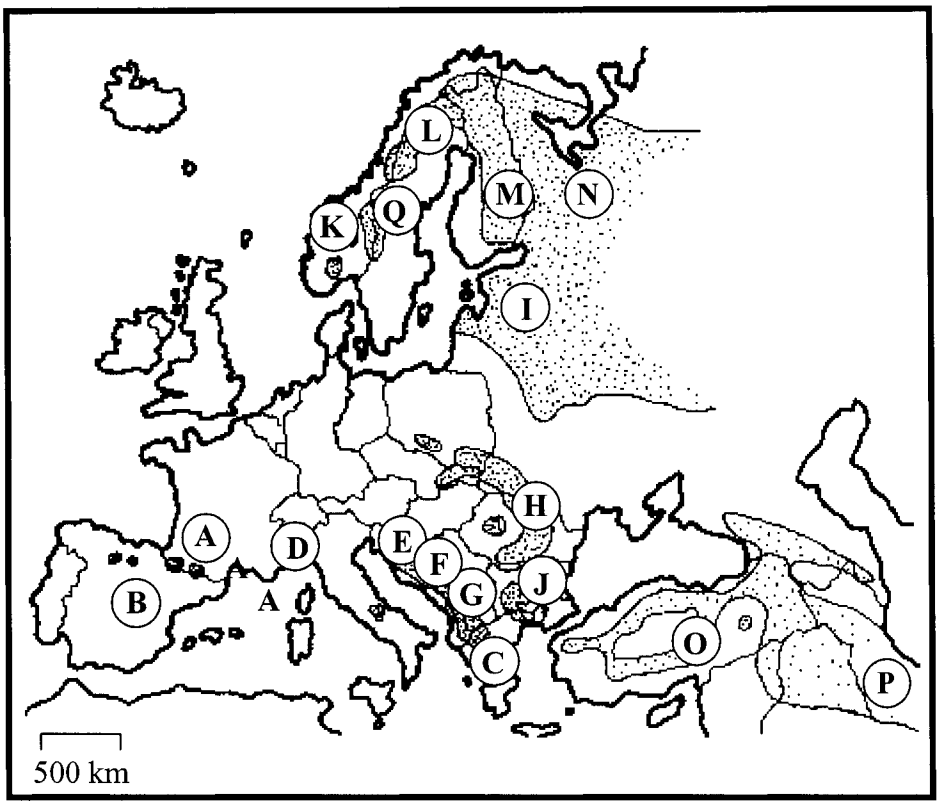

Fig. C.1a. Geographic sampling locations for Eurasian brown bears used in this study. The name and sample size for each sampling location are indicated in Table C.1. The current range of *U. arctos* is shaded in gray.

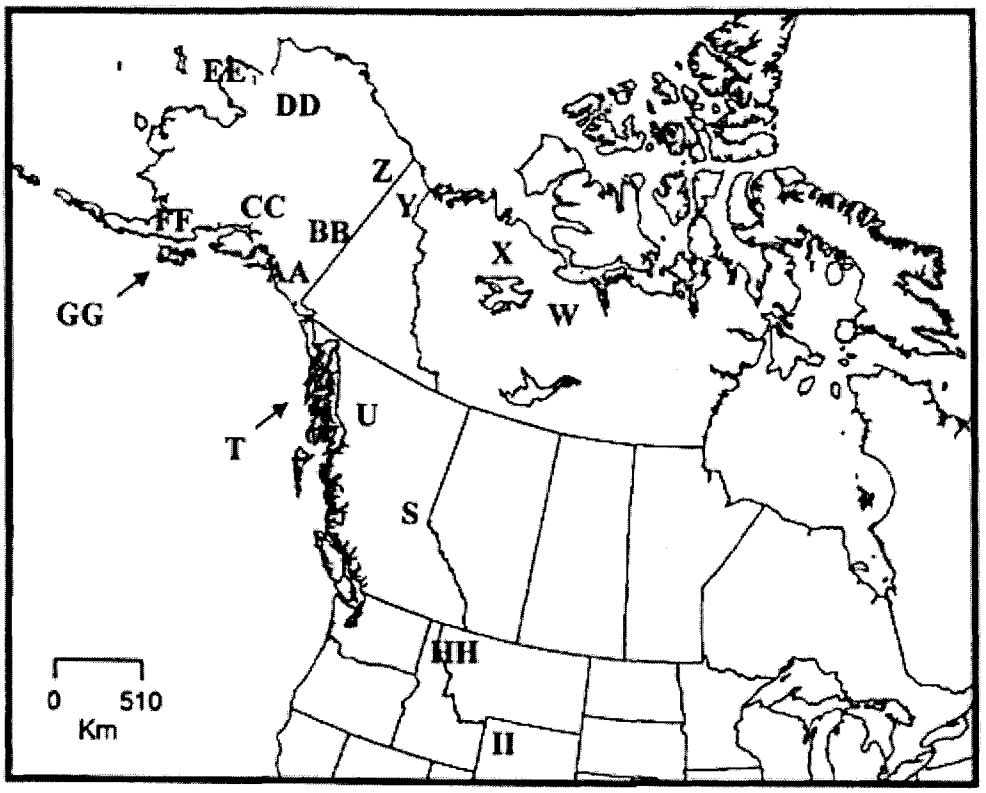

Fig. C.1b. Geographic sampling locations for North American brown bears used in this study. The name and sample size for each sampling location are indicated in Table C.1.

	A B C D	E F G H	I J K L	M N O P	Q R S T	U V W X	Y Z AA BB	CC DD EE FF	GG HH II JA	TI SI TOTAL
1	1 (3)									4
2					1(12)					13
3		1(3)								4
4		1(7)	(1)							9
5		1(2)								3
6	(4)	1 (25) (3) (1)								34
7		1(1)								2
8					4					4
9					2					2
10					7					7
11					5					5
12					3					3
17									1	1
18				2				2		2
19										2
20							2			2
21						(2)	1			3
22						2				2
23							2 7			9
24							2			2
25		1								1
26								1		1
27								1 2		3
28								1		1
29		(13)	(2) (13)	(2) (3)			3 3	7 5	32(21)	3 107
30								12		12
31								19		19
32								10		10
33		1								1
34		1(9)								1 11
35								1		1
36							2			2
37					(1)				2(16)	19
38					(39)				3(40)	82
30									1(2)	3
40									3(12)	15
41									1	1
42	1									1
43			1							1
44	2									2
45		1								1
46			2							2
47		1								1
48		1			4					5
49				1						1
50										1 1
51									5	5
52									1	1
53						2 3				5
54						1				1
55									2	2
56						3				3
57						12				12
58						6				6
59						1				1
60									2	2

Figure C.2. Distribution and frequency of brown bear mtDNA haplotypes by sampling locality. The sampling localities defined in Table C.1 and Fig. C.1 are on the horizontal axis, and the haplotype numbers are on the vertical axis. Haplotypes defined by Cyt*b* and control region sequences are above the dashed line. Numbers in italic brackets {} denote samples that were provisionally assigned to a combined data haplotype based on identical control region sequence, but Cyt*b* sequence was unavailable. Haplotypes 13 – 16 are polar bear haplotypes and are not presented on the diagram. Abbreviations: JA – Japan, TI – Tibet, and SI – Siberia.

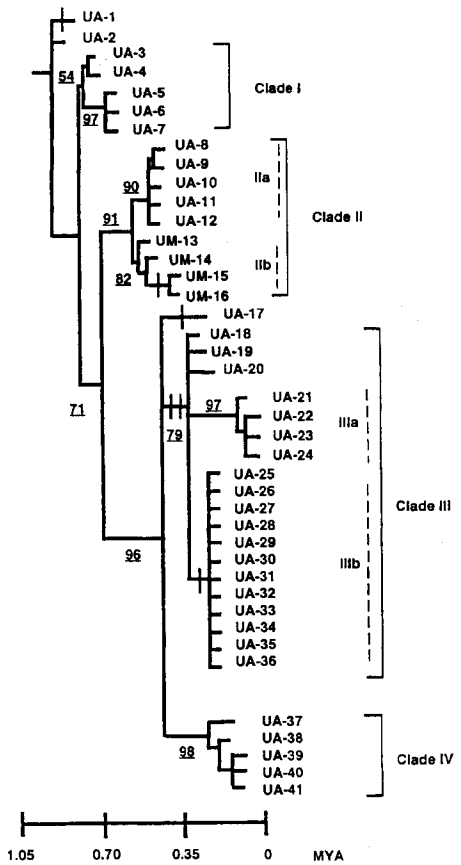

Fig. C.3. Best maximum likelihood and maximum parsimony (226 steps) topology for 37 brown (UA) and four polar (UM) haplotypes defined by control region and Cyt*b* sequences (558 nt; Table C.1) with spectacled bear as outgroup. Parsimony bootstrap values (50% majority rule, 1,000 replicates) are underlined, and branch lengths are drawn to scale from maximum likelihood analysis. A vertical line on the tree denotes an amino acid substitution.

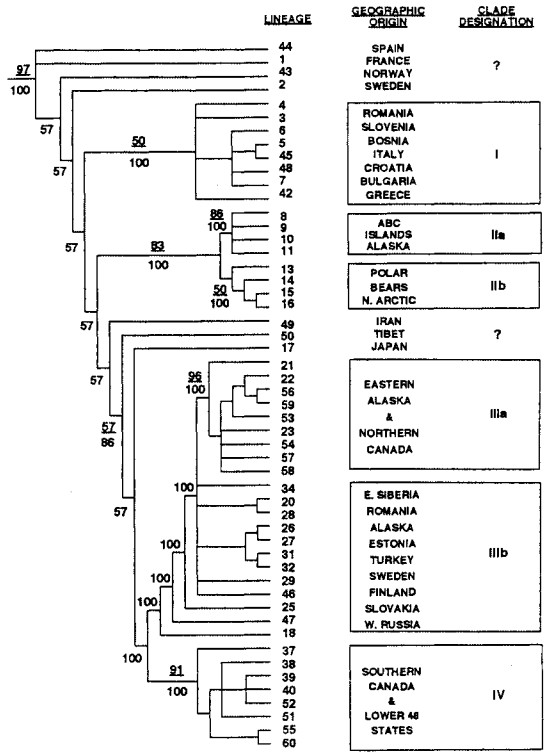

Fig. C.4. Majority-rule consensus tree of 48 brown bear haplotypes and 4 polar bear haplotypes defined by maximum parsimony analysis of 263 nt of the mtDNA control region using the spectacled bear as an outgroup. Numbers denote consensus tree values and underlined numbers denote parsimony bootstrap values (50% majority rule, 1,000 replicates) for groups with greater than 50% support.

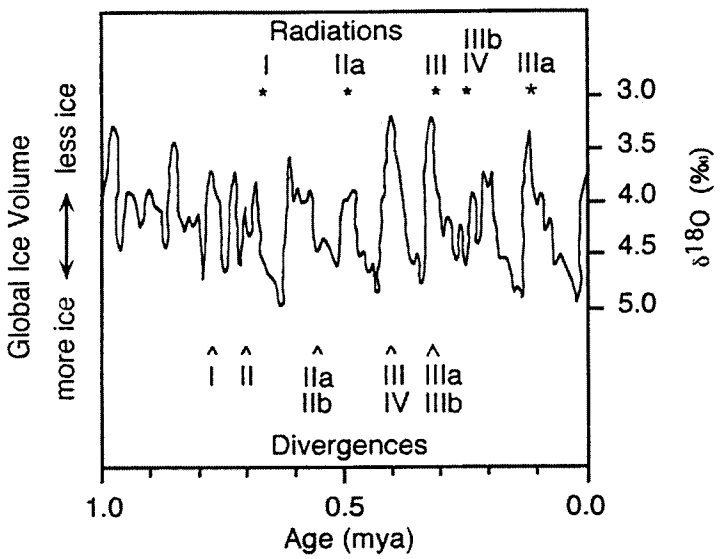

Fig. C.5. Fluctuations in global ice volume during the Pleistocene, as measured by $\delta^{18}O$ values, plotted in conjunction with the estimated dates of divergence and radiation for the major mtDNA clades defined by control region and Cyt*b* sequences observed in *U. arctos* (Fig. C.3). Divergence refers to the origin of an ancestral haplotype within a clade. Roman numerals identify Clades I-IV, as defined in Fig. C.3. Abbreviation: mya – million years ago.

Table C.1. Map location (Fig. C.1), locality, and sample size for each brown bear sampling location.

Map Location	Locality	Sample Size
A	France	4
B	Spain	2
C	Greece	1
D	Northern Italy	4
E	Slovenia	30
F	Bosnia/Croatia	4
G	Slovakia	14
H	Romania	27
I	Estonia	4
J	Bulgaria	1
K	Norway	1
L	Northern Sweden	13
M	Finland	2
N	Western Russia	3
O	Turkey	2
P	Iran	1
Q	Southern Italy	4
R	Southern Sweden	13
	Hokkaido, Japan (not on map)	1
	Tibet, China (not on map)	1
	Eastern Siberia (not on map)	3
	North America (Fig. C1B)	
S	Canadian Rockies	40
T	Admiralty, Baranof, and Chichagof (ABC) Islands	21
U	Southeast coast of Alaska	2
V	Kluane National Park	24
W	Coppermine	2
X	Paulatuk & Anderson	4
Y	Richardson	2
Z	Arctic National Wildlife Refuge (ANWR)	14
AA	South coast of Alaska	1
BB	Alaska Range	5
CC	Interior Alaska	4
DD	Brooks Range	9
EE	Seward Peninsula	17
FF	Izembek and Katmai	31
GG	Kodiak Island	53
HH	Idaho/Montana/Southern BC	43
II	Yellowstone ecosystem	47
	Total	455

Table C.2. Identical brown bear (UA) and polar bear (UM) haplotypes in the combined data set.

Control Region	Cytochrome *b*
UA-40, 41	UA-21, 22
UA-11, 12	UA-20, 24
UA-23, 24	UM-15, 16
UA-19, 29, 30, 33, 35, 36	UA-18, 19
	UA-5, 6, 7
	UA-37, 38, 39, 40
	UA-9, 10, 11, UM-13, 14
	UA-25, 26, 27, 28, 29, 31, 32, 34

Table C.3. Average Kimura sequence divergence (above underlined diagonal) and average number of pairwise differences (below diagonal and in parentheses) between and within (bold) mtDNA clades of brown bears based on 294 nt and 264 nt of the mtDNA control region and Cyt*b* gene, respectively.

	Clade 1	Clade II	Clade III	Clade IV
Clade I	**0.005 (2.7)**	0.035	0.055	0.055
Clade II	18.9	**0.010 (5.6)**	0.051	0.056
Clade III	29.3	27.3	**0.011 (6.1)**	0.035
Clade IV	29.1	29.6	19.0	**0.005 (2.8)**