SYSTEMATIC CLASSIFICATION OF BLACK BEARS IN THE SOUTHEASTERN UNITED STATES

by

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(ABSTRACT)

I assessed genetic variation within and between 18 American black bear (*Ursus americanus*) populations in the southeastern United States using multilocus DNA fingerprinting techniques (Hinf I restriction digests, Jeffreys' 33.15 probe). Band-sharing data indicated that American black bear populations (*U. a. americanus*) exhibit significantly less genetic similarity both within and between populations (similarity within=0.66, similarity between=0.40) than do the Louisiana (*U. a. luteolus*) (similarity within=0.75, similarity between=0.58) and Florida (*U. a. floridanus*) (similarity within=0.75, similarity between=0.57) black bear populations ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons). Louisiana and Florida black bear populations share the same degree of genetic similarity within and between populations, and are more closely related to each other

than they are to American black bear populations ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons). I conclude that, based on genetic data, separate subspecies status may or may not be warranted for the Florida and Louisiana black bears; however, both groups of populations should be considered as distinct population segments for listing under the Endangered Species Act of 1973.

I also assessed any genetic effects that may have been caused by translocation of bears from Minnesota to Louisiana and Arkansas. Analysis of band-sharing data indicated that any genetic impacts that may have been caused by the translocations were not statistically significant. The bear population in Cook County, MN exhibits less within-population genetic similarity (similarity within=0.57) than bear populations in Louisiana and Arkansas (similarities within=0.74). Populations in Louisiana and Arkansas are more closely related to each other (similarity between=0.53) than they are to the population in Minnesota (similarity between=0.34) ($p<0.001$, Mann-Whitney test). These findings are in agreement with previous genetic and morphological studies of black bear populations in the southeastern United States.

Table of Contents

List of Tables

List of Figures

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CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Current Classification

The American black bear (*Ursus americanus*) was originally distributed throughout nearly all of North America. Across this range, the American black bear has been divided into 16 subspecies, with 3 occurring in the southeastern United States: *U. a. americanus, U. a. floridanus*, and *U. a. luteolus* (Hall 1981). In the late 19th century, Merriam (1893) used 5 skulls taken from Morehouse Parish in northeastern Louisiana to describe the Louisiana black bear (*U. a. luteolus*). The original range of *U. a. luteolus* included eastern Texas, southern Mississippi, southern Arkansas, and all of Louisiana (Hall 1981). Merriam (1896) also described the Florida black bear (*U. a. floridanus*), using several skulls (exact number unknown) collected in southern Florida. The range of *U. a. floridanus*, as originally described, included the coastal plain of Georgia and Alabama, and all of Florida.

Status of the Black Bear

Originally, much of North America provided adequate vegetation and other habitat requirements needed to sustain

black bear populations (Pelton 1989). Bear habitat, however, was reduced significantly as land was cleared and developed for human activity, particularly in the southeastern United States. Currently, less than 10% of the original black bear range is believed to support bear populations (Maehr 1984), with most bears surviving on scattered publicly-owned lands (Pelton 1985).

With decreased habitat available to black bears in the coastal plain physiographic region of the Southeast, population sizes were greatly diminished (Pelton 1989). The Louisiana Department of Wildlife and Fisheries recognized that black bear numbers were very low within their state, and began a restocking program in the mid-1960s. Adult bears and cubs were trapped in Cook County, Minnesota, and transported to Louisiana, where 150-200 were released in Pointe Coupee Parish and in Tensas/Madison Parishes (Taylor 1971 cited in Pelton 1989). Recovery records of released bears were not conclusive regarding the success of the restocking program. The Louisiana populations of *U. a. luteolus* remain at notably low levels, as has the amount of suitable bear habitat. In 1981, the bear populations were estimated at 30-50 individuals in the Tensas River area, 30-50 individuals in the lower Atchafalaya Basin, with an unknown number in the upper

Atchafalaya Basin (State Survey, 1981; Louisiana Department of Wildlife and Fisheries, cited in Pelton 1989).

Black bear habitat in Florida has been reduced much as in Louisiana due to land clearing and development. This habitat fragmentation has left *U. a. floridanus* populations in broken patches of suitable habitat (Brady and Maehr 1985). Many of these broken patches can sustain only a small number of bears, especially in southern Florida.

Petition For Listing

The Endangered Species Act of 1973 was established to protect against the loss of living natural resources. Under the Act, Congress defined species to include "any subspecies of fish or wildlife or plants, and any distinct population segment of any species or vertebrate fish or wildlife which interbreeds when mature (emphasis added)." Under this definition, any subspecies or distinct population segment of *U. americanus* found to warrant protection could be protected; protection did not have to be justified at the species level.

With increasing concern over loss of black bear habitat and notably low bear populations, the U.S. Fish and Wildlife Service (USFWS) was petitioned by private citizens on March 6, 1987 and May 20, 1990 to list *U. a. luteolus*

and *U. a. floridanus*, respectively, as endangered species under the Endangered Species Act of 1973.

The USFWS consulted a report by Pelton (1989) in making preliminary recommendations. The report assembled genetic and morphological analyses of black bears from Louisiana, Florida, Tennessee, West Virginia, Virginia, Arkansas, and Minnesota. Genetic marker frequency data, including protein electrophoresis and mitochondrial DNA (mtDNA) data, failed to reveal significant inter-population differentiation, excepting data from 2 isozyme loci from Minnesota populations that may be distinct (Johns et al. 1989, Zimmerman 1989, both serving as appendices to Pelton 1989). Skull morphometrics, however, indicated that the Louisiana black bear was distinct from the American black bear (Kennedy 1989, appendix to Pelton 1989). The report also suggested that Louisiana and Florida black bears may be the same subspecies.

Based on these preliminary findings, the USFWS recommended listing the Louisiana black bear as "threatened", which it did officially in January of 1992 (57 Federal Register 588), while placing the Florida black bear in a "warranted but precluded" category (55 Federal Register 42223). "Warranted but precluded" indicated that the Florida black bear most likely warranted protection,

but due to a backlog of listing petitions and limited administrative resources, the Florida black bear was precluded from listing at that time.

Justification for Further Study

Mayr (1969) defines a subspecies as a group of phenotypically similar individuals of a species that occupy a geographic subdivision of the range of a species and are taxonomically distinct from other such groups. Biologists and managers have generally accepted *U. a. luteolus* and *U. a. floridanus* as legitimate subspecies of *U. americanus* under this definition. Many questions, however, were raised during the USFWS listing process concerning the validity of classifying *U. a. luteolus* and *U. a. floridanus* as distinct subspecies (Pelton 1989). Review of preliminary information, including the report by Pelton (1989), suggested that these 2 subspecies may not be distinct. The preliminary genetic data (protein electrophoresis and mitochondrial DNA) suggested that no significant differentiation existed among nominal subspecies, while the preliminary skull morphology data suggested that differentiation did exist.

In addition to the controversy over *U. americanus* classification, questions were raised about the potential

genetic impacts of the bears introduced into Louisiana from Minnesota during the 1960s. This introduction of the *U. a. americanus* gene pool into Louisiana could have altered the putatively unique gene pool of *U. a. luteolus*. Although preliminary genetic data collected after the restocking program found no significant genetic differentiation between the Minnesota and Louisiana populations (Johns et al. 1989), sample sizes were too small for any strong conclusions. The impacts of the introduced bears on the *U. a. luteolus* gene pool were largely unknown at the time of listing.

Biologists and managers need to gain a better understanding of *U. americanus* population subdivision and resolve the issue of *U. americanus* classification. Attempts to establish or confirm valid systematic classification of *U. americanus* can be approached using several techniques, including skull morphology analysis, mtDNA analysis, and nuclear DNA analysis. Systematic classification holds important biological and management implications. Even if distinct subspecies designations are found to be unjustified, any differentiation among distinct populations segments may be found significant.

Hypotheses

Given the background of declining bear populations in Louisiana and Florida and uncertainty regarding differentiation among black bear populations, the following null and alternate hypotheses were tested:

H_0: There are no genetic differences between black bear (*Ursus americanus*) populations in the southeastern United States.

H_1: *U. a. luteolus* and *U. a. floridanus* are genetically distinct from one another.

H_2: *U. a. luteolus*, *U. a. floridanus*, and *U. a. americanus* are all genetically distinct.

H_3: *U. a. luteolus* and *U. a. floridanus* are not genetically distinct from one another, but are genetically distinct from *U. a. americanus*.

Objectives

To test these research hypotheses, the objectives of this study were:

1. To analyze genetic variation found within and between populations of 3 putative subspecies of *Ursus americanus: U. a. americanus, U. a. luteolus*, and *U. a. floridanus*.

2. To interpret findings relevant to the systematics of *U. a. luteolus* and *U. a. floridanus* in light of implications for listing of these putative subspecies under the Endangered Species Act of 1973.

3. To examine the validity of current *U. americanus* classification.

CHAPTER 2: LITERATURE REVIEW

Genetic Stock Identification

Wildlife biologists and managers need to recognize and understand genetic variability within and between the populations that they are studying and managing. Most current management practices are conceived at the species level, with the assumption that populations of the same species are genetically homogeneous (Soule 1986). Genetic variation is not always homogeneous within a species, however, and genetically distinct populations often can be recognized (Nei 1987, Hartl 1988). Genetic subdivision of a species often is referred to as stock structure, a term that historically has been associated with fisheries, and to a lesser degree, with wildlife management (Chakraborty and Leimar 1987). In recent years, wildlife managers have realized that recognizing genetic subdivision is critical for the management and conservation of genetic resources. The process of recognizing structure within a species is commonly termed genetic stock identification (GSI) (Milner et al. 1985, Waples et al. 1990), and it has become an important concept both biologically and legally for wildlife managers, as exemplified by the current black bear

systematics issue in the southeastern United States.

Biologically, genetic stock identification (GSI) is critical for effective management of multiple bear populations. Previous studies have suggested that outbreeding depression, defined as reduced fitness due to expression of unfavorable gene combinations, can result from mixing of 2 distinct stocks, even if both stocks are of the same species (Templeton 1986). For instance, restocking efforts to enhance *U. a. luteolus* or *U. a. floridanus* populations by introducing relocated *U. a. americanus* individuals could potentially harm target populations through outbreeding depression. On the other hand, if isolated populations are not significantly distinct from one another, restocking programs could counteract inbreeding depression and random genetic drift. Inbreeding depression is defined as the decrease in fitness of a population due to matings of closely related individuals, and random genetic drift is defined as sampling error upon allele frequencies, which can result in differentiation of populations (Hartl 1988). Both inbreeding and random genetic drift can reduce the fitness of small, isolated populations (Nei et al. 1975, Ballou and Ralls 1982, Ralls et al. 1988, Laikre and Ryman 1991, Tave 1993). Biologists and managers working with black bears,

therefore, should be concerned with the effects of mixing stocks through nuisance relocations, enhancement introductions, or captive breeding programs. Genetic consequences of past bear introductions into Louisiana and Arkansas from Minnesota, for example, should be investigated. Future introductions should be considered from the viewpoint of possible genetic consequences. From a legal perspective, GSI is important in bear management because managers are obligated to protect "distinct population segments" under the Endangered Species Act of 1973. This protection may be warranted for subspecies or distinct populations of *U. americanus*. Additionally, bear managers may need to recognize genetic stocks when dealing with wildlife forensics cases, such as poaching of endangered bears or commercializa-tion of bear parts across state lines or international borders (Kirby 1992).

Many molecular genetic methods are available for use in GSI studies, including protein electrophoresis, mtDNA analysis, and nuclear DNA analysis. GSI in large mammals, however, can prove difficult. Large mammals tend to have limited stock structure, possibly due to their high mobility and potential for gene flow. Previous studies indicated that large mammals, including black bears, also tend to exhibit low levels of heterozygosity and overall

genetic variability (Manlove et al. 1980, Sheffield et al. 1985, Wathen et al. 1985). Because of limited genetic variability, protein electrophoresis has not proven an effective tool for GSI in bears, although DNA analysis methods may prove useful (Zimmerman 1989, appendix to Pelton 1989).

Currently, little information appears in the literature regarding GSI in *U. americanus*. Manlove et al. (1980) reported low levels of heterozygosity (H_0) for individual black bears in Tennessee ($H_0 = 0.015$), while Wathen et al. (1985) found higher levels of heterozygosity in the same region ($H_0 = 0.080$). Johns et al. (1989, appendix to Pelton 1989) conducted protein electrophoresis upon black bears from several states and found no significant differentiation among populations. Zimmerman (1989, appendix to Pelton 1989) also found no significant differentiation in mtDNA haplotypes (as determined using restriction fragment variation) from black bear populations sampled over several states. Cronin et al. (1991) examined interspecific and intraspecific mtDNA variation in North American bears (*Ursus spp.*), and found that genetic divergence was greater in black bears than in polar (*Thalarctos maritimus*) and grizzly (*Ursus horribilis*) bears. However, divergence among black bears was minimal

across North America, suggesting a high degree of gene flow.

GSI techniques also have been used with other large mammals. Sheffield et al. (1985) examined white-tailed deer (*Odocoileus virginianus*) populations in western Maryland using allozyme techniques and suggested that genetic stock structure did exist. Wayne et al. (1991) used protein electrophoresis and mtDNA analysis to examine genetic differentiation in gray wolves (*Canis lupus*) from Isle Royale and the Lake Superior area and found significant genetic stock structure (F_{ST}=0.19). Examining mtDNA variability, Wayne et al. (1992) detected genetic stock structure in *C. lupus* populations sampled across their worldwide range. Ashley et al. (1990) analyzed genetic differentiation among 3 populations of black rhinoceros (*Diceros bicornis*) and found no significant stock structure using mtDNA analysis. DNA fingerprinting conducted by Gilbert et al. (1990) revealed significant population subdivision among California Channel Island fox (*Urocyon littoralis*) populations.

DNA Fingerprinting

DNA fingerprinting originated with the research conducted by Jeffreys et al. (1985) on hypervariable

minisatellite regions of human DNA. Since the mid-1980s, DNA fingerprinting has been used widely as a means of assessing genetic identity among individual organisms. DNA fingerprints are individual-specific, much like the fingerprints on a human hand are individual-specific.

DNA fingerprinting, as described by Kirby (1992), generally is conducted using whole genomic DNA extracted from blood or tissue. The DNA is digested with a restriction enzyme that recognizes specific nucleotide sequences along the DNA molecule, cutting the DNA at these recognition sites. The product of cleavage by a restriction enzyme is a collection of DNA fragments of varying lengths. These fragments then are subjected to electrophoresis through an agarose gel. Migration distance is a function of the molecular weight of each fragment. The DNA fragments then are denatured into single strands and transferred to a nylon membrane, a process known as Southern blotting (Southern 1975). The Southern blot is hybridized with a probe, or known DNA sequence, that is radiolabelled or tagged with chemiluminescent agents. The hybridized regions of the membrane are observed as bands in an autoradiograph of the membrane made with X-ray film. The autoradiograph will display a series of bands for each individual represented on the Southern blot. The

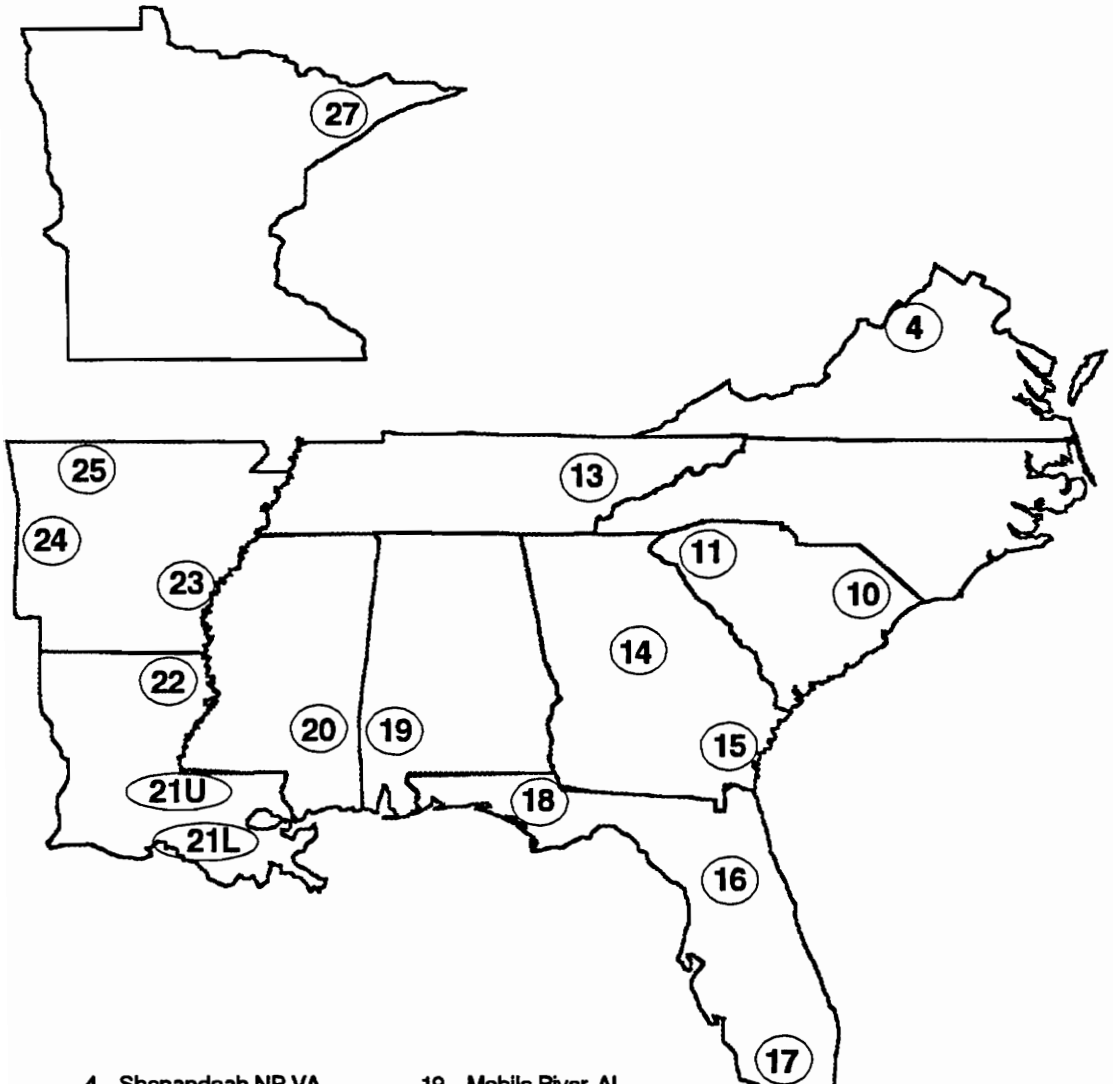
variability in the banding patterns provides information about the genetic similarities of the individuals being examined. The bands represent alleles at numerous genomic loci, and banding patterns are compared across individuals. The basis for the observed polymorphisms lies in the different numbers of repeats of simple DNA sequences at a given locus, yielding different-sized DNA fragments during restriction enzyme cleavage. These differences are responsible for the variability of bands observed on a DNA fingerprint autoradiograph.

CHAPTER 3: METHODS

Collection of Tissue

Blood and tissue (muscle, liver) samples were collected from 18 black bear populations, primarily located in the southeastern United States (Figure 1). Samples were collected from live-trapped, hunter-killed, and road-killed bears during the summers of 1992-1994.

Bears were live-trapped using Aldrich foot snares and culvert traps, baited with produce or meat scraps. Both cubby sets and trail sets were used in trapping (Hawthorne 1980). Captured bears were immobilized using blown darts loaded with a 2:1 mixture of ketamine hydrochloride and xylazine hydrochloride at 300 mg/ml with a dose of 1 cc per 45 kg of estimated body weight (Kasbohm and Vaughan 1993). In cases where bears were not caught securely, a carbon dioxide pistol equipped with Palmer darts (Palmer Chemical and Equipment, Co., Inc., Douglasville, GA) was used to administer the ketamine:xylazine mixture. Darts were targeted for intramuscular injections in the shoulder when possible, with the rump as an alternate target. Once immobilized, approximately 40 ml of whole blood was collected per bear from a femoral vein into 8 vacutainer



4. Shenandoah NP, VA
10. Myrtle Beach, SC
11. Sumter NF, SC
13. Great Smoky Mt. NP, TN
14. Ocmulgee River, GA
15. Okefenokee NWR, GA
16. Ocala NF, FL
17. Big Cypress NP, FL
18. Apalachicola NF, FL
19. Mobile River, AL
20. Mississippi
21U. Upper Atchafalaya Basin, LA
21L. Lower Atchafalaya Basin, LA
22. Tensas River NWR, LA
23. White River NWR, AR
24. Ouachita NF, AR
25. Ozark NF, AR
27. Cook County, MN

Figure 1. Black bear populations in the southeastern United States surveyed for genetic variability.

tubes half-filled with a sodium dodecyl sulphate (SDS) buffer (10 g sodium dodecyl sulphate, 9.5 g tris, 25 g EDTA, and distilled water to 500 ml, pH 8.0). A volume of blood equal to that of the SDS buffer was collected, and the blood/buffer mixture was gently mixed for 30 seconds. The SDS buffer allowed storage of whole blood at room temperature (Kirby 1992). In cases where SDS vacutainer tubes were not available, blood was collected in EDTA vacutainer tubes and kept frozen; frozen blood samples then were shipped on dry ice via overnight courier to the genetics laboratory. In addition to the blood samples, 2 9-mm diameter plugs of ear tissue were taken from each bear and stored in silica desiccant. A premolar was extracted from each bear for aging using cementum annuli techniques (Willey 1974). Bears then were weighed, measured for morphological analysis, ear-tagged, lip-tattooed, and given an intravenous injection of yohimbine (Yobine, Lloyd Laboratories, Shenandoah, IA) at 5 mg / 45 kg body weight as a reversing agent for the anesthesia. Bears were monitored until they recovered from the anesthesia and were able to leave the capture site.

Muscle or liver tissue was taken from road-killed or hunter-killed bears. When available, 30 g of tissue was taken per bear. Tissue samples were kept frozen and

shipped on dry ice to the genetics laboratory at Virginia Polytechnic Institute and State University. The targeted sample size for each population was a minimum of 10 tissue or blood samples (Table 1). Frozen blood and tissue samples were stored at -60 C. The SDS-buffered tubes of blood and desiccated ear plugs were stored at room temperature.

I assisted with the collection of blood and tissue samples by trapping bears in Pamlico County, N.C. and along the Ocmulgee River in south-central Georgia. In addition to the trapping efforts of Virgina Tech personnel, numerous other trapping teams from universities and government agencies assisted in collection of tissue and blood samples (see Acknowledgements).

DNA Preparation

Multilocus DNA fingerprinting was conducted to assess genetic differentiation within and among black bear populations.

Extraction of DNA

Whole genomic DNA was extracted from the SDS buffer mixture using the protocol described by D. Stanley (1993, personal communication): proteinase K (200 ul) was added

Table 1. Black bear populations in the southeastern United States surveyed for genetic variability.

Populations By Subspecies	Number of bears surveyed
American black bear, *Ursus americanus* (n=91)	
Cook County, MN	31
Ozark National Forest, AR	8
Ouachita National Forest, AR	8
Ocmulgee River, GA	9
Sumter National Forest, SC	6
Myrtle Beach, SC	6
Great Smoky Mountains National Park, TN	7
Shenandoah National Park, VA	16
Subtotal	91
Florida black bear, *U. a. floridanus* (n=106)	
Okefenokee National Wildlife Refuge, GA	20
Ocala National Forest, FL	21
Big Cypress National Park, FL	13
Apalachicola National Forest, FL	39
Mobile River, AL	11
Mississippi[1]	2
Subtotal	106

Table 1 Continued.

Populations By Subspecies	Number of bears surveyed
Louisiana black bear, *U. a. luteolus* (n=56)	
White River National Wildlife Refuge, AR	17
Tensas River National Wildlife Refuge, LA	16
Upper Atchafalaya River Basin, LA	11
Lower Atchafalaya River Basin, LA	12
Subtotal	56
Total number of bears surveyed	253

1 Due to small sample size, close proximity, and high genetic similarity, Mississippi was pooled with Mobile River, Alabama for statistical analyses.

to each sample (approximately 5 ml) and mixed well. Samples thenwere incubated at 37 C overnight. An equal volume of phenol/chloroform was added to the samples and mixed until samples were mud-brown in color. The samples then were centrifuged at 3,300 rpm (1380 g) for 30 minutes at room temperature. The supernatant and white interface from each sample was removed and placed in a clean tube. This step was repeated again using an equal volume of phenol/chloroform. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the samples and mixed gently, after which the samples were left to stand for 5 minutes. Samples were gently mixed again, then centrifuged at 3,300 rpm (1380 g) for 30 minutes at room temperature. The supernatant was removed from each centrifuged tube and placed in a clean tube on ice. DNA then was precipitated from the samples by adding 0.2X the volume of 10 M ammonium acetate and twice the volume of 95% ethanol and mixed gently. The tubes were left at -20 C overnight for further precipitation. After precipitation occurred, samples were centrifuged at 3,300 rpm (1380 g) for 60 minutes at 0 C. The ethanol was poured off, and the precipitated DNA pellets were washed with 70% ethanol and dried at room temperature. Once pellets were dry, 500 ul of 1X TE (10mM Tris-HCl, 1mM Na_2EDTA, pH 8.0) was added to each sample.

The tubes containing each sample were covered with cellophane and incubated overnight at 37 C. The dissolved, purified DNA was transferred to Eppendorf tubes and placed in the ultrafreezer at -60 C.

Whole genomic DNA was extracted from the frozen blood and tissue samples using the protocol described by the U.S. Fish and Wildlife Service National Wildlife Forensics Laboratory (Steven Fain, personal communication). The samples were mixed with an equal volume of lysis buffer (10mM NaCl, 5mM $MgCl_2$, 10mM Tris-HCl), then centrifuged at 3,300 rpm (1380 g) for 30 minutes. The supernatant was poured off and discarded, and the lysis treatment repeated 2 more times. Samples then were gently mixed with 30 ul of proteinase K in 3 ml of proteinase K buffer (0.1 M Tris, 0.05 M Na_2EDTA, 0.2 M NaCl, 0.5% Triton X-100, 2.0% SDS, pH8.0) and incubated at 50 C overnight. After incubation, the tissue/blood cell lysates were mixed with an equal volume of buffer-saturated phenol until emulsions formed. Each emulsion was centrifuged for 30 minutes at 3,300 rpm (1380 g) and the clear upper phase (aqueous) transferred into a clean tube, discarding the organic layer. An equal volume of buffer saturated phenol:chloroform was added to the samples and mixed until emulsions formed (approximately 5 minutes). Each emulsion was again centrifuged for 30

minutes at 3,300 rpm (1380 g) and the clear upper phase (aqueous) transferred into a clean tube. After centrifugation, an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added to each sample and mixed until emulsions formed. The samples then were centrifuged again for 30 minutes at 3,300 rpm (1380 g) and the clear upper phase (aqueous) transferred into clean tubes on ice. DNA was precipitated from the aqueous solution by adding 0.2X the volume of ammonium acetate and two volumes of cold 95% ethanol to each sample and gently mixing. The precipitated samples incubated at -20 C overnight to allow complete precipitation. The samples then were centrifuged for 30 minutes at 0 C at 3,300 rpm (1380 g). After centrifugation, the ethanol was decanted, being careful not to loosen the DNA pellets from the bottom of the tubes. DNA pellets were washed once with 500 ul of 70% ethanol, centrifuged for 4 minutes at 3,300 rpm (1380 g), and the ethanol decanted off. The residual ethanol was evaporated within 15-25 minutes, being careful to not overdry the samples. Once free of ethanol, the DNA pellets were dissolved in 500 ul of TE buffer and incubated overnight at 37 C. After incubation, the dissolved DNA samples were stored at -60 C.

DNA Concentration and Quality

Upon completion of DNA extraction from the blood and tissue samples, DNA concentrations were determined flourmetrically, using a TKO-100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Calf-thymus DNA was used to generate a standard absorbance-concentration curve. Concentrations were measured in ug/ml.

The quality of the DNA samples was estimated initially using ethidium bromide-stained electrophoretic mini-gels (Maniatis et al. 1982). These gels were used only to estimate the quality of the sample DNA by revealing the presence or absence of high molecular weight bands of DNA, approximately 50 kb pairs in length. If such bands were present and distinct, the DNA sample was classified as "good"; if the mini gel revealed a smear of DNA in the sample lane, the sample was considered excessively sheared and classified as "not good." By running preliminary check-out gels, it was possible to determine how many of the extracted samples were usable for DNA fingerprinting before conducting the actual process.

Before pouring the gel, the mini gelbox (E-C Minicell, E-C Apparatus Corp., St. Petersburg, FL) was set on a level surface, using 2 1 mm X 12 well combs for producing 24

sample wells. A 35 g, 1% (w/w) agarose gel in TAE buffer was prepared (Maniatis et al. 1982). The gel mixture was heated for approximately 10 minutes on a hot plate, using a stir-bar, until all solid agarose had dissolved and the solution had boiled. The gel was removed from heat and the gel temperature was allowed to drop to 60 C (with constant stirring during the cool down process). When the temperature reached 60 C, enough distilled water was added to the gel to return the gel weight back to 35 g. The gel then was allowed to cool to 55 C, with continuous stirring. The gel was poured and any air bubbles were removed using a fine-tipped pipette. After pouring, the gel sat at room temperature for approximately one hour. Once the gel had hardened, the combs were removed, exposing 2 rows of 12 wells each. With the gel positioned inside the electrophoretic box, 260 ml of TAE buffer was added to the box to fill the electrode wells and submerge the gel by 2 mm.

DNA samples were prepared for the check-out gels on a titer plate. Each well to be used on the plate was filled with 8 ul of TBE buffer, 2 ul of ethidium bromide loading dye (25 mg Orange G, 1.5 g Ficoll, 0.2 mg EtBr) and 2 ul of DNA sample. Each well was mixed gently with the pipette tip used to dispense the DNA. After mixing, the samples

were ready for loading on the gel. The prepared DNA samples were inserted into the wells on the gel using a pipette. One well was loaded with uncut phage lambda DNA, approximately 48 kb pairs in length, as a molecular weight standard. Once gel loading was completed, 50 volts at 40 mA was applied to the gel for approximately one hour using an electrophoresis power source (FB105, Fisher Scientific, Pittsburgh, PA). After electrophoresis, the gel was removed and photographed using an ultraviolet transilluminator (UVP, Inc., San Gabriel, CA) (wavelength = 302 nm) using black-and-white polaroid film (Polaroid MP4+ camera, Fotodyne Inc.). Ethidium bromide-stained DNA fragments fluoresced under the UV light, providing estimates of DNA fragment sizes. The DNA then was classified as "good" or "not good" as discussed previously.

Digestion of Purified DNA

Purified DNA was digested using the restriction enzyme, Hinf I (New England Biolabs, Beverly, MA). Digestions were conducted on 10-30 ug of DNA, to which 3-8 ul (30-80 units) of restriction enzyme were added. Buffer (NE 2, 10 X) was added to each digest to 10% of the total digest volume. Distilled water was added to bring the volume up to the desired total. Each digest was incubated

overnight at 37 C, and then loaded into a full-size agarose gel (175 g) for electrophoresis or frozen (-60 C) for future use.

Check-out gels were used to estimate the quality of the digests, and were constructed, loaded, and electrophoresed as described previously. For evaluating digested DNA, a smeared lane indicated varying DNA fragment lengths caused by restriction enzyme cleavage. Such a smear was classified as a "usable" digest. If a lane showed only a small number of prominent bands, the digest was classified as "not usable" due to lack of enzymatic cleavage.

DNA Fingerprinting

DNA fingerprinting was conducted using agarose gel electrophoresis, Southern blotting, and DNA-DNA hybridization using chemiluminescent probes for visualization (Southern 1975, Maniatis et al. 1982, Kirby 1992).

Electrophoresis

The first step of DNA fingerprinting was electrophoresis of the digested DNA samples in an agarose gel. Before pouring the gel, the gel-box (Model H4, GIBCO

BRL Life Technologies Inc., Gaithersburg, MD; or BioRad Unit, BioRad, Richmond, CA) was setup on a level surface, using a 0.75 or 1 mm comb designed for 20 wells. The 1% TBE gel (200 g - BRL Unit; 175 g - BioRad Unit) was heated on a hot plate, using a stir-bar, until all solid agarose had dissolved and the solution had boiled (approximately 20 minutes). Once all agarose had dissolved, the gel was removed from heat and the gel temperature was allowed to drop to 60 C (with constant stirring during the cool down process). When the temperature reached 60 C, enough distilled water was added to the gel to return the gel to the original weight. The gel was allowed to cool to 55 C, with continuous stirring. Once the gel had reached 55 C, the gel was poured and any air bubbles in the gel were removed using a fine-tipped pipette. After pouring, the gel sat at room temperature for approximately one hour. Once the gel had hardened, the comb was removed, exposing 1 row of 20 wells. With the gel positioned inside the electrophoretic gel-box, 1500 ml of TBE buffer was added to the box to fill the electrode wells and cover the gel by 2 mm.

The digested DNA was prepared for the gel by adding 5 ul of ethidium bromide loading dye to each sample. After loading dye had been added, 20-40 ul of the DNA/loading dye

mixture per sample was added to each well on the gel using a pipette. Lambda DNA cut with BstEII (New England Biolabs, Beverly, MA) was used as a molecular weight marker and placed in several lanes across each gel. Once loading was completed, voltage was applied to the gel. The voltage and current used varied from 20-30 V and 10-30 mA, depending on the length of time electrophoresis was conducted (72-96 hours). Electrophoresis was stopped when the seventh band of the Lambda cut with BstEII (3.7 kb) had migrated approximately 14 cm. The voltage/current/time combinations chosen allowed for all low molecular weight fragments 2 kilobase pairs (kb) or smaller to run off the gel. After electrophoresis, the gel was removed from the power source and prepared for Southern blotting.

Southern Blotting

Southern blotting (Southern 1975) protocols followed those suggested by the manufacturer of the Magnagraph nylon blotting membrane (Micron Separations Inc., Westboro, MA). For denaturing of DNA, the gel was incubated twice in 500 ml of 0.15 M NaCl/0.5 M NaOH for 30 minutes each wash. The second wash stage neutralized the DNA, and consisted of 2 30 minute washes using 500 ml of 0.5 M Tris-HCl/0.15 M NaCl (pH 7.7) for each wash. All washes were conducted under

continuous agitation using a Belly Dancer shaker™ (Stovall, Life Science, Inc., Greensboro, NC).

The DNA was transferred onto a 20 X 25 cm membrane for the BRL unit gels, and a 14.5 X 25 cm membrane for the BioRad unit gels. Before transfer occurred, the membrane was immersed in distilled water, then soaked in transfer buffer for 30 minutes.

DNA transfer was conducted by stacking (from bottom to top): 2 sheets of Whatman filter paper (20 X 35 cm, 14.5 X 35 cm), the gel, the nylon membrane, 2 more sheets of Whatman filter paper (19.5 cm X 24.5 cm, 14 X 24.5 cm), and 5 cm of paper towels. The stack was placed on a gel restrainer (GIBCO BRL Life Technologies Inc., Gaithersburg, MD) positioned in a buffer tray, and a weight was placed on top of the blot transfer apparatus for even compression. Approximately 1000 ml of buffer solution was added to the tray, and the edges of the bottom 2 sheets of filter paper were allowed to soak in the buffer. Soaking allowed a wicking action through the stack, transferring the DNA from the gel to the membrane. The Southern blot process was conducted for 24 hours to ensure complete transfer. Once transfer was completed, the DNA was cross-linked to the membrane using an ultraviolet GS Gene Linker™ (BioRad, Richmond, CA), executed in program mode C2 for dry

membranes and C3 for damp membranes.

DNA-DNA Hybridization

The DNA attached to the nylon membrane was single stranded at this point, allowing for hybridization using a Non-Isotopic Chemiluminescent Enhanced (NICE™) probe (Cellmark Diagnostics, Germantown, MD). The multi-locus DNA probe that was used was the minisatellite core sequence probe 33.15 (Jeffreys et al. 1987). The protocol used for hybridization and washes was established by Cellmark Diagnostics (Germantown, MD).

Before hybridization, the membrane was wetted with 1XSCC (0.015 M Tri-Sodium Citrate, 1.5 M NaCl, pH 7.0), then placed in a hybridization oven cylinder (Hybaid Oven, National Labnet Co., Woodbridge, NJ) with 50 ml of pre-warmed pre-hybridization buffer at 50 C for 3 hours with continuous agitation. After pre-hybridization, 16 ml of pre-warmed hybridization buffer and 10 ul of a NICE™ probe vial were added to the membrane in an oven cylinder at 50 C and agitated for 1 hour. After 1 hour, the membrane was washed by placing it in an oven cylinder with 200 ml of pre-warmed wash solution (0.5 M Na_2HPO_4, pH 7.2, 10% SDS) at 50 C for 20 minutes. This wash step was repeated again for an additional 20 minutes using fresh wash solution. Two

more washes were conducted for 10 minutes each at room temperature using 500 ml of a maleic acid solution (0.1 M maleic Acid/0.15 M NaCl, pH 7.5). Once the washes were completed, the membrane was placed on an acetate sheet, and 2 ul of Lumi-Phos™ 530 buffer were pipetted onto the membrane (DNA side up) and spread gently using a Kimwipe™. An acetate sheet was laid on top of the membrane, and any excess buffer was squeezed out between the sheets using a ruler. The membrane then was wrapped in plastic wrap and placed in a light-proof cassette against X-ray sensitive film. Two exposures were made for each hybridization. The first piece of film was exposed for approximately 40 hours, and the second piece of film was exposed for approximately 1 week. The X-ray film then was developed in a dark room using standard development procedures. Each piece of film was placed in developer for 2 minutes, washed in a stop bath (water) for 30 seconds, and agitated in fixer for 2 minutes. The film then was placed in a 10 minute water bath before drying at room temperature. The chemiluminescence revealed hybridization bands on the X-ray film.

Data Analysis

There were 2 phases involved in interpretation of DNA

fingerprints: scoring the banding patterns on the autoradiographs and statistical analysis of the findings.

Scoring Banding Patterns

The autoradiographs displayed a series of bands within a lane for each DNA sample (i.e., one lane per individual bear). These bands represented molecular weight-specific fragments of DNA and exhibited a high degree of variability, both within and among populations. Allelic relationships among bands in multilocus DNA fingerprints can not be readily inferred. Therefore, the relationship between 2 individual bears was quantified in terms of the number of bands of equal electrophoretic mobility exhibited by both individuals. Using the concept of shared bands, a measure of phenotypic similarity, **S**, was calculated for numerous pairs of individuals, both within and among populations (Bruford et al. 1992). **S** was calculated as:

$$S = 2n_{xy}/(n_x+n_y)$$

where n_{xy} = the number of bands exhibited by both individuals,
n_x = the total number of bands exhibited by individual x, and
n_y = the total number of bands exhibited by individual y.

The collection of **S** values allowed for comparison of within versus between population similarity. Comparisons of median similarity values were made between Louisiana,

Florida, Minnesota, and other North American black bear populations, providing a quantitative measure of genetic differentiation.

Statistical Analysis of Similarity Values

The band-sharing data from each DNA fingerprint represent a collection of non-independent data points (Wayne et al. 1991). The collection of **S** values is non-independent due to the pairwise nature of the similarity index calculation. The banding pattern of each individual was compared to that of every other individual on a DNA fingerprint, giving data points with correlated values.

The one-sample Kolgomorov-Smirnov test was conducted to determine if the distributions underlying the data from each fingerprint were Gaussian (normal). Many of the data sets were found to be not Gaussian (Appendix Table 1). Given the independence issue and the non-Gaussian data distributions, non-parametric statistical procedures were employed for data analysis.

At least 3 band-sharing data subsets were generated per DNA fingerprint blot: genetic similarity values within population 1, genetic similarity values within population 2, and the genetic similarity values between individuals of populations 1 and 2. The number of band-sharing data

subsets generated per fingerprint blot was dependent on the number of populations represented on that fingerprint (generally 2 populations). The Kruskal-Wallis (one way ANOVA by ranks) and Mann-Whitney tests were performed to determine whether the median similarity values within and between populations were significantly different. If significant differences were found, a non-parametric multiple comparisons procedure was performed to identify the specific differences.

Under the Kruskal-Wallis test, Mann-Whitney test, and multiple comparisons follow-up, data points are assumed to be independent. Although, the independence assumption was violated by the data from this study, review of the current genetics and statistics literatures yielded no alternative statistical methods for handling dependent data. The robustness of the Kruskal-Wallis and Mann-Whitney tests to dependence is largely unknown. After consultation with faculty from the Statistics Department at Virginia Tech as well as other geneticists faced with this statistics problem, we decided to continue with our non-parametric data analysis as previously described.

In addition to testing differences in median similarity values within and between populations, simple linear regression was conducted to investigate potential

correlations between band-sharing values and geographic distance between populations. Under the "isolation by distance model" (Wright 1943), gene flow is expected to be reduced as populations are further separated by geographic distance. If this was true for *U. americanus* populations, we would expect to see an inverse relationship between genetic similarity and geographic distance between populations.

CHAPTER 4: ASSESSMENT OF GENETIC EFFECTS OF TRANSLOCATING BEARS FROM MINNESOTA TO ARKANSAS AND LOUISIANA

Introduction

Translocation Background

The original range of the American black bear (*Ursus americanus*) included most of North America, including all of Louisiana and Arkansas. Across this range, the American black bear was divided into 16 subspecies (Hall 1981). The Louisiana black bear, *U. a. luteolus*, was originally described by Merriam (1893) on the basis of skull morphology. *U. a. luteolus* historically occupied eastern Texas, all of Louisiana, southern Mississippi, and the southern portion of Arkansas. The American black bear, *U. a. americanus*, occupied eastern and central North America, including central and northern Arkansas (Hall 1981).

U. a. luteolus and *U. a. americanus* populations declined significantly in Louisiana and Arkansas during the 1800s and early 1900s due to unregulated harvest and substantial loss of forest habitat (Pelton 1989, Smith and Clark 1994). The Arkansas Game and Fish Commission closed the bear hunting season in 1927 due to concerns over low population levels (Smith and Clark 1994). Because bear

populations were low, the Arkansas Game and Fish Commission began a restocking program in 1958. The main objective for the program was to increase populations to a level that would allow reopening bear hunting seasons (Smith and Clark 1994). In 1958, 40 bears (*U. a. americanus*) were captured in northeastern Minnesota (Lake and St. Louis Counties) and released in the Interior Highlands of Arkansas. Additional bears were captured in northeastern Minnesota and released in Arkansas during the summers of 1962 - 1968. Release sites were in the Ozark National Forest (Piney Creek Wildlife Management Area, Johnson County; and White Rock Wildlife Management Area, Crawford and Franklin Counties) and the Ouachita National Forest (Muddy Creek Wildlife Management Area, Montgomery and Scott Counties). Documentation on the bear releases is incomplete, but reports indicate that approximately 254 bears had been released in the Ozark and Ouachita National Forests from 1958 - 1968 (Rogers 1973 cited in Smith and Clark 1994). The precise number of bears released at each site cannot be determined due to unclear records. With increasing public concern over bear-human interactions and the high cost of relocating bears, the Arkansas restocking efforts ended in 1968 (Smith and Clark 1994).

The Louisiana Department of Wildlife and Fisheries

recognized that black bear numbers were low within their state and began a restocking program in 1964. Adult bears and cubs were trapped in northeastern Minnesota (Cook County), transported to Louisiana, and released in 2 areas. During 1964-1967, approximately 130 bears were released in the Upper Atchafalaya River Basin (Pointe Coupee Parish). Approximately 35 bears were released in the Tensas River Basin (Tensas and Madison Parishes) during 1965 - 1966 (Taylor 1971 cited in Pelton 1989).

The Arkansas bear populations increased considerably after restocking, with an estimated 2,500 bears now present in western and northern Arkansas, eastern Oklahoma, and southern Missouri (Smith and Clark 1994). With increasing population levels, the Arkansas Game and Fish Commission met the restocking objective, and reopened black bear hunting in western and northern Arkansas in 1980 (Pharris 1981 cited in Smith and Clark 1994). Hunting was still closed for a remnant population of native black bears near the White River National Wildlife Refuge in southeastern Arkansas. The current White River population is estimated at approximately 170 bears, but the population may have reached a low of approximately 25 bears during the 1940s (Dellinger 1942 cited in Smith and Clark 1994, Smith and Pelton 1990).

Recovery records of released bears could not provide conclusive evaluation of the success of the restocking programs in Louisiana (Taylor 1971 cited in Pelton 1989). Louisiana bear populations remain at notably low levels, as has the amount of suitable bear habitat. In 1981, the bear populations were estimated at 30-50 individuals in the Tensas River area, 30-50 individuals in the lower Atchafalaya Basin, with an unknown number of individuals in the upper Atchafalaya Basin (State Survey, 1981; Louisiana Department of Wildlife and Fisheries, cited in Pelton 1989).

Concerns Regarding Federal Protection

Under the Endangered Species Act of 1973, Congress defined species to include "any subspecies of fish or wildlife or plants, and any distinct population segment of any species or vertebrate fish or wildlife which interbreeds when mature (emphasis added)." Hence, any subspecies or distinct population segment of *U. americanus* found to warrant protection because of low numbers could be protected; protection did not have to be justified at the species level. With an increasing concern over loss of black bear habitat and notably low bear populations, the U.S. Fish and Wildlife Service (USFWS) listed the Louisiana

black bear as "threatened" in January of 1992 (57 Federal Register 588).

During the listing process, questions were raised about the potential genetic impacts of the bears introduced into Louisiana from Minnesota during the 1960s (Pelton 1989, 1991; Smith and Clark 1994). This introduction of bears expressing the *U. a. americanus* gene pool into Louisiana could have altered the putatively unique gene pool of *U. a. luteolus*. In making the listing decision, the USFWS consulted a report by Pelton (1989) that assembled preliminary genetic and morphological analyses of black bears from Louisiana, Tennessee, West Virginia, Virginia, Arkansas, and Minnesota. Genetic marker frequency data, including protein electrophoresis and mitochondrial DNA (mtDNA) data, failed to reveal significant inter-population differentiation, excepting 2 isozyme loci from Minnesota populations that may be distinct (Johns et al. 1989, Zimmerman 1989, both serving as appendices to Pelton 1989). The sample sizes for studies reported in Pelton (1989) were small (approximately 3 bears per population), however, limiting the strength of conclusions. Any genetic impacts of the introduced bears on the *U. a. luteolus* genome were unknown at the time of listing. The objective of this study, therefore, was to

assess the genetic effects of translocated bears from Minnesota on bear populations in Arkansas and Louisiana.

Methods

Sample Collection

Blood and tissue samples were collected from 7 black bear populations in Arkansas, Louisiana, and Minnesota (Figure 2). Bears were trapped and immobilized using standard procedures (Kasbohm and Vaughan 1993). Tissue samples were collected from live-trapped, hunter-killed, and road-killed bears by personnel from Virginia Polytechnic Institute and State University and many other agencies and universities (see Acknowledgements). Blood and tissue samples were stored at -60 C or mixed with a sodium dodecyl sulphate (SDS) buffer for storage at ambient temperatures (Kirby 1992).

DNA Fingerprinting

Whole genomic DNA was extracted from blood and tissue using standard proteinase K digestion and phenol/chloroform extraction techniques (Sambrook et al. 1989). Purified DNA was digested using the restriction enzyme, *Hinf I*, and the DNA samples were subjected to TBE agarose gel electrophoresis according to Sambrook et al. (1989).

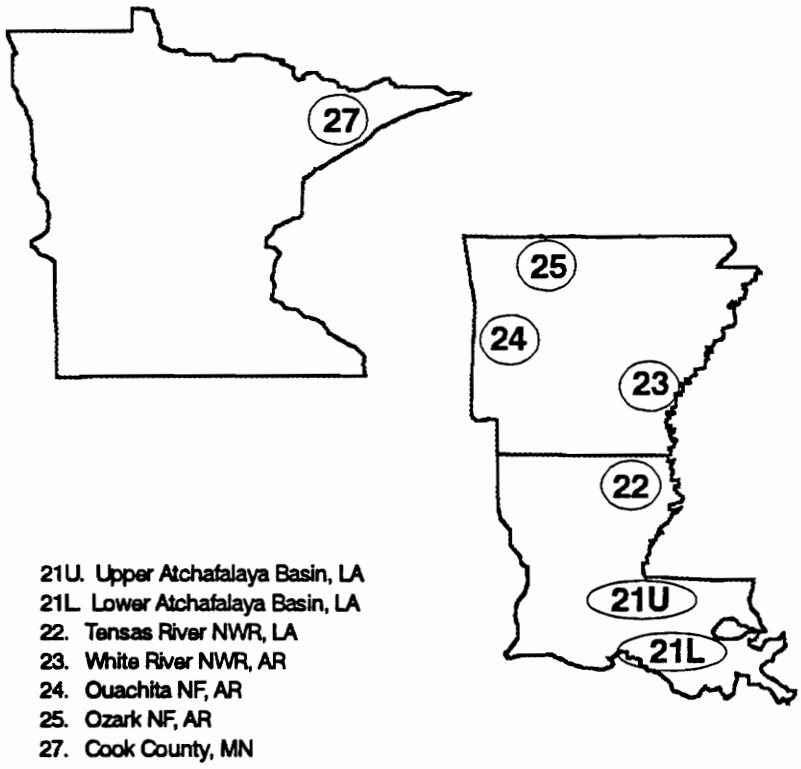


Figure 2. Black bear populations in Minnesota, Arkansas, and Louisiana surveyed for genetic variability.

Lambda DNA cut with BstEII (New England Biolabs, Beverly, MA) was used as a molecular weight marker and placed in several lanes across each gel. DNA fragments were transferred from the agarose gel to a Magnagraph nylon membrane (MSI, Westboro, MA) via Southern blotting (Southern 1975). Southern blots were hybridized using Jeffreys' minisatellite probe 33.15 (Jeffreys et al. 1987), tagged with chemiluminescent agents (Cellmark Diagnostics, Germantown, MD). After hybridization using the specific protocol established by Cellmark Diagnostics, each membrane was exposed to Kodak XAR5 X-omat X-ray film. The hybridized regions on each membrane were observed as banding patterns on the autoradiograph.

Interpretation and Analysis of DNA Fingerprints

The degree of relatedness between 2 individual bears was quantified in terms of the number of bands of equal electrophoretic mobility exhibited by both individuals in the 2.3 - 7.2 kb range of molecular weights. A measure of phenotypic similarity, **S**, was calculated for all pairings of individuals, allowing for comparisons within and between populations examined on the same autoradiograph (Bruford et al. 1992).

S was calculated as:

$$S = 2n_{xy}/(n_x+n_y)$$

where n_{xy} = the number of bands exhibited by both individuals,
n_x = the total number of bands exhibited by individual x, and
n_y = the total number of bands exhibited by individual y.

The **S** values were summarized and median similarity values were compared within and between the Arkansas, Louisiana, and Minnesota populations. Median **S** values were used instead of means because **S** data were found to be non-normally distributed (Appendix Table 1).

Due to the non-normal distribution of **S** values, non-parametric statistical procedures were used for data analysis. Mann-Whitney tests were performed to determine if median similarity values between or within populations were significantly different.

Results

One hundred three bears from 7 populations were screened using DNA fingerprinting techniques (Table 2). Median estimated similarity values within populations (reported along the diagonal of Table 3) ranged from 0.57 (Cook County, MN) to 0.81 (White River NWR, AR). Median estimated similarity values between populations ranged from

Table 2. Black bear populations in Minnesota, Arkansas, and Louisiana surveyed for genetic variability.

Populations By State	Number of bears surveyed
Minnesota	
Cook County	31
Arkansas	
Ozark National Forest	8
Ouachita National Forest	8
White River National Wildlife Refuge	17
Louisiana	
Tensas River National Wildlife Refuge	16
Upper Atchafalaya River Basin	11
Lower Atchafalaya River Basin	12
Total number of bears surveyed	103

Table 3. Matrix of median band-sharing values for genetic similarities of black bear populations in Minnesota, Arkansas, and Louisiana (Hinf I restriction digests, Jeffreys' 33.15 probe). Values along the diagonal represent estimated genetic similarities within populations; values below the diagonal represent estimated genetic similarities between populations. Asterisks indicate comparisons that were not conducted on the same Southern blot.

POPULATION[1]	COOK	OZAR	OUAC	WRIV	TENS	UATC	LATC
COOK	0.57						
OZAR	0.30	0.63					
OUAC	0.23	0.43	0.635				
WRIV	0.39	0.405	0.19	0.81			
TENS	0.41	****	****	0.70	0.78		
UATC	0.29	****	****	****	0.56	0.67	
LATC	****	****	****	****	****	0.50	0.78

[1] COOK = Cook County, Minnesota
OZAR = Ozark National Forest, Arkansas
OUAC = Ouachita National Forest, Arkansas
WRIV = White River National Wildlife Refuge, Arkansas
TENS = Tensas River National Wildlife Refuge, Louisiana
UATC = Upper Atchafalaya Basin, Louisiana
LATC = Lower Atchafalaya Basin, Louisiana

0.19 (White River NWR, AR versus Ouachita NF, AR) to 0.70 (White River NWR, AR versus Tensas NWR, LA) (Table 2, Figure 3).

Estimated genetic similarity within the Cook County, MN population was 0.57, while the median estimated genetic similarity within the Arkansas and Louisiana populations was 0.74 (Table 4). Within-population similarity values (0.57 versus 0.74) within the Minnesota and Arkansas/Louisiana populations were different ($p<0.001$, Mann-Whitney test). Among the 3 Arkansas populations, estimated genetic similarity was higher ($p<0.001$, Mann-Whitney test) within White River NWR (0.81) than within the Ozark and Ouachita NF populations (0.63 pooled) (Figure 3). Among the 3 Louisiana populations, estimated genetic similarity was lower ($p<0.001$, Mann-Whitney test) within the Upper Atchafalaya Basin (0.67) than within the Tensas River NWR and Lower Atchafalaya Basin populations (0.78 pooled) (Figure 3).

The estimated median genetic similarity between the Cook County, MN population and each population from Louisiana and Arkansas was 0.34 (5 inter-population comparisons). The estimated median genetic similarity between the Louisiana and Arkansas populations was 0.53 (6 inter-population comparisons) (Table 4). These two

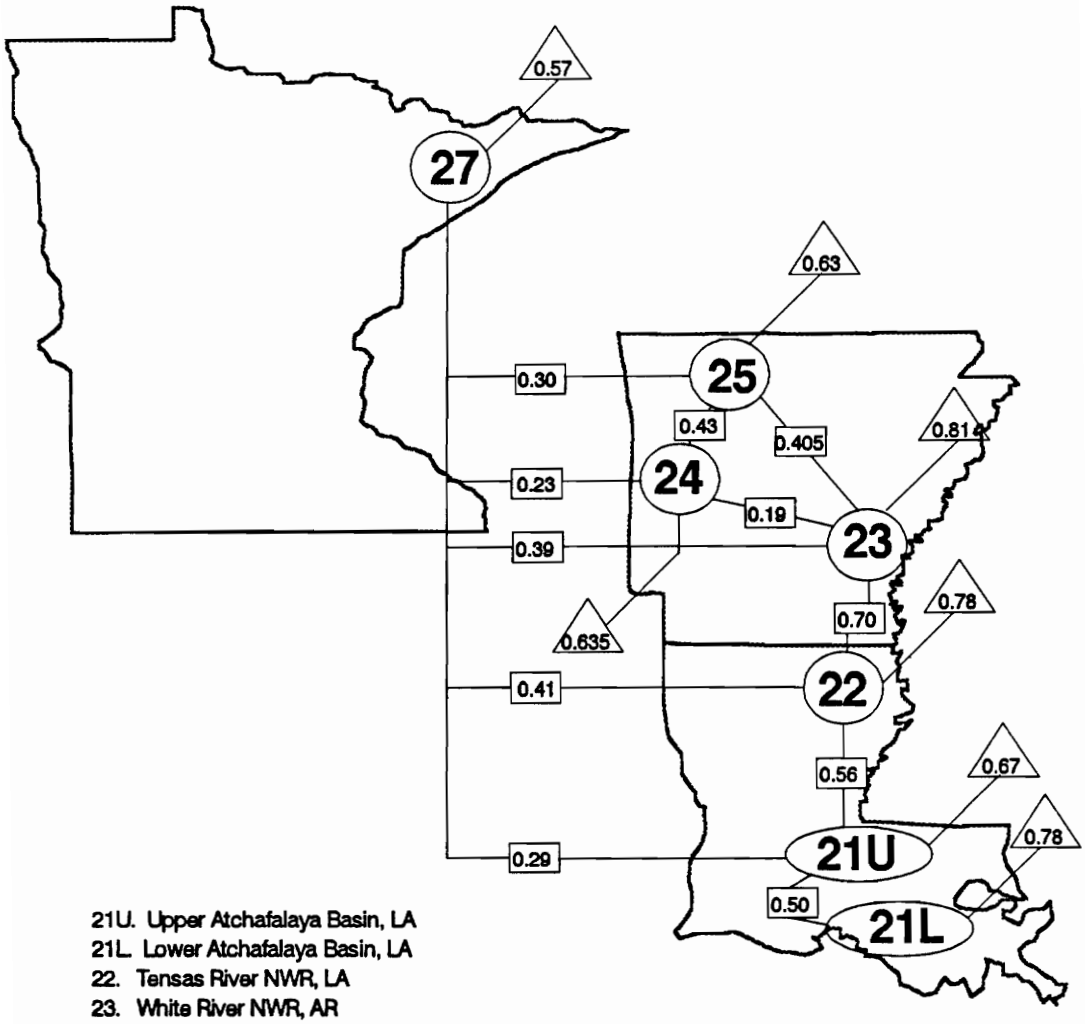


Figure 3. Median band-sharing values for genetic similarities within and between black bear populations in Minnesota, Arkansas, and Louisiana (Hinf I restriction digests, Jeffreys' 33.15 probe). Numbers enclosed in circles represent populations, numbers enclosed in triangles represent genetic similarities within populations, and numbers enclosed in rectangles represent genetic similarities between populations.

Table 4. Median band-sharing values for genetic similarities of black bear populations in Minnesota, Arkansas, and Louisiana (Hinf I restriction digests, Jeffreys' 33.15 probe). The Arkansas and Louisiana populations have been grouped for statistical purposes.

Population	Median Band-sharing
Within Individual Populations: ($p < 0.001$)	
Minnesota (n=31)	0.57
Louisiana/Arkansas (n=72)	0.74
Between Individual Populations: ($p < 0.001$)	
Minnesota versus Louisiana/Arkansas (n=77)	0.34
Louisiana/Arkansas (n=56)	0.53

between-population similarity values were different ($p<0.001$, Mann-Whitney test).

Discussion

Genetic Variation Within Populations

Band-sharing data indicate that the bear population in Cook County, Minnesota exhibits more genetic diversity than bear populations from Louisiana and Arkansas. The Minnesota population is much larger than the fragmented Louisiana or Arkansas populations (Jonkel 1978), and larger populations tend to show more genetic variation than smaller, fragmented populations (Allendorf and Leary 1986, Hartl 1988).

Among the 3 populations in Arkansas, the White River NWR population exhibited the highest within-population genetic similarity. Even though the White River NWR population may have declined to approximately 25 individuals in the 1940s (Dellinger 1942 cited in Smith and Clark 1994), this population was not part of the restocking program in Arkansas. The population remains at a low number relative to the Ozark and Ouachita NF populations (Smith and Clark 1994), and the White River NWR population may have lost genetic variation due to a genetic bottleneck and associated random genetic drift (Allendorf 1986, Hartl

1988). Smith and Clark (1994) estimate that approximately 2,500 bears now inhabit western and northern Arkansas and portions of eastern Oklahoma and southern Missouri. Restocking efforts may have reduced any effects of random genetic drift and be partly responsible for the higher genetic diversity within the Ozark and Ouachita NF populations. Alternatively, the rapid recovery from any genetic bottleneck may have minimized associated losses of genetic diversity (Nei 1975).

Among the 3 black bear populations in Louisiana, the Tensas River NWR and Lower Atchafalaya Basin populations showed less genetic variation. This may be due to a genetic bottleneck with associated random genetic drift (Hartl 1988), given that these populations were each estimated to have only 30 - 50 bears in 1981 (State Survey, 1981, Louisiana Department of Wildlife and Fisheries cited in Pelton 1989). Even though approximately 35 bears were translocated to the Tensas River NWR population during the restocking program, Nowak (1986 cited in Pelton 1989) speculated that none of the released bears survived or remained in the area. The Lower Atchafalaya Basin population was not part of the restocking program. The Upper Atchafalaya Basin exhibited the most within-population genetic variation. Restocking efforts may have

reduced the impacts of random genetic drift (Hartl 1988) and be partly responsible for the higher genetic diversity. The possibility that translocated black bears may have affected the *U. a. luteolus* gene pool, therefore, cannot be excluded.

Genetic Variation Between Populations

Estimated genetic similarities between black bear populations in Louisiana and Arkansas were higher than the estimated genetic similarities between the Cook County, Minnesota population and each population in Louisiana and Arkansas ($p<0.001$, Mann-Whitney test). These statistics indicate that bear populations in Louisiana and Arkansas are more closely related to each other than they are to the population in Minnesota. Although restocking efforts may have influenced levels of genetic variability within the Louisiana and Arkansas bear populations, any genetic impacts caused by these translocations were not significant enough to alter the overall genetic similarity between populations.

Within Arkansas, the White River NWR population was more similar to the Ozark NF population than to the Ouachita NF population. Bears use narrow river corridors for dispersal or migration (Pelton 1989) and potentially

can travel between the Ozark NF and White River NWR (approximately 250 km) using the White River corridor, providing a means for gene flow between these two populations. In contrast, travel between the Ouachita NF and White River NWR (approximately 175 km) may be limited by migration barriers, possibly including the Arkansas River, the city of Little Rock, and several major interstate roadways.

Estimated genetic similarity between the White River NWR, AR and Tensas River NWR, LA populations was high (0.70), suggesting gene flow between these 2 populations (Pelton 1989). Given the small sizes of the White River NWR and Tensas River NWR populations, sufficient migration would be necessary to keep the 2 populations from diverging genetically due to random genetic drift; the actual divergence of the populations would be a function of the number of migrants per generation and of means and variances of allele frequencies (Allendorf and Phelps 1981).

Statistical Considerations

Data on band-sharing, using DNA fingerprinting techniques, can provide valuable information when appropriate statistical procedures are used for analysis

(Lynch 1990, Bruford et al. 1992, Stephens et al. 1992, Scott and Williams 1994). Band-sharing data often are analyzed in the genetics literature using classical statistical procedures that assume normal data (Gilbert et al. 1990, Westneat 1990, Wayne et al. 1991, Triggs et al. 1992). However, non-parametric procedures should be used when data do not come from a normal distribution (Hollander and Wolfe 1973). Median values provide a more reliable representation of non-normal distributions than mean values. Therefore, median genetic similarity values were reported in this study instead of mean genetic similarity values, and non-parametric tests for differences in medians were used.

Given the pairwise nature of the similarity index calculation, band-sharing data from each DNA fingerprint represent a collection of non-independent data points (Wayne et al. 1991). Under the Mann-Whitney test, however, data points are assumed to be independent. Although the independence assumption is violated, the current genetics and statistics literatures offer no alternative statistical methods for handling dependent data. Although the robustness of the Mann-Whitney test to non-independence is unknown, after consultation with statisticians and geneticists, the non-parametric procedures discussed were

used for data analysis.

Lynch (1990) provided a method for partitioning the similarity index into within- and between-population components, but generous assumptions are needed to make the transition. Stephens et al. (1992) presented a method for calculating average heterozygosity using band-sharing data, provided that allele frequencies can be inferred from such data. These two statistical methods were not used in this study due to the underlying assumptions each required.

CHAPTER 5: GENETIC VARIABILITY IN FLORIDA, LOUISIANA, AND AMERICAN BLACK BEARS

Introduction

Classification and Status of the Black Bear

The original range of the American black bear (*Ursus americanus*) included nearly all of North America, except for portions of the Southwest. Across this range, the American black bear was divided into 16 subspecies (Hall 1981). The Louisiana black bear, *U. a. luteolus*, was originally described by Merriam (1893) on the basis of skull morphology. *U. a. luteolus* historically occupied eastern Texas, all of Louisiana, southern Mississippi, and the southern portion of Arkansas. The Florida black bear, *U. a. floridanus*, also described by Merriam (1896) on the basis of skull morphology, historically occupied Florida and the coastal plain of Georgia and Alabama (Hall 1981). The more widespread American black bear, *U. a. americanus*, occupied most of eastern and central North America.

Originally, much of North America provided adequate vegetation and other habitat requirements needed to sustain black bear populations (Pelton 1989). However, bear habitat was reduced significantly as land was cleared and

developed for human activity, particularly in the southeastern United States. Currently, less than 10% of the original black bear range is believed to support bear populations (Maehr 1984), with most bears surviving on scattered publicly-owned lands (Pelton 1985).

With decreased habitat available to black bears in the coastal plain physiographic region of the Southeast, population sizes were greatly diminished (Pelton 1989). The Louisiana Department of Wildlife and Fisheries recognized that black bear numbers were very low within their state, and they began a restocking program in the mid-1960s. Adult bears and cubs were trapped in Cook County, Minnesota, and transported to Louisiana, where 150-200 were released in Pointe Coupee Parish and in Tensas/Madison Parishes (Taylor 1971 cited in Pelton 1989). Recovery records of released bears were not conclusive regarding the success of the restocking program. The Louisiana populations of *U. a. luteolus* remain at notably low levels, as has the amount of suitable bear habitat. In 1981, the bear populations were estimated at 30-50 individuals in the Tensas River area, 30-50 individuals in the lower Atchafalaya Basin, with an unknown number in the upper Atchafalaya Basin (State Survey, 1981; Louisiana Department of Wildlife and Fisheries, cited in Pelton

1989).

Black bear habitat in Florida has been reduced due to land clearing and development. This habitat fragmentation has left *U. a. floridanus* populations in broken patches of suitable habitat (Brady and Maehr 1985). Many of these broken patches, especially in southern Florida, can sustain only a small number of bears. Population estimates for *U. a. floridanus* range between 500 - 1000 individual bears, mostly distributed in remote, forested areas (Brady and Maehr 1985).

Federal Protection of Black Bear Populations

The Endangered Species Act of 1973 was established to protect against the loss of living natural resources. Under the Act, Congress defined species to include "any subspecies of fish or wildlife or plants, and <u>any distinct population segment</u> of any species or vertebrate fish or wildlife which interbreeds when mature (emphasis added)." Under this definition, any subspecies or distinct population segment of *U. americanus* found to warrant protection could be protected; protection did not have to be justified at the species level.

With an increasing concern over loss of black bear habitat and notably low bear populations, the U.S. Fish and

Wildlife Service (USFWS) listed the Louisiana black bear as "threatened" in January of 1992 (57 Federal Register 588). The USFWS placed the Florida black bear in a "warranted but precluded" category (55 Federal Register 42223), indicating that the Florida black bear most likely warranted protection, but was precluded from protection at that time due to a backlog of petitions and limited administrative resources.

In making the listing decisions, the USFWS consulted a report by Pelton (1989) that assembled genetic and morphological analyses of black bears from several populations of *U. a. luteolus, U. a. floridanus*, and *U. a. americanus*. Genetic marker frequency data, including protein electrophoresis and mitochondrial DNA (mtDNA) data, failed to reveal significant inter-population differentiation (Johns et al. 1989, Zimmerman 1989, both serving as appendices to Pelton 1989). Skull morphometrics, however, indicated that *U. a. luteolus* was distinct from *U. a. americanus*, but similar to *U. a. floridanus* (Kennedy 1989, appendix to Pelton 1989). The sample sizes for studies reported in Pelton (1989) were small (approximately 3 bears per population), however, limiting the strength of conclusions. During the listing process, therefore, it became evident that the genetic

relationships within and between *U. americanus* populations were largely unknown. The objective of this study, therefore, was to analyze the genetic variation within and between *U. americanus* populations.

Methods

Sample Collection

Blood and tissue samples were collected from 18 black bear populations, 17 located in the southeastern United States (Figure 1, Chapter 3). The Cook County, MN population was included to account for any possible effect of translocations (see Chapter 4). Bears were trapped and immobilized using standard procedures (Kasbohm and Vaughan 1993). Tissue samples were collected from live-trapped, hunter-killed, and road-killed bears by personnel from Virginia Polytechnic Institute and State University and many other agencies and universities (see Acknowledgements). Blood and tissue samples were stored at -60 C or mixed with a sodium dodecyl sulphate (SDS) buffer for storage at ambient temperatures (Kirby 1992).

DNA Fingerprinting

Whole genomic DNA was extracted from blood and tissue using standard proteinase K digestion and phenol/chloroform

extraction techniques (Sambrook et al. 1989). Purified DNA was digested using the restriction enzyme *Hinf I*, and the DNA samples were subjected to TBE agarose gel electrophoresis according to Sambrook et al. (1989). Phage Lambda DNA cut with BstEII (New England Biolabs, Beverly, MA) was used as a molecular weight marker and placed in several lanes across each gel. DNA fragments were transferred from the agarose gel to a Magnagraph nylon membrane (MSI, Westboro, MA) via Southern blotting (Southern 1975). Southern blots were hybridized using Jeffreys' minisatellite probe 33.15 (Jeffreys et al. 1987), tagged with chemiluminescent agents (Cellmark Diagnostics, Germantown, MD). After hybridization using the specific protocol established by Cellmark, each membrane was exposed to Kodak XAR5 X-omat X-ray film. The hybridized regions on each membrane were observed as banding patterns on the autoradiograph.

Interpretation and Analysis of DNA Fingerprints

The degree of relatedness between 2 individual bears was quantified in terms of the number of bands of equal electrophoretic mobility exhibited by both individuals in the 2.3 - 7.2 kb range of molecular weights. A measure of phenotypic similarity, **S**, was calculated for all pairings

of individuals, allowing for comparisons within and between populations examined on the same autoradiograph (Bruford et al. 1992). **S** was calculated as:

$$S = 2n_{xy}/(n_x+n_y)$$

where n_{xy} = the number of bands exhibited by both individuals,
n_x = the total number of bands exhibited by individual x, and
n_y = the total number of bands exhibited by individual y.

The **S** values were summarized and median similarity values were compared within and between *U. a. luteolus*, *U. a. floridanus*, and *U. a. americanus* populations. Median **S** values were used instead of means because **S** data were found to be non-normally distributed (Appendix Table 1).

Due to the non-normal distribution of **S** values, non-parametric statistical procedures were used for data analysis. Kruskal-Wallis tests and multiple comparisons procedures were performed to determine if median similarity values within or between populations were significantly different. Standard least-squares regression was used to investigate any potential correlation between band-sharing values and geographic distance between populations.

Results

Two hundred and fifty-three bears from 18 populations

were screened using DNA fingerprinting techniques (Table 1, Chapter 3). Median estimated similarity values within all populations ranged from 0.57 (Cook County, MN) to 0.86 (Mobile River, AL and Mississippi pooled) (Figure 4, Table 5). Median estimated similarity values between all populations ranged from 0.19 (Ouachita NF, AR versus White River NWR, AR) to 0.70 (Tensas River NWR, LA versus White River NWR, AR) (Figure 5, Table 6).

Similarities Within Populations

Among the 3 subspecies, overall estimated median genetic similarities within *U. a. luteolus* and *U. a. floridanus* populations were both 0.75, higher than the estimated median genetic similarity of 0.66 within *U. a. americanus* populations ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 5).

Among *U. a. americanus* populations, estimated median genetic similarities within individual populations ranged from 0.57 (Cook County, MN) to 0.82 (Ocmulgee River, GA), representing 3 groups of different similarity values ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 5). Estimated median genetic similarities within individual populations of *U. a. floridanus* ranged from 0.69 (Okefenokee NWR, GA) to

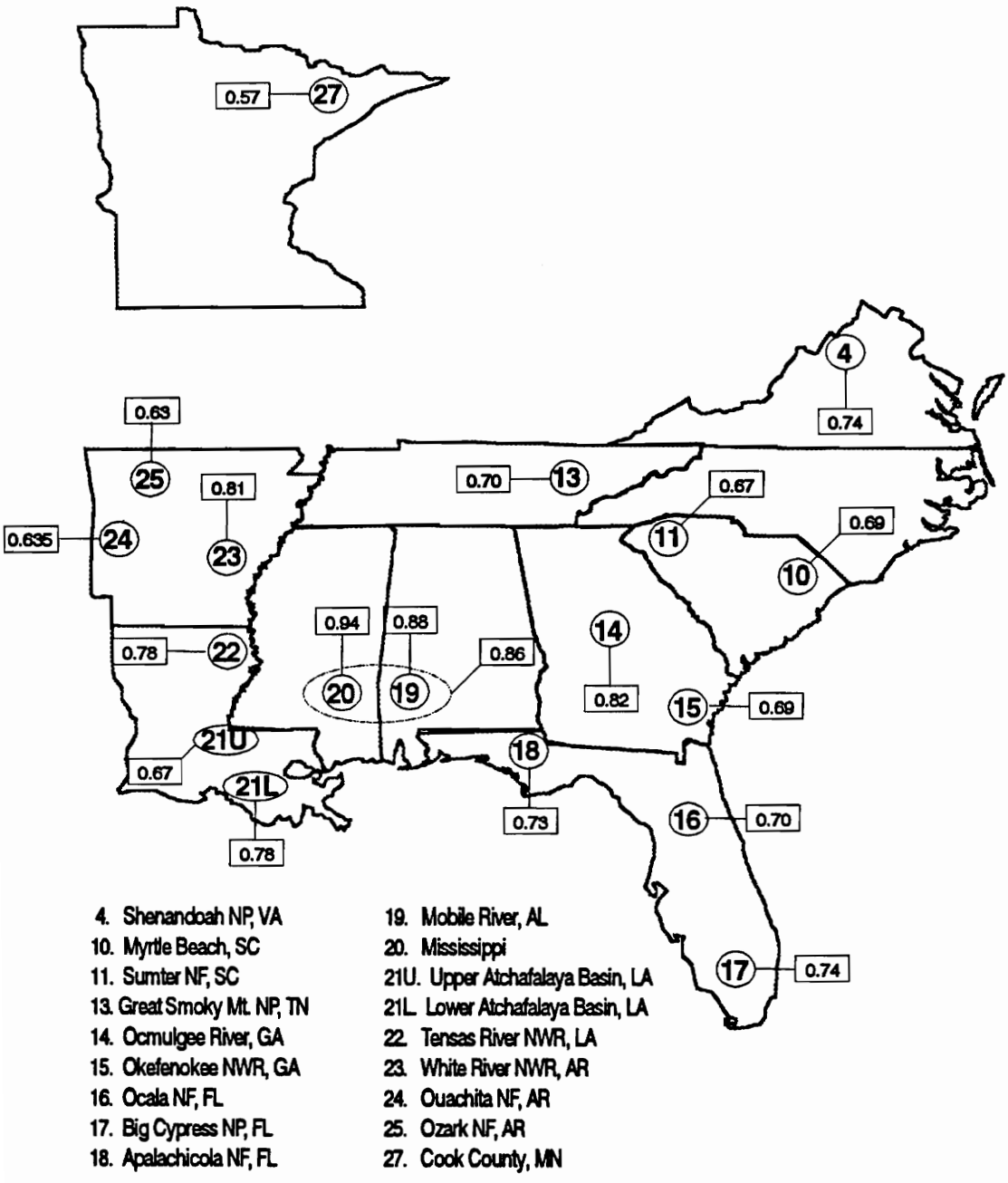


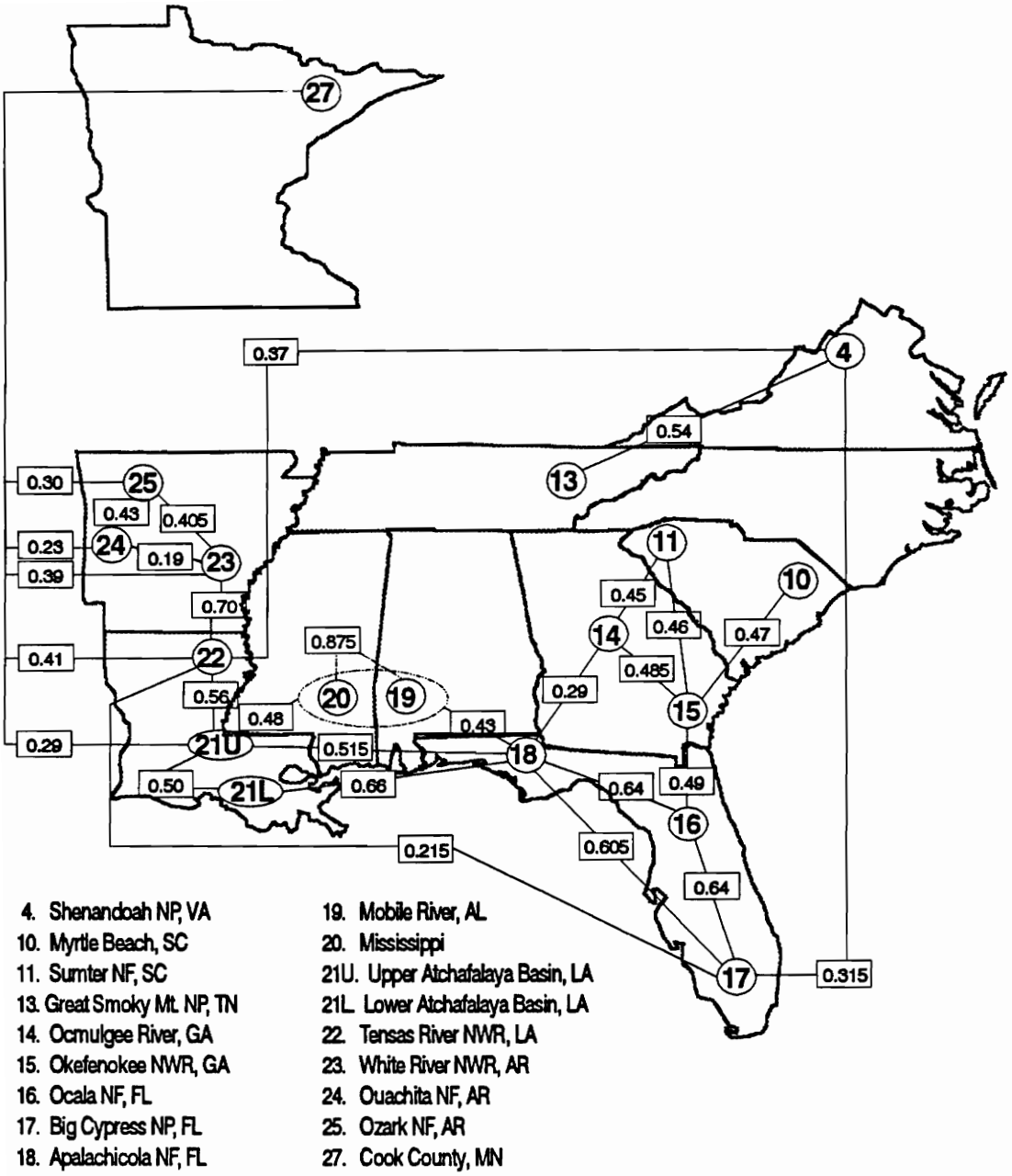
Figure 4. Median band-sharing values for genetic similarities within black bear populations in the southeastern United States (Hinf I restriction digests, Jeffreys' 33.15 probe). Numbers enclosed in circles represent populations, and numbers enclosed in rectangular blocks represent the genetic similarities within each population. Populations 19 and 20 were pooled due to high genetic similarity, close proximity, and small sample size.

Table 5. Median band-sharing values for genetic similarities within black bear populations in the southeastern United States (Hinf I restriction digests, Jeffreys' 33.15 probe). Similarity values are given in descending order within each subspecies. All Kruskal-Wallis tests (within subspecies) showed significant differences in median similarity values ($p < 0.001$). Median band-sharing values with the same superscripts were not different (Kruskal-Wallis test multiple comparisons, $\alpha = 0.05$).

Populations	Median Band-sharing
Genetic Similarity within *U. a. americanus*:	
Ocmulgee River, GA (n=9)	0.82^{a}
Shenandoah National Park, VA (n=16)	0.74^{ab}
Great Smoky Mountains National Park, TN (n=7)	0.70^{b}
Myrtle Beach, SC (n=6)	0.69^{b}
Sumter National Forest, SC (n=6)	0.67^{b}
Ouachita National Forest, AR (n=8)	0.635^{bc}
Ozark National Forest, AR (n=8)	0.63^{bc}
Cook County, MN (n=31)	0.57^{c}
Genetic Similarity within *U. a. floridanus*:	
Mobile River, AL and Mississippi (n=13)	0.86^{a}
Big Cypress National Park, FL (n=13)	0.74^{b}
Apalachicola National Forest, FL (n=39)	0.73^{b}
Ocala National Forest, FL (n=21)	0.70^{bc}
Okefenokee National Wildlife Refuge, GA (n=20)	0.69^{c}
Genetic Similarity within *U. a. luteolus*:	
White River National Wildlife Refuge, AR (n=17)	0.81^{a}
Tensas River National Wildlife Refuge, LA (n=16)	0.78^{a}
Lower Atchafalaya River Basin, LA (n=12)	0.78^{a}
Upper Atchafalaya River Basin, LA (n=11)	0.67^{b}

Table 5 continued.

Populations	Median Band-sharing
Genetic Similarity within populations grouped by subspecies:	
U. a. floridanus (n=106)	0.75[a]
U. a. luteolus (n=56)	0.75[a]
U. a. americanus (n=91)	0.66[b]



4. Shenandoah NP, VA
10. Myrtle Beach, SC
11. Sumter NF, SC
13. Great Smoky Mt. NP, TN
14. Ocmulgee River, GA
15. Okefenokee NWR, GA
16. Ocala NF, FL
17. Big Cypress NP, FL
18. Apalachicola NF, FL
19. Mobile River, AL
20. Mississippi
21U. Upper Atchafalaya Basin, LA
21L. Lower Atchafalaya Basin, LA
22. Tensas River NWR, LA
23. White River NWR, AR
24. Ouachita NF, AR
25. Ozark NF, AR
27. Cook County, MN

Figure 5. Median band-sharing values for genetic similarities between black bear populations in the southeastern United States (Hinf I restriction digests, Jeffreys' 33.15 probe). Numbers enclosed in circles represent populations, and numbers enclosed in rectangular blocks represent the genetic similarity between two populations. Populations 19 and 20 were pooled due to high genetic similarity, close proximity, and small sample size.

Table 6. Median band-sharing values for genetic similarities between black bear populations in the southeastern United States (Hinf I restriction digests, Jeffreys' 33.15 probe). Similarity values are given in descending order within each subspecies. All Kruskal-Wallis tests (within subspecies) showed significant differences in median similarity values ($p < 0.001$). Median band-sharing values with the same superscript were not different (Kruskal-Wallis test multiple comparisons, $\alpha = 0.05$).

Populations	Median Band-sharing
Genetic Similarity between *U. a. americanus* populations:	
Shenandoah NP, VA versus Great Smoky Mountains NP, TN (n=14)	0.54[a]
Sumter NF, SC versus Ocmulgee River, GA (n=12)	0.45[b]
Ozark NF, AR versus Ouachita NF, AR (n=16)	0.43[b]
Cook County, MN versus Ozark NF, AR (n=18)	0.30[c]
Cook County, MN versus Ouachita NF, AR (n=18)	0.23[c]
Genetic Similarity between *U. a. floridanus* populations:	
Ocala NF, FL versus Big Cypress NP, FL (n=14)	0.64[a]
Apalachicola NF, FL versus Big Cypress NP, FL (n=13)	0.605[a]
Apalachicola NF, FL versus Ocala NF, FL (n=15)	0.60[a]
Ocala NF, FL versus Okefenokee NWR, GA (n=14)	0.49[b]
Apalachicola NF, FL versus Mobile River, AL/Mississippi (n=14)	0.43[c]
Genetic Similarity between *U. a. luteolus* populations:	
White River NWR, AR versus Tensas River NWR, LA (n=13)	0.70[a]
Tensas River NWR, LA versus Upper Atchafalaya Basin, LA (n=13)	0.56[b]
Upp. Atchafalaya Basin, LA versus Low. Atchafalaya Basin, LA (n=18)	0.50[b]

Table 6 continued.

Populations	Median Band-sharing
Genetic Similarity between populations, within subspecies and between subspecies:	
U. a. luteolus (n=36)	0.58[a]
U. a. floridanus (n=66)	0.57[a]
U. a. luteolus versus *U. a. floridanus* (n=44)	0.51[b]
U. a. americanus (n=52)	0.40[c]
U. a. americanus versus *U. a. floridanus* (n=52)	0.40[cd]
U. a. americanus versus *U. a. luteolus* (n=72)	0.36[d]

0.86 (Mobile River, AL and Mississippi pooled), representing 3 groups of different similarity values ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 5). Among *U. a. luteolus* populations, estimated median genetic similarity within individual populations ranged from 0.67 (Upper Atchafalaya Basin, LA) to 0.81 (White River NWR, AR), representing 2 groups of different similarity values ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 5).

Similarities Between Populations

Estimated median genetic similarities between individual populations of *U. a. americanus* ranged from 0.23 (Cook County, MN versus Ouachita NF, AR) to 0.54 (Shenandoah NP, VA versus Great Smoky Mountains NP, TN), representing 3 groups of different similarity values ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 6). Within *U. a. floridanus*, estimated median genetic similarity between individual populations ranged from 0.43 (Apalachicola, FL versus Mobile River, AL and Mississippi pooled) to 0.64 (Ocala NF, FL versus Big Cypress NP, FL), representing 3 groups of different similarity values ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 6). Estimated median genetic

similarities between individual populations of *U. a. luteolus* ranged from 0.50 (Upper Atchafalaya Basin, LA versus Lower Atchafalaya Basin, LA) to 0.70 (White River NWR, AR versus Tensas River NWR, LA), representing 2 groups of different similarity values ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 6).

Within each subspecies, overall estimated median genetic similarities between populations were not significantly different for *U. a. luteolus* (0.58) and *U. a. floridanus* (0.57); however, both of these were higher than *U. a. americanus* (0.40) ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 6).

Estimated median genetic similarity between *U. a. luteolus* and *U. a. floridanus* populations (0.51) was higher than the estimated median genetic similarities between *U. a. luteolus* and *U. a. americanus* populations (0.36) and *U. a. floridanus* and *U. a. americanus* populations (0.40) ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 6). The estimated median genetic similarities between *U. a. luteolus* and *U. a. americanus* populations (0.36) and *U. a. floridanus* and *U. a. americanus* populations (0.40) were not different ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons) (Table 6).

Regression

The least-squares regression failed to reveal any significant correlation between band-sharing values and geographic distance between populations (adjusted R^2=0.225, Figure 6). Log, square, cube, square-root, and inverse transformations of either variable did not increase the adjusted R^2.

Discussion

Genetic Variation Within Populations

Band-sharing data indicate that *U. a. americanus* populations exhibited more genetic diversity than *U. a. luteolus* and *U. a. floridanus* populations. The American black bear populations tend to be larger than the fragmented Louisiana and Florida black bear populations (Jonkel 1978, Brady and Maehr 1985, Pelton 1989), and larger populations tend to show more genetic variation than smaller, fragmented populations (Allendorf and Leary 1986, Hartl 1988). Small populations can lose genetic diversity due to genetic bottleneck events and random genetic drift (Hartl 1988).

Among the *U. a. americanus* populations, Ocmulgee River, GA exhibited the highest within-population genetic similarity. The Ocmulgee River population exists at a low

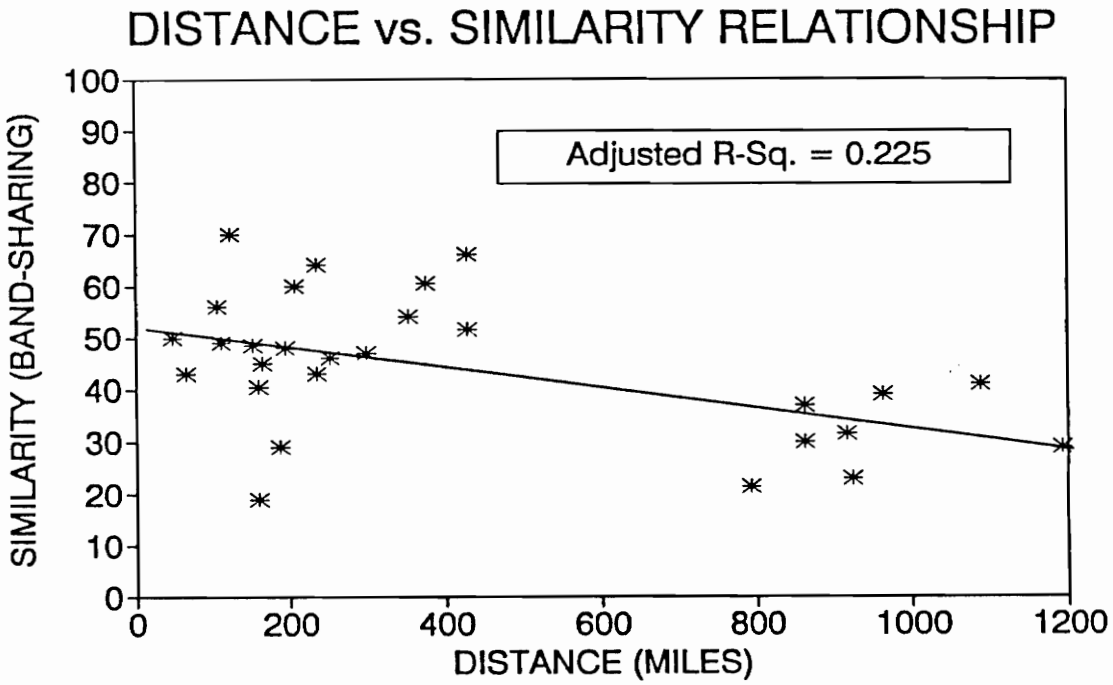


Figure 6. Least-squares regression of genetic similarity versus geographic distance between black bear populations in the southeastern United States. Genetic similarity was estimated using band-sharing calculations (Hinf I restriction digests, Jeffreys' 33.15 probe).

number with limited distribution (Maehr 1984), and inbreeding and random genetic drift may be responsible for the decreased genetic diversity (Hartl 1988). The Cook County, MN population exhibited the lowest within-population genetic similarity in *U. a. americanus*, likely attributable to its large population size. With populations as large as this, random genetic drift has a much weaker effect on genetic variation (Hartl 1988). All other *U. a. americanus* populations sampled exhibited intermediate within-population genetic similarities that were not significantly different (α=0.05).

Among the *U. a. luteolus* populations, genetic similarity within the Upper Atchafalaya Basin, LA was significantly lower than in the remaining 3 populations. Restocking efforts (Taylor 1971 cited in Pelton 1989) may have introduced genetic variability or reduced the impacts of random genetic drift (Hartl 1988) and be partly responsible for the higher genetic diversity in the Upper Atchafalaya Basin. The Tensas River NWR, LA and Lower Atchafalaya Basin, LA populations showed more within-population similarity than the Upper Atchafalaya Basin, possibly due to a genetic bottleneck and associated random genetic drift (Hartl 1988), given that these 2 populations were each estimated to have only 30 - 50 bears in 1981

(State Survey, 1981, Louisiana Department of Wildlife and Fisheries cited in Pelton 1989). The White River NWR, AR population exhibited the highest within-population similarity in *U. a. luteolus*, possibly due to a genetic bottleneck facilitated by the population reaching a low of approximately 25 bears in the early 1940s (Dellinger 1942 cited in Smith and Clark 1994).

Among the *U. a. floridanus* populations, genetic similarity was highest within the Mobile River, AL and Mississippi population. Mississippi was pooled with Mobile River, AL due to high genetic similarity, small sample size for the Mississippi population (n=2), and close geographic proximity. Given the small size of the AL/MS population (Kasbohm et al. 1994), inbreeding and random genetic drift may have contributed to limit genetic diversity (Hartl 1988). Many of the bears captured in AL/MS for this study expressed obvious morphological defects (e.g., kinked tails, missing tails, lack of scrotum, etc.) possibly resulting from inbreeding depression (Kasbohm et al. 1994). The Okefenokee NWR, GA population exhibited the lowest within-population genetic similarity, indicating the greatest genetic diversity within *U. a. floridanus*. According to the Georgia Game and Fish Division, the Okefenokee bear population is large and capable of

supporting a sustainable harvest (Bentzien 1991). The genetic similarities within the Florida populations (Big Cypress NP, Ocala NF, and Apalachicola NF) were not significantly different from one another, indicating that they each share the same degree of genetic diversity. Given that the original range of *U. a. floridanus* was continuous in Florida (Bentzien 1991), that these 3 populations are similar in size (Maehr 1984), and that isolation is relatively recent, the degree of genetic similarity within populations would be expected to be comparable.

Genetic Variation Between Populations

Within each subspecies, overall estimated genetic similarities between populations were not significantly different for *U. a. luteolus* and *U. a. floridanus*, indicating that these two sets of populations share the same degree of genetic variation. The overall estimated genetic similarity between populations of *U. a. americanus*, however, was significantly lower than the corresponding values for *U. a. luteolus* and *U. a. floridanus*. Louisiana and Florida black bear populations are smaller and more fragmented than American black bear populations (Maehr 1984, Brady and Maehr 1985, Pelton 1989). Smaller,

fragmented populations tend to lose genetic variation (Allendorf and Leary 1986, Hartl 1988), especially loss of rare alleles, which may explain the high degree of similarity between populations of *U. a. luteolus* and *U. a. floridanus*.

Band-sharing data from comparisons of populations between subspecies indicated that *U. a. luteolus* and *U. a. floridanus* were more closely related to each other than either was related to *U. a. americanus*. Our findings correspond to those of previous studies using other genetic techniques. Zimmerman (1989, appendix to Pelton 1989), who conducted a study of black bear populations in the southeastern United States using protein electrophoresis, described two genetic groupings of bears: coastal bears (including *U. a. luteolus* and *U. a floridanus*) and inland bears (*U. a. americanus*). In a similar report, Kennedy (1989, appendix to Pelton 1989) studied skull morphometrics in black bear populations from the southeastern United States and indicated that *U. a. luteolus* was distinct from *U. a. americanus*, but similar to *U. a. floridanus*.

Least-squares regression failed to reveal any significant correlation between band-sharing values and geographic distance between populations, suggesting that genetic similarity between 2 *U. americanus* populations is

not a simple function of the geographic distance between them. Under the "isolation by distance model" (Wright 1943), gene flow is expected to be reduced as populations are further separated by geographic distance. The band-sharing data from this study do not fit the "isolation by distance" pattern, possibly due to the varying degree of genetic diversity found among the populations surveyed.

Statistical Considerations

Data on band-sharing using DNA fingerprinting techniques can provide valuable information when appropriate statistical procedures are used for analysis (Lynch 1990, Bruford et al. 1992, Stephens et al. 1992, Scott and Williams 1994). Band-sharing data often are analyzed in the genetics literature using classical statistical procedures that assume normal data (Gilbert et al. 1990, Westneat 1990, Wayne et al. 1991, Triggs et al. 1992). However, non-parametric procedures should be used when data do not come from a normal distribution (Hollander and Wolfe 1973). Median values provide a more reliable representation of non-normal distributions than mean values. Therefore, median genetic similarity values were reported in this study instead of mean genetic similarity values, and non-parametric tests for differences in medians

were used.

Given the pairwise nature of the similarity index calculation, band-sharing data from each DNA fingerprint represent a collection of non-independent data points (Wayne et al. 1991). Under the Kruskal-Wallis test, however, data points are assumed to be independent. Although the independence assumption is violated, the current genetics and statistics literatures offer no alternative statistical methods for handling dependent data. Although the robustness of the Kruskal-Wallis test to non-independence is unknown, after consultation with statisticians and geneticists, the non-parametric procedures discussed were used for data analysis.

Lynch (1990) provided a method for partitioning the similarity index into within- and between-population components, but generous assumptions are needed to make the transition. Stephens et al. (1992) presented a method for calculating average heterozygosity using band-sharing data, provided that allele frequencies can be inferred from such data. These two statistical methods were not used in this study due to the underlying assumptions each required.

CHAPTER 6: GENERAL DISCUSSION: SYSTEMATIC CLASSIFICATION OF BLACK BEAR POPULATIONS IN THE SOUTHEAST

Introduction

With increasing concern over loss of black bear (*Ursus americanus*) habitat and notably low bear populations, the U.S. Fish and Wildlife Service (USFWS) listed the Louisiana black bear, *U. a. luteolus*, as "threatened" in January of 1992 (57 Federal Register 588) and placed the Florida black bear, *U. a. floridanus*, in a "warranted but precluded" category (55 Federal Register 42223). During the listing process, questions were raised regarding the classification of black bear populations in the southeastern United States (Pelton 1989, 1991). If bear populations or subspecies do not exhibit significant genetic differences, translocation programs could be used to maintain populations without the need for federal protection under the Endangered Species Act of 1973.

This study was conducted to assess the genetic variation within and between *U. americanus* populations in the southeastern United States. Multilocus DNA fingerprinting techniques were employed to estimate genetic similarity within and between 18 black bear populations.

The band-sharing data indicated that American black bear populations (*U. a. americanus*) exhibit significantly less genetic similarity within and between populations (similarity within=0.66, similarity between=0.40) than do the Louisiana (similarity within=0.75, similarity between=0.58) and Florida (similarity within=0.75, similarity between=0.57) black bear populations ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons). Louisiana and Florida black bear populations share the same degree of genetic variation within and between populations, and they are more closely related to each other than they are to American black bear populations ($p<0.001$, Kruskal-Wallis test, $\alpha=0.05$ for multiple comparisons).

During the listing process, concern was expressed that translocation of bears from Minnesota to Louisiana and Arkansas might have altered any unique genetic characters of *U. a. luteolus*. Analysis of band-sharing data indicated that any genetic impacts that may have been caused by translocations of bears from Minnesota to Louisiana and Arkansas were not significant. The bear population in Cook County, MN exhibits less within-population genetic similarity (similarity within=0.57) than bear populations in Louisiana and Arkansas (similarity within=0.74). Populations in Louisiana and Arkansas are more closely

related to each other (similarity between=0.53) than they are to the population in Minnesota (similarity between=0.34) ($p<0.001$, Mann-Whitney test). These findings are in agreement with previous genetic and morphological studies of black bear populations in the southeastern United States (Johns et al. 1989, Kennedy et al. 1989, Zimmerman et al. 1989, all serving as appendices to Pelton 1989).

Recommendations Regarding Current Taxonomy and Classification

U. a. luteolus was originally described by Merriam (1893) on the basis of 5 skulls collected in northeastern Louisiana (Morehouse Parish). Merriam (1896) also described *U. a. floridanus* based on several skulls (exact number unknown) collected in southern Florida (Key Biscayne). During the USFWS listing process, however, questions were raised about the validity of this classification and taxonomy (Pelton 1989). Kennedy (1991, cited in Bentzien 1991) questioned the taxonomic status of *U. a. luteolus* and *U. a. floridanus* because original descriptions were based on skulls representing only a portion of the geographic range for each subspecies. In another report, Johns et al. (1989, appendix to Pelton 1989) used protein

electrophoresis and found very little genetic differentiation among *U. a. americanus*, *U. a. luteolus*, and *U. a. floridanus* populations. Zimmerman (1989, appendix to Pelton 1989) used mitochondrial DNA (mtDNA) analysis and found that bear populations could be divided into 2 general groups based on mtDNA restriction patterns: coastal bear populations (*U. a. luteolus* and *U. a. floridanus*) and inland bear populations (*U. a. americanus*). Kennedy (1989, appendix to Pelton 1989) analyzed morphological characteristics of bear skulls from several widespread populations and concluded that *U. a. luteolus* was more similar to *U. a. floridanus* than *U. a. americanus*, but was significantly different from both.

The genetic and morphological data, therefore, did not provide an obvious framework for systematic classification of *U. americanus* populations. Genetic data collected prior to this study suggested that no significant differentiation exists among *U. americanus* populations or subspecies, while the skull morphology data suggested that differentiation does exist. When classifying populations, no one criteria should be used exclusively. Decisions should be based on numerous criteria, including genetic variation, morphology, behavior, ecology, and geographic distribution (Phillips and Henry 1992, Cronin 1993). Difficulties arise, however,

when findings based on the multiple criteria used for systematic classification are not in agreement, as was the case for the isozyme, mtDNA, and morphology data collected from *U. americanus* populations.

The genetic data from our study suggest that *U. a. luteolus* and *U. a. floridanus* are different from the more widespread *U. a. americanus*, but very similar to one another (see Chapter 5). This data is in agreement with findings from morphological data presented by Kennedy (1989, appendix to Pelton 1989). At what point, however, should morphological or genetic differences be used to delineate distinct populations or subspecies? Amadon (1949) and Mayr (1969) have established numeric rules for verifying subspecific status of populations based on differences in morphological characters. Based on these rules, Kennedy (1989, appendix to Pelton 1989) classified *U. a. luteolus* as a "valid" subspecies (classification of *U. a. floridanus* and *U. a. americanus* was not reported). Although rules regarding morphological characters are available, no such rules exist for genetic analyses using multilocus DNA fingerprinting. Even if a specific measure of genetic variation could be established as the threshold between subspecific or distinct population status (i.e., subspecies are distinguished if < X% band-sharing is

observed between individuals compared from both groups using multilocus DNA fingerprinting, restriction enzyme A, probe B, etc.), collection of all genetic data for classification purposes would have to be standardized. This standardization would require the use of the same techniques and would have to be species-specific. General agreement on and acceptance of such a standardized protocol seems unrealistic. Instead, multilocus fingerprinting data is most useful as a tool to reveal relative degrees of genetic variation within and between populations. When this approach is taken, emphasis is shifted from the estimated similarity values to the relative difference between similarity values within and between populations. For example, when viewed in isolation, the median estimated similarity value of 0.29 between the Apalachicola NF, FL and Ocmulgee River, GA populations does not reveal much about the genetic similarity between these 2 populations (Figure 5, Chapter 5). However, when this similarity value is compared to the median estimated similarity value of 0.60 between the Apalachicola NF, FL and Ocala NF, FL populations, the data now support inferences regarding genetic relationships between populations (Figure 5, Chapter 5): these similarity values suggest that the Apalachicola NF, FL population is more related to the Ocala

NF, FL population than to the Ocmulgee River, GA population. When this type of comparison is made for numerous populations over a large geographic area, groupings of populations can be established based on relative genetic similarities. These groupings should be combined with data on morphology, ecology, and behavior when trying to recognize and establish subspecies or distinct population segment status.

Should *U. a. luteolus* and *U. a. floridanus* be considered as subspecies of *U. americanus*, and if so, are they distinct from each other? Based on the relative genetic similarities from our study and the previous morphological analysis conducted by Kennedy (1989, appendix to Pelton 1989), populations of *U. a. luteolus* and *U. a. floridanus* appear to be distinct from the more widespread *U. a. americanus*. However, populations of *U. a. luteolus* and *U. a. floridanus* appear to be very similar; estimated genetic similarities between *U. a luteolus* and *U. a. floridanus* populations are greater than genetic similarities among *U. a. americanus* populations (see Chapter 5). Separate subspecific status for *U. a. luteolus* and *U. a floridanus* may be unwarranted; therefore, these two might be combined as a single subspecies, distinct from *U. a. americanus*. Final determination of the taxonomic

status of *U. a. luteolus* and *U. a. floridanus*, however, must await consensus among mammalian taxonomists based on several independent lines of evidence.

Listing Recommendations

Under the Endangered Species Act of 1973, Congress defined species to include "any species of fish or wildlife or plants, and any distinct population segment of any species or vertebrate fish or wildlife which interbreeds when mature." Under this definition, any subspecies or distinct population segment of *U. americanus* found to warrant protection could be protected.

In recent years, disagreements over systematic classification and taxonomy of some subspecies has raised questions about the validity of protecting subspecies under the Endangered Species Act. Examples of concern have included the red wolf (*Canis rufus*), the Florida panther (*Felis concolor coryi*), and the dusky seaside sparrow (*Ammodramus maritimus nigrescens*) (O'Brien and Mayr 1991, Dowling et al. 1992, Nowak 1992, Phillips and Henry 1992, Wayne 1992). Past disagreements over the systematic classification and taxonomy of the Louisiana black bear (*U. a. luteolus*) and Florida black bear (*U. a. floridanus*) also have raised concern regarding federal protection of

subspecies (Pelton 1989, Bentzien 1991).

Despite questions over classification and taxonomy, the U.S. Fish and Wildlife Service (USFWS) did officially list the Louisiana black bear, *U. a. luteolus*, as "threatened" in January of 1992 (57 Federal Register 588) and placed the Florida black bear, *U. a. floridanus*, in a "warranted but precluded" category (55 Federal Register 42223). Given the limited black bear habitat and small population sizes remaining in Louisiana and Florida (Brady and Maehr 1985, Pelton 1989), the *U. a. luteolus/U. a. floridanus* grouping appears to warrant federal protection. Unique morphological and genetic characteristics expressed in these populations (Kennedy 1989, Zimmerman 1989, both serving as appendices to Pelton 1989) (see Chapter 5) may be lost if protection is not provided under the Endangered Species Act of 1973. The degree of protection (i.e., endangered versus threatened) should be determined by biologists most familiar with recent trends in local habitat loss and population levels of *U. a. luteolus/U. a. floridanus*.

The Mobile River, AL and Mississippi population may warrant protection as a "distinct population" under the Endangered Species Act. This population had a very high median estimated genetic similarity (similarity

within=0.86), and bears from this population express obvious morphological defects (Kasbohm et al. 1994), possibly due to inbreeding depression. Relatedness of this population and other nearby populations was relatively low (median estimated genetic similarity of 0.43 and 0.48 with the Apalachicola NF, FL and Upper Atchafalaya Basin, LA populations, respectively), supporting the distinct character of this population.

Suggested Directions for Future Research

Many questions regarding genetic variation within and between *U. americanus* populations remain to be investigated. Future research directions include investigations at the population and regional levels.

Black bear populations should be sampled more extensively along the "boundary" of *U. a. luteolus/U. a. floridanus* and *U. a. americanus* to determine any geographic "boundary zone" between these 2 subspecies. Knowing which specific populations are endangered or threatened will be critical if protective measures are to be implemented.

Although federal protection for *U. a. americanus* has not been considered necessary, the bear population along the Ocmulgee River in central Georgia may warrant protection as a "distinct population" due to low population

sizes and a high degree of within-population similarity. Additional study of this population is strongly encouraged.

A study might be conducted to investigate genetic variation between coastal and inland bear populations from a broad area. The study should go beyond the Southern populations already sampled, and explore the possibility of coastal versus inland differences throughout the mid-Atlantic and New England States. Preliminary genetic analyses using multilocus DNA fingerprinting suggested that coastal and inland bear populations in the eastern United States might have a low degree of genetic similarity (Barb Lubinski, Virginia Polytechnic Institute and State University, personal communication).

Under the "isolation by distance" model (Wright 1943), gene flow is expected to be reduced as populations are further separated geographically. A study could be conducted to investigate clinal changes in genetic variation between populations sampled from all regions of North America. In addition to using nuclear DNA analysis, data could incorporate mtDNA analysis of black bear populations, conducted by Dr. Steven Fain (U.S. Fish and Wildlife Service National Forensics Laboratory, Ashland, OR). Trends in genetic similarity that support the "isolation by distance" model, as well as unusual trends,

could be assessed using regression techniques. In areas where similarity and geographic distance are not correlated, potential obstacles to gene flow might be identified.

A study could be conducted to look for a correlation between genetic similarity within a population and the size of the population. Given the diversity of DNA samples currently in storage, a broad range of population sizes could be incorporated in this study. Of special interest would be the northern populations (New Brunswick, Newfoundland, New York, etc.) that have been shown by our preliminary analysis to have limited genetic variation, despite the large size of these populations. Preliminary analysis of these populations was not reported as part of this study.

Advances in molecular technology, such as the polymerase chain reaction (PCR) technique and single-locus DNA fingerprinting, have become available recently. Future studies of *U. americanus* should seriously consider adoption of these techniques, which allow for more powerful data analyses.

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Appendix

Appendix Table 1. Kolgomorov-Smirnov test (Stephen's modification) for normality of genetic similarity values within and between black bear populations in the southeastern United States. Populations found to have non-normal band-sharing data are indicated by an asterisk ($p < 0.15$, standard significance level for normality determination). Sample sizes refer to number of pair-wise comparisons.

Population	n	p-Value
Cook County, MN	109	$p > 0.15$
Ozark NF, AR (*)	12	$0.15 > p > 0.10$
Ouachita NF, AR	12	$p > 0.15$
White River NWR, AR	48	$p > 0.15$
Tensas River NWR, LA (*)	66	$p < 0.01$
Upper Atchafalaya, LA (*)	108	$p < 0.01$
Lower Atchafalaya, LA (*)	52	$p < 0.01$
Mobile River, AL and MS (*)	120	$p < 0.01$
Apalachicola NF, FL (*)	123	$p < 0.01$
Ocala NF, FL (*)	77	$0.05 > p > 0.025$
Big Cypress NP, FL (*)	56	$0.10 > p > 0.05$
Okefenokee NWR, GA (*)	64	$p < 0.01$
Ocmulgee River, GA (*)	36	$0.10 > p > 0.05$
Sumter NF, SC (*)	15	$0.15 > p > 0.10$
Myrtle Beach, SC	21	$p > 0.15$
Great Smoky Mountains NP, TN (*)	21	$0.05 > p > 0.025$
Shenandoah NP, VA	63	$p > 0.15$
Cook County, MN versus Ozark NF, AR	44	$p > 0.15$
Cook County, MN versus Ouachita NF, AR (*)	44	$p < 0.01$
Cook County, MN versus White River NWR, AR (*)	69	$p < 0.01$
Cook County, MN versus Upper Atchafalaya, LA	42	$p > 0.15$
Cook County, MN versus Tensas River NWR, LA (*)	84	$0.15 > p > 0.10$

Appendix Table 1 continued.

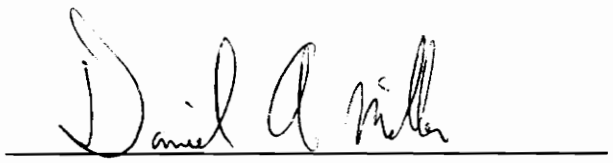
Population	n	p-Value
Ozark NF, AR versus Ouachita NF, AR	32	$p > 0.15$
Ozark NF, AR versus White River NWR, AR	16	$p > 0.15$
Ouachita NF, AR versus White River NWR, AR (*)	16	$0.025 > p > 0.01$
White River NWR, AR versus Tensas River NWR, LA (*)	42	$p < 0.01$
Tensas River NWR, LA versus Upper Atchafalaya, LA	42	$p > 0.15$
Upper Atchafalaya, LA versus Lower Atchafalaya, LA	77	$p > 0.15$
Upper Atchafalaya, LA versus Apalachicola NF, FL	36	$p > 0.15$
Lower Atchafalaya, LA versus Apalachicola NF, FL (*)	42	$0.10 > p > 0.05$
Mobile River, AL/MS versus Upper Atchafalaya, LA	49	$p > 0.15$
Apalachicola NF, FL versus Big Cypress NP, FL (*)	42	$0.15 > p > 0.10$
Apalachicola NF, FL versus Ocala NF, FL (*)	56	$0.15 > p > 0.10$
Ocala NF, FL versus Big Cypress NP, FL (*)	48	$0.025 > p > 0.01$
Ocala NF, FL versus Okefenokee NWR, GA (*)	49	$0.15 > p > 0.10$
Apalachicola NF, FL versus Ocmulgee River, GA	42	$p > 0.15$
Ocmulgee River, GA versus Okefenokee NWR, GA	36	$p > 0.15$
Sumter NF, SC versus Ocmulgee River, GA	36	$p > 0.15$
Sumter NF, SC versus Okefenokee NWR, GA (*)	36	$0.15 > p > 0.10$
Myrtle Beach, SC versus Okefenokee NWR, GA (*)	56	$p < 0.01$
Mobile River, AL/MS versus Apalachicola NF, FL (*)	49	$0.10 > p > 0.05$
Tensas River NWR, LA versus Shenandoah NP, VA (*)	24	$0.15 > p > 0.10$
Shenandoah NP, VA versus Great Smoky Mountains NP, TN	49	$p > 0.15$

Appendix Table 1 continued.

Population	n	p-Value
Tensas River NWR, LA versus Big Cypress NP, FL	20	$p > 0.15$
Shenandoah NP, VA versus Big Cypress NP, FL	50	$p > 0.15$

Vita

Daniel Anthony Miller was born to Bill and Florence Miller on June 22, 1970, in Washington, D.C. He was raised in Oxon Hill, Maryland, where he attended and graduated from Oxon Hill Senior High School in June 1988. After high school, he attended Virginia Tech, where he received his Bachelor of Science in Forestry and Wildlife in May 1992. After working for a year, he returned to Virginia Tech for graduate study and plans to receive his Master's of Science in Wildlife Sciences in June 1995.



Daniel A. Miller